Acquired resistance to TRAIL-induced apoptosis in human ovarian cancer cells is conferred by increased turnover of mature caspase-3

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Abstract
Little is known on how cancer cells can acquire resistance to tumor necrosis factor–related apoptosis-inducing ligand (TRAIL). In this study, we established TRAIL-resistant cells from the TRAIL-sensitive human ovarian carcinoma cell line OVCAR3 to evaluate the potential mechanisms of acquired resistance to TRAIL. The selected resistant cells were cross-resistant to Fas ligand but remained sensitive to drug-induced apoptosis. Expression of TRAIL receptors was not altered in TRAIL-resistant OVCAR3 cells. Cleavage of caspase-8 and caspase-3 occurred in both TRAIL-resistant and TRAIL-sensitive cells. However, mature caspase-3 fragments were not detected by immunoblot in TRAIL-resistant cells and caspase-3 activity was significantly inhibited in these cells. The addition of proteasome inhibitors significantly increased TRAIL-induced apoptosis in resistant cells and enhanced the accumulation of mature caspase-3 fragments. Pretreatment with cycloheximide showed that active caspase-3 fragments have a high turnover rate in OVCAR3 R350 cells. X-linked inhibitor of apoptosis down-regulation by RNA interference also increased the accumulation of cleaved caspase-3 intermediates and resensitized TRAIL-resistant cells. Our findings show that altered turnover of mature caspase-3 may lead to acquired TRAIL resistance in ovarian cancer cells. Proteasome and X-linked inhibitor of apoptosis inhibitors could have a role in clinical situations to potentiate the cytotoxic effects of TRAIL in resistant tumor cells. [Mol Cancer Ther 2006;5(3):509–21]

Introduction
Tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) is a member of the tumor necrosis factor family that triggers rapid apoptosis in vitro and in vivo in various tumor cell types (1–5). TRAIL binds to its death receptors, TRAIL-R1 and TRAIL-R2, which have a cytoplasmic death domain that signal downstream caspase activation to mediate TRAIL-induced apoptosis. In contrast to these death signaling receptors, TRAIL-R3 and TRAIL-R4 have a truncated intracellular domain or no intracellular domain and are unable to transduce death signals, thus acting as decoy receptors (6–8). Cross-linking of TRAIL to its death receptors leads to the recruitment of Fas-associated death domain, procaspase-8, and procaspase-10 to form the death-inducing signaling complex (9–11). When recruited to the death-inducing signaling complex, procaspase-8 is activated through a series of proteolytic cleavage steps, subsequently activating downstream effector caspases, such as caspase-3, leading to apoptosis. Activation of procaspase-8 can directly result in cleavage of caspase-3 to execute apoptosis (type I cells) or cleave Bid to produce a truncated form, which induces the release of cytochrome c from the mitochondria that leads to caspase-9 and subsequently caspase-3 activation (type II cells). In addition, the release of Smac from the mitochondria promotes caspase-3 activation by inhibiting inhibitor of apoptosis (IAP) family proteins (12, 13). X-linked IAP (XIAP), one of the members of the IAP family, is highly expressed in many tumor cells and its expression has been associated with resistance to chemotherapy and radiation (14). Although data from XIAP knockout mice have suggested that IAP family members may be redundant with XIAP, it may not always be the case (15). XIAP has been shown to bind in vitro to caspase-3 and caspase-7 as well as caspase-9. XIAP is thus a direct inhibitor of caspase-3 (16). It binds to the active form of caspase-3 and promotes the proteasomal degradation of this protease (15, 17). The binding of XIAP to caspase-3, however, requires the initial procaspase-3 cleavage (17).

Ovarian carcinoma is the leading cause of death from gynecologic cancers in North America (18). The long-term survival for patients with advanced high-grade ovarian cancer has been limited by the frequent occurrence of resistance to chemotherapeutic drugs (19). Ovarian cancer generally responds well to drug-based therapy; unfortunately, the initial response is not durable and tumors become resistant to therapy (acquired resistance). In this context, tumor cells can acquire resistance to apoptosis through alterations of molecules controlling the apoptotic cascade. TRAIL may represent an alternative therapeutic molecule for this type of cancer. We and others have shown...
that intrinsic (natural) resistance to TRAIL-induced apoptosis occurs in ~50% of ovarian carcinoma cell lines (20–23). The molecular mechanisms underlying this resistant TRAIL phenotype in human ovarian carcinoma cells is still unclear. Little is known also on how ovarian cancer can acquire resistance to TRAIL. If TRAIL is to be used for treatment of this type of cancer, it is important to understand how tumor cells may survive to repeated exposure to TRAIL. Understanding acquired TRAIL resistance may enable us to design better strategies to prevent the occurrence of resistance to TRAIL and improve its therapeutic potential.

The aim of this study was to understand how ovarian cancer cells may develop resistance to TRAIL-induced apoptosis. We therefore established a TRAIL-resistant human isogenic ovarian cell line, OVCAR3 R350, from the parental TRAIL-sensitive OVCAR3 cells. We show that R350 cells were cross-resistant to Fas ligand (Fas L) but remained sensitive to drug-induced apoptosis. Acquired resistance to TRAIL is not mediated by modulation of the expression of TRAIL receptors and other well-established regulators. When compared with parental TRAIL-sensitive OVCAR3 cells, resistant cells display a decreased caspase-3 activity with a corresponding lack of active caspase-3 subunits due to a rapid turnover rate of these subunits. Acquired resistance to TRAIL-induced apoptosis may be overcome by down-regulation of XIAP and the addition of proteasome inhibitors. Our studies define a new mechanism that may lead to acquired TRAIL resistance in ovarian cancer cells.

