

# X-linked Inhibitor of Apoptosis (XIAP) Blocks Apo2 Ligand/Tumor Necrosis Factor-related Apoptosis-inducing Ligand-mediated Apoptosis of Prostate Cancer Cells in the Presence of Mitochondrial Activation: Sensitization by Overexpression of Second Mitochondria-derived Activator of Caspase/Direct IAP-binding Protein with Low pI (Smac/DIABLO)

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## Abstract

The resistance to Apo2 ligand (Apo2L)/tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-mediated apoptosis could be overcome by treatment with subtoxic concentrations of actinomycin D (Act D) in prostate tumor cells. Furthermore, the sensitization to Apo2L/TRAIL-mediated apoptosis by Act D positively correlated with selective down-regulation of X-linked inhibitor of apoptosis (XIAP). In this study, we examined whether second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low pI (Smac/DIABLO), a known inhibitor of apoptosis (IAP)-neutralizing protein, sensitizes resistant prostate tumor cells to Apo2L/TRAIL-mediated apoptosis. The prostate tumor cell line CL-1 was treated with Apo2L/TRAIL, Act D, or a combination of the two. The apoptosis-mediated signaling pathway was examined by Western blotting and flow cytometry. Furthermore, CL-1 cells transfected with the anti-IAP inhibitor Smac/DIABLO were examined for sensitivity to Apo2L/TRAIL. Whereas Apo2L/TRAIL induced the release of cytochrome c and endogenous Smac/DIABLO in the CL-1 tumor cells, the cytosolic levels of both molecules were not sufficient to induce apoptosis. Transient transfectants with a Smac/DIABLO cDNA encoding a neutralizing inhibitor of IAPs were sensitized to Apo2L/TRAIL-mediated apoptosis. The sensitization to Apo2L/TRAIL by Smac/

DIABLO overexpression was a result of synergistic activation of caspases-3, -9, and -8. Treatment of the Smac/DIABLO transient transfectant with Apo2L/TRAIL enhanced the release of Smac/DIABLO from mitochondria and led to reduction of IAP family proteins (XIAP, c-IAP1, and c-IAP2). These results show that Smac/DIABLO can sensitize CL-1 tumor cells to Apo2L/TRAIL-mediated apoptosis. Thus, up-regulation of Smac/DIABLO and sensitization to Apo2L/TRAIL-mediated apoptosis are of potential clinical application in the immunotherapy of drug-/Apo2L/TRAIL-resistant tumors.

## Introduction

The TNF<sup>3</sup> ligand superfamily plays an important role in the host immune defense against cancer as an antitumor death-inducing agent (1). The TNF ligand members induce tumor programmed cell death or apoptosis by binding to their cognate death receptors on the cell surface (2–6). Recombinant TNF ligands have been widely explored as potential therapeutic agents against several types of cancer (7). Among the current members, Apo2L/TRAIL is the most promising for therapeutic use because it has been shown to kill a wide variety of malignant tumors while eliciting little systemic toxicity in experimental animal models (8, 9).

Similar to TNF- $\alpha$  and Fas, Apo2L/TRAIL induces apoptosis in sensitive tumor target cells by the death receptor pathway (9, 10). Upon cross-linking with the death receptors TRAIL-R1/DR4 and/or TRAIL-R2/DR5, Apo2L/TRAIL initiates aggregation of the death receptors, recruitment of the adaptor molecule Fas-associated death domain (FADD), and activation of initiator caspase-8 (11–13). The active caspase-8 or caspase-10 triggers a caspase activation cascade by directly activating effector caspase-3 (type I pathway) or by diverting

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<sup>3</sup> The abbreviations used are: TNF, tumor necrosis factor; Ab, antibody; Act D, actinomycin; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; GFP, green fluorescence protein; IAP, inhibitor of apoptosis; MTS, mitochondrial targeting sequence; PARP, poly(ADP-ribose) polymerase; Smac/DIABLO, second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low pI; XIAP, X-linked inhibitor of apoptosis; Apo2L, Apo2 ligand; FBS, fetal bovine serum; 7-AAD, 7-amino-actinomycin D.

the death signal to mitochondria (type II pathway; Refs. 14–16). The type II pathway, which involves the release of cytochrome *c* from mitochondria, further amplifies the death signal by activation of caspase-9 and ensuing activation of caspase-3 (15–18).

The Apo2L/TRAIL apoptotic signaling pathway is subjected to several levels of inhibitory regulation. These include surface expression of two decoy receptors (TRAIL-R3/DcR1 and TRAIL-R4/DcR2; Ref. 10); FLIP, a dominant negative form of caspase-8 that lacks the caspase catalytic site (19, 20); and, at the mitochondrial level, Bcl-2 antiapoptotic members (Bcl-2, Bcl-xL, Bfl-1/A1, and Mcl-1; Ref. 21). Finally, at the caspase level, IAP family members (c-IAP1, c-IAP2, XIAP, and survivin) bind to caspases-9, -3, and -7 and prevent the onset of the caspase activation cascade (22–24). All of these antiapoptotic molecules render cells insensitive to various apoptotic stimuli, including Apo2L/TRAIL. Their overexpression has been shown to be associated with tumor resistance to apoptosis-inducing tumor therapies (25).

One of the major problems in cancer treatment today is the development or acquisition of tumor resistance (25). Because many conventional cancer therapies such as chemotherapy and radiation eradicate tumors by apoptosis, high expression of antiapoptotic molecules will render tumor cells resistant to the conventional therapies. However, novel therapeutics may also use apoptosis to eradicate tumors. Experimental immunotherapeutic approaches based on immune cytotoxic molecules such as Apo2L/TRAIL will also most likely be ineffective against chemoresistant tumor cells. Therefore, the tumor resistance to apoptosis must be reversed to enhance the efficacy of cancer therapy.

