Uveal Melanoma Expression of Tumor Necrosis Factor–Related Apoptosis-Inducing Ligand (TRAIL) Receptors and Susceptibility to TRAIL-Induced Apoptosis

David H. Ren, Elizabeth Mayhew, Christina Hay, Haochuan Li, Hassan Alizadeh, and Jerry Y. Niederkorn

PURPOSE. The study had two purposes: to examine the expression of tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) receptors on uveal melanoma cells and metastases arising from uveal melanoma and to determine the susceptibility of uveal melanoma cells to TRAIL-induced apoptosis.

METHODS. Nine human uveal melanoma cell lines and three cell lines derived from uveal melanoma metastases were examined for TRAIL receptor expression by flow cytometry. In vitro apoptosis assays were performed to determine the relative susceptibility of uveal melanoma cells to TRAIL-induced apoptosis. Annexin V staining was also used to determine the capacity of either cycloheximide or interferon-β to enhance TRAIL-induced apoptosis.

RESULTS. Five of the nine uveal melanoma cell lines expressed TRAIL-R2 on more than 60% of the cells. All three of the cell lines derived from uveal melanoma metastases expressed TRAIL-R2 on more than 50% of the cells. Cycloheximide exerted a profound effect in enhancing TRAIL-induced apoptosis in all but two of the uveal melanoma cell lines and in all three of the metastases cell lines. Interferon-β produced a similar enhancement of TRAIL-induced apoptosis, even in cell lines that were previously shown to be resistant.

CONCLUSIONS. TRAIL is a potentially useful therapeutic modality for the management of uveal melanomas and their metastases. Moreover, pharmacological agents and biological response modifiers that independently display antineoplastic properties can enhance TRAIL-induced apoptosis in resistant uveal melanoma cells. (Invest Ophthalmol Vis Sci. 2004;45:1162–1168) DOI:10.1167/iovs.03-1285

It has been recognized for more than a century that many inflammatory and immune-mediated processes are excluded from the eye—a phenomenon known as immune privilege. Multiple anatomic, physiologic, and immunoregulatory processes contribute to ocular immune privilege. It has been proposed that the ocular immune apparatus is designed either to inhibit or minimize immune-mediated processes that might inflict irreparable injury to ocular tissues that are notoriously incapable of regeneration.

It seems that ocular immune privilege would create a “blind spot” in the host’s immune surveillance apparatus and favor the emergence of intraocular neoplasms, such as uveal melanoma. However, even though 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 six cases per million in the white population. Several conditions may restrict the development of intraocular neoplasms. Even though inflammation and immune responses are dampened in the eye, ocular immune privilege can be circumvented as demonstrated by the development of idiopathic uveitis in humans and experimental autoimmune uveitis in rodents. Moreover, immune rejection of intraocular tumors has been reported in several animal models.

T-cell–independent mechanisms may also function to limit the emergence of intraocular neoplasms. Recently, our laboratory and Lee et al. have demonstrated that tumor necrosis factor-related apoptosis inducing ligand (TRAIL) is constitutively expressed in the eyes of mice and humans. TRAIL, also known as Apo2 ligand, is a member of the TNF family of transmembrane proteins, which are capable of inducing programmed cell death (apoptosis). Unlike other members of the TNF family, TRAIL induces apoptosis in a wide variety of tumor cells, but does not seem to be cytotoxic to normal cells. TRAIL transmits a proapoptotic signal to cells expressing either TRAIL-R1 (DR4) or TRAIL-R2 (DR5). Another group of TRAIL receptors, TRAIL-R3 (DcR1) and TRAIL-R4 (DcR2), lack a death domain, do not induce apoptosis, and act as decoys to block TRAIL-induced apoptosis. Two separate studies using different tumor models demonstrated that TRAIL-R2 bearing tumors undergo TRAIL-dependent resolution in the eyes of allogeneic and syngeneic mice. Moreover, ocular cells mediate TRAIL-induced apoptosis in TRAIL-R2–bearing tumor cells in vitro. Thus, the eye has both T-cell–dependent and T-cell–independent mechanisms for controlling intraocular tumors. At the present time, there are no chemotherapeutic or immunotherapeutic agents that have been shown to be effective in the treatment of uveal melanoma metastases. Moreover, that the 5-year survival time for patients with uveal melanoma has not improved in the past 30 years underscores the urgency for identifying new therapeutic modalities. Considerable enthusiasm has surrounded the potential of TRAIL as a therapeutic agent for treating a wide variety of neoplasms. The susceptibility of various nonocular tumors to TRAIL-mediated apoptosis and the widespread expression of TRAIL in the human and mouse eye prompted us to examine the expression of TRAIL receptors on uveal melanomas and to determine the susceptibility of uveal melanoma cells and their metastases to TRAIL-induced apoptosis.

