Resistance and Susceptibility of Human Uveal Melanoma Cells to TRAIL-Induced Apoptosis

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Objective: To evaluate the resistance and susceptibility of human uveal melanoma cells to apoptosis induced by tumor necrosis factor–related apoptosis-inducing ligand (TRAIL).

Methods: The sensitivity of 11 human uveal melanoma cell lines was analyzed by flow cytometry for the expression of TRAIL receptors and the antiapoptotic protein survivin. Caspase-8 and caspase-10 expression was also examined using reverse transcriptase–polymerase chain reaction and Western blot analysis.

Results: Only 4 melanoma cell lines were sensitive to TRAIL-induced apoptosis. Positive correlation was observed between resistance and expression of survivin. Up-regulation of survivin by gene transfer enhanced resistance to TRAIL-induced apoptosis, whereas transfection with survivin antisense rendered resistant melanoma cells susceptible to TRAIL-induced apoptosis. Survivin expression and susceptibility to TRAIL-induced apoptosis could also be manipulated by treatment with actinomycin D, which produced a 30% to 50% decrease in the expression of survivin (P < .01) and a 5- to 7-fold increase in TRAIL-induced apoptosis (P < .001).

Conclusions: Resistance of uveal melanoma cells to TRAIL-induced apoptosis is regulated by antiapoptotic proteins such as survivin.

Clinical Relevance: Manipulation of apoptosis regulatory proteins, such as survivin, may have therapeutic applications in the management of uveal melanomas.


**UVEAL MELANOMA IS THE most common intraocular tumor in adults. It represents less than 1% of the annual cancer registrations and occurs with an incidence of only 6 cases per million in the white population.** It is among the most lethal neoplasms because of its extremely high propensity to metastasize to the liver. Approximately half of the patients will die of metastases 10 to 15 years after diagnosis. Despite diagnostic advances and the introduction of new treatment modalities, the rate of metastatic disease from these tumors has not been substantially reduced. Many chemotherapeutic agents act by inducing apoptosis, and it has been suggested that the low therapeutic efficacy in uveal melanoma might be due to an inherent resistance to apoptosis.

Tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) is a member of the TNF family of type 2 membrane proteins and has been shown to be a potent inducer of apoptosis in various cancer cells. The TRAIL can bind 2 death-inducing receptors, TRAIL-R1 (DR4) and TRAIL-R2 (DR5), resulting in receptor cross-linking, caspase activation, and apoptosis induction. Two other receptors, TRAIL-R3 (DcR1) and TRAIL-R4 (DcR2), bind to TRAIL but do not induce apoptosis. In addition, the soluble receptor osteoprotegerin (TRAIL-R5) can also bind to TRAIL. The TRAIL-R3, TRAIL-R4, and soluble TRAIL-R5 are believed to act as decoy receptors and inhibit TRAIL-induced apoptosis by competing with TRAIL-R1 and TRAIL-R2. The expression of TRAIL-R1 and TRAIL-R2 is necessary to induce the death signal of TRAIL. However, it is possible that the melanoma cells survive by mechanisms that inhibit TRAIL-mediated apoptosis. Multiple regulatory components of the TRAIL/TRAIL receptor system affect TRAIL-induced apoptosis at different loci in the apoptosis pathways. Therefore, an understanding of the cellular mediators of apoptosis may lead to the development of new cancer therapies.

Apoptosis induced by TRAIL is mediated by recruitment and activation of caspase-8, which subsequently activates a downstream caspase cascade involving...
Loss of caspase-8 confers resistance of neuroblastoma and Ewing sarcoma cells to TRAIL-mediated apoptosis. However, treatment with demethylation agents restores caspase-8 expression and promotes apoptosis in TRAIL-resistant cells. Ren et al. showed that some of the melanoma cell lines were not susceptible to TRAIL-induced apoptosis. We hypothesized that an intracellular regulator of apoptosis protein, such as survivin, is involved in this process.