Materials and Methods

Reagents

Recombinant human TRAIL was purchased from PeproTech, Inc. (Rocky Hill, NJ). Recombinant super–Fas L was obtained from Apotech Corp. (Hornby, Ontario, Canada). The tetrapeptide caspase inhibitors, z-DEVD-fmk and z-IETD-fmk, and anti-XIAP antibody were obtained from R&D Systems (Minneapolis, MN). Cis-diamminedichloroplatinum (cisplatin) was from Wayne Pharma, Inc. (Montreal, Quebec, Canada) and staurosporine was purchased from Sigma Canada Ltd. (Oakville, Ontario, Canada). Anti-human caspase-8, anti-caspase-9, anti-procaspase-3, anti-active caspase-3 (does not recognize p12), and anti-poly(ADP-ribose) polymerase (PARP) antibodies were purchased from Cell Signaling (Beverly, MA). Anti-TRAIL-R1 to R4 used for Western blot were from R&D Systems. Antibodies used for flow cytometry were from Alexis Biochemicals (San Diego, CA). Anti-XIAP antibody was a rabbit polyclonal from R&D Systems. Anti-c-FLIP, antibody was purchased from Calbiochem (La Jolla, CA). Anti-Bcl-2 antibody was obtained from DAKO (Carpinteria, CA), anti-tubulin was obtained from Sigma Canada, and anti-Bax was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Horseradish peroxidase–conjugated anti-mouse and anti-rabbit antibodies were purchased from GE Healthcare (Baie d’Urfé, Quebec, Canada) and Cell Signaling, respectively. Phenazine methosulfate and pro-pidium iodide (PI) were obtained from Sigma Canada. The proteasome inhibitors clasto-lactacystin β-lactone and z-Leu-Leu-B(OH)2 were obtained from A.G. Scientific (San Diego, CA). Cycloheximide was purchased from Sigma Canada.

Cell Culture

The OVCAR3 cell line was obtained from the American Type Culture Collection (Manassas, VA). OVCAR3 cells were maintained in RPMI 1640 (Wisent, St-Bruno, Quebec, Canada) with 20% fetal bovine serum and insulin (10 mg/L). OVCAR3 R350 cells are a TRAIL-resistant isogenic cell line that was obtained by exposing the TRAIL-sensitive OVCAR3 cell line to stepwise increases in TRAIL concentrations (10–350 ng/mL) over a period of 4 months to select for cells capable of growing at high concentrations of TRAIL. To limit the founder effects associated with cloning, the R350 cell line is a pool of all surviving colonies after TRAIL selection. R350 cells were maintained in RPMI 1640 in 10% fetal bovine serum and 10 mg/L insulin with continuous exposure to 200 ng/mL TRAIL.

Apoptosis Assays

Caspase-3 fluorogenic protease assay was done according to the manufacturer’s protocol (R&D Systems). In brief, 3 × 104 cells were lysed in 250 μL cell lysis buffer, and total cell lysates were incubated with 50 μmol/L DEVD-AFC substrate for 1 hour. Caspase-3 activity was measured on a Versa Fluor fluorometer (Bio-Rad, Hercules, CA). Protein concentration of the lysates was measured with Bio-Rad protein assay kit according to the manufacturer’s recommendations. To determine the sub-G0-G1 DNA content, floating and adherent cells were harvested, washed with PBS, and fixed with cold ethanol overnight. Cell pellets were resuspended, washed with PBS/1% bovine serum albumin, filtered on nylon mesh membrane (40-μm mesh) to remove cell aggregates, and counted. Cells were then incubated with PI [final concentration of 50 μg/mL in 38 mmol/L sodium citrate (pH 7.0)] and boiled RNase A (1 mg/mL) for 30 minutes at 37°C. Cells were analyzed on a FACScan flow cytometer (Becton Dickinson, Mississauga, Ontario, Canada).

Immunoblot Analysis

Whole-cell extracts were prepared in lysing buffer containing protease inhibitors and separated by 12% SDS-PAGE gels. Proteins were transferred to polyvinylidene difluoride membranes (Roche, Laval, Quebec, Canada) by electroblotting, and immunoblot analysis was done as described previously (24). All primary antibodies were incubated overnight at 4°C. Proteins were visualized by enhanced chemiluminescence (GE Healthcare).

Cytotoxicity Assays

Cytotoxicity and cell survival were determined by the 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide inner salt (XTT) assay. Briefly, cells were plated at 20,000 per well in 96-well plates. The next day, cells (confluence 60–70%) were treated with human TRAIL, Fas L, or other cytotoxic drugs and incubated for 48 hours. In some experiments, synthetic caspase inhibitors (25 μmol/L z-DEDV-fmk or z-IETD-fmk) were added...
1 hour before the addition of 100 ng/mL TRAIL. At the termination of the experiment, the culture medium was removed and a mixture of PBS and fresh medium (without phenol red) containing phenazine methosulfate and XTT (Sigma Canada) was added for 30 minutes. The absorbance of each well was determined using a microplate reader at 450 nm (Tecan Sunrise, Research Triangle Park, NC). The percentage of cell survival was defined as the relative absorbance of treated versus untreated cells. All assays were done in triplicate and repeated thrice.