METHODS

Uveal Melanoma Cell Lines

Nine primary uveal melanoma cell lines designated OCM1, OCM3, OCM8, MEL202, MEL270, MEL285, MEL290, 92.1, and OM431, and three cell lines isolated from metastatic lesions, designated OMM1, OMM1.5, and OMM2.3 were used. OCM1, OCM3, and OCM8 were kindly provided by June Kan-Mitchell (University of California, San Francisco).
Human recombinant TRAIL was purchased from R&D Systems (Minneapolis, MN). Anti-human TRAIL, anti-TRAIL-R1, -R2, -R3, and -R4 were purchased from R&D Systems.

Flow Cytometric Analysis
Expression of human TRAIL and TRAIL receptors was assessed by flow cytometry, as previously described. Single melanoma cell suspensions were prepared and washed in fluorescence-activated cell sorter (FACS) buffer consisting of phosphate buffered saline (PBS; pH 7.2) containing 1% bovine serum albumin (BSA) and 0.02% sodium azide. Cells were incubated with anti-human TRAIL, or anti-human TRAIL-R1, -R2, -R3, or -R4 monoclonal antibody (1 µg/mL) for 30 minutes on ice; washed three times; incubated with FITC-labeled secondary antibody; fixed in 1% paraformaldehyde; and assessed for fluorescence in a flow cytometer (FACScan; BD Biosciences).

Annexin V Assay for Apoptosis
Apoptosis in the melanoma cells was also measured by detection of annexin V binding to phosphatidylserine expressed on the cell membranes of apoptotic cells. Melanoma cells were collected after incubation with recombinant human TRAIL (300 ng/mL) for 36 hours at
37°C and the number of annexin-V–stained cells was determined using a commercially available apoptosis detection kit (TACS Annexin V-FITC; R&D Systems). Briefly, the target cells were harvested separately by gently trypsinizing with 0.05% trypsin-EDTA, centrifuged at 1500 rpm for 5 minutes, and washed once with 3 mL ice-cold PBS by centrifugation at 1500 rpm for 5 minutes. The cells were then gently resuspended in 100 μL ice-cold annexin V incubation reagent containing 10 μL 1× binding buffer, 10 μL propidium iodide (PI), and 1 μL annexin V-FITC (annexin V conjugated to FITC, both provided in the kit), and incubated in the dark at room temperature for 15 minutes. Three hundred microliters of ice-cold 1× binding buffer (a 1:10 dilution of 10× binding buffer provided in the kit was made in deionized H2O) was then added to each sample, and the samples were analyzed by flow cytometry within 1 hour to obtain the maximum signal. In this assay, annexin V-FITC binding to phosphatidylserine (PS) was used as an indicator for apoptotic cells. Although PS is normally confined to the inner leaflet of the plasma membrane (PM), it appears on the external leaflet of the PM during apoptosis, preceding even the nuclear changes that typically characterize apoptosis.20 PI staining, in contrast, was used to identify cells that had lost membrane integrity and were thus classified as being necrotic rather than apoptotic. During flow cytometric analysis, cells that were positive for annexin V-FITC fluorescence (FL1) only were identified as being apoptotic, whereas cells that were positive for both PI fluorescence (FL3) and annexin V-FITC fluorescence (FL1) were identified as being necrotic.

Statistics
Student’s t-test was used to evaluate the differences between the control and experimental groups. P < 0.05 was considered statistically significant. Each assay was performed at least twice with similar results.