To overcome tumor resistance to Apo2L/TRAIL, we have used Act D to sensitize immunoresistant prostate tumor cells to Apo2L/TRAIL-mediated apoptosis (26). We have also shown that sensitization to Apo2L/TRAIL killing is associated with the preferential down-regulation of XIAP (27). Recently, a mitochondrial molecule named Smac/DIABLO has been documented to be a neutralizing inhibitor of the apoptotic inhibitor IAP family proteins (28, 29). Upon receiving a death signal, mitochondria release Smac/DIABLO into the cytoplasm, in addition to the release of cytochrome *c* (28, 29). Cytoplasmic Smac/DIABLO binds to IAP family members and relieves the IAP-mediated inhibition of caspases-9 and -3 (28–30). In this study, we examined whether the overexpression of Smac/DIABLO, which down-regulates the level of XIAP, sensitizes CL-1 tumor cells to Apo2L/TRAIL-mediated apoptosis. This study also compared the apoptotic signaling events induced by Act D, Smac/DIABLO overexpression, and Apo2L/TRAIL.

## Materials and Methods

**Cells and Reagents.** The immunoresistant prostate tumor cell line CL-1 was kindly provided by Dr. Arie Beldegrun (University of California Los Angeles). The establishment and characterization of CL-1 cells have been described previously (31). The prostate cells were cultured in RPMI 1640 (Mediatech, Herndon, VA) supplemented with 10% FBS (Gemini Bioproducts, Woodland, CA). Act D was purchased

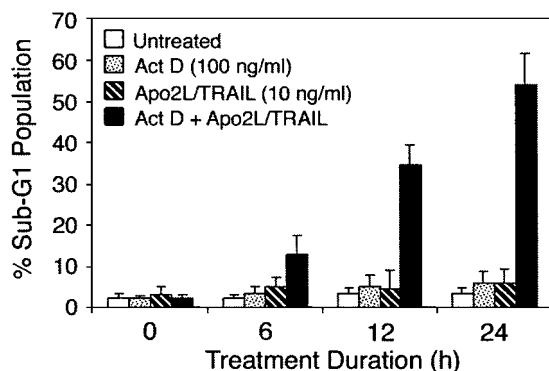
from Sigma (St. Louis, MO). Recombinant human Apo2L/TRAIL was purchased from Peprotech (Rocky Hill, NJ).

**Constructs.** The construct pcDNA-FLAG-smac overexpressing Smac/DIABLO was kindly provided by Dr. Xiaodong Wang (University of Texas Southwestern Medical Center, Dallas, TX). The production of the expression construct was described by Du *et al.* (28). The control vector pcDNA3.1 was purchased from Invitrogen (Carlsbad, CA). The pEGFP-N1 expression construct encoding GFP was purchased from Clontech (Palo Alto, CA).

**Abs.** The monoclonal anti-FLAG Ab M2 clone was purchased from Sigma. The polyclonal anti-Smac/DIABLO Ab was kindly provided by Dr. Xiaodong Wang. The polyclonal anti-survivin Ab was purchased from Pro-Sci (San Diego, CA). The monoclonal anti-actin Ab was purchased from Chemicon (Temecula, CA). The polyclonal Abs against IAP family proteins (c-IAP1, c-IAP2, and XIAP) were purchased from Trevigen (Gaithersburg, MD). The anti-Bcl-2 monoclonal Abs and anti-c-FLIP Abs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and Upstate Biotechnology (Lake Placid, NY), respectively. Monoclonal anti-cytochrome *c* Ab was purchased from BD PharMingen (San Diego, CA).

**Propidium Iodide-based DNA Fragmentation Assay by Flow Cytometry.** The propidium iodide-based flow cytometric DNA fragmentation assay was adopted from Nicoletti *et al.* (32). Approximately  $2 \times 10^5$  prostate tumor cells were seeded in each well in 12-well plates in RPMI 1640 supplemented with 10% FBS overnight. Recombinant Apo2L/TRAIL (10 ng/ml) and Act D (100 ng/ml) were simultaneously added to the cell cultures, and the samples were collected at various time periods after the addition of Act D and Apo2L/TRAIL. Measurement of DNA fragmentation in propidium iodide-stained cells was performed using an Epics XL flow cytometer (Coulter Electronics, Inc., Miami, FL). Region markers were drawn for sub-G<sub>1</sub>, G<sub>0</sub>-G<sub>1</sub>, S, and G<sub>2</sub>-M populations for quantitation of the cell populations by the flow cytometer. The sub-G<sub>1</sub> population represents the cells containing DNA hypodiploidy, a characteristic of apoptotic cells undergoing DNA fragmentation.

**Dual-Color Fluorescence Analysis of Apoptotic Cells by Flow Cytometry.** The dual-color fluorescence analysis of apoptotic cells by flow cytometry was modified from the original method. Briefly, the CL-1 prostate tumor cells were previously transfected with pEGFP-N1, followed by 24 h of humidified incubation at 37°C for the expression of GFP. The next day, the cells were treated with or without recombinant Apo2L/TRAIL molecules for additional 24-h incubation. After 48 h, the cells were harvested using PBS supplemented with 0.5 mM EDTA. The detached cells were checked under a fluorescence microscope to ensure expression of GFP in the cells. The cells were then centrifuged down and washed once with cold PBS, followed by resuspension of the cells in cold 0.5 ml of 20 μg/ml 7-AAD solution in PBS in each tube. The cells were incubated at 4°C for 20 min. After the incubation, the samples were analyzed on an Epics XL flow cytometer (Coulter Electronics, Inc.). The fluorescence of GFP was analyzed in the FL1 channel (green fluorescence). The green-positive cells were gated and further analyzed in