Members of the inhibitors of apoptosis protein family, especially survivin, affect the resistance and susceptibility of tumor cells to TRAIL and other apoptosis-inducing agents. Survivin is expressed or up-regulated in most human cancers but is undetectable or found at a very low levels in normal adult tissues. In several tumor cell lines, the presence of survivin correlates with resistance to apoptosis and is associated with increased malignancy and decreased patient survival. It has been shown that the transcription inhibitor actinomycin D increases tumor cell sensitivity to several apoptosis death signals, such as TNF, Fas-L, and TRAIL. Moreover, treatment with actinomycin D down-regulates survivin expression and sensitizes tumor cells to TRAIL-induced apoptosis.

We suspected that the up-regulation of survivin in human uveal melanoma cells was consistent with the hypothesis that survivin plays an important role in inhibiting TRAIL-induced apoptosis in human uveal melanoma cells. Thus, down-regulation of survivin expression with actinomycin D therapy might render uveal melanoma cells susceptible to TRAIL-induced apoptosis. Resistance of uveal melanomas to current treatment protocols still remains a major obstacle. The present study is based on the hypothesis that resistance of uveal melanoma cells to TRAIL-induced apoptosis can occur at different points in the apoptosis pathways. Thus, targeting these sites may provide therapeutic strategies that can overcome the resistance of uveal melanomas to chemotherapeutic agents that act by inducing apoptosis.

**METHODS**

**MELANOMA CELL LINES**

Eleven human uveal melanoma cell lines were used. The OCM1 and OCM8 cell lines were provided by June Kan-Mitchell, MD (University of California, San Diego). The MEL202, MEL285, MEL270, MEL290, OMM1.5, and OMM2.3 cell lines were provided by Bruce Ksander, MD (Schepps Eye Research Institute, Boston, Mass). The 92-1 cell line was provided by Martine J. Jager (Leiden University Hospital, Leiden, the Netherlands). The OM431 cell line was provided by Daniel Albert, MD (University Medical Center of Wisconsin, Madison). The OMM1 cell line is a subcutaneous metastasis originating from a uveal melanoma and was provided by Gregorius P. Luyten, MD (University Hospital Rotterdam, Rotterdam, the Netherlands). Melanoma cells were cultured as previously described elsewhere. Jurkat cells (human acute lymphoblastic T-cell leukemia) were obtained from the American Type Culture Collection (Rockville, Md) (clone E6.1). Cells were cultured in RPMI (Roswell Park Memorial Institute) medium (Mediatech, Inc, Herndon, Va) supplemented with 10% fetal calf serum.

**ANALYSIS OF CASPASE-8 AND CASPASE-10 GENES AND PROTEIN**

Melanoma cell messenger RNA (mRNA) was analyzed using reverse transcriptase–polymerase chain reaction for caspase-8 and caspase-10 expression as previously described elsewhere. Melanoma cells were cultured in 24-well plates (1×10^6 cells/well) with and without recombinant human TRAIL (300 ng/mL) and anti-6X histidine monoclonal antibody (10 µg/mL) for 36 hours according to the manufacturer’s instructions (R&D Systems Inc, Minneapolis, Minn). Briefly, cells were harvested separately by gently trypsinizing with 0.05% trypsin/EDTA, were centrifuged at 1300 rpm for 3 minutes, and then were washed once with 3 mL of ice-cold phosphate-buffered saline solution by centrifugation at 1500 rpm for 5 minutes. Cells were washed 3 times in Hanks balanced salt solution, were resuspended in Cytofix/Cytoperm solution (permeabilization buffer) (BD Biosciences PharmMingen, San Diego, Calif), and then were washed in Perm-Wash buffer (BD Biosciences PharmMingen). Cells were stained with phycoerythrin-labeled rabbit anti-caspase-3 antibody (BD Biosciences PharmMingen) for 20 minutes in the dark. The samples were analyzed using flow cytometry (FACScan; BD Biosciences, Franklin Lakes, NJ). For each sample, 5000 to 10 000 un gated events were acquired, and the results were analyzed using a software program (CellQuest; BD Biosciences). The results are expressed as the percentage of cells that stained positively with anti–caspase-3 antibody.

