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IFN-β Pretreatment Sensitizes Human Melanoma Cells to TRAIL/Apo2 Ligand-Induced Apoptosis[1]

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All human melanoma cell lines (assessed by annexin V and TUNEL assays) were resistant to apoptosis induction by TRAIL/Apo2L protein. TRAIL/Apo2L activated caspase-8 and caspase-3, but subsequent apoptotic events such as poly(ADP-ribose) polymerase cleavage and DNA fragmentation were not observed. To probe the molecular mechanisms of cellular resistance to apoptosis, melanoma cell lines were analyzed for expression of apoptosis regulators (apoptotic protease-associated factor-1, FLIP, caspase-8, caspase-9, caspase-3, cellular inhibitor of apoptosis, Bcl-2, or Bax); no correlation was observed. TRAIL/Apo2L was induced in melanoma cell lines by IFN-β and had been correlated with apoptosis induction. Because IFN-β induced other gene products that have been associated with apoptosis, it was postulated that one or more IFN-stimulated genes might sensitize cells to TRAIL/Apo2L. Melanoma cell lines were treated with IFN-β for 16–24 h before treatment with TRAIL/Apo2L. Regardless of their sensitivity to either cytokine alone, >30% of cells underwent apoptosis in response to the combined treatment. Induction of apoptosis by IFN-β and TRAIL/Apo2L in combination correlated with synergistic activation of caspase-9, a decrease in mitochondrial potential, and cleavage of poly(ADP-ribose) polymerase. Cleavage of X-linked inhibitor of apoptosis following IFN-β and TRAIL/Apo2L treatment was observed in sensitive WM9, A375, or WM3211 cells but not in resistant WM35 or WM164 cells. Thus, in vitro IFN-β and TRAIL/Apo2L combination treatment had more potent apoptotic and anti-growth effects when compared with either cytokine alone in melanoma cells lines. ***The Journal of Immunology*****, 2002, 169: 847–855.**

Tumor necrosis factor apoptosis-inducing ligand/Apo2L is a transmembrane protein that shares homology in its extracellular domains with other members of the TNF family (1, 2). A subset of these ligands, including TNF-α, Fas ligand, death receptor (DR)[4]3, and TRAIL/Apo2L initiates cellular death cascades. Both in vitro and in vivo studies have demonstrated tumoricidal activity without significant toxicity toward normal cells or tissues (3). TRAIL/Apo2L binds to TRAIL-R1 (DR4) and TRAIL-R2 (DR5) (4, 5) and mediates its effects by activating both NF-κB (antiapoptotic) and caspase-8 (proapoptotic) pathways. Once activated, DRs bind the cytoplasmic adapter molecule Fas-associated death domain protein, which in turn recruits either an initiator cysteine protease (caspase-8) or the receptor-interacting protein and TNFR-associated factor 2. Recruitment of caspase-8 initiates the apoptotic cascade, whereas receptor-interacting protein and TNFR-associated factor 2 activate NF-κB (6). In contrast, two other TRAIL receptors, TRAIL-R3 and TRAIL-R4 (DcR1, DcR2), lack a functional death domain and cannot transduce apoptotic signals (4, 5).

IFNs transcriptionally regulate >100 genes (7, 8). Some of these IFN-stimulated genes have been associated with induction of apoptosis[5] (9), including TRAIL/Apo2L (10–12). IFN-β preferentially induced TRAIL/Apo2L and had greater antiproliferative (13) and apoptotic effects in vitro in melanoma cells when compared with IFN-α2 (11). A rough correlation was observed in induction of TRAIL/Apo2L by IFN-β and apoptosis in melanoma cell lines. IFN-β induced apoptosis by activating the caspase cascade, releasing cytochrome *c* from mitochondria, and promoting DNA fragmentation. However, this activation occurred late (>72 h), implicating an intermediate cellular effector(s). Neutralizing experiments using Ab to TRAIL or dominant negative mutant of TRAIL-R2 (DR5) confirmed a functional role of TRAIL/Apo2L in IFN-β-mediated apoptosis in melanoma cells (11) and in multiple myeloma cells (12). Furthermore, all cell lines that underwent apoptosis in response to IFN-β exhibited TRAIL/Apo2L induction. However, a subset of melanoma cell lines, including WM35, exhibited TRAIL/Apo2L induction but did not apoptose in response to IFN-β. These data suggested that TRAIL/Apo2L was necessary but not sufficient to mediate IFN-β-induced apoptosis.

Enhanced antitumor activity of TRAIL/Apo2L in combination with chemotherapeutic agents that disrupt cellular metabolism and mitotic activity has been reported (14–18). We postulated that IFNs might sensitize melanoma cells to TRAIL/Apo2L, because IFN-β induced other genes associated with apoptosis. In this work, we report that IFN-β and TRAIL/Apo2L in combination synergistically induced apoptosis and caspase activation in melanoma cell lines. This occurred at least in part by cleavage of the X-linked inhibitor of apoptosis (XIAP).

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[4] Abbreviations used in this paper: DR, death receptor; IAP, inhibitor of apoptosis; XIAP, X-linked IAP; cIAP, cellular IAP; Apaf-1, apoptotic protease-associated factor-1; XAF1, XIAP-associated factor-1; PI, propidium iodide; HFF, human foreskin fibroblast.

[5] D. W. Leaman, M. Chawla-Sarkar, K. Vyas, A. Ozdemir, and E. C. Borden. Greater potency of IFN-β compared with IFN-α2 in inducing IFN stimulated genes in melanoma: identification of new ISGs by oligonucleotide microarray. *Submitted for publication*.

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Materials and Methods

Cell culture and cytokine treatments

Human melanoma cell lines WM9, WM35, WM3211, WM793, WM164 (19), A375, FEMX, Guilliams, and Minors (American Type Culture Collection, Manassas, VA) were grown in DMEM (Life Technologies, Rockville, MD) supplemented with heat-inactivated 10% FCS (HyClone Laboratories, Logan, UT) in a humidified chamber of 95% air/5% CO_2 at 37°C. Collection of biopsies and preparation of primary melanoma cell culture was conducted following Institutional Review Board guidelines and approval. Low passage, melanoma cells (CCFMel-1H, CCFMel-2B), primary astrocytes (CCF-TEN, CCF-Bon) (20), human foreskin fibroblasts (HFF) (Cleveland Clinic Foundation, Cleveland, OH), HUVECs, and human fibroblast cell line WI-38 (American Type Culture Collection) were cultured in DMEM-F12 medium supplemented with 10% FCS. Cells were periodically confirmed as mycoplasma free.

IFN-α2b (intron A; Schering-Plough, Kenilworth, NJ) or IFN-β (Rebif; Ares-Serono, Geneva, Switzerland) used in the study were of equivalent specific activity (2×10^8 U/mg protein). All experiments were done using different preparations of recombinant human TRAIL/Apo2L; that from Genentech (San Francisco, CA) is denoted by (G) and that from PeproTech (Rocky Hill, NJ) is denoted by (P) in the text. TRAIL/Apo2L (G) consisted of >99% trimeric protein with Zn^{2+} (21). Presence of Zn^{2+} has been reported as necessary for its optimal activity (4).