RESULTS
Expression of TRAIL Receptors on Uveal Melanoma Cells and their Metastases
Nine uveal melanoma cell lines and three cell lines derived from uveal melanoma metastases were examined for the expression of TRAIL receptors. It was important to determine the expression of TRAIL-R1 and -R2, because these receptors are involved in the transmission of proapoptotic signals. Typical flow cytometry profiles of TRAIL-R1–positive and -negative cells are shown in Figure 1. Examples of TRAIL-R2–positive and -negative uveal melanoma cells are depicted in Figure 2. Five of the nine uveal melanoma cell lines expressed TRAIL-R2 on more than 60% of the cells (Table 1). All three of the cell lines derived from uveal melanoma metastases expressed TRAIL-R2 on more than 50% of the cells.

Effect of Cycloheximide on TRAIL-Induced Apoptosis in TRAIL-Resistant Uveal Melanoma Cells
The expression of TRAIL-R1 and -R2 indicated that nine of the 12 melanoma cell lines were potentially vulnerable to TRAIL-induced apoptosis. Accordingly, the uveal melanoma and metastases cell lines were tested for their susceptibility to TRAIL-
TRAIL and uveal melanoma

induced apoptosis. Cells were incubated with recombinant human TRAIL (300 ng/mL) for 36 hours and tested for apoptosis using a standard annexin V assay. The results showed that three of the nine uveal melanoma cell lines (MEL285, MEL290, and OMM431; Fig. 3) and one of the three metastases cell lines (OMM1; Fig. 4) underwent TRAIL-induced apoptosis.

Expression of the FADD-like IL-1β-converting enzyme inhibitory protein (FLIP) correlates with the resistance of cutaneous melanoma cells to TRAIL-induced apoptosis. However, cycloheximide downregulates FLIP expression and renders many tumor cells susceptible to TRAIL-induced cell death. Therefore, experiments were performed to determine whether cycloheximide treatment would render TRAIL-resistant melanoma cells susceptible to TRAIL-induced apoptosis and also increase apoptosis in those cell lines that were already susceptible to TRAIL. Melanoma cells were incubated in cycloheximide (10 μg/mL), recombinant human TRAIL (300 ng/mL), medium, or a combination of cycloheximide and TRAIL for 36 hours at 37°C. The results indicate that cycloheximide exerted a profound effect in enhancing TRAIL-induced apoptosis (Figs. 3, 4). In the case of MEL202, cycloheximide produced a 10-fold increase in TRAIL-induced apoptosis (Fig. 3). Only two cell lines, 92.1 and OCM8, did not demonstrate increased susceptibility to TRAIL after cycloheximide treatment (Fig. 3). An important observation is that two of the three metastases cell lines responded to cycloheximide treatment and were significantly more susceptible to TRAIL-induced apoptosis than were cells treated with TRAIL alone (Fig. 4).

Effect of Cycloheximide on TRAIL Receptor Expression on Uveal Melanoma Cells

The capacity of cycloheximide to enhance TRAIL-mediated apoptosis in OCM1 and OCM3 cells was puzzling, as these cell lines failed to express quantities of TRAIL-R1 or -R2 that were detectable by flow cytometry. Accordingly, we considered the possibility that cycloheximide treatment induces the upregulation of either TRAIL-R1 or -R2. Both cell lines were incubated in cycloheximide (10 μg/mL), cycloheximide and TRAIL (300 ng/mL), or medium for 36 hours and then assessed by flow cytometry for TRAIL-R1 and -R2 expression. As before, neither cell line displayed TRAIL receptor expression above the background control. However, exposure to cycloheximide alone resulted in a greater than two-fold increase in the number of

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**TABLE 1.** Expression of Trail Receptors on Uveal Melanoma Cell Lines and Cell Lines of Uveal Melanoma Metastases