**FLOW CYTOMETRY ANALYSIS OF TRAIL AND TRAIL RECEPTORS**

The TRAIL and surface expression of TRAIL receptors were assessed using flow cytometry as previously described using anti-human TRAIL monoclonal antibody (R&D Systems Inc) (1 µg/mL) or goat anti–human TRAIL-R1, TRAIL-R2, TRAIL-R3, and TRAIL-R4. Stained cells were assessed for fluorescence in a flow cytometer (FACScan). For each sample, 5000 to 10 000 un gated events were acquired, and the results were analyzed using a software program (CellQuest). Assays were performed in triplicate.

**SURVIVIN EXPRESSION**

Cytoplasmic expression of survivin was determined by flow cytometry after permeabilization of the cell membranes with Cytofix/Cytoperm (permeabilization buffer) and incubation with rabbit anti–human survivin or an isotype control IgG (R&D Systems Inc) at a dilution of 1:100. The samples were analyzed by flow cytometry as described in the “Analysis of Caspase-8 and Caspase-10 Genes and Protein” subsection. The results are expressed as a percentage of cells stained positively with anti-survivin antibody.

**TRANSFECTION OF UVEAL MELANOMA CELLS WITH SURVIVIN AND ANTISENSE SURVIVIN**

Plasmids that encode human survivin (pcDNA3-survivin) or human antisense survivin (pcDNA3-antisense) driven by the human cytomegalovirus promoter were provided by Dario C. Altieri, MD (Department of Cancer Biology and Cancer Center, University of Massachusetts Medical School, Worcester). Human melanoma cell lines were transfected with plasmids that contained survivin, survivin antisense, or an empty vector (pcDNA3) as previously described elsewhere. Forty-eight hours after transfection, cells were collected and survivin expression was determined using flow cytometry as described in the “Analysis of Caspase-8 and Caspase-10 Genes and Protein” subsection.
STATISTICS ANALYSIS

Statistical significance was calculated using the paired t test. A P < .05 was considered statistically significant.

RESULTS

TRAIL-INDUCED APOPTOSIS IN UVEAL MELANOMA CELL LINES

The susceptibility of uveal melanoma cells to TRAIL-induced apoptosis was tested in vitro. Eleven human uveal melanoma cell lines were incubated with or without TRAIL, and apoptosis was determined by using caspase-3 assays. Jurkat cells were used as a control. Cell lines were considered susceptible to TRAIL-induced apoptosis if the percentage of apoptosis was greater than 10% above background levels. (This level was selected arbitrarily.) Only 4 of 11 melanoma cell lines were sensitive to TRAIL-induced apoptosis, whereas 7 cell lines were resistant (Figure 1). The results are presented as the mean of 3 experiments.

EXPRESSION OF TRAIL AND TRAIL RECEPTORS IN HUMAN UVEAL MELANOMA CELL LINES

The differential expression of TRAIL receptors may determine whether a tumor cell is resistant or susceptible to TRAIL-induced apoptosis. Thus, TRAIL receptor expression on 11 uveal melanoma cell lines was analyzed using flow cytometry. The TRAIL was not expressed in any of the uveal melanoma cell lines. The TRAIL-R1 was expressed at high levels in 2 of 4 susceptible cell lines (MEL270 and OM431) and not at all or at very low levels in resistant cell lines (Table). The TRAIL-R2 was expressed at high levels in most of the cell lines. In contrast, TRAIL-R2 expression was low or undetectable on OCM1, OCM8, and OM431 cell lines. None of the cell lines expressed detectable amounts of TRAIL-R3 (DcR1), whereas 7 cell lines expressed detectable amounts of TRAIL-R4 (DcR2). Moreover, there was no correlation between the expression of TRAIL-R3 or TRAIL-R4 and the sensitivity or resistance to TRAIL-mediated apoptosis. Collectively, the results suggest that TRAIL resistance of most uveal melanoma cell lines is not due to failure of the cells to express TRAIL-R2, but it is likely that other regulatory mechanisms are involved.