Cells were treated with IFN-β and/or TRAIL/Apo2L for different time periods based on the experiment. For analysis of early apoptosis events such as annexin V positivity, cells were treated with TRAIL/Apo2L for 16–24 h and for late apoptosis TUNEL or antiproliferative effects (24–36 h). To analyze enzymes or proteins involved in initiation and execution of apoptosis pathway, cells were treated with TRAIL/Apo2L for <16 h (2, 6, or 12 h) to avoid too many dead cells. Doses and time for IFN (100 and 500 U/ml) used in this study were based on a previous report (12). All treatments were performed at 37°C in a humidified chamber of 95% air/5% CO_2.

Antiproliferative assays

Cells were plated at a cell density of 10,000 cells/well in 96-well plates and IFN-α2 or IFN-β were added in different dilutions (100 and 500 U/ml) to the assay plate. Quadruplicates of each treatment were performed. After 24 h, recombinant human TRAIL/Apo2L (P) was added at different concentrations. After 36 h, plates were fixed with 10% TCA (4°C) for 1 h, rinsed with water, and allowed to air dry. Cell numbers were estimated by staining with 0.4% sulforodamine B (w/v) (Sigma-Aldrich, St. Louis, MO) and measuring the absorbance at 570 nm (22). Results were calculated as follows: % growth = $(OD_{exp} - OD_{ini})/(OD_{fin} - OD_{ini}) \times 100$, where OD_{fin} corresponds to A_{570} of wells with no treatment, OD_{ini} corresponds to 0% growth, and OD_{exp} corresponds to wells treated with different concentrations of IFN. The multiple drug-effect analysis method of Chou and Talalay (23) was used to measure interaction between IFN-β and TRAIL/Apo2L.

Gel electrophoresis and immunoblot analyses

Whole cell lysates were prepared in 1× lysis buffer (50 mm Tris-Cl (pH 8), 1% Triton X-100, 10% glycerol, 1 mM EDTA, 250 mM NaCl, 1 mM DTT, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 10 μg/ml pepstatin) for subsequent immunoblotting studies (11). SDS-PAGE was conducted by using Laemmli buffer system on 12% polyacrylamide gels, and proteins separated on gels were transferred onto a polyvinylidene difluoride membrane by the semidry method (Trans Blot SD; Bio-Rad, Hercules, CA). Binding of the primary and secondary Abs was performed according to standard protocols (11). Membranes were immunoblotted with the mAb to apoptotic protease-associated factor-1 (Apaf-1), Bcl2, Bax (Santa Cruz Biotechnology, Santa Cruz, CA), or with the polyclonal Ab to caspase-3 (BD PharMingen, San Diego, CA), cellular inhibitor of apoptosis (cIAP)1, cIAP2 (Santa Cruz Biotechnology), and XIAP (BD PharMingen), followed by incubation with HRP-conjugated secondary Abs (Pierce, Rockford, IL). Immunoreactive bands were visualized by using ECL (PerkinElmer, Boston, MA). Equal protein loading was confirmed by reprobing with actin mAb (Sigma-Aldrich). All the immunoblots in this study were repeated two to three times with reproducible results.

EMSA

A375 cells were treated with IFN-α2 (24 h), IFN-β (24 h), or TRAIL/Apo2L (2 h), or were treated with IFN (24 h) followed by TRAIL/Apo2L (2 h), and cytoplasmic extracts were prepared. NF-κB binding consensus (5′-AGTTGAGGGGACTTTCCCAGGC-3′) sequence from the IFN-β gene promoter was end labeled with [γ^{32}-P]dATP (3000 Ci/mol) using T4 polynucleotide kinase. DNA binding reactions were performed in a 20-μl volume containing 10 μg nuclear protein, 20 mM HEPES, 10 mM KCl, 0.1% Nonidet P-40, 0.5 mM DTT, and 10% glycerol. The binding reaction was performed for 20 min at room temperature. Complexes were separated from the free probe on a 6% nondenaturing polyacrylamide gel in 0.5× Tris-borate EDTA buffer at 200 V for 2 h. Gels were dried and exposed to film.

Caspase activity assay

Caspase-3, caspase-8, and caspase-9 activities were measured using a commercially available ApoAlert assay kit (Clontech Laboratories, Palo Alto, CA). Briefly, cells treated with IFN-β (40 h), TRAIL/Apo2L (2, 6, or 16 h), or IFN-β (24 h), followed by TRAIL/Apo2L for 2, 6, or 16 h, were washed twice with cold PBS and lysed on ice in 50 μl of cold lysis buffer. Cell lysates were centrifuged at 10,000 × *g* for 10 min to precipitate cellular debris. Assay was performed in triplicate on a 96-well plate based on the manufacturer's protocol.

TUNEL assay

DNA fragmentation was detected in IFN-β- and TRAIL/Apo2L-treated cells by TUNEL staining using the APO-BRDU kit (BD PharMingen) as per the manufacturer's protocol. The percentage of FITC-positive cells was analyzed by FACS (FACSVantage; BD Biosciences, San Diego, CA).

Annexin V/PI assay

Annexin V staining of exposed membrane phospholipid phosphatidylserine was done using the annexin V assay kit (BD PharMingen) following the manufacturer's protocol. The percentages of annexin V- and propidium iodide (PI)-positive cells were analyzed by FACS (FACSVantage).

Results

IFN pretreatment sensitizes cells to TRAIL/Apo2L-induced apoptosis

Defects in TRAIL/Apo2L induction by IFNs (11) were postulated to be a factor mediating resistance to apoptosis by IFNs in melanoma cell lines. Based on the hypothesis that exogenous TRAIL/Apo2L might induce apoptosis in resistant melanoma cells, functional in vitro studies were performed with recombinant TRAIL/Apo2L protein. Based on previous studies (24, 25), increasing doses of TRAIL/Apo2L were tested in A375 melanoma cells. No significant (<10%) apoptosis (assessed by annexin V/PI staining) was observed at concentrations ranging from 25 to 200 ng/ml (24–48 h). To analyze sensitivity of other melanoma cell lines, cells were treated with TRAIL/Apo2L (100 ng/ml) for 24 h and IFN-β (500 U/ml) for 40 h (11). No significant cytotoxic effects were observed in response to TRAIL/Apo2L in WM9, WM3211, A375, WM35, and WM164 melanoma cells (Fig. 1). At 40 h, IFN-β alone induced 13–16% apoptosis in WM9 and WM3211 cells. To confirm that cells responded to TRAIL/Apo2L, they were cotreated with the metabolic inhibitor actinomycin D (10 ng/ml) and TRAIL/Apo2L. TRAIL/Apo2L induced apoptosis in most melanoma cells in the presence of actinomycin D (Fig. 1), confirming that they expressed functional TRAIL receptors and downstream apoptotic components. All the apoptosis assays in this study were repeated at least three times and a variation of ±5% between individual experiments was considered acceptable.

