Preferential Induction of Apoptosis by Interferon (IFN)-β Compared with IFN-α2: Correlation with TRAIL/Apo2L Induction in Melanoma Cell Lines

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ABSTRACT

On the basis of *in vitro* inhibition of tumor cell growth, IFNs have been generally considered to be antiproliferative proteins. To probe further the potential mechanisms of the antitumor effects of IFNs, we have assessed apoptosis in response to IFN-α2 and IFN-β in cell lines of varied histologies, with a focus on melanomas. Many of the cell lines tested underwent apoptosis in response to IFN-β, as assessed both by Annexin V and terminal deoxynucleotidyl transferase-mediated nick end labeling staining. In general, IFN-β had greater growth inhibitory and proapoptotic effects than IFN-α2 on all cell lines. The melanoma cell line WM9, sensitive to growth inhibition by IFNs, had a greater degree of apoptosis than A375 melanoma cells, which were largely resistant to antigrowth effects of IFNs. IFN-β-induced apoptosis was dependent on activation of the caspase cascade with cleavage of caspases 3, 8, and 9 and of the caspase 3 substrate, poly(ADP-ribose) polymerase. Caspase inhibitors benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone or benzyloxycarbonyl-Asp-Glu-Val-Asp-fluoromethyl keton, inhibited IFN-β-induced apoptosis. Other changes associated with apoptosis, including the movement of cytochrome *c* from mitochondria to cytoplasm and DNA fragmentation, were also identified in response to IFN-β. *Apo2L ligand* [tumor necrosis factor-related apoptosis-inducing ligand *(TRAIL)*] was one of the early genes induced by IFN-β in apoptosis-sensitive WM9 cells. Other sensitive melanoma cell lines had a similar IFN-β-specific induction of *TRAIL*. Neutralizing antibody to TRAIL inhibited IFN-β-induced apoptosis in WM9 cells. In resistant A375 cells, IFN-β did not induce *TRAIL/Apo2L* expression. Thus, induction of *TRAIL* by IFNs in some tumor types may initiate the apoptotic cascade. This study offers another mechanism for the antitumor effects of IFNs.

INTRODUCTION

IFNs comprise a family of multifunctional polypeptides with defined antiviral, antiproliferative, and immunomodulatory properties. IFNs play a central role in antiviral and antitumor host defense (1, 2). Although IFNs were introduced clinically more than 15 years ago to treat malignancies, viral infections, and multiple sclerosis, only a portion of patients respond therapeutically. A better understanding of the mechanisms that underlie the antitumor effects of IFNs and the identification of the factors responsible for resistance to IFNs could lead to improved use in malignant and other diseases.

The potent and pleiotropic gene modulatory effects of IFNs must underlie clinical activity (3). Both type I and type II IFNs use distinct signaling pathways to drive the expression of specific ISGs.[3] Some IFN-induced genes, such as the *dsRNA-dependent serine/threonine protein kinase PKR*, *2–5 A synthetase*, and *p78* partially explain the antiviral effects. These induced proteins may also mediate some aspects of the antiproliferative response by decreasing protein synthesis (3). Down-regulation of cdc25A, phosphatases, and cyclins (D3, E and A subtypes), which in turn are implicated in activation of cyclin-dependent kinases, may also contribute to the antiproliferative effects by prolonging different phases of the cell cycle (4). Greater cell growth-inhibitory effects of IFN-β compared with IFNs-α, particularly in nonhematopoietic cells lines, have been repeatedly identified (5–8).

It is our working hypothesis that the regulation of gene expression within tumor cells by IFNs results in antitumor effects for most tumor types. IFNs prolong all phases of the cell cycle (1, 2), contributing to what has been defined as an antiproliferative effect. However, we had made observations of detached and condensed cells in culture and DNA fragmentation consistent with apoptosis, particularly after prolonged treatment with IFN-β. Therefore we postulated that cell loss may contribute to the antiproliferative effects of IFNs, and in the present

