Differential Localization and Regulation of Death and Decoy Receptors for TNF-Related Apoptosis-Inducing Ligand (TRAIL) in Human Melanoma Cells

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Differential Localization and Regulation of Death and Decoy Receptors for TNF-Related Apoptosis-Inducing Ligand (TRAIL) in Human Melanoma Cells

Xu Dong Zhang, Agustin V. Franco, Tam Nguyen, Christian P. Gray, and Peter Hersey

Induction of apoptosis in cells by TRAIL, a member of the TNF family, is believed to be regulated by expression of two death-inducing and two inhibitory (decoy) receptors on the cell surface. In previous studies we found no correlation between expression of decoy receptors and susceptibility of human melanoma cells to TRAIL-induced apoptosis. In view of this, we studied the localization of the receptors in melanoma cells by confocal microscopy to better understand their function. We show that the death receptors TRAIL-R1 and R2 are located in the trans-Golgi network, whereas the inhibitory receptors TRAIL-R3 and -R4 are located in the nucleus. After exposure to TRAIL, TRAIL-R1 and -R2 are internalized into endosomes, whereas TRAIL-R3 and -R4 undergo relocation from the nucleus to the cytoplasm and cell membranes. This movement of decoy receptors was dependent on signals from TRAIL-R1 and -R2, as shown by blocking experiments with Abs to TRAIL-R1 and -R2. The location of TRAIL-R1, -R3, and -R4 in melanoma cells transfected with cDNA for these receptors was similar to that in nontransfected cells. Transfection of TRAIL-R3 and -R4 increased resistance of the melanoma lines to TRAIL-induced apoptosis even in melanoma lines that naturally expressed these receptors. These results indicate that abnormalities in “decoy” receptor location or function may contribute to sensitivity of melanoma to TRAIL-induced apoptosis and suggest that further studies are needed on the functional significance of their nuclear location and TRAIL-induced movement within cells. The Journal of Immunology, 2000, 164: 3961–3970.

Several members of the TNF family have been shown to induce apoptosis in susceptible cells by activation of the caspase pathways (1–3). Induction of apoptosis appears to be restricted to receptors that contain “death domains” such as those for Fas ligand, TNF-α, TNF-related apoptosis-inducing ligand (TRAIL)/apo-2 ligand (4, 5), and apo-3 ligand (4–6). TRAIL appears of particular importance in that it is able to induce apoptosis in a wide range of transformed cell lines but not normal cells (4, 5). mRNA for TRAIL can be detected in most normal tissues, and in view of this it has been postulated that regulation of TRAIL-induced apoptosis occurs at the level of receptor expression (3). Apoptosis induced by TRAIL appears to be mediated by two receptors referred to as DR4 (TRAIL-R1) (7, 8) and DR5/TRAIL-R2/TRICK 2 (9 –11). These receptors, like TRAIL, were found to be widely expressed on normal tissues, but the latter are believed to be protected from apoptosis by two additional receptors, TRAIL-R3/TRID/DcR1/LIT (12–15) and TRAIL-R4/DcR2/TRUNDD (16–18). The mechanisms of protection by these receptors was postulated to involve competition for TRAIL (i.e., act as decoys) or activation of antiapoptotic signals perhaps via activation of NF-kB (3, 18). A fifth receptor, osteoprotegerin exists in a secreted form and appears to inhibit TRAIL-induced apoptosis by competitive inhibition of binding of TRAIL to the death receptors TRAIL-R1 and -R2 (19).

We have shown previously that TRAIL but not other members of the TNF family was able to induce varying degrees of apoptosis in approximately two-thirds of the melanoma lines tested (20–23). Resistance of some lines to TRAIL was due to absence of all receptors for TRAIL, whereas other cell lines had mRNA for TRAIL-R but lacked cell surface expression of the receptors. The latter applied particularly to TRAIL-R3 and -R4 decoy receptors, which appeared located predominantly within the cell (23). Their location within the cell suggested that expression of the receptors may involve regulated movement from intracellular compartments to the membrane.

In the present studies we have used confocal microscopy and flow cytometry to identify the cellular location of the receptors and their movement in response to TRAIL. The results indicate that TRAIL-R3 and -R4 are located predominantly in the nucleus, whereas TRAIL-R1 and -R2 are located in the Golgi apparatus. TRAIL-R1 and -R2 appear to orchestrate relocation of the decoy receptors from the nucleus to the cytoplasm and cell membrane. The site and location of decoy receptors after exposure to TRAIL may have an important bearing on resistance of melanoma cells to TRAIL-induced apoptosis.