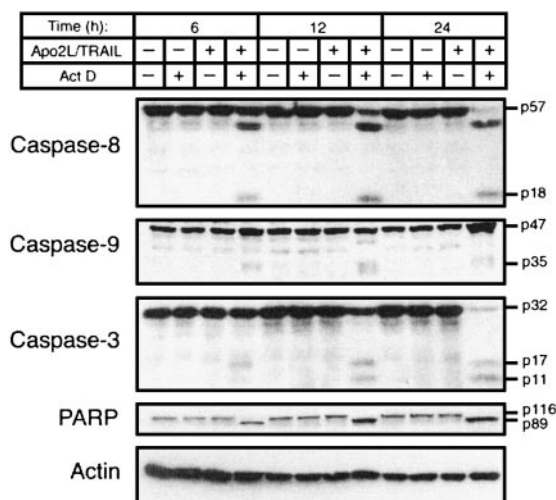
**Fig. 1.** Synergistic induction of apoptosis in prostate tumor CL-1 cells by combination treatment with Apo2L/TRAIL and Act D. The cells were pretreated with or without Act D (100 ng/ml) for 6 h, followed by the addition of Apo2L/TRAIL (10 ng/ml) for the various times indicated (6, 12, and 24 h), or left untreated. The percentages of apoptotic cells were determined by a flow cytometric cell cycle analysis based on propidium iodide staining (see "Materials and Method"). The bars represent the percentages of sub-G<sub>1</sub> populations  $\pm$  SD ( $n = 3$ ) at various incubation periods with Apo2L/TRAIL. The cells were harvested and fixed at different time intervals, and then all samples were accumulated and quantitated in a flow cytometer concurrently.

the FL3 channel (red fluorescence). The apoptotic cells are more permeable to 7-AAD, and thus they appear to be more fluorescent in red and can be distinguished from the live cells.

**Transient Transfection of Tumor Cells.** The transfection of CL-1 tumor cells was performed using the polycationic liposome reagent LipofectAMINE 2000 (Life Technologies, Inc., Rockville, MD). The transfection was done according to the manufacturer's instructions. The vector DNA and pcDNA3-Smac/DIABLO were first mixed with the liposome reagent in a ratio of 2.0  $\mu$ l of LipofectAMINE 2000:1.25  $\mu$ g of DNA in 0.5 ml of serum-free RPMI 1640 (Mediatech) for 20 min at room temperature. The 0.5-ml liposome-DNA mixture was then added to each well of cells plated on 24-well plates for transfection. After 6 h, the transfection medium was removed, and fresh medium containing 10% FBS was added to allow the recovery of the cells.

**Immunoblotting.** This was determined as we described previously (27).

**Isolation of the Cytosolic Fraction and Determination of the Release of Cytochrome *c* and Smac/DIABLO.** CL-1 tumor cells were grown to near confluence (approximately  $8.0 \times 10^6$  cells) in  $100 \times 20$ -mm culture dishes. After the treatments, both the floating and attached cells were collected. The cell pellets were washed twice in cold PBS and resuspended in 2 volumes of homogenization buffer [0.5% (w/v) 3-[[3-cholamidopropyl]dimethylammonio]-1-propane-sulfonic acid, 250 mM sucrose, 20 mM HEPES (pH 7.4), 10 mM KCl, 1.5 mM MgCl<sub>2</sub>, 1 mM sodium EDTA, 1 mM sodium EGTA, 1 mM DTT, and a tablet of Mini complete protease inhibitor mixture (Roche, Indianapolis, IN) per 10 ml of homogenization buffer]. Cells were incubated on ice for 30 min to let the cells swell and lyse. Cell homogenates were prepared by disrupting the cells with 40 strokes in a 2-ml Dounce glass homogenizer with a loose-size pestle. Unbroken cells and



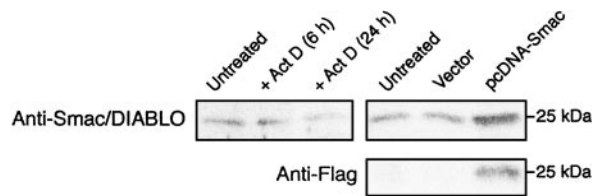
**Fig. 2.** Apo2L/TRAIL-induced caspase activation in Act D-treated CL-1 cells. The cells were pretreated with Act D (100 ng/ml) for 6 h and then treated with Apo2L/TRAIL (10 ng/ml) for the different incubation periods indicated (6, 12, and 24 h), and lysates were collected at those times for immunoblotting analysis. A panel of polyclonal Abs detected the following caspase proteins and PARP: caspase-8 proform (p57) and its cleaved activated subunit (p18); zymogen pro-caspase-9 (p47) and cleaved activated intermediate (p35); zymogen pro-caspase-3 (p32) and cleaved activated subunits (p17 and p11); and intact PARP protein (p116) and PARP cleavage product (p89).  $\beta$ -Actin was also detected to ensure equal loading of the samples. Total protein extracts were separated on a 15% polyacrylamide gel for the detection of PARP and caspase cleavage. Thus, the migration of PARP is much smaller ( $M_r$  166,000 to  $M_r$  89,000) compared with that of caspase-3 ( $M_r$  32,000 to  $M_r$  11,000). The PARP bands are located near the top of the gel, whereas caspases ran much further down.

nuclei were centrifuged down ( $2,500 \times g$ , 4°C, 5 min) twice. Supernatants, which contained mitochondria, were collected and subjected to further centrifugation at  $16,000 \times g$  at 4°C for 30 min to pellet down the mitochondrial fraction. The postmitochondrial supernatants were successively filtered through 0.2 and 0.1  $\mu$ m Ultrafree MC filters (Millipore, Bedford, MA) to yield a cytosolic fraction. The cytosolic fraction was then subjected to Western blotting analysis for cytochrome *c* and Smac/DIABLO.