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[3] The abbreviations used are: ISG, IFN-stimulated gene; cyt *c*, cytochrome *c*; mAb, monoclonal antibody; AMC, aminomethylcoumarin; Ac, acetyl; fmk, fluoromethyl ketone; z, benzyloxycarbonyl; VAD, Val-Ala-Asp; DEVD, Asp-Glu-Val-Asp; IETD, Ile-Glu-Thr-Asp; LEHD, Leu-Glu-His-Asp; PARP, poly(ADP-ribose) polymerase; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; Tdt, terminal deoxynucleotidyl transferase; TUNEL, Tdt-mediated nick end labeling; Br-dUTP, bromolated deoxyribonucleotide triphosphate; FACS, fluorescent activated cell scanning; PI, propidium iodide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT-PCR, reverse transcription-PCR; TNF, tumor necrosis factor; FasL, Fas ligand; FasR, Fas receptor.

study, we investigated the possible role of apoptosis in mediating the antigrowth effects of IFNs. To probe underlying molecular mechanisms, we focused on melanoma cells and assessed activation of the proteolytic caspase cascade.

MATERIALS AND METHODS

Cell Culture and IFN. Melanoma cell lines, WM9 (9), A375, FemX, and Guilliams and Minors (American Type Culture Collection) were grown in DMEM medium (Life Technologies, Inc., Rockville, MD) supplemented with heat-inactivated 10% FCS (HyClone, Logan, UT) in a humidified chamber of 95% air and 5% CO_2 at 37°C. Cells were treated with IFN-α2b (Intron A; Schering-Plough) or IFN-β (Rebif; Serono) produced by recombinant DNA technology for the indicated time periods. They were of equivalent specific activity, 2×10^8 units/mg protein. All other cell lines tested were grown either in RPMI 1640 (Daudi, Jurkat, U937, and NIHOVCAR3) or in DMEM (ACHN, MCF-7, HeLa, MDA-MB231, and T47D) supplemented with 10% FCS. Periodically, cells were confirmed as *Mycoplasma*-free.

Antiproliferative Assays. Cell monolayers were harvested with trypsin EDTA, washed with PBS, and resuspended in media containing 10% serum. Cells were plated in 200 μl of medium in 96-well plates at a cell density of 2000 cells/well. Cells were allowed to adhere to the plate for 6–8 h, and then IFN-α2 or IFN-β was added in different dilutions (10, 100, 200, 400, and 1000 units/ml) to the assay plate. One plate was fixed with 10% TCA at the time of IFN addition to serve to as an initial control. Quadruplicates of each treatment were performed. After 96 h, the plates were fixed with 10% TCA (at 4°C) for 1 h, rinsed with water, and allowed to air dry. Cell numbers were estimated by staining with 0.4% sulforhodamine B (w/v; Sigma Chemical Co., St. Louis, MO) in 1% acetic acid for 1 h. Excess dye was removed by washing the wells with 1% acetic acid. Dye was eluted from cells with 100 μl of Tris-HCl (pH 10.5), and absorbance was measured at 570 nm (10). Results were calculated as the percentage of 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.

Gel Electrophoresis and Immunoblotting Analysis. Cell lysates were prepared by resuspending in 1× lysis buffer [50 mm Tris-HCl (pH 8.0), 1% Triton X-100, 10% Glycerol, 1 mM EDTA, 250 mM NaCl, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 10 μg/ml pepstatin] and incubating on ice for 20 min, with subsequent centrifugation at 12,000 [times *g* for 10 min (11). The supernatant was used as whole cell extracts for subsequent immunoblotting studies. For PARP cleavage, the cell extracts were prepared according to the manufacturer's protocol (Biomol, Plymouth Meeting, PA).

For immunoblot analyses, cells were treated with IFN-α2 or IFN-β for different time periods, and whole cell lysates were prepared. SDS-PAGE was conducted by using the Laemmli buffer system on 12% polyacrylamide gels. Protein was estimated by using the Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA), and 20 μg of protein extract/sample was used for all immunoblotting experiments. Proteins separated on gels were transferred onto polyvinylidene difluoride membrane by the semidry method (Trans Blot SD; Bio-Rad). Binding of the primary and secondary antibodies was performed in 1× Tris-buffered saline (pH 7.4) containing 5% (w/v) nonfat dry milk and 0.2% (v/v) Tween 20 for 1 h at room temperature. Membranes were immunoblotted with a mAb to PARP (Biomol) or polyclonal antibody to caspase 3 (PharMingen, San Diego, CA), and cyt *c* (PharMingen), with subsequent incubation with horseradish peroxidase-conjugated secondary antibodies (Pierce, Rockward, IL). Immunoreactive bands were visualized by using enhanced chemiluminescence (NEN). Equal protein loading was confirmed by reprobing with Actin mAb (Sigma Chemical Co.).