ROLE OF CASPASE-8 AND CASPASE-10 IN TRAIL-INDUCED APOPTOSIS OF UVEAL MELANOMA CELLS

Susceptibility to apoptosis may be affected at different points in the apoptosis pathways. It has been shown that apoptosis induced by TRAIL is mediated by the activation of caspase-8 and caspase-10, which subsequently activate a downstream caspase cascade involving caspase-3, caspase-6, and caspase-7. Experiments were designed to determine whether resistance or susceptibility of uveal melanoma cells to TRAIL-induced apoptosis was correlated with the expression of caspase-8 and caspase-10. Expression of caspase-8 and caspase-10 was analyzed using reverse transcriptase–polymerase chain reaction in 3 susceptible and 4 resistant melanoma cell lines but was not found to correlate with susceptibility to TRAIL-induced apoptosis. That is, significant caspase-8 mRNA expression was found in 3 of the 4 TRAIL-resistant melanoma cell lines, and caspase-10 mRNA was expressed in varying levels in all 4 of the TRAIL-resistant cell lines but not in MEL270 and OM431 (Figure 2). Caspase-8 mRNA expression correlated with caspase-8 protein levels in all of the cell lines except OM431.
MEL270, and OMM1, which failed to express caspase-8 levels that were detectable by Western blot (data not shown). In contrast, caspase-10 protein levels correlated with mRNA expression in all but 1 cell line, OM431, which demonstrated high caspase-10 mRNA expression but undetectable caspase-10 protein expression (data not shown). These results suggest that caspase-8 and caspase-10 mRNA and protein levels in uveal melanoma cells do not correlate with susceptibility to TRAIL-induced apoptosis.

CORRELATION BETWEEN SURVIVIN LEVELS AND RESISTANCE TO APOPTOSIS IN UVEAL MELANOMAS

Members of the inhibitors of apoptosis protein family, especially survivin, are involved in the inhibition of apoptosis and the control of cell division. The presence of survivin is correlated with resistance to apoptosis in many tumors. Thus, survivin expression was examined in 11 melanoma cell lines using flow cytometry. All 7 TRAIL-resistant melanoma cell lines expressed high levels of survivin (ie, >20% positivity), whereas survivin levels in the 4 TRAIL-sensitive melanomas were low (Figure 3). The detection of high levels of survivin in the TRAIL-resistant cell lines suggests that intracellular survivin may affect uveal melanoma susceptibility to TRAIL-induced apoptosis. Moreover, Jurkat cells expressed low levels of survivin when examined by Western blot analysis (data not shown).

It has been shown that treatment with actinomycin D down-regulates survivin expression and sensitizes tumor cells to TRAIL-induced apoptosis. Thus, experiments were performed to determine whether actinomycin D treatment would render TRAIL-resistant melanoma cells susceptible to TRAIL-induced apoptosis and also increase apoptosis in cell lines that were already susceptible to TRAIL. Accordingly, 2 TRAIL-resistant melanoma cell lines (92-1 and OMM2.3) and 1 TRAIL-sensitive cell line (MEL290) were treated with TRAIL in the presence or absence of actinomycin D (20 ng/mL), and apoptosis was determined by using caspase-3 assays. Pretreatment of TRAIL-resistant cells with actinomycin D induced a 5- to 10-fold increase in sensitivity to TRAIL-mediated apoptosis (Figure 4). In contrast, the addition of actinomycin D to TRAIL-sensitive melanoma cells (MEL290) only slightly increased sensitivity to TRAIL-induced apoptosis. These results indicate that resistance to TRAIL-induced apoptosis is regulated by the apoptotic cascades that are sensitive to actinomycin D treatment.