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. FH, CV, LT, AT, and RMu were
from lymph nodes. MC and MM were from skin. RM and JG were from bowel. The cell lines had been in culture for 2–6 mo at the time of these studies. MM200, Me1007, Me10538, and Me4405 were from primary melanoma. The derivation of MM200, Me1007, Me10538, and Me4405 is described elsewhere (23). All melanoma cell lines were positive for tyrosinase and MART-1 (melanoma Ag recognized by T cells) mRNA by RT-PCR tests described elsewhere (24), except for Me-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 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 huTRAIL-M271, Lot 7136–07), TRAIL-R2 (IgG1 huTRAIL-R2-M413, Lot 5274–96), TRAIL-R3 (IgG1 huTRAIL-R3-M430, Lot 7313–17), TRAIL-R4 (IgG1 huTRAIL-R4-M444, Lot 7136–15), and the mAb against the leucine zipper on TRAIL (M15) were also supplied by Immunex. The specificity of the Abs are described elsewhere (25). Isotype control mAbs used were the ID4,5 (IgG2a) mAb against Salmonella typhi supplied by Dr. L. Ashman (IMVS, Adelaide, Australia) and the 107,3 IgG1 mAb purchased from PharMingen (San Diego, CA). The Golgi was identified with rhodamine-labeled (100 mg/ml) wheat germ lectin (Sigma, Castle Hill, NSW Australia; catalogue no. L-5266) and the trans-Golgi network (TGN) with rabbit anti-polyuboside A to p230 kindly supplied by Dr. P. A. Gleeson (Monash University, Melbourne, Australia) and described elsewhere (26). Rabbit IgG purchased from Sigma (catalogue no. IS006) was used as control. Abs (goat IgG) against the transferrin receptor (CD71) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA; catalogue no. SC7088). Control Abs were goat IgG purchased from Sigma (catalogue no. I5256). Second Abs were TRITC-conjugated rabbit anti-goat IgG (Sigma; catalogue no. T6028) and Alexa 594 goat anti-rabbit IgG (Molecular Probes, Eugene, OR). Brefeldin A (BFA) was supplied by Sigma (catalogue no. B-7651). It was dissolved in ethanol and used at 5 μg/ml. The calpain proteosome inhibitor, N-acetyl-leucinyl, leucinyl, nor leucinyl (LLnL) was supplied by Sigma (catalogue no. L-6388). It was dissolved in DMSO and made up in a stock solution of 25 mM. Cycloheximide (CHX) and actinomycin D (Act-D) were supplied by Sigma (catalogue no. 7698 and A1410, respectively). The pan caspase inhibitor, zVAD-fmk (benzoyloxycarbonyl-Asp-Glu-Val-Asp-fluoromethyl ketone), was purchased from Calbiochem (La Jolla, CA; catalogue no. 627610).

**Immunofluorescence and confocal microscopy**

Melanoma cells were seeded onto sterile glass coverslips in 24-well plates (Falcon 3047; Becton Dickinson, Lane Cove, NSW, Australia) 16–24 h before fixation. In studies on relocalization of TRAIL receptors, cells were pretreated with recombinant TRAIL (200 ng/ml) at 37°C for 30 min. Cells were washed in PBS, fixed in 4% paraformaldehyde, permeabilized in 0.1% saponin in permeabilization buffer (4°C) for 45 min. After washing with PBS, cells were incubated with Alexa 488 goat anti-mouse IgG conjugate (Molecular Probes) or FITC-conjugated sheep anti-mouse (Sileneus; catalogue no. SC7088). Control Abs were goat IgG purchased from Sigma (catalogue no. IS006). Second Abs were TRITC-conjugated rabbit anti-goat IgG (Sigma; catalogue no. T6028) and Alexa 594 goat anti-rabbit IgG (Molecular Probes, Eugene, OR). Brefeldin A (BFA) was supplied by Sigma (catalogue no. B-7651). It was dissolved in ethanol and used at 5 μg/ml. The calpain proteosome inhibitor, N-acetyl-leucinyl, leucinyl, nor leucinyl (LLnL) was supplied by Sigma (catalogue no. L-6388). It was dissolved in DMSO and made up in a stock solution of 25 mM. Cycloheximide (CHX) and actinomycin D (Act-D) were supplied by Sigma (catalogue no. 7698 and A1410, respectively). The pan caspase inhibitor, zVAD-fmk (benzoyloxycarbonyl-Asp-Glu-Val-Asp-fluoromethyl ketone), was purchased from Calbiochem (La Jolla, CA; catalogue no. 627610).

**Flow cytometry**

Analysis was conducted using a Becton Dickinson (Mountain View, CA) FACScan flow cytometer. In studies on relocalization of TRAIL receptors, cells were treated with recombinant TRAIL (100 ng/ml) at 37°C for 30 min. Cells were washed in PBS, fixed in 4% paraformaldehyde for 5 min, and permeabilized with 0.1% saponin in PBS containing 10% human AB serum for 10 min. Cells were then incubated with primary Abs diluted in PBS containing 1% human AB serum at 4°C for 45 min. After washing with PBS, cells were incubated with Alexa 488 goat anti-mouse IgG conjugate (Molecular Probes) or FITC-conjugated sheep anti-mouse (Silenus; catalogue no. SC7088). Control Abs were goat IgG purchased from Sigma (catalogue no. IS006). Second Abs were TRITC-conjugated rabbit anti-goat IgG (Sigma; catalogue no. T6028) and Alexa 594 goat anti-rabbit IgG (Molecular Probes, Eugene, OR). Brefeldin A (BFA) was supplied by Sigma (catalogue no. B-7651). It was dissolved in ethanol and used at 5 μg/ml. The calpain proteosome inhibitor, N-acetyl-leucinyl, leucinyl, nor leucinyl (LLnL) was supplied by Sigma (catalogue no. L-6388). It was dissolved in DMSO and made up in a stock solution of 25 mM. Cycloheximide (CHX) and actinomycin D (Act-D) were supplied by Sigma (catalogue no. 7698 and A1410, respectively). The pan caspase inhibitor, zVAD-fmk (benzoyloxycarbonyl-Asp-Glu-Val-Asp-fluoromethyl ketone), was purchased from Calbiochem (La Jolla, CA; catalogue no. 627610).