Caspase Activity Assay. Caspase 3 activity was measured by using a commercially available caspase-3 assay kit (PharMingen). Briefly, cells were treated with IFN (0–96 h), washed twice with cold PBS, and lysed on ice in 1× lysis buffer [10 mM Tris-HCl (pH7.5), 130 mM NaCl, 1% Triton X-100, and 10 mM NaPPi]. Cell lysates were centrifuged at 10,000 × *g* for 10 min, and total protein concentration was estimated using Bio-Rad protein assay reagent. Assay was performed in 96-well plates, and triplicates were taken for each sample. For each reaction, 20 μg of protein extract, 200 μl of 1× HEPES buffer, and 5 μg of Ac-DEVD-AMC (a fluorogenic substrate) were mixed and incubated at 37°C for 1 h. As controls, cell lysates or substrate alone were incubated in parallel. Where indicated in the text, the extracts were preincubated with the caspase 3 inhibitor Ac-DEVD-CHO (0.5 μg) for 5 min before the addition of the substrate. Caspase enzymatic hydrolysis was measured by AMC liberation from Ac-DEVD-AMC at 380 nm/460 nm using spectrofluorometer. Relative fluorescence of substrate control was subtracted as background emission. Caspases 8 and 9 activities were measured using Ac-IETD-AMC and Ac-LEHD-AMC substrates (Biomol) as described for caspase 3 assay.

cyt *c* Release Analysis. Cells (1×10^7/sample) were washed twice with 1× PBS and incubated on ice for 20 min in hypotonic buffer [20 mM HEPES-KOH (pH 7.2), 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 250 mM sucrose, and protease inhibitors]. The cell suspension was Dounce-homogenized and centrifuged at 750 × *g* for 5 min. The supernatant was centrifuged further at 10,000 × *g* for 15 min. The resulting pellet, containing the mitochondrial fraction, was designated as P-10. The supernatant was subjected to ultracentrifugation at 100,000 × *g* for 45 min. The resulting pellet (membrane) and supernatant (cytosol), were designated as P-100 and S-100 respectively. P-10 and S-100 fractions were immunoblotted with cyt *c* antibody (PharMingen).

TUNEL Assay. DNA fragmentation was detected in IFN-α2- and IFN-β-treated cells by TUNEL staining using the APO-BrdUrd kit (PharMingen). Briefly, IFN-treated cells were washed with cold PBS, trypsinized, and fixed in 1% paraformaldehyde for 15 min on ice. Fixed cells were washed twice with PBS, pelleted, and suspended in 70% ethanol. The cells were kept for 12–24 h at 20°C in 70% ethanol. For labeling the cells with Br-dUTPs, cells were washed twice with PBS and labeled with Br-dUTP by enzyme Tdt for 2 h at 37°C. After labeling, cells were washed and stained with FITC-conjugated anti-BrdUrd mAb for 30 min in a low-light environment. RNase-PI was added, and samples were incubated for an additional 30 min