**Flow Cytometry for TRAIL Receptor Expression**
Cell monolayers were detached using EDTA, washed with PBS, and fixed with 2% paraformdehyde in PBS for 1 hour. Cells were incubated with the following unlabeled primary antibodies for 1 hour at 4°C: human anti-TRAIL-R1 to R4 (Alexis Biochemicals). The isotypic control antibody was a normal mouse IgG (Santa Cruz Biotechnology). After three washes with PBS, cells were incubated with phycoerythrin-conjugated donkey anti-mouse antibody (Jackson ImmunoResearch, West Grove, PA) for 45 minutes at 4°C. Cells were analyzed immediately using a FACScan (Becton Dickinson).

**Transfection with Small Interfering RNA Oligonucleotides**
XIAP small interfering RNA (siRNA) oligonucleotides (Signal Silence XIAP siRNA) were obtained from Cell Signaling. The luciferase GL2 duplex, which was used as a control, was purchased from Integrated DNA Technologies (Coralville, IO). Cells (6 × 10^6) were transfected in suspension with a mixture containing Oligofectamine (Invi- trogen/Life Technologies, Burlington, Ontario, Canada), Opti-MEM (Life Technologies), and siRNA oligonucleotides (25 μmol/L) according to the protocol suggested by the manufacturer. Cells were incubated for 48 hours before the addition of TRAIL.

**Results**

**TRAIL-Resistant OVCAR3 R350 Ovarian Cancer Cells Are Cross-Resistant to Fas L**
We have reported previously that some ovarian cancer cell lines can be killed by TRAIL; however, a significant proportion of the tumor cells tested were resistant to TRAIL-induced apoptosis (20). Although many molecules are known to regulate the TRAIL signaling cascade, the mechanisms by which tumor cells can escape TRAIL-induced cell death is still not clear. To gain a better understanding of the molecular alterations that can lead to acquired TRAIL resistance, we developed a TRAIL-resistant OVCAR3 cell line from the TRAIL-sensitive parental cell line. To this end, we submitted OVCAR3 cells to stepwise increases in TRAIL concentrations (10–350 ng/mL) over a period of 4 months to select cells capable of growing at high concentrations of TRAIL. TRAIL induced cell death in OVCAR3 cells in a dose-dependent manner (Fig. 1A). In contrast, exposure of OVCAR3 R350 cells at concentrations of TRAIL up to 500 ng/mL resulted in <20% cell death as determined by XTT assays. At a TRAIL concentration of 200 ng/mL, OVCAR3 cells were rounded and were floating in the medium, suggesting massive cell death, whereas OVCAR3 R350 cells formed a typical epithelioid monolayer (Fig. 1B). Consistent with these results, cell cycle distribution by flow cytometry analysis combined with PI staining showed that whereas R350 cells showed no specific pattern of cell cycle distribution, OVCAR3 cells exhibited an increase in sub-G0-G1 DNA contents, indicating apoptotic nuclear fragmentation (Fig. 1C). To assess whether OVCAR3 R350 cells were also cross-resistant to Fas L, these cells and the parental OVCAR3 cell line were exposed to increasing concentrations of Fas L and cell survival was determined by XTT (Fig. 1A). Similarly to what we have observed with TRAIL, Fas L induced dose-dependent cell death in OVCAR3 with <50% of cell survival at Fas L concentrations of 50 ng/mL (Fig. 1A and B). However, >85% of OVCAR3 R350 cells remained viable at this concentration of Fas L.

**TRAIL-Resistant OVCAR3 R350 Cells Remain Sensitive to Cytotoxic Drugs**
We determined whether OVCAR3 R350 cells were also resistant to cytotoxic drugs. OVCAR3 R350 and the parental cell line were incubated with increasing concentrations of two cytotoxic drugs, cisplatin and staurosporine. We found that both TRAIL-resistant and TRAIL-sensitive cells were efficiently killed by these drugs (Fig. 2A). Less than 20% of cell survival was observed at high concentrations of cisplatin in both OVCAR3 and OVCAR3 R350. Although OVCAR3 R350 seemed slightly less sensitive than OVCAR3 to staurosporine, treatment with this drug also resulted in a dose-dependent cell death in both TRAIL-resistant and TRAIL-sensitive cells. Furthermore, a significant cell detachment was observed at 48 hours following the addition of cisplatin (10 μg/mL) or staurosporine (100 ng/mL) in both OVCAR3 and OVCAR3 R350 (Fig. 2B). Analysis of sub-G0-G1 DNA contents by flow cytometry in OVCAR3 and OVCAR3 R350 cells treated with cisplatin also indicated a similar susceptibility to this drug (Fig. 2C). The data show that TRAIL- and Fas L-resistant OVCAR3 R350 cells remain sensitive to cytotoxic drugs.