**FIGURE 1.** Effect of exposure of Me4405 cells to TRAIL (100 ng/ml) for 30 min on TRAIL-R surface expression indicated as R1, R2, R3, and R4 in the figure. The histograms show reduced expression of TRAIL-R2 and increased expression of TRAIL-R3 and -R4.
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 registered in a logarithmic scale. At least 10^6 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.

**Transfection of melanoma cells with cDNA for TRAIL-R1, -R3, and -R4**

Mel-MC, which lacks mRNA expression for TRAIL-R3 and -R4 receptors, Mel-SP, which expressed only TRAIL-R3 (23), MM200, and Me4405 were transfected by electroporation (650 V/cm, 960 μF) with 40 μg p-TARGET (Promega) encoding full-length cDNA of TRAIL-R3 receptor. The pDC409 vector carrying TRAIL-R4 cDNA was a gift from Immunex. Target (Promega) encoding full-length cDNA of TRAIL-R3 receptor. In view of the predominant intracellular location of some of the receptors shown in our previous studies, we investigated ligand-induced regulation of TRAIL-R expression.

**Results**

**Ligand-induced regulation of TRAIL-R expression**

In view of the predominant intracellular location of some of the TRAIL receptors shown in our previous studies, we investigated whether binding of TRAIL may regulate surface expression of its receptors in melanoma cells. Melanoma cells were pretreated with TRAIL (200 ng/ml) in DMEM at 37°C for 30 min, and then both surface and total expression of receptors were examined. As shown in Fig. 1 for studies on Me4405, TRAIL induced marked down-regulation of TRAIL-R2 expression, whereas there was an increase in the surface expression of TRAIL-R3 and -R4. Exposure to TRAIL did not appear to have significant effects on TRAIL-R1 expression in cells from the Mel-FH line.

These studies were repeated on the panel of 13 melanoma cells shown in Table I. The pattern of changes in TRAIL-R expression illustrated in Fig. 1 were seen to varying degrees in most of the lines. The main exceptions were small or no reduction in TRAIL-R2 surface expression in Me1007 and Mel-LT, which were resistant to TRAIL-induced apoptosis. TRAIL-R3 and -R4 surface expression increased in all the lines except in the TRAIL-resistant cell line Mel-LT and the sensitive line Mel-JG. TRAIL-R4 was also not up-regulated in MM200 cells, and TRAIL-R3 was not up-regulated in Mel-SP cells, which lacked TRAIL-R1 and -R2 (23). TRAIL-R1 showed an increase in the TRAIL-resistant lines Me1007 and Mel-LT but was otherwise unchanged (Mel-FH, Mel-AT, and Mel-JG) or showed a decrease similar to TRAIL-R2 (Me4405, SK-Mel28, Mel-CV, and Mel-RMu).

Studies on permeabilized cells showed that most of the changes induced by TRAIL were due to relocation of the receptors as total expression in permeabilized cells was relatively unchanged (Table I). A small reduction in TRAIL-R2 expression was still evident after exposure to TRAIL (e.g., Mel-FH, MM200), but this was not as marked as the down-regulation noted in studies on intact cells. Similarly, changes in TRAIL-R1, -R3, and -R4 expression induced by TRAIL were small compared with changes observed in studies on intact cells. The results shown are representative of at least two studies on the cell lines.

### Table I. Expression of TRAIL receptors on melanoma cells before and after exposure to TRAIL

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Pretreatment with TRAIL (100 ng/ml)</th>
<th>% Apoptosis Induced by TRAIL</th>
<th>% Surface Expression</th>
<th>% Total Expression (permeabilized cells)</th>
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<tr>
<td></td>
<td></td>
<td>R1</td>
<td>R2</td>
<td>R3</td>
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<tr>
<td>Me1007</td>
<td>-a</td>
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<td></td>
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</tr>
<tr>
<td>+</td>
<td>13.5</td>
<td>10.4</td>
<td>3.2</td>
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</tr>
<tr>
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<td>+</td>
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<td>5.3</td>
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<td>0</td>
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<td>32</td>
<td>17.7</td>
<td>29.3</td>
<td>19.6</td>
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<tr>
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<td>+</td>
<td>21</td>
<td>40.3</td>
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<tr>
<td>+</td>
<td>83</td>
<td>76</td>
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<tr>
<td>MM200</td>
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<td>0</td>
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<td>32</td>
<td>13.6</td>
<td>14.1</td>
<td>14.1</td>
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<tr>
<td>Mel-SP</td>
<td>-</td>
<td>16.8</td>
<td>6.9</td>
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<tr>
<td>+</td>
<td>20</td>
<td>14.6</td>
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<tr>
<td>SK-Mel-28</td>
<td>-</td>
<td>56.4</td>
<td>68.2</td>
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<tr>
<td>+</td>
<td>90</td>
<td>57.2</td>
<td>32.8</td>
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<tr>
<td>Mel-JG</td>
<td>-</td>
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<td>72</td>
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<td>+</td>
<td>41</td>
<td>31.5</td>
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<tr>
<td>Mel-RMu</td>
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<td>68</td>
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<tr>
<td>+</td>
<td>15</td>
<td>17.5</td>
<td>0</td>
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</table>