To further probe the interaction of IFNs and TRAIL/Apo2L, a melanoma cell line (A375) defective in endogenous *TRAIL* gene induction by IFNs and resistant to IFN-β-induced apoptosis was chosen. Cells were either left untreated or treated in triplicate with IFN-β (500 U/ml for 40 h) or TRAIL/Apo2L (100 ng/ml for 16 h). In parallel, cells were cotreated with IFN-β and TRAIL/Apo2L (16 h) or pretreated with IFN-β for 8 or 24 h followed by TRAIL/Apo2L (16 h). Apoptotic cell death was measured by annexin V/PI

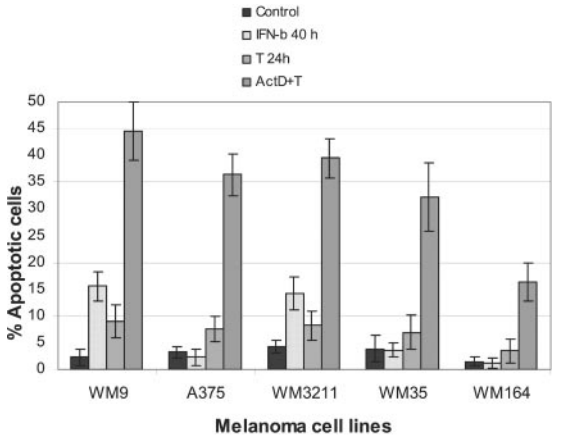

FIGURE 1. Melanoma cells are resistant to apoptotic effects of TRAIL/Apo2L. Melanoma cell lines sensitive (WM9, WM3211) or resistant (A375, WM35, WM164) to IFN-β-induced apoptosis were treated with either IFN-β (500 U/ml for 40 h) or TRAIL/Apo2L (100 ng/ml for 24 h) or were cotreated with actinomycin D (10 ng/ml) and TRAIL/Apo2L (24 h). The percentage of apoptotic cells was measured by annexin V/PI staining. Error bars represent SEM from three separate experiments.

staining followed by bivariate FACS analysis. No significant apoptosis was observed in cells treated with either cytokine as a single agent or together. However, cells pretreated with IFN-β for 8 h followed by TRAIL/Apo2L had an 8–10% increase in annexin V/PI positivity. This increased to >30% in cells pretreated with IFN-β for 24 h (Fig. 2*A*). Similar results were obtained with TUNEL analyses (Fig. 2*B*).

Other studies using recombinant soluble TRAIL/Apo2L protein had reported sensitivity of melanoma cells to TRAIL/Apo2L alone (24, 25). To confirm that the observations were not specific to one TRAIL preparation, two melanoma cell lines, WM-9 (sensitive to IFN-β-induced apoptosis) and A375 (resistant to IFN-β-induced apoptosis) were treated in parallel with recombinant TRAIL/Apo2L (P) or with Zn^{2+} (G). Cells were treated with similar doses as described previously and assessed by annexin V/PI staining. Again, both cell lines showed synergistic increase in apoptosis following IFN-β pretreatment. Neither of the TRAIL/Apo2L preparations alone induced apoptosis in either of the cell lines (Fig. 3).

To determine whether IFN-β sensitized other cells to TRAIL/Apo2L-induced apoptosis, annexin V/PI and TUNEL staining were performed. TRAIL/Apo2L (16–24 h) alone had no significant effect on apoptosis (3–9%) in most melanoma cells. However, IFN-β (40 h) induced partial apoptosis (12–15%) in a subset of melanoma cell lines. IFN-β pretreatment sensitized seven of nine melanoma cell lines (WM3211, FeMX, WM793, CCFMel2H, CCFMelB) to TRAIL/Apo2L-induced cytotoxicity. However, a subset (two of nine) of cell lines (WM35, WM164) that were resistant to IFN-β-induced apoptosis was also resistant to proapoptotic effects of the IFN-β and TRAIL/Apo2L combination (Table I). Primary nonmalignant human cells, HUVECs, HFF, WI-38, CCF-TEN, and CCF-BON, were resistant to apoptotic effects of TRAIL/Apo2L alone as well as in combination with IFNs (Table I).

IFN-β is more potent then IFN-α2 in sensitizing cells to TRAIL/Apo2L-induced antiproliferative effects

Short-term antiproliferative assays (60 h) were performed with IFN-α2 and IFN-β with TRAIL/Apo2L in combination to assess growth inhibition. A375 cells were pretreated with either IFN-α2

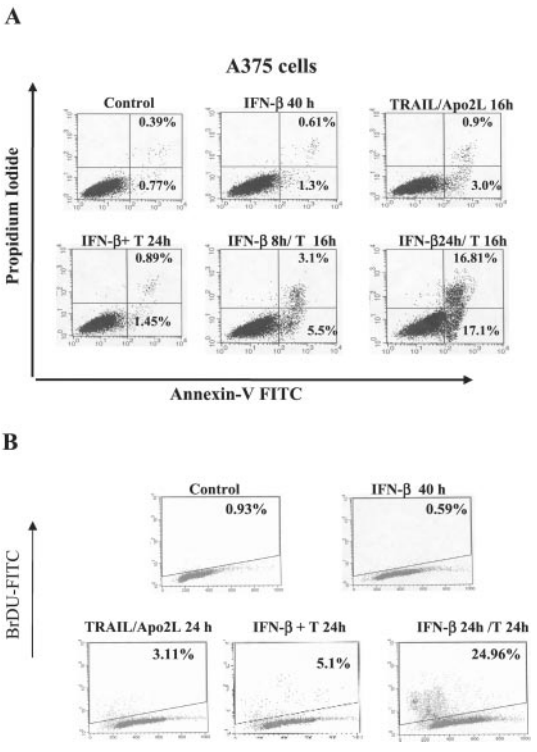

FIGURE 2. *A*, IFN pretreatment but not cotreatment sensitizes cells to TRAIL/Apo2L-induced apoptosis. A375 cells treated with TRAIL/Apo2L (100 ng/ml for 16 h), IFN-β (500 U/ml for 40 h), IFN-β plus TRAIL/Apo2L (24 h), IFN-β (8 h)/TRAIL (16 h), and IFN-β (24 h)/TRAIL (16 h) were stained with annexin V/PI and subjected to bivariate FACS analysis. The percentage of annexin V- and PI-positive cells (representative of three separate experiments) are shown in the *lower right* and *upper right panels*, respectively. *B*, DNA fragmentation, a late-stage apoptosis marker, was detected by TUNEL analysis. A375 cells treated with IFN-β (500 U/ml for 40 h), TRAIL/Apo2L (100 ng/ml for 24 h), and IFN-β (24 h)/TRAIL (24 h) were fixed, labeled with bromo-dUTP by the enzyme TdT, and then stained with FITC-labeled anti-5-bromo-2′-deoxyuridine mAb. The percentage of FITC-positive cells was assessed by FACS analysis.

or IFN-β (100 and 500 U/ml) for 24 h followed by increasing doses (0–200 ng/ml) of TRAIL/Apo2L for 36 h. Cells were fixed and relative cell numbers were assessed by sulforhodamine B

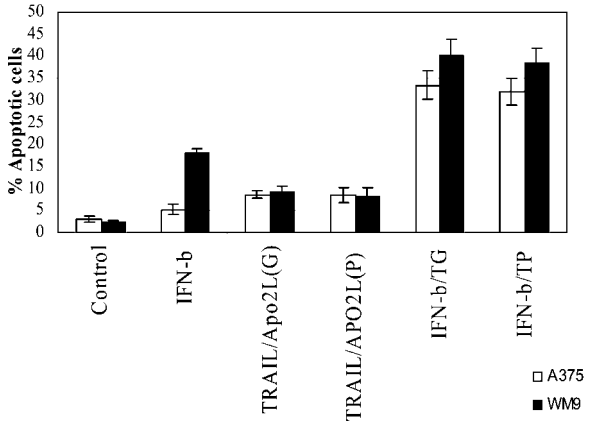

FIGURE 3. Comparative cytotoxic effects of TRAIL/Apo2L preparations from Genentech (G) or PeproTech (P) on melanoma cells. A375 and WM9 cells were treated with TRAIL/Apo2L (G) or (P) and IFN-β, stained with annexin V and PI, and subjected to bivariate FACS analysis as described in Fig. 2. Error bars represent SEM from three separate experiments.