**Statistical Analysis.** All quantitative assays were set up in triplicates, and the results were expressed as the mean  $\pm$  SD. Statistical significance for comparison between two groups of data was determined using Student's *t* test. Significant differences were considered to exist for those probabilities  $<5\%$  ( $P < 0.05$ ).

## Results

**Pretreatment with Act D Sensitizes CL-1 Tumor Cells to Apo2L/TRAIL-mediated Apoptosis.** Titration of both TRAIL and Act D for optimal cytotoxicity revealed that 100 and 10 ng/ml Act D were the most optimal concentrations for synergistic killing of the androgen-independent CL-1 prostate tumor cells (27). The cells were pretreated with Act D (100 ng/ml) for 6 h, followed by the addition of human recombinant Apo2L/TRAIL (10 ng/ml). The cells were sampled at various time points during the Apo2L/TRAIL treatment (6, 12, and 24 h) to assess apoptosis. The combination treat-



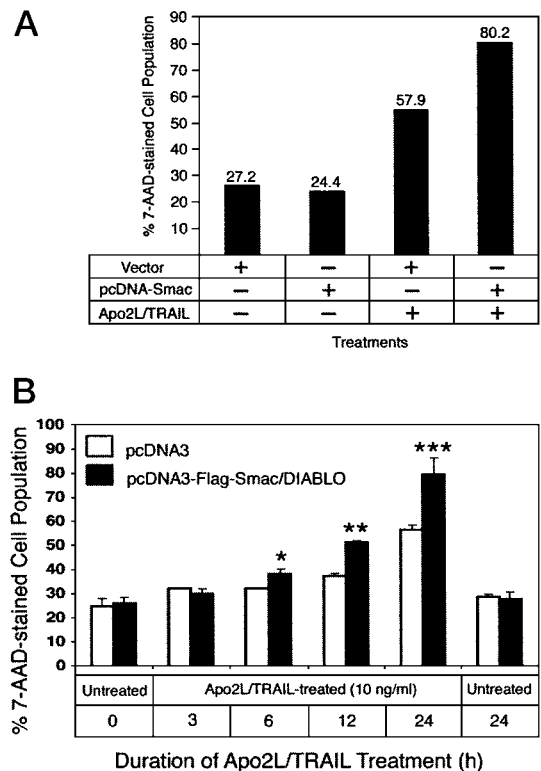
**Fig. 3.** Expression of Smac/DIABLO in prostate tumor cells. The level of endogenous Smac/DIABLO mature protein ( $M_r$  25,000) was determined in untreated cells, Act D-treated cells, and cells transfected with pcDNA3 (control vector) or pcDNA-FLAG-Smac/DIABLO using a polyclonal anti-Smac/DIABLO Ab in immunoblotting analysis. The duration of Act D treatment is indicated (6 or 24 h). The expression of FLAG-tagged Smac/DIABLO in the transfectants was detected using a monoclonal anti-FLAG Ab.

ment resulted in significant enhancement of apoptosis with up to 55.0% of apoptotic cells displaying DNA hypoploidy, whereas treatment with Act D or Apo2L/TRAIL alone resulted in little DNA fragmentation at the 24 h time point (Fig. 1).

**Pretreatment with Act D, followed by Treatment with Apo2L/TRAIL, Activates Caspases-8, -9, and -3.** The synergistic apoptotic killing by Act D and Apo2L/TRAIL determined by the propidium iodide flow cytometric DNA fragmentation assay was confirmed by immunoblotting analyses for caspase activation (Fig. 2). After pretreating the cells with Act D (100 ng/ml) for 6 h, followed by 6 h of treatment with Apo2L/TRAIL (10 ng/ml), noticeable levels of caspases-8, -9, and -3 and cleavage of PARP (a cellular substrate of caspase-3) were observed in the combination-treated cells. The levels of activated caspases and cleaved PARP products became more apparent at the later time points (12 and 24 h) of the Apo2L/TRAIL treatment (Fig. 2).

**Overexpression of Smac/DIABLO Enhanced Apo2L/TRAIL-mediated Apoptosis in Prostate Tumor Cells Based on a 7-AAD Staining Assay.** We examined whether the overexpression of Smac/DIABLO, a neutralizing inhibitor of IAPs, could enhance Apo2L/TRAIL-mediated killing. The transient transfectants were generated with a cDNA encoding the full-length Smac/DIABLO (pcDNA3.1-Smac/DIABLO), attached with a FLAG tag at the COOH terminus of the protein. The protein level of Smac/DIABLO in the transfectants was determined by immunoblotting analysis to ensure that the cells transfected with pcDNA3-Smac/DIABLO were overexpressing the protein (Fig. 3). The level of mature Smac/DIABLO in the transfectants was also compared with that in untreated and Act D-treated cells. Indeed, the Smac/DIABLO transfectants expressed more Smac/DIABLO protein than the untreated or Act D-treated cells (Fig. 3).