*a Mabs against TRAIL-R were used at 10 μg/ml.
*b Indicates mRNA for the receptor not present in the cells (23).
Subcellular localization of TRAIL receptors in melanoma cells

The subcellular localization of TRAIL receptors was studied on melanoma cells from the Mel-FH and Me4405 cell lines by confocal microscopy. The photomicrographs in Fig. 2 show that TRAIL-R2 was located on the membrane of the cells and within intracellular organelles. The latter was identified as the Golgi network by colocalization studies with wheat germ lectin, which is known to stain the Golgi network (31). In contrast, TRAIL-R3 and -R4 showed virtually no membrane expression on Mel-FH cells and appeared to be entirely located in the nucleus, as shown by colocalization studies with propidium iodide (Fig. 2). The expression was diffuse throughout the nucleoplasm but not in nucleoli.

**FIGURE 2.** Confocal appearance of TRAIL receptors in Me4405 melanoma cells before and after exposure to TRAIL. The panels under A indicate appearance of receptors stained with mAbs to the receptors alone at 10 μg/ml. The panels under B indicate appearance of Golgi network (TRAIL-R1 and -R2) or nucleus (TRAIL-R3 and -R4). The panels under C indicate appearance of merged images. The yellow color indicates colocalization of the structures shown in the panels under A and B. Magnification, ×1260.

**FIGURE 3.** Western blot studies on extracts from isolated nuclei and cytoplasm of the Mel-FH and Me4405 melanoma cells. Twenty micrograms was loaded per lane. mAb M431 identified a band at 27 kDa consistent with that of TRAIL-R3, and mAb 444 identified a band at 42 kDa consistent with that of TRAIL-R4 in extracts of nuclei but not in cytoplasmic extracts.

**FIGURE 4.** The colocalization of TRAIL-R2 in Me4405 cells shown in green in A with Abs to the TGN (p230) shown in red in B is illustrated by the yellow color in the merged images in C. The perinuclear location of the Golgi network after treatment with BFA (5 μg/ml) is shown in green in D. The nucleus is shown by staining with propidium iodide. Magnification, ×1260.
TRAIL-R1 in Me4405 cells displayed the same staining pattern as for TRAIL-R2 with expression in the cell membrane and the Golgi apparatus (Fig. 2). This pattern of expression was found in studies on Me1007, Mel-JG, and Mel-RMU. In contrast, TRAIL-R1 was predominantly within the nucleus of Mel-FH. The reason for this different distribution in Mel-FH is not known. The nuclear location of TRAIL-R3 and -R4 was confirmed by Western blot studies on extracts from isolated nuclei from Mel-FH and Me4405, which showed characteristic bands at 27 and 42 kDa, respectively (Fig. 3).

Further studies with Abs against the TGN (p230) in cells from the Me4405 line showed that TRAIL-R1 and -R2 were co-localized in the TGN. Fig. 4, A—C, shows the results for TRAIL-R2. BFA is a fungal metabolite which is known to inhibit the export of

Table II. Expression of TRAIL-R in melanoma cells transfected with cDNA for TRAIL-R1, -R3, or -R4

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>% Apoptosis Induced by TRAIL</th>
<th>% Surface Expression</th>
<th>% Total Expression</th>
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<tbody>
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<td></td>
<td>R1</td>
<td>R2</td>
<td>R3</td>
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<td>Mel-MC</td>
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<td>38.1</td>
<td>12.3</td>
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* Parental cells transfected with vector alone.

b = indicates no mRNA was present for the TRAIL-R (23).
proteins from the Golgi network and causes the different Golgi compartments to redistribute in a characteristic manner (32). Culture of Me4405 cells in BFA (5 \( \mu \)g/ml) resulted in disruption of the Golgi network and appearance of a perinuclear staining pattern characteristic of the TGN (32, 33) (see Fig. 4D). Colocalization studies with mAbs against TRAIL-R1 and -R2 in Me4405 treated with BFA showed an identical localization with the TGN (data not shown). No staining was evident with the isotype controls.