Table I. *Effect of IFN-β pretreatment on TRAIL/Apo2L-induced apoptosis in melanoma cell lines and primary nonmalignant human cell lines*[a]

Histopathology	Cell Line	Apoptotic Cells (%)			
		Control	IFN-β (48 h)	TRAIL (24 h)	IFN-β (24 h)/TRAIL (24 h)
Melanoma	WM3211	3.02 ± 0.92	13.2 ± 2.8	8.8 ± 1.6	33.5 ± 3.3
	FeMX	1.78 ± 1.3	11.8 ± 1.9	6.25 ± 0.99	32.9 ± 2.9
	WM793	3.1 ± 0.72	7.5 ± 2.1	6.5 ± 1	20.5 ± 2.7
	CCF-Mel2H	4.4 ± 1.2	14.6 ± 2.7	8.5 ± 1.5	37.5 ± 4.1
	CCF-Mel1B	6.5 ± 1.6	10.6 ± 2.1	9.7 ± 1.4	28.3 ± 3.2
	WM35	3.3 ± 0.7	5.4 ± 1.6	8.36 ± 1.39	14.3 ± 2.1
	WM164	2.02 ± 0.6	3.12 ± 0.96	4.5 ± 0.42	3.89 ± 1.3
Primary or nonmalignant cells	HUVECS	2.5 ± 1.0	5.4 ± 1.54	4.2 ± 0.91	3.7 ± 1.9
	WI-38	3.2 ± 0.91	4.9 ± 1.09	5.2 ± 1.23	5.6 ± 1.9
	HFF	5.6 ± 1.6	6.0 ± 0.75	5.1 ± 1.5	4.9 ± 1.27
	CCF-BON	4.3 ± 0.8	3.9 ± 1.9	5.9 ± 1.5	7.2 ± 2.1
	CCF-TEN	3.5 ± 1.09	4.8 ± 0.9	4.6 ± 1.4	5.4 ± 1.7

[a] Cell lines or primary human cells were treated with IFN-β, TRAIL/Apo2L, or the combination and stained with annexin V/PI as described in *Materials and Methods*. Percentages of apoptotic cells represent the sum of annexin V-positive and annexin V/PI double-positive cells from three separate experiments.

staining. Unlike apoptotic effects, TRAIL/Apo2L had growth inhibitory effects on A375 melanoma cells (ID_{50} of ~200 ng/ml). Compared with IFN-α2, IFN-β was more potent in sensitizing cells to TRAIL/Apo2L. The IFN-β and TRAIL/Apo2L combination effect was synergistic (23). At an IFN concentration of 100 U/ml, the ID_{50} for TRAIL/Apo2L was 25 ng/ml with IFN-β but was 200 ng/ml for IFN-α2-treated cells (data not shown). With an increased concentration of IFNs (500 U/ml), the ID_{50} for TRAIL/Apo2L was 12.5 ng/ml and 100 ng/ml with IFN-β and IFN-α2, respectively (Fig. 4*A*). The differences in potency of IFN-α2 and IFN-β alone ($p \leq 0.05$) or in combination with TRAIL/Apo2L were statistically significant ($p \leq 0.01$).

To compare effects of IFN-α2 and IFN-β pretreatment on TRAIL-induced apoptosis, A375 cells were treated with either IFN-α2 and IFN-β (100 and 500 U/ml) or TRAIL/Apo2L (100 ng/ml) alone or were pretreated with IFN followed by TRAIL/Apo2L. Apoptosis was assessed by annexin V/PI staining. As observed in antiproliferative assays, IFN-β pretreatment was more potent in inducing apoptosis (24–34%) when compared with IFN-α2 (7–12%) at equivalent TRAIL/Apo2L concentrations (Fig. 4*B*).

IFN-β did not modulate activation of NF-κB or expression of proteins involved in DR-mediated apoptotic signaling

An IFN-β-dependent inhibition of NF-κB activation might sensitize cells to TRAIL-induced apoptosis. To test this possibility, A375 cells treated with IFN-α2, IFN-β, or TRAIL/Apo2L alone or with the IFN and TRAIL/Apo2L in combination were assessed for NF-κB activation by EMSA. IFN alone did not induce NF-κB activation. TRAIL/Apo2L induced equivalent amounts of NF-κB in untreated or IFN-pretreated A375 cells (Fig. 5*A*).

To examine the effects of IFN treatment on other intracellular pathways associated with apoptosis, Western blot studies were performed. Basal and IFN (24 h)-dependent expression of FLIP, Apaf-1, cIAP-1, cIAP2, Bcl2, Bax, caspase-8, caspase-9, and caspase-3 was analyzed in melanoma cell lines. Although expression levels varied from cell to cell, comparable expression of all proteins examined was observed in cells that were resistant (WM35, WM164) or sensitive (WM-9, WM3211, A375) to the combined cytotoxic effects of IFN-β and TRAIL/Apo2L. IFN-β treatment did not induce or inhibit expression of any of these proteins (Fig. 5*B*).

IFN-β and TRAIL/Apo2L in combination synergistically activated caspase-9

To identify the mechanism of synergism of IFN and TRAIL/Apo2L, we assessed cleavage of key death substrates that indicate activation of the cell death machinery. To test caspase activation, time course study (2–16 h) was done following TRAIL/Apo2L treatment. A375 cells were treated with either IFN-β (500 U/ml for 40 h) or TRAIL/Apo2L (100 ng/ml) alone or pretreated with IFN-β (24 h) followed by TRAIL/Apo2L (2, 6, and 16 h). Cell extracts were analyzed for caspase-3, caspase-8, and caspase-9 activity using specific fluorogenic caspase tetrapeptide substrates. IFN-β alone had no effect on any of the caspases in A375 cells. Consistent with a previous report (24), at 6- and 16-h time points, TRAIL/Apo2L alone resulted in 10- to 30-fold increased caspase-8 and caspase-3 activity. However, IFN-β treatment further augmented caspase-3 and caspase-8 activity by 2-fold (Fig. 6, *A* and *B*). Furthermore, despite increased caspase-3 activity, poly(ADP-ribose) polymerase, an apoptotic protease downstream of caspase-3, was not cleaved in cells treated with TRAIL/Apo2L alone (data not shown).