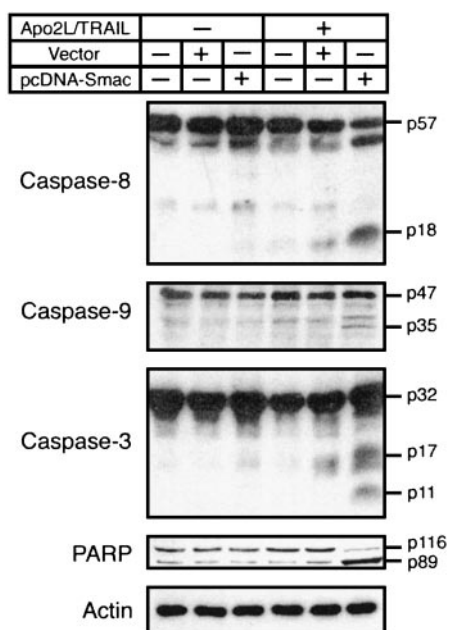
In the following flow cytometry-based apoptosis assay, an expression construct encoding GFP was cotransfected at a 1:5 ratio with Smac/DIABLO or control constructs to track the Smac/DIABLO transfectants. The transfection efficiency was between 13% and 34%, as measured by flow cytometry (data not shown). After treatment with Apo2L/TRAIL, we further measured the level of apoptosis in the GFP-positive transfectants by 7-AAD staining. The amount of apoptosis is reflected in the increased staining of the semipermeable red fluorescent dye 7-AAD (33, 34). In a log-red fluorescence



**Fig. 4.** Synergistic induction of apoptosis in prostate tumor CL-1 cells by overexpression of Smac/DIABLO and treatment with Apo2L/TRAIL. The cells were transfected with either the control vector (pcDNA3) or pcDNA3-FLAG-Smac/DIABLO and cotransfected with pEGFP-N1 to track GFP-expressing transfectants. The cells were incubated at 37°C for 24 h after transfection and then treated with recombinant human Apo2L/TRAIL (100 ng/ml) at various time points. The percentages of apoptotic cells were determined by a flow cytometric analysis based on 7-AAD uptake. To quantify the amount of apoptotic cells in the transfectants, the total GFP-expressing cell populations that display higher green fluorescence were gated and further analyzed for red fluorescence. The 7-AAD-stained cells are those cells that have undergone apoptosis and displayed a red fluorescent population in a forward scatter versus red fluorescence histogram. The percentage of 7-AAD-stained cells was gated and counted by flow cytometry. **A**, apoptotic effects of 24-h Apo2L/TRAIL treatment on Smac/DIABLO transfectants and control transfectants. The X axis indicates the treatment conditions [transfection with either control vector or pcDNA3-FLAG-Smac/DIABLO, followed by 24-h treatment with or without Apo2L/TRAIL (10 ng/ml)]. The bars represent the number of 7-AAD-stained cells (apoptotic cells). **B**, apoptotic effects of Apo2L/TRAIL treatment (10 ng/ml) on Smac/DIABLO transfectants and control transfectants at various time intervals. The bars represent the mean  $\pm$  SD ( $n = 3$ ) of the percentages of the cells stained with 7-AAD (the apoptotic cells). The time intervals (0, 3, 6, 12, and 24 h) at the bottom indicate the duration of Apo2L/TRAIL treatment. Black bars indicate Smac/DIABLO transfectants, and white bars indicate cells transfected with the control vector pcDNA3. Untreated transfectants at the 0 and 24 h time points were included for comparison. Statistical significance: \*,  $P < 0.01$ ; \*\*,  $P < 0.001$ ; \*\*\*,  $P < 0.05$ .

versus forward scatter histogram, the late apoptotic cells displayed brighter 7-AAD red fluorescence and smaller cell size (forward scatter).

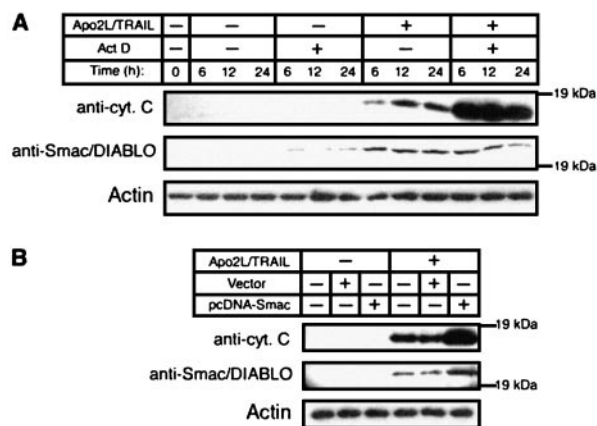
Using this flow cytometry-based protocol, we determined that the expression of Smac/DIABLO enhances Apo2L/TRAIL-mediated apoptosis. When the vector- or pcDNA-Smac/DIABLO-transfected cells were left untreated for 48 h after transfection, 27.2% and 24.4% of the total green cells



**Fig. 5.** Apo2L/TRAIL-induced caspase activation in Smac/DIABLO transfectant and control vector transfectant. The cells were transfected with pcDNA3-FLAG-Smac/DIABLO or control vector (pcDNA3) and then treated with Apo2L/TRAIL (10 ng/ml). After a 24-h treatment with Apo2L/TRAIL, lysates were collected for immunoblotting analysis. Detection of active caspase enzymatic subunits is similar to that in Fig. 2.

underwent apoptosis, respectively (Fig. 4A). When the Smac/DIABLO-transfected cells were treated with Apo2L/TRAIL for 24 h after the 24-h transfection, the cells became more apoptotic (57.9% for the vector-transfected cells and 80.2% for the pcDNA-Smac/DIABLO transfected cells; Fig. 4A). The killing of transfectants by Apo2L/TRAIL was further examined at various time points (3, 6, 12, and 24 h). The increase in the percentage of apoptotic cells in the Smac/DIABLO-transfected cells was observed as early as 6 h after treatment (5% increase; Fig. 4B). At 24 h, maximally enhanced Apo2L-mediated killing by Smac/DIABLO overexpression was observed (22% increase).