Subcellular localization of TRAIL-R after exposure to TRAIL

Mel-FH and Me4405 cells, grown on coverslips, were pretreated with TRAIL (200 ng/ml) for 30 min, and the localization of TRAIL-R examined by confocal microscopy. As shown in Fig. 2, exposure to TRAIL resulted in reduced surface expression of TRAIL-R2 and appearance of punctate staining throughout the cytoplasm. The Golgi-associated staining pattern of TRAIL-R2 appeared to be retained. TRAIL-R1 in Me4405 displayed the same changes in expression as noted for TRAIL-R2 following exposure to TRAIL. Marked changes were seen in the distribution of TRAIL-R3 and -R4 in that the receptors in the nucleus became dispersed throughout the cytoplasm and cell membrane in all lines except Mel-AT, Mel-JG, and, in the case of TRAIL-R4, MM200. The subcellular location of TRAIL-R3 and -R4 in the cytoplasm after exposure to TRAIL remains under study. The pattern of distribution of TRAIL-R3 was punctate whereas that of TRAIL-R4 was more granular, but the organelles involved have not been identified.

Studies on cell lines transfected with cDNA for TRAIL-R

To confirm the localization of TRAIL-R3 and -R4 in the nucleus, Mel-MC that lacks mRNA expression for TRAIL-R3 and -R4 (23) was transfected with cDNA for TRAIL-R3 and -R4 or vector alone. The levels of expression of the receptors to TRAIL, assessed by flow cytometry are shown in Table II. TRAIL-R4 but not TRAIL-R3 was detected on the cell surface. This correlated with the level of apoptosis induced by TRAIL in that there was a marked reduction in the cells transfected with TRAIL-R4 but only a slight reduction in the TRAIL-R3 transfected cells. As shown in Fig. 5, TRAIL-R3 and -R4 were identified in the nuclei of the transfected but not the nontransfected Mel-MC cells. Some perinuclear staining with mAbs to TRAIL-R4 was also evident. Mel-SP cells, which expressed only TRAIL-R3 (23), were transfected with cDNA for TRAIL-R1. Despite good levels of surface expression (Table II), the Mel-SP cells remained insensitive to TRAIL-induced apoptosis. Fig. 5 shows that the transfected TRAIL-R1 were located in the Golgi network, as expected from studies on the nontransfected cell lines. Control studies showed no reactivity of mAbs against TRAIL-R1 in the parental Mel-SP cells or mAbs against TRAIL-R3 and R4 in the parental Mel-MC cells. The isotype control mAbs 104.5 (IgG2a) and 107.3 (IgG1) did not react with the transfected Mel-SP or Mel-MC cells (data not shown).

MM200 cells (similar to Mel-LT and Mel-JG) did not express TRAIL-R4 on the surface after exposure to TRAIL despite its
presence in the nucleus of the cells. After transfection of TRAIL-R4, however, TRAIL-R4 was detected on the cell surface and apoptosis induced by TRAIL was decreased (Table II). Transfection of cDNA for TRAIL-R3 in this line also resulted in increased TRAIL-R3 surface expression, but this resulted in only a small decrease in apoptosis (Table II). Transfection of cDNA for TRAIL-R3 and -R4 into Me4405 cells resulted in increased levels of expression within the cell, but there were only small increases in surface expression of TRAIL-R3 and -R4. Similarly, there were only small reductions in apoptosis induced by TRAIL in these cells (Table II). Confocal microscopy indicated that the location of the receptors were as shown in Fig. 2. The results shown are representative of at least two experiments on the transfected and parental cell, vector alone, combinations.

**TRAIL-R1 and -R2 undergo internalization after exposure to TRAIL**

As indicated in Table I, the total expression of each receptor assessed in permeabilized melanoma cells remained relatively constant after treatment with TRAIL, which suggested the changes in surface expression were the result of relocation of the receptors into or out of the interior of the cell. The down-regulation of TRAIL-R1 and -R2 from the cell surface in Mel-RMu is shown in Fig. 6A. This indicates the kinetics of the down-regulation of TRAIL-R1 and -R2 following exposure to TRAIL. Similar results were found in a repeat of the experiment. Thirty minutes after exposure to TRAIL, the level of expression was relatively stable. Fig. 6B indicates that TRAIL, identified by mAb against the leucine zipper could be detected in low amounts on the cell surface but in greater amounts within the cell, indicating internalization of the ligand. TRAIL within the cell appeared to be within endosomes, as shown by colocalization of mAbs to TRAIL and the Abs against the transferrin receptor (CD71) which identified endosomes (Fig. 7) (34).

BFA is known to inhibit export of proteins from the Golgi network. We therefore pretreated melanoma cells from the Mel-RMu line with BFA to determine whether the continuing presence of TRAIL on the surface of the cells was due to expression of new receptors for TRAIL. As shown in Fig. 6B, there was a gradual reduction in the amount of TRAIL on the cell surface so that by 30 min the levels were barely detectable. This suggests that the continued detection of TRAIL on the cell surface was due to continuing expression of TRAIL-R2, even though the TRAIL/TRAIL-R2 complex was being endocytosed. The results shown are representative of three similar experiments.