**Expression of TRAIL Receptors Is Not Altered in OVCAR3 R350 Cells**
Alterations in the expression levels of TRAIL-R1 and TRAIL-R2 or TRAIL decoy receptors R3 and R4 have been associated with the modulation of TRAIL-induced cell death in various cell types (25–30). We thus determined the expression of the different TRAIL receptors in OVCAR3 and OVCAR3 R350 cell lines in the presence or absence of TRAIL. We did not observe any marked decrease of TRAIL-R1 and TRAIL-R2 in OVCAR3 R350 cells by Western blot (Fig. 3A). Assessment of death receptor expression levels on the cell surface by flow cytometry showed that TRAIL-R1 was slightly decreased in OVCAR3 R350 compared with the parental cell line (Fig. 3B). There was also a slight increase in expression of decoy receptor R4 in OVCAR3 R350 as determined by Western blot (Fig. 3A), but cell surface expression of this receptor was similar in both TRAIL-resistant and TRAIL-sensitive OVCAR3 cells (Fig. 3B).
We determined the expression of antiapoptotic proteins Bcl-XL, Bcl-2, and c-FLIP, and the proapoptotic protein Bax by Western blot (Fig. 3A). These proteins were expressed at similar levels in OVCAR3 and OVCAR3 R350 cells.

Processing of Caspase-3 to Active Enzyme Is Altered in OVCAR3 R350

To gain further insight on the molecular basis of TRAIL resistance, we assessed the protein levels and processing of caspases in OVCAR3 and OVCAR3 R350 cell lines. The processing of procaspase-8 and procaspase-3 was observed in both TRAIL-sensitive and TRAIL-resistant cell lines following exposure to TRAIL (Fig. 4A, lanes 2 and 4) despite the observation that procaspase-8 was expressed at lower levels in R350 cells, suggesting that resistance to TRAIL in R350 cells is regulated downstream of caspase-8. Caspase-3 is synthesized as an inactive proenzyme (p32) consisting of a 3-kDa prodomain, the large and the small subunit. Maturation is executed by an initial proteolytic

Figure 1. Sensitivity of OVCAR3 and OVCAR3 R350 cells to TRAIL- and Fas L-induced apoptosis. A, dose-dependent effect of TRAIL (0–500 ng/mL) and Fas L (0–50 ng/mL) on cell viability in OVCAR3 and OVCAR3 R350 (R350) was determined by XTT assay 48 h after addition of TRAIL or Fas L. Representative of three independent experiments done in triplicate. Points, mean cell viability (%) of treated cells relative to untreated cells; bars, SE. B, morphologic analysis of parental and resistant R350 cells treated with TRAIL (100 ng/mL) or Fas L (25 ng/mL). Cells were analyzed after 48 h under a phase-contrast microscope. Representative of two independent experiments. C, apoptosis of parental and resistant R350 cells was determined by PI staining and flow cytometry. Cells were treated with TRAIL (100 ng/mL) for 24 h and stained with PI, and the sub-G0-G1 DNA content was analyzed by flow cytometry. Representative of two independent experiments.
cleavage mediated via an initiator caspase (caspase-8), and in the second step, preactivated caspase-3 self-catalyzes removal of its prodomain from the large subunit (p20) to generate the p17 and p12 active fragments. Interestingly, the fate of active caspase-3 fragments differed between OVCAR3 and R350 cells (Fig. 4A). We failed to detect mature caspase-3 fragments (p17) in R350 cells, which would be consistent with the more rapid turnover of mature caspase-3 in these cells. In contrast, activated caspase-3 fragments (p17) were detected at higher levels in OVCAR3 following treatment with TRAIL. In line with this observation, cleavage of PARP, a substrate of caspase-3, was completely cleaved in TRAIL-sensitive OVCAR3 cells, whereas PARP cleavage was strongly inhibited in TRAIL-resistant cells. To confirm these results, we monitored caspase-3 activity at different time points in extracts from OVCAR3 and R350 cells treated with TRAIL using the fluorogenic substrate DEVD-AFC (Fig. 4B). For OVCAR3 cells, there was a progressive increase in caspase-3 activity with time after exposure to TRAIL. In contrast, there was very limited increase of caspase-3 activity over time in R350 cells. Taken together, the data show that the initial cleavage step of caspase-3 occurs in both sensitive and resistant cells, but caspase-3 activity is significantly decreased in resistant cells.

XIAP is a substrate for caspase-3. In the presence of activated caspase-3, the full-length 53-kDa protein is depleted and concomitant generation of a 30-kDa fragment occurs (31). We observed that endogenous full-length XIAP was cleaved and a fragment of ~30 kDa was generated during treatment of OVCAR3 cells with TRAIL, which is consistent with the idea that XIAP is cleaved by activated caspase-3 during TRAIL-induced apoptosis (Fig. 4C, lane 3). The addition of an irreversible pan-caspase inhibitor (z-VAD-fmk) completely abrogated

Figure 2. TRAIL-resistant R350 cells are not cross-resistant to cisplatin and staurosporine. A, parental OVCAR3 and R350 cells were treated with increasing concentrations of cisplatin (0 – 100 µg/mL) or staurosporine (0 – 100 ng/mL) and cell viability was determined by XTT assay 48 h after addition of the drugs. Representative of three independent experiments done in triplicate. Points, mean cell viability (%) of treated cells relative to untreated cells; bars, SE. B, morphologic analysis of parental and resistant R350 cells treated with cisplatin (10 µg/mL) or staurosporine (100 ng/mL). Cells were analyzed 24 h under a phase-contrast microscope. Representative of two independent experiments. C, apoptosis of parental and resistant R350 cells was determined by PI staining and flow cytometry. Cells were treated with cisplatin (10 µg/mL) for 24 h and stained with PI, and the sub-G0-G1 DNA content was analyzed by flow cytometry. Representative of two independent experiments.
XIAP cleavage. XIAP cleavage was strongly inhibited in R350 cells treated with TRAIL (Fig. 4C), which is consistent with the observation that caspase-3 activity is significantly lower in these cells.