**Activation of Caspases by the Combination of Overexpression of Smac/DIABLO and Treatments with Recombinant Apo2L/TRAIL.** The level of caspase activation was examined by the combination of Smac/DIABLO overexpression and Apo2L/TRAIL treatment. As shown in Fig. 5, the transfection process itself did not induce any detectable caspase-dependent apoptotic events. Upon 24-h treatment with Apo2L/TRAIL, slight activation of caspases-8 and -3 was observed in vector-transfected cells, and higher levels of caspases-8, -9, and -3 and cleavage of PARP, a substrate of caspase-3, were seen in the Smac/DIABLO-transfected cells (Fig. 5). Although Apo2L/TRAIL slightly activates caspase-3 in the vector-transfected cells, it was not sufficient to result in significant cleavage of PARP, compared with the untreated controls. Because Apo2L/TRAIL could activate a full apoptotic program involving caspases in the Smac/DIABLO-transfected cells, Smac/DIABLO is effective in sensitizing CL-1 tumor cells to Apo2L/TRAIL-mediated apoptosis.



**Fig. 6.** Apo2L/TRAIL-induced release of cytochrome c and Smac/DIABLO from mitochondria. **A**, treatments with Apo2L/TRAIL (10 ng/ml) increased the level of cytochrome c and mature Smac/DIABLO in the cytoplasm in both the Act D-pretreated cells and untreated cells. Prostate tumor cells were pretreated with or without Act D for 6 h, followed by treatment with Apo2L/TRAIL for the various time periods indicated (6, 12, and 24 h). At each time point, cells were harvested, and cytosolic fractions were extracted. The cytosolic extract was later subjected to immunoblotting analysis. Cytochrome c and mature Smac/DIABLO were detected as  $M_r$  15,000 and  $M_r$  25,000 protein bands, respectively. **B**, treatments with Apo2L/TRAIL (10 ng/ml) increased the level of cytochrome c and mature Smac/DIABLO in the cytoplasm in all samples (nontransfectant, control vector transfectant, and Smac/DIABLO transfectant). Cytosolic fractions were isolated after a 24-h treatment with Apo2L/TRAIL (10 ng/ml) and later subjected to immunoblotting analysis for detection of cytochrome c and mature Smac/DIABLO.  $\beta$ -Actin was detected to ensure equal loading of protein samples.

**Apo2L/TRAIL Induces the Release of Cytochrome c and Smac/DIABLO in Untreated Cells, Act D-treated Cells, and Smac/DIABLO-transfected Cells.** The synergistic activation of caspase-9 by Apo2L/TRAIL is clearly evident in both the Act D-sensitized cells and cells overexpressing Smac/DIABLO. The activation of caspase-9 relies on the formation of apoptosome induced by cytoplasmic cytochrome c and may also require Smac/DIABLO in the cytoplasm to counteract the IAP inhibition on caspase-9 (30). Thus, we compared the release of cytochrome c and Smac/DIABLO from the mitochondria induced by Apo2L/TRAIL in both Act D-treated cells and Smac/DIABLO transfectants. As shown in the *top panels* of Fig. 6, **A** and **B**, Apo2L/TRAIL alone induces the release of cytochrome c in all of the untreated, Act D-treated, and Smac/DIABLO-transfected cells. However, Apo2L/TRAIL can only activate caspases in Act D-treated cells and Smac/DIABLO transfectants, and not in untreated cells (Figs. 2 and 5), indicating that the release of cytochrome c induced by Apo2L/TRAIL alone is not sufficient to induce apoptosis.

Next, we examined the release of Smac/DIABLO from the mitochondria induced by Apo2L/TRAIL in both experimental systems (Fig. 6, **A** and **B**, *bottom panels*). Similar to the release of cytochrome c, Apo2L/TRAIL also induced the release of Smac/DIABLO from the mitochondria in all samples, including the untreated cells. However, Apo2L/TRAIL-induced release of Smac/DIABLO, in conjunction with the release of cytochrome c, was not sufficient to induce caspase activation and apoptosis (Figs. 2, 4, and 5). Only when the cells were pretreated with Act-D for 6 h or over-

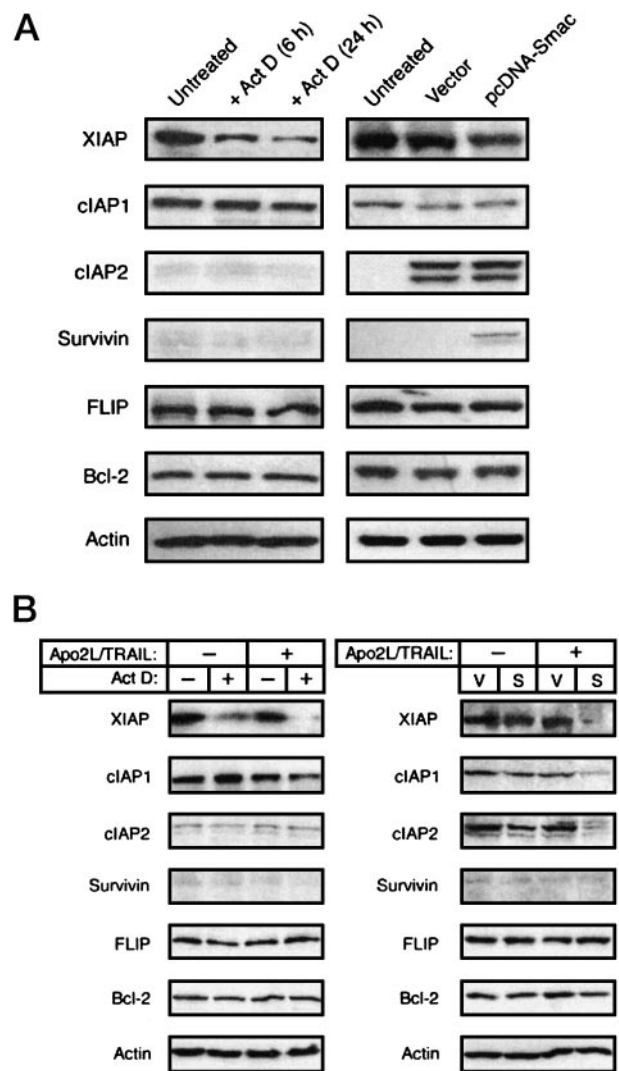
expressed Smac/DIABLO were the cells sensitized to undergo Apo2L/TRAIL-mediated apoptosis. Upon stimulation with Apo2L/TRAIL, the Smac/DIABLO transfectants were able to release a higher level of Smac/DIABLO protein in comparison with the untreated or Act D-treated cells. The higher level of Smac/DIABLO in the cytoplasm might be responsible for the sensitization of Smac/DIABLO transfectants to Apo2L/TRAIL-mediated apoptosis. Thus, the result suggests that the endogenous level of Smac/DIABLO was not sufficient to render cells sensitive to Apo2L/TRAIL-mediated apoptosis and that increased expression of Smac/DIABLO might be necessary for inactivation of IAPs and sensitization to Apo2L/TRAIL-mediated apoptosis.