In contrast to caspase-3 and caspase-8, TRAIL/Apo2L alone had no significant effect on caspase-9 activity at 2 or 6 h and a <5-fold increase in activity was detected following 16 h of treatment. However, when A375 cells were pretreated with IFN-β (24 h) followed by TRAIL/Apo2L (2 h), a marked increase (>20-fold) in caspase-9 activity resulted. This was further enhanced with increasing time (Fig. 6*C*). In IFN-β-sensitive WM9 cells, enhanced caspase-9 activity was observed in cells treated with IFN-β or IFN-β plus TRAIL/Apo2L. TRAIL/Apo2L alone had no significant effect on caspase-9 activity (Fig. 6*D*). These data suggested that failure to activate caspase-9 resulted in the lack of TRAIL/Apo2L-induced apoptosis.

To ascertain the role of caspase-9 in mediating IFN-β pretreatment effects, A375 cells were treated either with the caspase-9 inhibitor (LEHD-fmk) alone or with the combination of IFN-β and TRAIL/Apo2L. The caspase-3 inhibitor (DEVD-fmk) was also used with IFN-β and TRAIL/Apo2L in combination as a positive control. Caspase inhibitors alone had no significant cytotoxic effects. IFN-β followed by TRAIL/Apo2L resulted in >30% apoptotic cells, but in the presence of the caspase-9 inhibitor (LEHD-fmk) only 7–8% cells were annexin V/PI positive. As expected, the caspase-3 inhibitor (DEVD-fmk) blocked IFN-β- and TRAIL/Apo2L-mediated apoptosis completely (Fig. 6*E*).

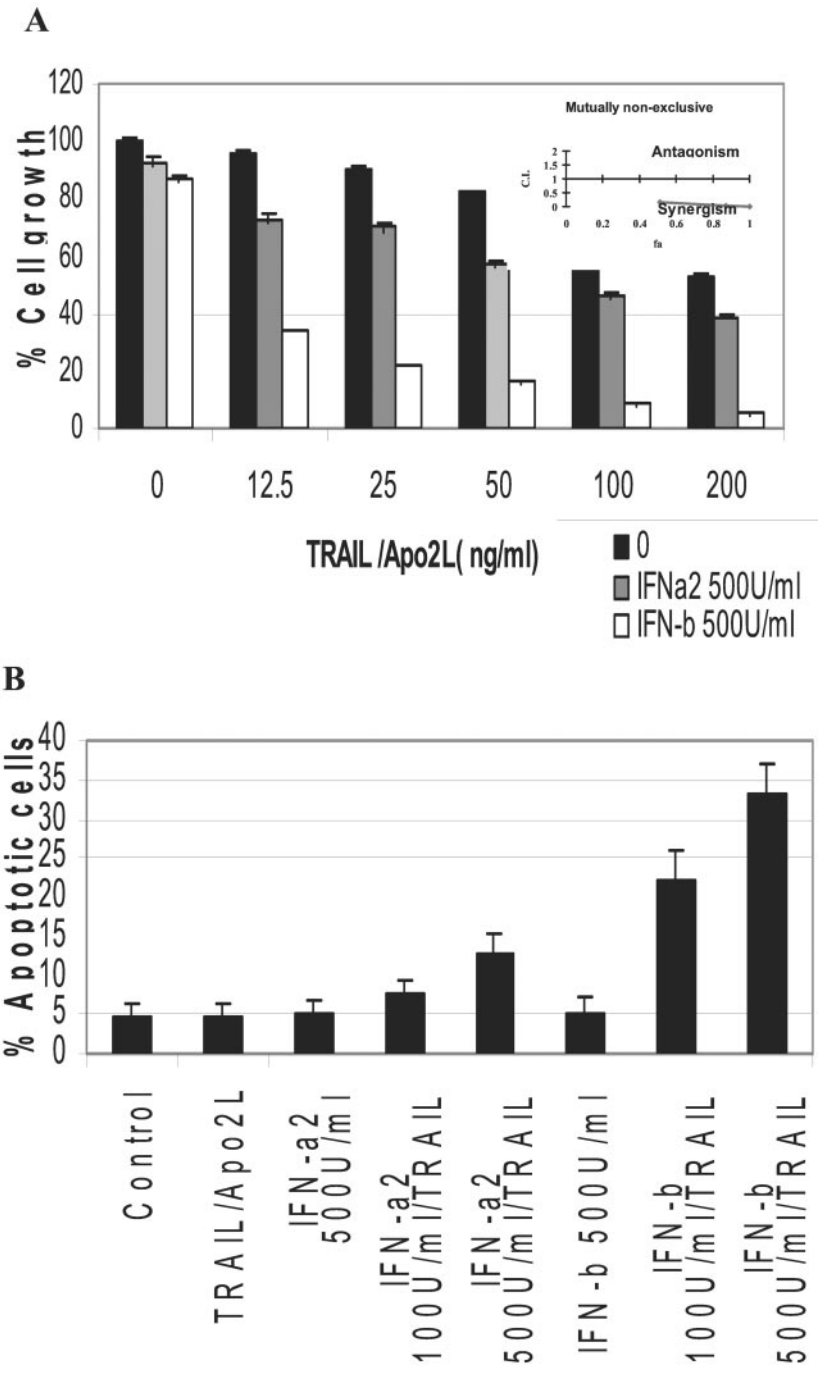

FIGURE 4. IFN-β is more potent than IFN-α2 in sensitizing cells to TRAIL/Apo2L-induced antiproliferative and apoptotic effects. *A*, A375 cells were treated in quadruplicate with IFN-α2 or IFN-β at 500 U/ml for 24 h followed by increasing doses of TRAIL/Apo2L (12.5–200 ng/ml) for 36 h. Cell growth was analyzed by sulforhodamine B staining as described in *Materials and Methods*. *B*, A375 cells were treated with TRAIL/Apo2L (100 ng/ml) and IFN-β (100 or 500 U/ml) or IFN-α2 (100 or 500 U/ml), stained with annexin V/PI, and subjected to bivariate FACS analysis. The percentage of apoptotic cells indicated is the sum of annexin V and annexin V/PI double-positive cells. Error bars represent SEM from three separate experiments.

Caspase-3 cleavage was confirmed in resistant and sensitive melanoma cells by immunoblot analyses. Consistent with the fluorogenic caspase assays, the 32-kDa procaspase-3 was partially cleaved to a 20-kDa active form after TRAIL/Apo2L (P) or (G) treatment of cells both sensitive (A375, WM9) and resistant (WM35) to the combination treatment. However, the p17 and p11 subunits resulting from the autocatalytic activity of caspase-3 were observed only in sensitive (A375, WM9, WM3211) cell lines following treatment with IFN-β and TRAIL/Apo2L in combination (Fig. 7).

IFN-β and TRAIL/Apo2L in combination synergistically induced cleavage of XIAP in apoptosis-sensitive melanoma cell lines

Because TRAIL/Apo2L resulted in cleavage of caspase-3, a block in apoptosis downstream of caspase-3 was postulated. XIAP is a potent inhibitor of apoptosis (IAP) that binds to and inhibits caspase-9 and caspase-3 activity (26) and has been associated with TRAIL/Apo2L resistance in melanoma cells (27). Thus, the effect of IFN and TRAIL/Apo2L in combination on XIAP was examined. A375, WM9, WM3211, WM35, and WM 164 cells were treated with IFN-β (40 h), TRAIL/Apo2L (P) or (G) (12 h), and the combination IFN-β and TRAIL/Apo2L (P) or (G). Immunoblot analysis with XIAP Ab revealed synergistic cleavage of XIAP to an inactive 29-kDa fragment in WM9, WM3211, and A375 cells following combination treatment with IFN-β and TRAIL/Apo2L (Fig. 8). No cleavage was observed in resistant WM35 or WM164 cells. Both preparations of TRAIL/Apo2L (P) and (G) had comparable activity. IFN-β-sensitive cells WM9 and WM3211 had slight cleavage of XIAP with IFN-β alone. TRAIL/Apo2L alone had no significant effect on XIAP expression in melanoma cell lines.