Because treatment with actinomycin D rendered resistant melanoma cells susceptible to TRAIL-induced apoptosis, it was important to determine whether there was a commensurate down-regulation of survivin protein expression in the actinomycin D–treated cells that displayed increased susceptibility to TRAIL-induced apoptosis. Flow cytometric analysis of intracellular survivin protein expression revealed that actinomycin D treatment resulted in a 30% to 50% decrease in survivin expression in the TRAIL-resistant cell lines but had no effect on the already low intracytoplasmatic expression of survivin protein in the TRAIL-susceptible MEL290 cell line.

(Figure 5). These results indicate that pharmaceutical manipulation of apoptosis-inhibitor proteins, such as survivin, can have a significant effect on the efficacy of therapeutic apoptosis-inducing agents such as TRAIL.

In the case of MEL290, actinomycin D produced a significant increase in TRAIL-induced apoptosis, which is correlated with down-regulation of survivin after actinomycin D treatment (Figures 4 and 5).

IN Volvement of Survivin in TRAIL-Induced Apoptosis

The results at this point indicate that elevated expression of survivin correlates with increased resistance to TRAIL-induced apoptosis. To determine whether survivin is directly involved in the resistance of uveal melanomas to TRAIL-induced apoptosis, 2 TRAIL-sensitive melanoma cell lines (OMM1 and MEL270) were transfected with either a control plasmid or a plasmid containing survivin complementary DNA. After transfection with the survivin gene, the melanoma cells were evaluated using flow cytometry for the expression of cytoplasmic survivin. Transfection with the control plasmid did not affect the expression of survivin in either of the TRAIL-sensitive melanoma cell lines (Figure 6A). However, transfection with the survivin plasmid resulted in a 4-fold increase in the number of survivin-expressing OMM1 cells and a 25% increase in the number of MEL270 cells expressing survivin. The increased expression of survivin correlated with a sharp decrease in susceptibility to TRAIL-induced apoptosis (Figure 6B).

If survivin protects melanoma cells from TRAIL-induced apoptosis, it might be possible to disarm its protective effect by introducing survivin antisense into high survivin-expressing cells. Accordingly, 2 cell lines that expressed significant levels of survivin were transfected with either a control plasmid or a plasmid containing survivin antisense. After in vitro transfection, both cell lines were examined using flow cytometry for the expression of sur-
vivin protein. Transfection with the control plasmid did not affect survivin expression in either cell line (Figure 7A). In contrast, introduction of the survivin antisense resulted in a 50% to 70% reduction in the number of survivin-expressing 92-1 and OMM1 melanoma cells (Figure 7A). The decreased expression of survivin coincided with an approximately 3-fold increase in the susceptibility of both of the previously resistant cell lines to TRAIL-induced apoptosis (Figure 7B).

These results support the notion that manipulating the expression of antiapoptosis genes, such as survivin, can markedly enhance the efficacy of therapeutic agents that act through apoptosis pathways.

**COMMENT**

The TRAIL induces apoptosis of many cancer cell lines in vitro, and its tumoricidal activity has been confirmed in different animal models of human cancer. Although most human cancer cell lines express death receptors for TRAIL receptors 1 and 2, many remain resistant to
TRAIL-induced apoptosis.\textsuperscript{37} Identifying the signals responsible for protecting tumor cells against TRAIL-induced apoptosis may have a major effect on using TRAIL in the treatment of uveal melanoma.

In this study, we showed that 7 of 11 melanoma cell lines are resistant to TRAIL-induced apoptosis. However, TRAIL-R1 was expressed at high levels in 2 cell lines and at very low levels in 9 cell lines. The presence of TRAIL-R1 in 2 of the TRAIL-sensitive cell lines suggests that TRAIL-R1 may be involved in TRAIL-mediated apoptosis in some uveal melanomas. In contrast, the expression of TRAIL-R2 did not correlate with susceptibility to TRAIL-induced apoptosis and suggests that the level of TRAIL receptor expression alone is not sufficient to render melanoma cells sensitive to TRAIL-induced apoptosis and implies that additional intracellular factors that regulate apoptosis pathways are involved. It has been shown that TRAIL decoy receptors are expressed in most normal tissues but are not extensively expressed on tumor cells, implying that expression of these receptors provides resistance to TRAIL-induced apoptosis.\textsuperscript{11-13} Our results indicate that all of the melanoma cell lines expressed at least some level of the TRAIL decoy receptor (DcR2). The expression of DcR2 alone does not guarantee resistance to TRAIL-induced apoptosis because 2 of the 4 TRAIL-sensitive melanoma cell lines expressed detectable levels of DcR2. In agreement with this, DcR1 and DcR2 have been detected in a variety of other cancer cell lines that are sensitive to TRAIL-mediated apoptosis.\textsuperscript{10,38}