**Protein Level of Various Antiapoptotic Molecules in Act-D-treated Cells and Smac/DIABLO-transfected Cells before and after Treatment with Apo2L/TRAIL.** Previously, Smac/DIABLO has been shown to bind to the IAP family proteins (c-IAP1, c-IAP2, XIAP, and survivin) and relieves the inhibition of caspase-3 and -9 (28–30). The binding of Smac/DIABLO to IAPs will disable the direct binding of IAPs to active caspases. Once IAPs are free from caspase by Smac/DIABLO, they can be easily ubiquitinated and degraded by proteasome (35). The modulation of the protein levels of various antiapoptotic factors by Act D treatment and overexpression of Smac/DIABLO was compared. As seen in Fig. 7A, Act D selectively down-regulated the level of XIAP protein after 6 h of treatment, whereas other antiapoptotic proteins (c-IAP1, c-IAP2, survivin, FLIP, and Bcl-2) remained largely unaffected. On the other hand, the transfection with Smac/DIABLO cDNA did result in significant reduction of XIAP (Fig. 7A). In addition, the transfection with Smac/DIABLO cDNA and vector alone resulted in a slight reduction of c-IAP1 and a significant induction of c-IAP2 (Fig. 7A). These changes are most likely caused by the nonspecific effects of liposomal transfection. The overexpression of Smac/DIABLO also resulted in a slight induction of survivin (Fig. 7A). Despite the up-regulation of survivin, Apo2L/TRAIL-mediated apoptosis of prostate tumor cells was still enhanced, suggesting that survivin is not a dominant resistance factor against Apo2L/TRAIL-mediated apoptosis in prostate tumor cells or that the level of induction was not sufficient to block the enhancement of killing.

Because the release of Smac/DIABLO from mitochondria can only be induced by treatment with Apo2L/TRAIL (Fig. 6), we examined whether the high level of Smac/DIABLO release in the Smac/DIABLO transfectant induced by Apo2L/TRAIL would greatly reduce the level of IAP family proteins. As seen in Fig. 7B, right panels, IAP family proteins (XIAP, c-IAP1, and c-IAP2) were reduced by Apo2L/TRAIL treatment in the Smac/DIABLO transfectants. Such changes were not observed in the untreated cells and vector transfectants. Other antiapoptotic proteins (FLIP, and Bcl-2) were not affected. In Act D-pretreated cells, Apo2L/TRAIL also reduced the level of c-IAP-2 slightly, whereas the levels of other antiapoptotic proteins were unchanged.

## Discussion

In this study, we found that overexpression of Smac/DIABLO could render resistant tumor cells sensitive to Apo2L/TRAIL-



**Fig. 7.** Comparison of various antiapoptotic protein levels in Act D-treated cells and Smac/DIABLO transfectants. **A**, effects of Act D (100 ng/ml) and Smac/DIABLO overexpression on the protein levels of various antiapoptotic proteins. Protein lysates were prepared from cells treated with Act D (100 ng/ml) for 6 or 24 h and cells transfected with pcDNA3 (vector) and pcDNA3-FLAG-Smac/DIABLO for 24 h. The protein levels of IAP family members (XIAP, c-IAP1, c-IAP2, and survivin), FLIP, and Bcl-2 were examined by immunoblotting analysis.  $\beta$ -Actin was determined to ensure equal loading of protein lysates. **B**, effects of Apo2L/TRAIL (10 ng/ml) on the protein levels of various antiapoptotic proteins (XIAP, c-IAP1, c-IAP2, survivin, FLIP, and Bcl-2) in Act D-pretreated cells and Smac/DIABLO transfectants. +, cells pretreated with Act D (100 ng/ml) for 6 h before the addition of Apo2L/TRAIL (10 ng/ml). Lanes V and S indicate cells transfected with the vector pcDNA3 (Lanes V) or pcDNA-FLAG-Smac/DIABLO (Lanes S). Lysates were prepared after a 6-h treatment with Apo2L/TRAIL and then subjected to immunoblotting analysis.