Proteasome Inhibition Increases TRAIL-Mediated Apoptosis in OVCAR3 R350

The ubiquitin-proteasome pathway has been shown to regulate the activity of caspase-3 (32). Caspase-3 ubiquitination promotes a proteasome-dependent degradation of the active forms of caspase-3 but not procaspase-3. We thus explored the potential role the ubiquitin-proteasome pathway in the regulation of TRAIL-induced apoptosis in our system. When R350 cells were cultured in the presence of clasto-lactacystin, a proteasome inhibitor (33), TRAIL-induced cell death was significantly increased (40–50%), whereas clasto-lactacystin (5 μmol/L) alone without TRAIL did not affect cell viability (Fig. 5A). Similar results were obtained with another proteasome inhibitor, z-Leu-Leu-B(OH)2. The addition of z-Leu-Leu-B(OH)2 (5 μmol/L) to R350 cells treated with TRAIL (200 ng/mL) resulted in a 50% increase in cell death compared with R350 cells treated with TRAIL only (data not shown). The addition of caspase inhibitors, z-IETD-fmk or z-DEVD-fmk, almost completely abrogated TRAIL-induced apoptosis in the presence of clasto-lactacystin, confirming the role of caspases in this process (Fig. 5A). Microphotographs of OVCAR3 R350 cells

Figure 3. Comparison of TRAIL receptors and other apoptosis regulators in resistant and sensitive OVCAR3 cells. Parental and R350 cells were treated in the presence or absence of TRAIL (100 ng/mL) for 24 h. A, total cell lysates were isolated and subjected to SDS-PAGE and immunoblotting. Western blot analysis of endogenous levels of TRAIL-R1 to R4, antiapoptotic proteins Bcl-X_L, Bcl-2, and c-FLIP_L, and proapoptotic protein Bax was done. Tubulin was immunoblotted to ensure equivalent loading of proteins in each lane. B, fluorescence-activated cell sorting analysis of cell surface expression of TRAIL-R1 to R4 with or without TRAIL (100 ng/mL) for 24 h in nonpermeabilized OVCAR3 and TRAIL-resistant R350 cells.
treated with TRAIL in the presence of clasto-lactacystin confirmed the sensitizing effect of this proteasome inhibitor. In the presence of clasto-lactacystin, R350 cells treated with TRAIL were rounded and floating, showing cell death (Fig. 5B), whereas cells formed a regular epithelioid monolayer when treated with TRAIL alone. Taken together, these data suggest that TRAIL-induced apoptosis is regulated by a proteasome-dependent pathway in OVCAR3 R350 cells.

**Sensitization to TRAIL-Induced Apoptosis by Proteasome Inhibitors Is Regulated at the Caspase-3 Level**

We first examined whether the inhibitor of the proteasome clasto-lactacystin modulates the processing of procaspase-8 and procaspase-3. As shown in Fig. 6A, exposure of OVCAR3 R350 cells to TRAIL induced activation of procaspase-8 and procaspase-3 in both OVCAR3 and R350 cells as shown previously. The addition of clasto-lactacystin in combination with TRAIL had little effect on the levels of procaspase-8 and procaspase-3 compared with cells treated with TRAIL alone. However, treatment of R350 cells with clasto-lactacystin increased the accumulation of active caspase-3 subunits (p17) in the presence of TRAIL when compared with treatment with TRAIL alone (Fig. 6A). Consistent with these results, clasto-lactacystin-treated R350 cells displayed much greater accumulation of cleaved PARP and cleaved XIAP compared with untreated cells in the presence of TRAIL (Fig. 6A). The cleavage of XIAP in R350 cells was similar to that of parental OVCAR3 cells, showing that treatment with clasto-lactacystin restored caspase-3 activity in R350 cells. Of note, clasto-lactacystin treatment alone did not significantly alter XIAP levels. Clasto-lactacystin treatment in combination with TRAIL increased DEVD-AFC hydrolysis, a measure of caspase-3 activity, in OVCAR3 R350 cells, whereas caspase-3 hydrolytic activity was significantly reduced in the absence of clasto-lactacystin (Fig. 6B).

To directly assess the turnover rate of active caspase-3 fragments and XIAP, R350 cells were pretreated with cycloheximide to block protein synthesis in combination or not with the proteasome inhibitor clasto-lactacystin. Active caspase-3 fragments (p17) were produced within 4 hours after the addition of TRAIL and rapidly decreased on addition of cycloheximide, indicating that active caspase-3 fragments have a high turnover rate in OVCAR3 R350 cells (Fig. 6D, middle). In contrast, the expression of XIAP remained unchanged on treatment with cycloheximide, thereby increasing the ratio of XIAP to caspase-3.
Active caspase-3 fragments (p17) accumulated over time on treatment with proteasome inhibitor (Fig. 6D, left), which promoted XIAP cleavage. Furthermore, in the presence of both cycloheximide and clasto-lactacystin, the turnover rate of active caspase-3 fragments was significantly decreased (Fig. 6D, right), and a cleaved XIAP fragment was detectable at 8 hours. Thus, inhibition of the proteasome clearly decreases the turnover rate of caspase-3. In addition, the accumulation of active caspase-3 fragments is associated with XIAP cleavage, suggesting that caspase-3 mediates XIAP cleavage. Taken together, our findings indicate that TRAIL-induced accumulation of active caspase-3 subunits found in sensitive cells is lacking in TRAIL-resistant R350 cells. These data are consistent with a rapid turnover of active caspase-3 fragments in R350 cells.