**Regulation of TRAIL-R3 and -R4 is mediated by signals from TRAIL-R1 and -R2**

As shown above in Table I, there was no up-regulation of receptors for TRAIL. As shown in Fig. 6, there was a gradual reduction in the amount of TRAIL on the cell surface so that by 30 min the levels were barely detectable. This suggests that the continuing detection of TRAIL on the cell surface was due to continuing expression of TRAIL-R2, even though the TRAIL/TRAIL-R2 complex was being endocytosed. The results shown are representative of three similar experiments.

**Regulation of TRAIL-R3 and -R4 is mediated by signals from TRAIL-R1 and -R2**

As shown above in Table I, there was no up-regulation of TRAIL-R3 in cells which expressed this receptor alone (Mel-SP). To further delineate the signals involved in up-regulation of TRAIL-R3 and -R4 by TRAIL, we conducted studies on the cell

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**Table III. Effect of hypertonicity on TRAIL-induced down-regulation of TRAIL-R1 and -R2**

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Pretreatment with TRAIL</th>
<th>Culture Condition</th>
<th>% Surface Expression</th>
</tr>
</thead>
</table>
|           |                         |                   | TRAIL-R1 | TRAIL-R2 | TRAIL |%
| Mel-RMu   | –                       | DMEM + FCS        | 67.98    | 48.7     | 5.1   |
|           | +                       | DMEM + FCS        | 24.6     | 0        | 29    |
|           | +                       | DMEM + FCS + sucrose | 44.4    | 21.8     | 59.3  |
| Mel-FH    | –                       | DMEM + FCS        | 10.7     | 57.2     | 35.2  |
|           | +                       | DMEM + FCS        | 9.3      | 11.4     | 35.2  |
|           | +                       | DMEM + FCS + sucrose | 8.8     | 32.5     | 62.3  |

*Identified with mAb M15 against the leucine zipper on TRAIL (10 μg/ml).

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**Table IV. Up-regulation of TRAIL-R3 and -R4 in cells exposed to TRAIL is mediated by signals from TRAIL-R1 and R2**

<table>
<thead>
<tr>
<th>Anti-TRAIL-R1 M271 (10 μg/ml)</th>
<th>Anti-TRAIL-R2 M413 (10 μg/ml)</th>
<th>% Apoptosis</th>
<th>% Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mel-RM</td>
<td>–</td>
<td>–</td>
<td>0</td>
</tr>
<tr>
<td>Mel-RM + TRAIL</td>
<td>–</td>
<td>–</td>
<td>76 ± 3.1</td>
</tr>
<tr>
<td>Mel-RM + TRAIL</td>
<td>–</td>
<td>+</td>
<td>2.9 ± 0.9</td>
</tr>
<tr>
<td>Me4405</td>
<td>–</td>
<td>–</td>
<td>0</td>
</tr>
<tr>
<td>Me4405 + TRAIL</td>
<td>–</td>
<td>–</td>
<td>61 ± 5.2</td>
</tr>
<tr>
<td>Me4405 + TRAIL</td>
<td>+</td>
<td>–</td>
<td>16.5 ± 1.6</td>
</tr>
<tr>
<td>Me4405 + TRAIL</td>
<td>–</td>
<td>+</td>
<td>32.4 ± 2.4</td>
</tr>
<tr>
<td>Me4405 + TRAIL</td>
<td>+</td>
<td>+</td>
<td>0</td>
</tr>
</tbody>
</table>

*Results shown are the mean ± 1 SE of three experiments.

*Mel-RM expressed mRNA for TRAIL-R2 and -R3 only (23).
line Mel-RM that expresses TRAIL-R2 on the surface and TRAIL-R3 within the nucleus (23). However, it lacks mRNA for TRAIL-R1 and -R4. Previous studies have shown that preincubation of cells with the mAb M413 for 1 h at 37°C blocks the binding of TRAIL to TRAIL-R2 (25). TRAIL-induced apoptosis of Mel-RM cells was completely abrogated by the mAb M413. Under the same experimental conditions the surface expression of TRAIL-R3 induced by exposure to TRAIL was also completely inhibited (Table IV). This indicated that binding of TRAIL to TRAIL-R2 is required for TRAIL to induce expression of TRAIL-R3 on the cell surface.

Similar experiments were conducted to investigate whether TRAIL-R1 also induced up-regulation of TRAIL-R3 and -R4 in cells from the Me4405 line. Me4405 cells were treated with the mAb M271 that blocks binding of TRAIL to TRAIL-R1, mAb M413, or a combination of both mAbs before exposure of the cells to TRAIL. As shown in Table IV, pretreatment with either mAb attenuated TRAIL-induced apoptosis, and TRAIL-induced up-regulation of TRAIL-R3 and -R4. When the two mAbs were used in combination, TRAIL failed to induce apoptosis and completely inhibited relocation of TRAIL-R3 and -R4. It was of note that TRAIL-R1 mAb M271 appeared to have greater effect on TRAIL-induced apoptosis and receptor relocation than the mAb M413 against TRAIL-R2. Whether this reflects differing affinities of the mAbs or activity of the receptors is not known.