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>TRAIL-R1</th>
<th>TRAIL-R2</th>
<th>TRAIL-R3</th>
<th>TRAIL-R4</th>
</tr>
</thead>
<tbody>
<tr>
<td>92.1</td>
<td>1.5 ± 2.4</td>
<td>69.2 ± 22.7</td>
<td>5.1 ± 1.9</td>
<td>13 ± 1.6</td>
</tr>
<tr>
<td>MEL202</td>
<td>2.1 ± 2.9</td>
<td>59.6 ± 31.3</td>
<td>2.1 ± 0.7</td>
<td>7.8 ± 6.3</td>
</tr>
<tr>
<td>MEL270</td>
<td>17.1 ± 24.2</td>
<td>84 ± 13.5</td>
<td>5.1 ± 0.08</td>
<td>35.6 ± 22.2</td>
</tr>
<tr>
<td>MEL285</td>
<td>0 ± 0</td>
<td>65.4 ± 0</td>
<td>2.2 ± 0</td>
<td>9.8 ± 0</td>
</tr>
<tr>
<td>MEL290</td>
<td>0.78 ± 1.1</td>
<td>62.9 ± 1.7</td>
<td>0.65 ± 0.92</td>
<td>2.17 ± 0</td>
</tr>
<tr>
<td>OCM1</td>
<td>2.7 ± 3.4</td>
<td>0.99 ± 1.7</td>
<td>1.6 ± 2.6</td>
<td>1.7 ± 1.9</td>
</tr>
<tr>
<td>OCM3</td>
<td>3.0 ± 2.6</td>
<td>1.6 ± 1.4</td>
<td>1.9 ± 1.7</td>
<td>5.7 ± 0.43</td>
</tr>
<tr>
<td>OCM8</td>
<td>0.71 ± 0.63</td>
<td>4.4 ± 5.5</td>
<td>5.6 ± 7.1</td>
<td>10.5 ± 7.8</td>
</tr>
<tr>
<td>OM431</td>
<td>21.9 ± 20.8</td>
<td>0 ± 0</td>
<td>2.2 ± 0</td>
<td>2.2 ± 2.7</td>
</tr>
<tr>
<td>OMM1 (metastasis)</td>
<td>1.4 ± 1.3</td>
<td>69.3 ± 7.4</td>
<td>2.7 ± 4.2</td>
<td>25.3 ± 4.2</td>
</tr>
<tr>
<td>OMM1.5 (metastasis)</td>
<td>0.28 ± 0.39</td>
<td>53.0 ± 12.4</td>
<td>7.6 ± 1.9</td>
<td>19.1 ± 4.2</td>
</tr>
<tr>
<td>OMM2.3 (metastasis)</td>
<td>2.8 ± 2.4</td>
<td>62.6 ± 0.74</td>
<td>3.6 ± 4.3</td>
<td>2.9 ± 0.41</td>
</tr>
</tbody>
</table>

Data are expressed as % positive cells; mean ± SD.

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**FIGURE 3.** Effect of cycloheximide treatment on TRAIL-induced apoptosis in human uveal melanoma cells. Melanoma cells were incubated in cycloheximide (10 μg/mL), recombinant human TRAIL (300 ng/mL), medium, or a combination of cycloheximide and TRAIL for 36 hours at 37°C. Apoptosis was determined by flow cytometry with annexin V staining used as an indicator of apoptosis. PI-positive cells were removed by gating before determining annexin-V-positive cells. The results shown are typical of those in three independent assays. Probabilities determined by Student’s t-test.
and up to 95% of the patients who die of uveal melanoma have liver metastases.\textsuperscript{55,56} Although the treatment of primary uveal melanoma has improved substantially over the past two decades, there have been no significant advances in the management of metastases, and, as such, the 5-year survival time for uveal melanoma patients has not changed in more than 25 years.\textsuperscript{53,54} Regrettably, there is still no effective treatment for uveal melanoma metastases.\textsuperscript{27}

Although cutaneous and uveal melanomas arise from neural crest progenitors, they differ significantly in their epidemiologic,\textsuperscript{57,58} cytogenetic,\textsuperscript{29} metastatic,\textsuperscript{25,26,50} and immunologic characteristics.\textsuperscript{5,31} One of the most striking differences between skin and uveal melanomas is in their metastatic behavior. Skin melanoma metastasizes to almost any organ in the body and is one of the few cancers that metastasize to the heart.\textsuperscript{52} By contrast, uveal melanoma displays a strong propensity to metastasize to the liver.\textsuperscript{53-55}

The present study was based on three propositions: (1) new therapeutic modalities are desperately needed for the management of uveal melanomas; (2) uveal melanomas are fundamentally different from other neoplasms, including cutaneous melanomas, in their responsiveness to various therapeutic agents; and (3) the availability of a diverse array of primary human uveal melanoma cell lines and cell lines derived from uveal melanoma metastases provides an opportunity to examine the
heterogeneity in the susceptibility of uveal melanomas to TRAIL-induced apoptosis.