mediated apoptosis. We compared the apoptotic signaling events in the Apo2L/TRAIL-mediated apoptosis pathway (release of cytochrome c and caspase activation) in both Act D-sensitized cells and Smac/DIABLO transfectants. Apo2L/TRAIL activated caspases-8, -9, and -3 in both Act D-sensitized cells and Smac/DIABLO transient transfectants, suggesting that activation of caspases was necessary to result in apoptosis. However, Apo2L/TRAIL induced the re-

lease of cytochrome *c* and Smac/DIABLO in both resistant control cells and sensitized cells. This result shows that the predominant resistance of prostate cancer cells is present downstream of the mitochondrial events, suggesting that IAP family proteins are the prime candidate proteins that confer prostate tumor immunoresistance. By comparing the protein expression patterns induced by Apo2L/TRAIL in Act D-sensitized cells and Smac/DIABLO transient transfectants, we found that down-regulation of XIAP was the common feature found in both cell systems. Thus, XIAP could be a potential target for overcoming Apo2L/TRAIL resistance, and up-regulation of Smac/DIABLO may serve as a potential effective modifying signal to immunosensitize resistant prostate tumor cells to apoptosis.

The identification of Smac/DIABLO as a neutralizing inhibitor of IAP family proteins has revealed additional complexities in the regulation of tumor sensitivity to apoptosis. Similar to cytochrome *c*, Smac/DIABLO is characterized as a mitochondrial protein, and it is released from the mitochondria when the cells received proper death signals (28, 29). Normally, Smac/DIABLO is produced as a precursor protein that contains a MTS and remains nonapoptotic. The proapoptotic function of Smac/DIABLO is attained when its MTS is cleaved after being transported to the mitochondria. Further analysis revealed that the 5-amino acid peptide AVPIA at the NH<sub>2</sub> terminus, which is exposed after cleavage of MTS, is thought to be responsible for the interaction with the baculovirus IAP repeat 3 domain of XIAP and inhibition of IAP function (36). Contrary to this finding, another report documented that the proapoptotic function of Smac/DIABLO does not depend on its IAP-neutralizing domain (37). In light of this study, whereas our results have suggested that Smac/DIABLO may exert its sensitizing effect by down-regulating XIAP, inhibition of other non-IAP resistance factors may be important for sensitization by Smac/DIABLO. Further detailed analysis of IAP-Smac/DIABLO interaction will determine whether indeed XIAP is one of the predominant resistance factors that were down-regulated by Smac/DIABLO.

The containment of Smac/DIABLO inside the mitochondria could be also important for the inhibition of apoptosis. It is unclear at this point whether there is a difference in the mechanisms for the release of cytochrome *c* and Smac/DIABLO. In CL-1 tumor cells, Apo2L/TRAIL was able to induce simultaneous release of cytochrome *c* and Smac/DIABLO, but the release of both molecules was still not sufficient to induce apoptosis. Therefore, the endogenous level of Smac/DIABLO in prostate tumor cells may not be adequate to neutralize the negative regulation on apoptosis. When the level of Smac/DIABLO was up-regulated in the transient transfectants, the sensitivity to Apo2L/TRAIL was restored.

The inability to induce apoptosis by translocation of cytochrome *c* and endogenous Smac/DIABLO induced by Apo2L/TRAIL highlights the importance of the inhibition of downstream apoptotic events in tumor resistance. In our studies, the induction of apoptosis and activation of effector caspases are possibly dictated by the level of IAPs. In CL-1 tumor cells, sensitization to Apo2L/TRAIL-mediated apoptosis is associated with down-regulation of IAPs, in particular, XIAP, by Act D or a high level of Smac/DIABLO. Other studies

have also shown that overexpression of IAP family proteins is associated with poor responsiveness to apoptosis-inducing therapies. In leukemia patients, a high level of XIAP in leukemia tumor cells correlates with poor survival rates (38). In the same study, the expression of XIAP and c-IAP-1 was found to be the most predominant in a panel of 60 human tumor cell lines (38). Furthermore, XIAP was also found to be highly expressed in resistant ovarian cancer cells to chemotherapy and radiation, and treatment of ovarian cancer cells with antisense XIAP reversed the tumor resistance (39, 40). In addition to the downstream inhibitors such as XIAP, upstream resistance factors such as Akt, which affects BID cleavage, are also critical for Apo2L/TRAIL resistance (41, 42). These findings, together with our study, underscore the importance of death signal amplification through the mitochondrial pathway in the killing of prostate tumor cells by Apo2L/TRAIL.

Tumor resistance to conventional therapies remains a major problem today. To solve this problem, we previously proposed an approach that involves two complementary signals to restore a functional apoptotic pathway that can be used by immunotherapy (27, 43). Similar to the approach in which we used Act D and Apo2L/TRAIL as our two complementary signals to kill resistant prostate tumor cells, the combination of Smac/DIABLO overexpression and Apo2L/TRAIL treatment is an equally effective option. This finding confirms that the success of this combinatory therapeutic approach requires the identification and direct reversal of the apoptotic signaling block. We predict that any method that counteracts the function of the identified resistance factor should serve as an effective complementary signal with Apo2L/TRAIL to cause a potent anti-prostate tumor killing. The immunosensitization by direct down-regulation of resistant proteins serves as a potential approach to enhance both chemotherapeutic and immunotherapeutic responses.

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# Molecular Cancer Therapeutics

**X-linked Inhibitor of Apoptosis (XIAP) Blocks Apo2 Ligand/Tumor Necrosis Factor-related Apoptosis-inducing Ligand-mediated Apoptosis of Prostate Cancer Cells in the Presence of Mitochondrial Activation: Sensitization by Overexpression of Second Mitochondria-derived Activator of Caspase/Direct IAP-binding Protein with Low pI (Smac/DIABLO) 1 Supported by Department of Defense/United States Army DAMD Grant 170210023 and in part by Tumor Immunology Training Grant NIH 5T32CA09120-22,23 (to C-P. N.).**

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