Down-Regulation of XIAP in OVCAR3 R350 Increases Sensitivity to TRAIL-Induced Apoptosis

XIAP, a member of the human IAP family proteins, has been shown to regulate drug-induced apoptosis in ovarian cancer cells (34). XIAP binds to the active form of caspase-3 but not to procaspase-3 and promotes the proteasomal degradation of this enzyme (30, 31, 35). Furthermore, XIAP is the most potent caspase inhibitor of the IAP family (30). To investigate the role of XIAP in proteasome-dependent degradation of caspase-3, we used RNA interference to specifically down-regulate expression of XIAP protein in OVCAR R350 cells. As shown in Fig. 7A, XIAP protein was down-regulated by XIAP-specific siRNA, whereas control siRNA-treated or lipid alone–treated cells expressed similar levels of XIAP proteins. To determine whether XIAP siRNA might increase apoptosis, R350 cells were transfected either with oligofectamine alone, control siRNA (Luc), or XIAP siRNA. Significant augmentation in apoptosis occurred only in cells transfected with XIAP siRNA as determined by microscopic appearance of cells and XTT assay (Fig. 7B and C). Compared with TRAIL alone or Luc siRNA-transfected cells treated with TRAIL, XIAP siRNA-transfected R350 cells had greater number of cells rounded and floating in the medium (Fig. 7B). Increasing concentrations of TRAIL resulted in a significant increase in cell death in XIAP siRNA-transfected R350 cells compared with controls (Fig. 7C). These observations are consistent with the idea that mature caspase-3 turnover is regulated by XIAP.

Depletion of XIAP Increases the Levels of Mature Caspase-3 and PARP Cleavage

To address the question of whether the depletion of XIAP in R350 cells promotes the accumulation of activated
caspase-3 fragments, R350 cells were transfected with either control (Luc) or XIAP siRNA and treated with TRAIL (100 ng/mL), and the cleavage of caspase-3 and PARP was determined by Western blot. Treatment of R350 cells with XIAP siRNA almost completely abolished the expression of XIAP (Fig. 8A, lanes 5 and 6). Importantly, following the depletion of XIAP, TRAIL-induced procaspase-3 cleavage was similar to that of controls (Fig. 8A, lanes 2, 4, and 6). However, depletion of XIAP significantly increased the levels of activated caspase-3 fragments compared with controls, indicating that increased levels of activated caspase-3 are not the results of an increase in the processing of procaspase-3. The accumulation of activated caspase-3 fragments correlated with nearly complete cleavage of PARP, whereas little caspase-3-mediated PARP cleavage occurred in R350 cells treated with TRAIL alone (Fig. 8A). These data suggest that inhibition of XIAP decreases the turnover rate of active caspase-3 fragments.

Release of proteins from the mitochondria, such as Smac/DIABLO and HtrA2/Omi, lead to caspase-9 activation by inhibiting the antiapoptotic function of several IAPs, particularly XIAP. To assess whether the defect in R350 cells was at the mitochondrial level, we examined the processing of caspase-9 in both OVCAR3 and R350 cells following treatment with TRAIL or cisplatin. In cells treated with TRAIL, processing of caspase-9 could not be detected (Fig. 8B). In contrast, in cells treated with

![Figure 6.](Mol Cancer Ther 2006;5(3). March 2006)
cisplatin, caspase-9 was processed as shown by the decrease in the levels of procaspase-9. Consistently, the cleavage of PARP was observed in both cell lines treated with cisplatin (Fig. 8B). Interestingly, however, PARP cleavage also occurred in OVCAR3 cells treated with TRAIL, although caspase-9 was not activated. These results suggest that the mitochondria do not seem to contribute to TRAIL sensitivity.

Discussion

Resistance to cytotoxic agents is a major problem in the treatment of many tumors that clinicians have to face on a regular basis. Drug resistance may be intrinsic or acquired during a course of therapy. Intrinsic resistance is observed when tumors are first exposed to anticancer agents, whereas acquired drug resistance is seen in tumors that no longer respond to the drugs to which they were initially sensitive. Ovarian cancer is a good example where acquired drug resistance is common. Although there is a high rate of initial response to cytotoxic agents, relapses with ovarian tumors that have become resistant to the agents are very common. The molecular alterations leading to TRAIL resistance have been mostly studied in the context where cell lines were intrinsically resistant to this cytotoxic agent. However, little is known about the molecular mechanisms that may contribute to the development of acquired resistance during treatment with TRAIL. This aspect is obviously important as TRAIL may be used for treatment of human cancers. In this study, we have developed an isogenic ovarian cancer cell model to study the molecular alterations leading to acquired resistance to TRAIL. We identified a new mechanism by which tumor cells can develop TRAIL resistance. We have shown that cells resistant to TRAIL are also resistant to Fas L but remained sensitive to chemotherapeutic agents, such as cisplatin. The death-inducing signaling complex apparently remained functional, as we did not find any difference in activation of caspase-8 between TRAIL-resistant and TRAIL-sensitive cells. Furthermore, analysis of cell surface expression of TRAIL receptors and total protein expression of these receptors did not provide any explanation to account for TRAIL resistance in R350 cells. However, the processing of procaspase-3 into active fragments was altered in selected TRAIL-resistant cells. The ability of activated caspase-3 to cleave natural and synthetic substrates was significantly decreased in TRAIL-resistant cells. The rapid degradation of activated caspase-3 fragments via the ubiquitin-proteasome pathway. This idea is supported by the fact that pretreatment of R350 cells with cycloheximide showed a rapid turnover rate of active caspase-3 fragments. Furthermore, incubation with proteasome inhibitors resensitized resistant cells to TRAIL-induced apoptosis and significantly enhanced the accumulation of activated fragments of caspase-3 and their activity. The rapid degradation of caspase-3 may reflect the cell’s adaptation to limit the cellular damage.
induced by TRAIL. Degradation of caspase-3 fragments is likely mediated, at least in part, by XIAP in resistant cells, because down-regulation of XIAP by RNA interference led to significant augmentation of activated caspase-3 fragments, PARP cleavage, and apoptosis.