Apoptosis induced by TRAIL is mediated by recruitment and activation of caspase-8 and caspase-10, which subsequently activate a downstream caspase cascade involving caspase-3, caspase-6, and caspase-7.\textsuperscript{15} It has been shown that the loss of caspase-8 confers resistance to TRAIL-mediated apoptosis in neuroblastoma and Ewing sarcoma cells.\textsuperscript{16,17} Our results show that caspase-8 is expressed at low levels in most uveal melanoma cell lines and suggests that sensitivity to TRAIL is not correlated with low levels of caspase-10 expression. Thus, our results indicate that the TRAIL signaling pathway circumvents caspase-8 or caspase-10 activation in uveal melanomas, which is in agreement with studies performed with other tumors.\textsuperscript{10,40}

The results of the present study indicate that low levels of TRAIL receptors and caspase-8 or caspase-10 are not sufficient to account for the resistance of uveal melanomas to TRAIL-induced apoptosis and that additional inhibitory mechanisms are involved.
Because many melanoma cells are resistant to TRAIL-induced apoptosis, we hypothesized that TRAIL-induced apoptosis may also be regulated at the intracellular level. A member of the inhibitors of apoptosis protein family, designated survivin, was recently identified and has been shown to play an important role in the inhibition of apoptosis.33,41 The present results indicate that survivin protects uveal melanoma cells from TRAIL-induced apoptosis. All 7 TRAIL-resistant uveal melanoma cell lines expressed high levels of survivin. In addition, actinomycin D decreased the levels of survivin and increased the susceptibility of the uveal melanoma cells to TRAIL-induced apoptosis. Transfection of TRAIL-sensitive cell lines with survivin complementary DNA resulted in up-regulation of survivin and significant inhibition of apoptosis induced by TRAIL. Moreover, results from survivin antisense transfection confirm that survivin is involved in the resistance of uveal melanoma cells to TRAIL-induced apoptosis. It has been shown that survivin inhibits apoptosis induced by a variety of chemotherapeutic agents by interfering with caspase-3, caspase-7, and caspase-9.41,42 Because activation of caspases is involved in TRAIL-induced apoptosis, it is possible that survivin may contribute to the regulation of caspase activation pathways. Overexpression of survivin has been reported in several human tumors, such as skin melanomas.22 Although our data indicate that survivin is a potential inhibitor of TRAIL-induced apoptosis, expression of survivin cannot dictate the resistance of all cell lines to TRAIL-induced apoptosis. It has been shown that other inhibitors of apoptosis protein family members, such as FLIP and BCL-2, may also play a role in this process.31,33,44 In conclusion, actinomycin D sensitized resistant cell lines to TRAIL-induced apoptosis by modulating survivin expression in uveal melanomas. Therefore, manipulation of apoptosis regulatory proteins by chemotherapeutic agents or gene transfer may be useful adjuncts for the treatment of uveal melanoma.

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Figure 7. Effect of survivin antisense transfection on uveal melanomas. Tumor necrosis factor–related apoptosis-inducing ligand (TRAIL)–resistant melanoma cells were transfected with survivin antisense complementary DNA (cDNA), and the mean expression of survivin (A) and TRAIL-induced apoptosis (B) was determined by using caspase-3 assays. Asterisk, dagger, and double dagger indicate significantly different from untreated controls: P<.05, P<.01, and P<.001, respectively. RPMI indicates Roswell Park Memorial Institute medium. Error bars represent standard error.

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