The results of these in vitro studies indicate that most primary human uveal melanoma cell lines express either TRAIL-R1 or -R2 and are susceptible to TRAIL-induced apoptosis. However, the extent of TRAIL-induced apoptosis varied among the melanoma cell lines. A variety of antiapoptotic genes affect apoptosis, including cFLIP, which is known to protect skin melanoma cells from TRAIL-induced apoptosis.21 The results shown in the current study indicate that the susceptibility of both primary uveal melanoma cells and metastases was dramatically enhanced by pretreatment with cycloheximide, an agent that is known to downregulate cFLIP gene expression.22 The inability of cycloheximide to enhance TRAIL-mediated apoptosis in 92.1 melanoma cells may be due to a preexisting elevation in cFLIP expression. That is, these cells may constitutively express cFLIP at maximum levels. Conversely, it is possible that 92.1 melanoma cells fail to express the cFLIP gene or they express a mutated form of the gene. By contrast, the failure of cycloheximide to enhance TRAIL-induced apoptosis in OCM8 melanoma cells was most likely due to the virtual absence of TRAIL-R1 and -R2 expression on these cells; thus, downregulation of cFLIP in these cells would have no effect, as there were no receptors for TRAIL to activate. However, in other cell lines, there was evidence that cycloheximide treatment could induce increased expression of TRAIL-R1 and -R2 on uveal melanoma cells, thereby accounting for the susceptibility of cycloheximide-treated OCM1 and OCM3 melanoma cells to TRAIL-induced apoptosis. The presence of recombinant human TRAIL further augmented the cycloheximide upregulation of TRAIL-R1 and -R2 on OCM1 cells. We are at a loss to explain this interesting and reproducible finding.

Interferons are potent biological response modifiers that regulate more than 100 different genes.36 IFN-γ upregulates TRAIL-R2 (DR5) and can sensitize tumor cells to TRAIL-induced apoptosis.37 IFN-β is significantly more potent than IFN-γ in enhancing TRAIL-induced apoptosis.36 However, unlike cycloheximide and IFN-γ, IFN-β enhances TRAIL-induced apoptosis by upregulating caspase-3 and -8, but has no effect on the expression of cFLIP; Apaf-1; caspase-9, -8, and -3; cIAP-1 and -2; or TRAIL-R2.36 In the present study, IFN-β alone induced apoptosis in eight of the nine human uveal melanoma cell lines and two of the three cell lines derived from uveal melanoma metastases. However, the most striking effect was found when IFN-β was combined with TRAIL.

We have recently demonstrated the efficacy of IFN-β in reducing liver metastases in mice harboring intracocular B16L89 melanomas.38 The efficacy of IFN-β in optimizing TRAIL-induced apoptosis in uveal melanoma cells and its antimetastatic properties suggest that combining these two agents offers glimmers of hope as a new therapeutic strategy for managing liver metastases in patients with uveal melanoma. This ap-

![Figure 6](https://iovs.arvojournals.org/) Effect of IFN-β on TRAIL-induced apoptosis in uveal melanoma cells. Uveal melanoma cells were incubated at 37°C with human IFN-β (500 U/mL) for 24 hours. Cells were incubated at 37°C for an additional 36 hours in the presence of recombinant human TRAIL (300 ng/mL), and apoptosis was assessed by flow cytometry with annexin V.

![Figure 7](https://iovs.arvojournals.org/) Effect of IFN-β on TRAIL-induced apoptosis in cell lines derived from uveal melanoma metastases. Melanoma cells were incubated with human IFN-β (500 units/mL) for 24 hours before addition of recombinant human TRAIL (300 ng/mL). Apoptosis was assessed 36 hours later by flow cytometry with annexin V.
proach warrants serious consideration, given the low toxicity of both of these agents and the current absence of effective therapeutic modalities for treating liver metastases in uveal melanoma.

References