The protein XIAP has been shown to act as an inhibitor of caspase-3, caspase-7, and caspase-9 activities by directly binding to these proteases (17, 36). Binding of XIAP to caspase-3 requires initial procaspase-3 cleavage, enabling XIAP to target activated caspase-3 for ubiquitin-mediated degradation (15, 17). In addition, cleavage of XIAP by caspase-3 reduces the ability of this protein to suppress apoptosis in Fas L-treated cells (31) and cisplatin-treated ovarian cancer cells (34). Consistent with these results, we found that cleavage of XIAP occurred in TRAIL-sensitive cells. Rapid cleavage of XIAP is probably necessary in these cells to circumvent its inhibitory action on activated caspase-3 fragments and prevent proteasome-mediated degradation of these caspase-3 fragments. The addition of proteasome inhibitor clasto-lactacystin increases the accumulation of activated caspase-3 fragments and enhances TRAIL-induced apoptosis (Fig. 6). Although the processing of procaspase-3 was similar between TRAIL-sensitive and TRAIL-resistant cells, the activity of caspase-3 was strongly inhibited in TRAIL-resistant cells. In addition, the accumulation of activated caspase-3 fragments was decreased in the presence of TRAIL when compared with sensitive cells. Consequently, the cleavage of XIAP was also significantly reduced in resistant cells. This constitutes a novel mechanism by which ovarian tumor cells can develop resistance to TRAIL-induced apoptosis. The ubiquitin-proteasome pathway likely plays an important role in this process because the addition of clasto-lactacystin resensitizes resistant cells to TRAIL-induced apoptosis. The degradation of caspase-3 fragments was prevented in the presence of clasto-lactacystin, and the cleavage of XIAP and PARP was increased. The importance of XIAP in the regulation of caspase-3 activity was confirmed by the fact that the down-regulation of XIAP protein led to higher levels of activated caspase-3 fragments and enhance TRAIL-induced apoptosis in resistant cells. Importantly, increased levels of activated caspase-3 are not the results of an increase in the processing of procaspase-3 as shown by the observation that procaspase-3 expression levels in the presence of TRAIL are similar in controls and XIAP siRNA-treated cells (Fig. 8). Therefore, we speculate that in resistant cells XIAP targets activated caspase-3 fragments for proteasomal degradation, thereby preventing apoptosis, whereas the caspase-inhibitory activity of XIAP is blocked in sensitive cells. How is this process regulated at the molecular levels requires further studies. This question is particularly relevant because the basal levels of XIAP protein were similar between parental OVCAR3 and R350 cell lines (Fig. 4C). The intracellular concentration of XIAP relative to active caspase-3 fragments may determine, at least in part, the catalytic activity of caspase-3 as suggested by Fig. 6D. Alternatively, it is possible that XIAP proteins are not functionally equivalent in terms of their ubiquitin-protein ligase activities in the sensitive versus the resistant cell line. Alternatively, other proteins might compete with XIAP for binding to caspase-3, thereby decreasing the inhibitory effect of XIAP on caspase-3. In this regard, HtrA2/Omi, a protein released from the mitochondria to the cytosol during cisplatin-induced apoptosis, was shown to increase, whereas XIAP was down-regulated, thus enhancing cisplatin-induced apoptosis (37). As HtrA2/Omi can bind directly to XIAP and inhibit its antiapoptotic function (38), down-regulation of HtrA2/Omi would provide a way for tumor cells to enhance the antiapoptotic effect of XIAP. Smac/DIABLO may be released from mitochondria during TRAIL-induced apoptosis and is a potent inhibitor of XIAP (12, 13, 39). Therefore, resistant cells might require the release of Smac/DIABLO and/or HtrA2/Omi from the mitochondria for inhibition of XIAP. However, there are several observations that argue against a defect at the level of the mitochondria in the R350 cells. First, the expression levels of several important regulators of the mitochondrial pathway, such as Bcl-2, Bcl-XL, and Bax, do not differ from parental OVCAR3 and OVCAR3 R350 cells (Fig. 3A). Second, caspase-9 is not