Relocalization of TRAIL-R3 and -R4 from the nucleus is blocked by the proteasome inhibitor LLnL and by BFA but not by the pan caspase inhibitor zVAD-fmk or protein synthesis inhibitors

TRAIL-R1 and -R2 are known to be complex receptors that can activate several different signal pathways. One of these leads to activation of caspases and apoptosis, whereas others lead to activation of NF-κB (8). To assess the role of caspases in the relocation of TRAIL-R3 and -R4 we used the Pan caspase inhibitor zVAD-fmk (at 20 nM) to block the caspase pathway (25). We found that although this completely inhibited apoptosis, it had no effect on TRAIL induced relocation of TRAIL-R3 and -R4 (data not shown). To assess the role of NF-κB, the proteasome inhibitor LLnL was used to prevent degradation of IκB and hence activation of NF-κB (36). It was found that treatment of cells with LLnL before exposure of cells to TRAIL blocked the up-regulation of TRAIL-R4 and to a lesser extent, TRAIL-R3 (Table V). However, there was no effect on TRAIL-induced down-regulation of TRAIL-R2. This experiment was repeated with the Mel-CV line with similar results, i.e., there was no effect on the down-regulation of TRAIL-R1 and -R2 but up-regulation of TRAIL-R3 and -R4 was inhibited by LLnL. (Percent expression of TRAIL-R3 and R4 induced by TRAIL in absence of LLnL was 13.1 and 13.4, respectively, and in presence of LLnL, 5.4 and 2.5, respectively.) Similarly BFA, which blocks export of proteins from the Golgi apparatus, was found to have no effect on the expression of TRAIL receptors on the cell surface in cells not exposed to TRAIL but partially inhibited the up-regulation of TRAIL-R3 and -R4 after exposure to TRAIL (Table V). This may be secondary to effects of BFA on TRAIL-R2 in that pretreatment with BFA was found to further decrease the expression of TRAIL-R2 induced by TRAIL. This may indicate that TRAIL-R2 levels on the cell are contributed to by export from the Golgi and when the latter is blocked by BFA the TRAIL-R2 receptor levels are reduced (see also Fig. 6B). To determine whether TRAIL induced relocalization of TRAIL receptors involved protein synthesis, we cultured Mel-FH cells or Me4405 cells in the presence of CHX (100 μg/ml) to block protein synthesis or Act-D (3 ng/ml) to block transcription, before adding TRAIL. It was found that neither CHX nor Act-D treatment affected TRAIL-induced relocalization of the receptors, which suggested the relocation of TRAIL-R3 and -R4 is a posttranslational process.

**Discussion**

In previous studies we found a correlation between expression of TRAIL death receptors on melanoma and their susceptibility to TRAIL induced apoptosis but were unable to show any correlation with TRAIL decoy receptor expression (23). This study showed that TRAIL decoy receptors R3 and R4 were located predominantly within the cell, whereas TRAIL-R2 was detected equally well on intact or permeabilized melanoma cells (23). In the present studies we have extended these findings by use of confocal microscopy and mAbs to individual TRAIL-R to identify the cellular localization of the receptors and the effect of interaction with TRAIL on their localization. Colocalization studies using mAbs to identify TRAIL-R and reagents to identify the Golgi and TGN indicated that TRAIL-R2 was located predominantly in the cell membrane and the TGN of the melanoma cells. Localization in the TGN was also shown by the pattern of staining following treatment with BFA. The latter is known to block export of proteins from the Golgi and to cause a characteristic redistribution of the TGN to the microtubule organizing center in a perinuclear location, whereas other compartments of the Golgi redistribute into the endoplasmic reticulum, giving a cytoplasmic staining pattern (32). TRAIL-R2 in BFA-treated cells was localized predominantly around the nucleus and colocalized with the TGN, consistent with its location in the TGN.

The appearance of TRAIL-R1 in most of the melanoma cells was identical to TRAIL-R2 except in cells from the Mel-FH line, where TRAIL-R1 was located in the cell membrane and nucleus rather than the Golgi network. The distribution of TRAIL-R1 in melanoma cells that had been transfected with cDNA for TRAIL-R1 was typically that associated with the TGN and suggests that this is
the normal location of both death receptors. The reason for the different distribution of TRAIL-R1 in the Mel-FH cells is unknown.

These results are similar to studies suggesting that TNF-R1 in human endothelial and monocyte lines was predominantly located in the TGN (33). Similarly, p53 activation was reported to result in increased expression of Fas on vascular smooth muscle cells due to transport from the TGN (37). These and our own findings suggest that localization in the TGN may be a common feature of TNF family receptors inducing apoptosis. Another similarity between TRAIL-R1 and -R2 and TNF-R1 appears to be internalization of the receptor/ligand complex. This was shown previously for the TNF-R1/TNF complex (33, 38) and was suggested in the present studies by internalization of TRAIL and a decrease in TRAIL-R1 and -R2 expression on the membrane, shown by confocal microscopy and flow cytometry. Confocal microscopy indicated colocalization of TRAIL and markers of endosomes, suggesting that the TRAIL/TRAIL-R2 complex was endocytosed. Flow cytometry revealed a rapid increase of TRAIL within the cell in permeabilized cells. It was also noted that treatment of cells exposed to TRAIL with BFA resulted in inhibition of the surface expression of TRAIL-R1 and -R2 on Me4405 cells, which may indicate that TRAIL-R1 and -R2 undergo continual export from the Golgi after interaction with TRAIL.