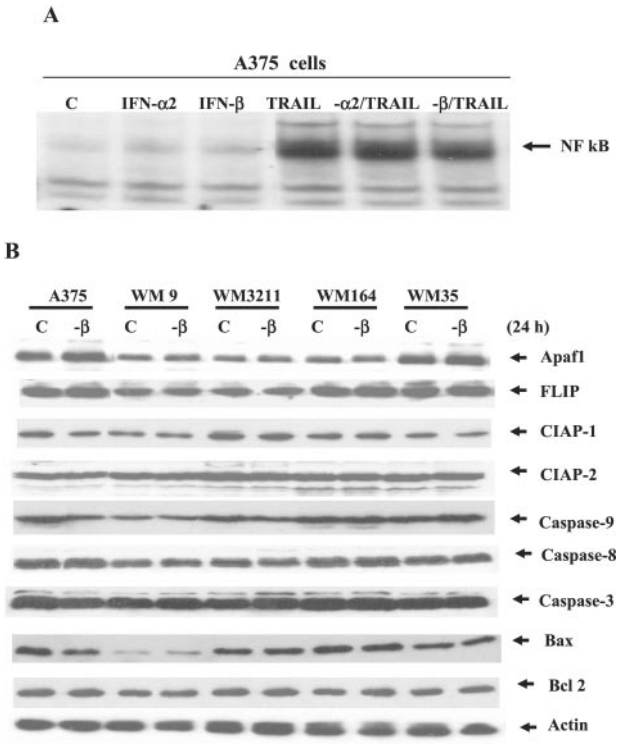

FIGURE 5. *A*, Effect of IFN pretreatment on TRAIL/Apo2L-induced NF-κB. A375 cells were treated with IFN-α2 (24 h), IFN-β (24 h), TRAIL/Apo2L (2 h), or IFN (24 h) followed by TRAIL/Apo2L (2 h). Cell extracts were subjected to EMSA using a [γ^{32}-P]ATP-labeled NF-κB consensus binding sequence as a probe. Protein-DNA complexes were resolved on a 6% polyacrylamide gel. *B*, Effect of IFN-β on apoptosis-related proteins. Melanoma cells were left untreated or were treated with IFN-β (24 h). Cell extracts were subjected to Western blot analysis of Apaf-1, FLIP, cIAP-1, cIAP-2, Bcl-2, Bax, caspase-3, caspase-8, and caspase-9 as described in *Materials and Methods*. Signal was detected using an ECL system. Data are representative of two separate experiments.

Discussion

Melanoma cells were previously reported to be susceptible to TRAIL/Apo2L-induced apoptosis (24, 25). Postulating that recombinant TRAIL/Apo2L might be effective in IFN-resistant melanoma cells, cytotoxic effects of TRAIL/Apo2L were tested on IFN-resistant and IFN-sensitive melanoma cells. Consistent with a previous report (3), when treated for 24–48 h TRAIL/Apo2L had growth inhibitory effects on melanoma cell lines (ID_{50} of ~100–200 ng/ml). However, no apoptotic effects of recombinant TRAIL/Apo2L protein produced in *Escherichia coli* by two independent sources (Genentech and PeproTech) were observed in any of the melanoma cell lines.

In contrast to results obtained with TRAIL/Apo2L as a single agent, combination of TRAIL/Apo2L with the metabolic inhibitor actinomycin D induced apoptosis, suggesting that TRAIL receptors, apoptotic initiators, and caspases were functional in these melanoma cells. Other tumor cell types resistant to TRAIL/Apo2L have been rendered sensitive by cotreatment with chemotherapeutic agents such as actinomycin D, CPT11, or 5-fluorouracil (14–18). IFN-α2 also has been suggested to sensitize cells to TNF-α-mediated apoptosis (28). Thus, it was postulated that IFNs might sensitize melanoma cells to TRAIL/Apo2L-mediated apoptosis.

Cell lines sensitive (WM9) and resistant (A375) to IFN-β-induced apoptosis were treated concomitantly with IFN-β and TRAIL/Apo2L. Unlike metabolic inhibitors such as actinomycin D, concomitant treatment with IFN-β and TRAIL/Apo2L had no greater effects compared with either single agent. Irrespective of sensitivity to either cytokine alone, when cells were pretreated with IFN-β for 12–48 h followed by TRAIL/Apo2L apoptosis was augmented in most of the melanoma cell lines. In vitro, IFN-α2 was weak compared with IFN-β in inducing apoptosis alone (11) or in sensitizing cells to TRAIL/Apo2L in melanomas. However, IFN-α2 induced apoptosis by activating the DR-mediated caspase pathway in multiple myeloma cells (12). Similarly, the IFN-γ and TRAIL/Apo2L combination was 2-fold less potent (15–20% apoptosis) compared with IFN-β (30–40%) in melanoma cells (data not shown). Unlike IFN-α2 or IFN-β, IFN-γ may sensitize cells to DR-mediated apoptosis by up-regulation of DR5 (29). Similar observations regarding augmentation of TRAIL/Apo2L-induced apoptosis by IFN-β was reported in breast carcinoma cells (30). Very little cytotoxicity was observed with IFN-β or TRAIL/Apo2L alone or with the IFN-β/TRAIL combination on primary nonmalignant human cells such as HUVECs, fibroblasts (HFF, WI-38), and astrocytes (CCF-TEN, CCF-BON).

TRAIL/Apo2L activates the NF-κB-mediated prosurvival signaling pathway. Inhibition of NF-κB activation by IFN-α2 has been reported as the mechanism for IFN-α2-dependent sensitivity to TNF-α in Daudi cells (28). However, IFN-β neither activated nor inhibited TRAIL-induced NF-κB, suggesting no direct role of NF-κB in mediating the effects of IFN-β on TRAIL/Apo2L sensitivity.

Resistance to TRAIL/Apo2L has been attributed to differential expression of DRs (31), defects in caspase-8 (32), higher expression of FLIP (33) or XIAP (27), or defects in Apaf-1 (34) expression. Because treatment of melanoma cell lines with actinomycin D rendered them TRAIL sensitive, a possible role of downstream inhibitory proteins like FLIP and IAPs that bind to Fas-associated death domain protein or other proteins in the caspase pathway was postulated. Expression of these apoptotic regulators was assessed in various melanoma cell lines. Although constitutive expression of these proteins varied from cell to cell, there was no correlation between levels of expression and sensitivity to apoptosis induced by IFN-β alone or the combination. Furthermore, IFN-β treatment (24 h) did not alter expression of FLIP, Apaf-1, caspase-9, caspase-8, caspase-3, cIAP-1, or cIAP-2 in melanoma cells. No correlation with apoptotic sensitivity has been observed in expression of DRs (TRAIL-R1, TRAIL-R2) and decoy receptors and in resistance to TRAIL/Apo2L in melanoma cells including WM9, WM35, WM3211, WM793, and WM-981 (25). IFN-β treatment had no effect on transcript levels of TRAIL-R1 and TRAIL-R2 in sensitive and resistant melanoma cells (11).