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**Figure 8.** Down-regulation of XIAP by siRNA increases PARP cleavage. **A**, R350 cells were transfected with the respective siRNA (25 μmol/L) and 48 h later, the cells were incubated with TRAIL (100 ng/mL). After 48 h, cell lysates were obtained and analyzed for PARP expression. The membrane was also probed with anti-tubulin antibody to ensure equal loading. **B**, OVCAR3 and R350 cells were treated with TRAIL (100 ng/mL), cisplatin (10 μg/mL), or no drug for 24 h. Lysates were then obtained and analyzed for caspase-9 and PARP expression by Western blot.
processed following the addition of TRAIL in both OVCAR3 and R350, suggesting that the mitochondrial pathway is not activated in neither of the cell lines (Fig. 8B). Activation of the mitochondria induces the release ofSmac/DIABLO, HtrA2/Omi, and cytochrome c. As processing of caspase-9 is induced by the release of cytochrome c from the mitochondria, the lack of caspase-9 processing is likely to reflect the lack of mitochondrial activation. Therefore, the activation of the mitochondrial pathway in OVCAR3 cells does not seem to be necessary for inactivation of XIAP. As the mitochondria does not seem to contribute to TRAIL sensitivity, it is unlikely that a defect at the mitochondrial level would confer TRAIL resistance. Third, processing of caspase-9 is observed in both OVCAR3 and R350 following treatment with cisplatin, a DNA-damaging agent, and correlated with PARP cleavage (Fig. 8B). These data suggest that R350 cells do not harbor any defect in the mitochondrial pathway compared with the parental OVCAR3 cell line. Other IAPs may contribute to the regulation of caspase-3 activity in R350 cells; however, recent data showed that IAPs are functionally nonequivalent and regulate effector caspases through distinct mechanisms (40). The cIAP1 and cIAP2 associate with caspase-9 or caspase-7 but not caspase-3 to regulate apoptosis (30, 40). The specificity of IAPs for binding to effector caspases is conferred by IAP-binding motifs (40).

Although our studies identified XIAP as a possible mediator of acquired TRAIL resistance, the development of drug resistance usually involves multiple alterations of gene expression, and XIAP may be only one of the mechanisms by which ovarian cancer cells acquire resistance to TRAIL. Treatment with proteasome inhibitors or RNA interference to block XIAP antiapoptotic function resulted only in partial resensitization of TRAIL-induced apoptosis in resistant cells. The small heat shock protein family, such as Hsp27 or its related protein aB-crystallin, can block the proteolytic activation of caspase-3 (41) by binding to the p24 partially processed caspase-3. Because the proteins function in a manner similar to XIAP, they may contribute to TRAIL resistance in R350 cells. Overexpression of aB-crystallin protein has been shown to inhibit apoptosis induced by various stimuli, including TRAIL, by disrupting the proteolytic activation of caspase-3 (42, 43). Although aB-crystallin does not inhibit the initial cleavage of pro-caspase-3 and binds to activated caspase-3 like XIAP, aB-crystallin protein is not cleaved by caspase-3 (42) and may therefore act by a different mechanism to inhibit caspase-3. In support of a potential role for this protein in TRAIL resistance, microarray DNA expression analysis has revealed a 3-fold increase in expression of aB-crystallin in R350 cells.2

It is interesting to note that selection for resistance to TRAIL did not concomitantly confer resistance to other cytotoxic agents, such as cisplatin. This is consistent with previous observations where ovarian cancer cells that were resistant to TRAIL remained sensitive to chemotherapeutic drugs (20). Alterations of the final common pathway of apoptosis as we have observed in R350 cells would be expected to confer some resistance to drugs, such as cisplatin. The discrepancy between our data and previous reports (34, 44, 45) on the action of cisplatin lead us to speculate that cisplatin-induced apoptosis can occur through caspase-3-dependent and caspase-2-independent mechanisms in ovarian cancer cells. Cisplatin-induced apoptosis by a caspase-3-independent pathway has been described previously in ovarian cancer cells (46). It is widely accepted that cisplatin causes cross-linking in intrastrands and interstrands of DNA, which ultimately lead to cell death (47). Cisplatin-induced DNA damage activates several pathways that may be caspase dependent or independent. For example, activation of the mitogen-activated protein kinase-extracellular signal-regulated kinase pathway by cisplatin may facilitate apoptosis independently of caspases (47). Therefore, it is conceivable that pathways activated by cisplatin to induce apoptosis may vary depending on the cell context. In contrast to these findings, cells selected for resistance to TRAIL were completely resistant to Fas L. This suggests that the selected TRAIL-resistant cells showed specific alterations to death receptor–induced apoptosis.

In conclusion, our studies indicate that the emergence of TRAIL resistance is mediated, at least in part, by rapid turnover of mature caspase-3 fragments, and XIAP may exert a caspase-inhibitory role. The effect of proteasome inhibitors on TRAIL-induced apoptosis occurs through stabilization of active caspase-3. Our studies define a new mechanism that contributes to acquire TRAIL resistance in ovarian cancer cells. Understanding how tumor cells can develop resistance during treatment with TRAIL is important and may help design better treatment strategies to circumvent the problem of resistance. In that respect, our findings that proteasome inhibitors resensitize cells that have acquired resistance to TRAIL may be of interest from a clinical standpoint. Proteasome inhibitors could be used in clinical situations to potentiate the cytotoxic effects of TRAIL in resistant tumor cells.

References

2 Unpublished data.
Acquired resistance to TRAIL-induced apoptosis in human ovarian cancer cells is conferred by increased turnover of mature caspase-3

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