In contrast to the localization of the death receptors for TRAIL in the Golgi network, the TRAIL decoy receptors were localized in the nucleus. After exposure to TRAIL there was rapid relocation of TRAIL-R3 and -R4 to the cytoplasm and cell membrane in all but two of the lines. The cytoplasmic staining was punctate or granular in pattern but apart from some colocalization in the Golgi network the organelles involved have not been identified. This pattern of expression was also shown in cell lines transfected with cDNA for TRAIL-R3 and -R4. We do not believe the nuclear location of TRAIL-R3 and -R4 was merely due to overexpression of the proteins as similar transfection of TRAIL-R1 resulted only in localization to the TGN and the transfected cells had identical staining patterns to normally expressed TRAIL-R3 and -R4. The transfected receptors were also functional, and in the case of the TRAIL-R4 transfectants were more resistant to TRAIL-induced apoptosis than the untransfected cells. The TRAIL-R3 transfectant had only slightly reduced sensitivity to TRAIL-induced apoptosis. These results, showing increased resistance to apoptosis in cells transfected with TRAIL-R3 and -R4, are consistent with previous studies (13, 18) but at variance with studies on nontransfected melanoma cells where there was no correlation between expression of TRAIL-R3 and -R4 and sensitivity to TRAIL-induced apoptosis (23).

Several inferences can be made from the studies on transfected cells. In studies on the MM200 line the endogenous TRAIL-R4 was not relocated from the nucleus after exposure to TRAIL, whereas the transfected receptors underwent the expected relocation. Such a result would be consistent with aberrant structure of the endogenous receptor and account in part for the susceptibility of this line to TRAIL-induced apoptosis. In the studies on the Me4405 line there was very little increase in the surface expression of receptors in the transfected cells and a small decrease in apoptosis, even though there was a marked increase in receptor content within the cell. It is not clear why the transfected TRAIL-R3 and -R4 were not expressed at higher levels in these cells, but cell surface rather than nuclear expression of the receptors may be needed for inhibition of apoptosis. The ability of the cell to up-regulate TRAIL-R3 and -R4 may therefore be as important as the total amount of receptors in the cell.

Signals from TRAIL-R1 and -R2 appear responsible for relocation of TRAIL-R3 and -R4. This was suggested by the observation that melanoma cells that expressed only TRAIL-R3 receptors and not TRAIL-R1 or -R2 did not undergo relocation from the nucleus and was confirmed by use of mAbs which block interaction of TRAIL with TRAIL-R1 and -R2 (25). In one line (Mel-RM) which expressed only TRAIL-R2 and -R3 the relocation of R3 was completely inhibited by mAb against TRAIL-R2. Similarly, in a line (Me4405) which expressed all TRAIL-R, inhibition of TRAIL-R2 alone was insufficient to stop relocation of TRAIL-R3 and -R4, and it was also necessary to inhibit TRAIL-R1. These results indicate that the signals for relocation of the decoy receptors are dependent on signals from TRAIL-R1 or -R2. A precedent for these findings is the shedding of the TNF-R2 receptor from the cell surface, which was shown to be dependent on signals from TNF-R1 (38).

The nature of the signals from TRAIL-R1 and -R2 responsible for the relocation of TRAIL-R3 and -R4 were investigated on the basis of the signal pathways known to be activated by TRAIL receptors, i.e., the caspases and NF-kB (8). Inhibitors against activation of NF-xB but not those against activation of the caspase pathway suggested that the relocation of the decoy receptors may be dependent on activation of the transcription factor NF-xB by TRAIL-R1 and/or TRAIL-R2. This was more evident for up-regulation of TRAIL-R4 than TRAIL-R3. The proteosome inhibitor LLaL used in these studies is not specific for NF-xB, and more specific inhibitors of NF-xB, such as mutated forms of IxB which are resistant to degradation (39), are needed to confirm these results. However, involvement of NF-xB would be consistent with its role in inhibition of apoptosis (40). TRAIL-R4 was reported to activate NF-xB (3, 18) so that it is possible the relocation to the cell membrane may serve to amplify signals from TRAIL-R3 and -R4 which activate NF-xB. The latter is believed to inhibit apoptosis by mechanisms such as induction of inhibitors of apoptosis 1 and 2 (40).

In summary, the present studies show that the death and decoy receptors for TRAIL have different locations and undergo different movement within the cells on contact of the death receptors with TRAIL. They also raise the possibility that abnormalities in the localization and movement of the receptors may underlie the failure of the decoy receptors to protect against TRAIL-induced apoptosis. The basis for the nuclear localization and export from the nucleus remains to be studied, but it is of interest that TRAIL-R3 and -R4 have sequences compatible with those needed to bind to exportin 1, which is a nuclear export factor (41, 42).

References