Activities of the initiator (caspase-8) and the executioner (caspase-3) caspases were analyzed in A375 cells following TRAIL/Apo2L, IFN-β, and the combination treatment. IFN-β failed to induce TRAIL/Apo2L in A375 cells (11) and had no significant effect on caspase activity. Both caspase-8 and caspase-3 resulted in cleavage of their specific fluorogenic substrates in response to TRAIL/Apo2L (35). However, TRAIL/Apo2L alone couldnot activate caspase-9 without prior IFN-β treatment. Activation of caspase-9 may have provided additional apoptotic signals to enhance disruption of mitochondrial functions. Synergistic cytochrome *c* release from mitochondria followed disruption of mitochondrial potential ($\Delta\Psi_m$) with the combination treatment with IFN-β and TRAIL/Apo2L (data not shown).

IAP proteins are defined by a novel conserved motif termed the baculoviral IAP repeat (36). XIAP (also known as human IAP-likeprotein/minor histocompatibility Ag), the most potent caspase

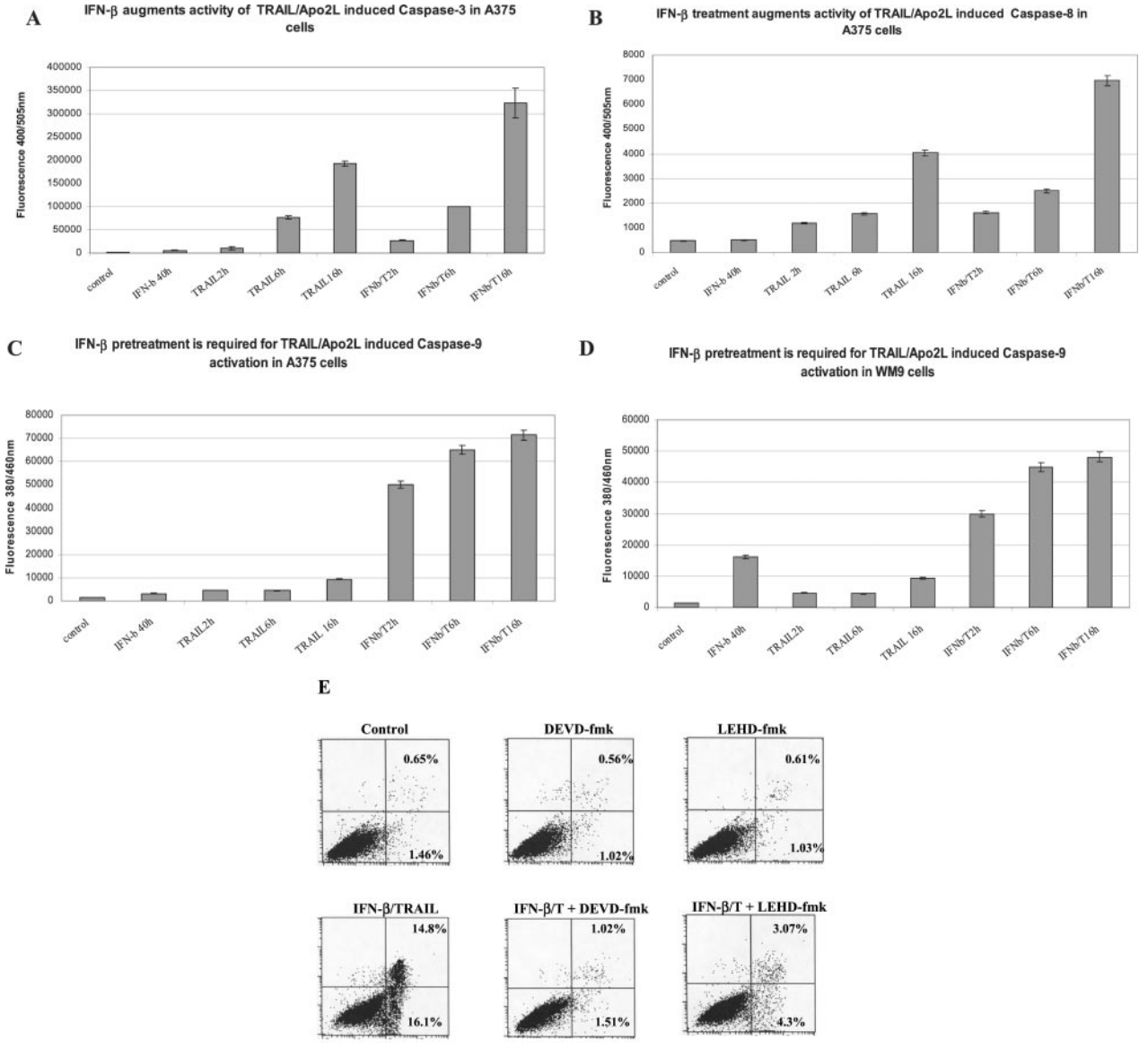

FIGURE 6. The IFN-β and TRAIL/Apo2L combination mediates apoptosis by synergistically activating caspase-9. Cells were treated with IFN-β (500 U/ml) for 40 h, TRAIL/Apo2L (100 ng/ml) for 2, 6, and 16 h, and IFN (24 h)/TRAIL for 2, 6, and 16 h. Cell lysates containing an equivalent amount of protein were assayed for protease activity toward the fluorogenic caspase-3 (*A*), caspase-8 (*B*), or caspase-9 (*C* and *D*) peptide substrates Ac-DEVD-AFC, Ac-IETD-AFC, and Ac-LEHD-AMC, respectively. Relative fluorescence was measured at 400/505 nm and 380/460 nm. Error bars represent SEM from three separate experiments. *E*, Inhibition of IFN-β and TRAIL/Apo2L combination-mediated apoptosis in A375 cells by caspase-9 inhibitor. A375 cells were either left untreated or treated with IFN-β, TRAIL/Apo2L, caspase inhibitors (DEVD-fmk and LEHD-fmk), IFN-β/TRAIL, or IFN-β/TRAIL in combination with caspase-9 inhibitor (LEHD-fmk) for 16 h followed by annexin V/PI staining. Caspase-3 inhibitor (DEVD-fmk) was used in parallel as a positive control. Data shown represent two independent experiments.

inhibitor, directly binds and inhibits caspase-3, caspase-9, and caspase-7 but not caspases-1, caspase-6, caspase-8, or caspase-10 (26, 37). TRAIL/Apo2L alone has been implicated in cleavage of XIAP in TRAIL-sensitive melanoma cell lines (27). No significant effects on expression or cleavage of XIAP were observed following treatment with TRAIL/Apo2L alone in this study. However-,when cells were pretreated with IFN-β followed by TRAIL/Apo2L treatment, XIAP, but not cIAP-1 or cIAP-2, was cleaved to its inactive 29-kDa form. Cleavage of XIAP could contribute to activation of caspase-9 and cleavage of poly(ADP-ribose) polymerase and Bid following the combination treatment.

TRAIL/Apo2L binds to its DRs, activates caspase-8, and results in cleavage of procaspase-3 to active caspase-3 (p20). However, the p17 subunit resulting from autocatalytic activity of active caspase-3 was detectable only in cells pretreated with IFN-β. It seems probable that active XIAP bound to the p20 subunit of caspase-3 prevents the second catalytic cut that is necessary for downstream events (27, 38). These results implicated XIAP as a significant inhibitor of TRAIL/Apo2L-induced apoptosis.

IFN-β may modulate TRAIL/Apo2L-mediated cleavage of XIAP through induction of XIAP-associated factor-1 (XAF1) (41), a negative regulator of XIAP (39, 40). XAF1 protein was strongly up-regulated in WM-9, A375, and WM3211 cells but not in resistant WM35 and WM164 cells (41). IFN-β alone had no effect on XIAP expression. The mechanism involved in cleavage of XIAP may not be induction of XAF1 by IFN but rather caspase-3 and caspase-9, freed of inhibitory effects of XIAP. It is conceivable that potentiation of activities of caspase-3 and caspase-9 may ren-

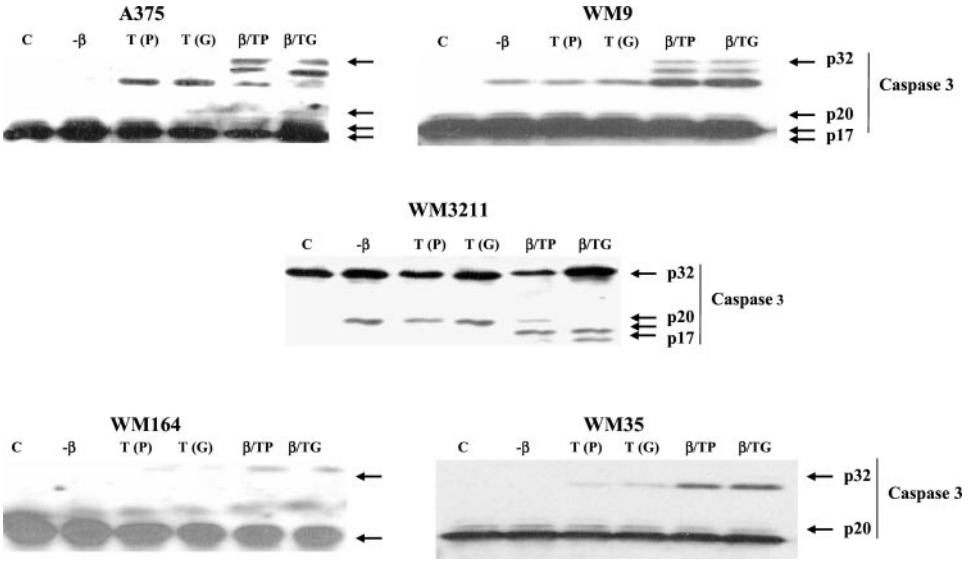

FIGURE 7. Effect of IFN-β and TRAIL/Apo2L in combination on caspase-3 cleavage. Melanoma cell lines sensitive (A375, WM9, WM3211) or resistant (WM164, WM35) to IFN/TRAIL-induced apoptosis were left untreated or were treated with IFN-β (40 h), TRAIL/Apo2L (12 h), or IFN-β (24 h)/TRAIL (12 h) (P) and (G). Cell extracts were subjected to Western blot analysis using caspase-3 Ab followed by HRP-conjugated secondary Ab. The blots were developed using an ECL system. Data shown represent three separate experiments. Procaspase 3 (32 kDa) was cleaved to a active 20-kDa protein following TRAIL/Apo2L treatment in A375, WM9, WM3211, and WM35 cells. However, IFN-β/TRAIL resulted in cleavage of a 20-kDa fragment to smaller active peptides only in sensitive A375, WM3211, and WM9 cells. IFN-β alone cleaved caspase-3 in WM9 and WM3211 cells. No significant cleavage of caspase-3 was observed in WM164 cells.

der resistant cells sensitive to TRAIL/Apo2L. Thus, one or more novel IFN-stimulated genes, such as XAF1, may have an important role in IFN-mediated sensitization to TRAIL/Apo2L. These studies provide a novel role of IFNs in mediating sensitivity to DR-mediated apoptosis in vitro by modulating IAPs. Further in vivo studies with IFN and TRAIL/Apo2L in combination are needed to ascertain its antitumor effects.

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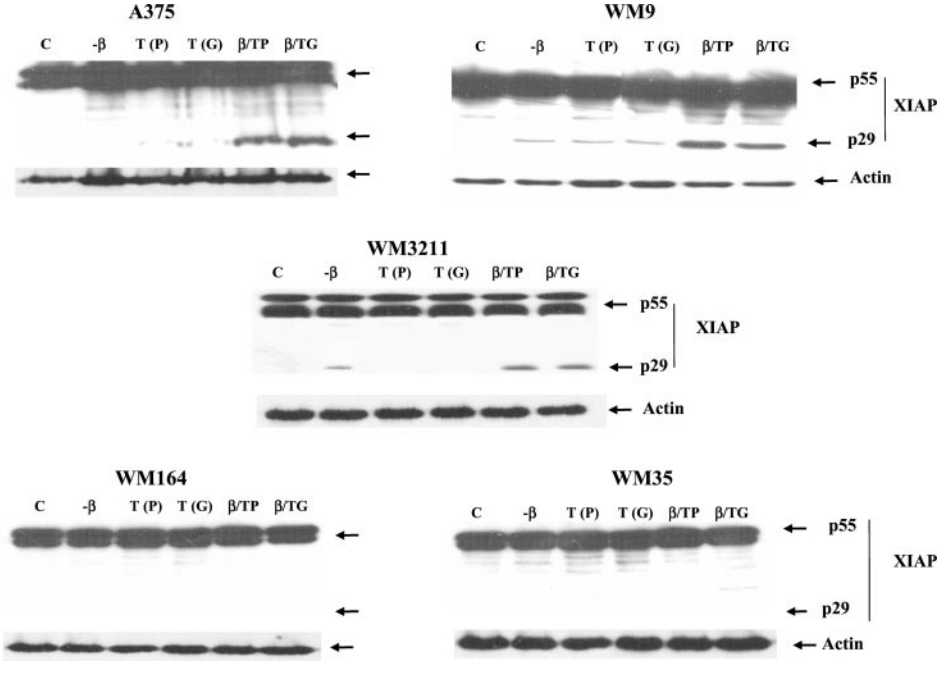

FIGURE 8. The IFN-β and TRAIL/Apo2L combination synergistically induces cleavage of XIAP. Western blot analysis was performed using XIAP mAb. XIAP was cleaved to the inactive-29 kDa fragment following IFN-β/TRAIL combination treatment in A375, WM9, and WM3211 cells. IFN-β-sensitive cells WM9 and WM3211 showed cleavage of XIAP with IFN-β treatment. No cleavage of XIAP was observed in resistant cells WM35 or WM164 following IFN-β, TRAIL/Apo2L, or the combination treatment.

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