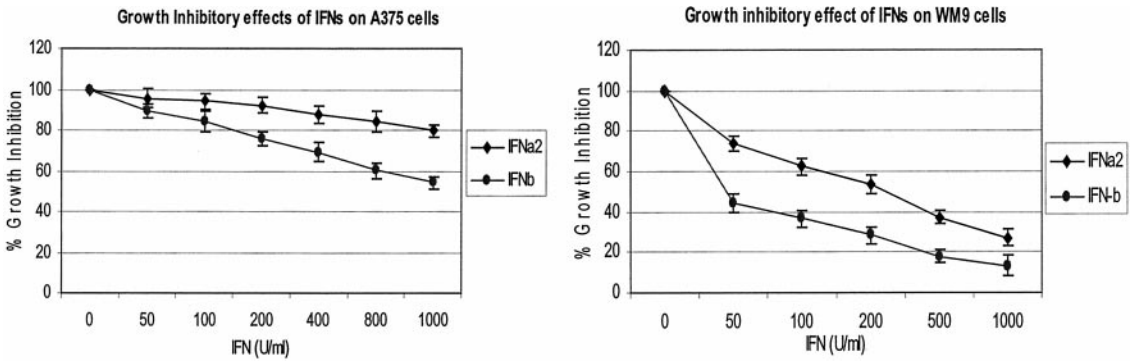

Fig. 1 IC_{50}s for IFN-α2 and IFN-β in melanoma cell lines. WM9 and A375 melanoma cell lines were treated with different doses of IFN-α2 and IFN-β for 4 days. The percentage of inhibition of cell proliferation was calculated taking cell growth in untreated cultures as 100% proliferation. Values are the means of three separate experiments with SD <10%. The concentration of IFN (units/ml) required for 50% inhibition (IC_{50}) in cell growth compared with the untreated controls was determined after sulforhodamine β staining by regression analysis.

(at room temperature). The percentage of FITC-positive cells were analyzed by FACS (Becton Dickinson Facsvantage).

Annexin V/PI Assay. Annexin V staining of exposed membrane phospholipid phosphatidylserine was done using the Annexin V assay kit (PharMingen). Briefly, cells were harvested according to the manufacturer's protocol, washed with 1× PBS twice, and resuspended in 100 μl 1× binding buffer [10 mM HEPES (pH 7.4), 140 mM NaCl, and 2.5 mM $CaCl_2$]. Annexin V-FITC and PI were added to individual samples and incubated for 15 min in a low-light environment. The reaction was stopped by adding 3–4 volumes of 1× binding buffer. Cells were analyzed by FACS (Becton Dickinson Facsvantage).

RNase Protection Assay. Total RNA was isolated from cells after IFN treatment by using Trizol reagent (Life Technologies, Inc.). RNase protection assay was done using the Riboquant system with a multiprobe template set from PharMingen. The hAPO-3c template set was used for the T7 polymerase-directed synthesis of high specific activity [^{32}P]UTP-labeled antisense RNA probes. The probe set contained 13 probes, including two housekeeping genes, *GAPDH* and *L32*. Probe (4×10^5 cpm) was hybridized with each RNA (10 μg) sample overnight at 56°C. RNA samples were digested with RNase A and T1, purified, and resolved on 6% denaturing polyacrylamide gels. Internal housekeeping genes were analyzed to confirm equal RNA loading.

RT-PCR. Total RNA was isolated from cells after IFN treatment by using Trizol reagent (Life Technologies, Inc.). RNA (1 μg/sample) was annealed with random primers at 70°C, quick-chilled, and reverse-transcribed with Molony murine leukemia virus Reverse transcriptase (Promega Corp., Madison, WI) at 42°C for 45 min. One-tenth of each reverse transcription reaction was used as template for PCR using TRAIL- (5′-TTTACCAACGAGATGAAGCAG-3′; 5′-CTATGTTGGTCACTATGGTC-3′) and GAPDH- (5′-AAATCCCATCACCATCTTC-3′; 5′-CACCACCCTGTTGCTGTAG-3′) specific primers. PCR was carried out for 15 cycles to assure linear amplification. The PCR reaction was separated on 1% agarose gel and transferred to nylon membrane by capillary blotting. The membrane was hybridized with [^{32}P]CTP-labeled TRAIL and GAPDH probes.

RESULTS

Effects of IFN-α2 and IFN-β on Growth of Melanoma Cell Lines. To provide a basis for assessing the mechanisms responsible for the variation in IFN-regulated cell growth, melanoma cell lines were treated with IFN-α2 and IFN-β in short term (4-day) antiproliferative assays (12). Cells were exposed to increasing doses of IFN-α2 and IFN-β in parallel, and relative cell numbers were assessed by sulforhodamine β staining after 96 h. Concentration of IFNs (units/ml) required to inhibit cell growth by 50% (IC_{50}) was determined for each cell line. The IC_{50}s for IFN-β were less when compared with IFN-α2 in all melanoma cell lines. Two cell lines, WM9 (IC_{50}, 10 units/ml) and A375 (IC_{50}, 1000 units/ml), which were "sensitive" or "relatively resistant" to antigrowth effects of IFNs, respectively, were used in this study (Fig. 1). Both A375 and WM9 cells are equally sensitive to IFN-α2 or -β-mediated JAK-STAT activation or induction of classical ISGs. After 72 h, IFN-β-treated WM9 cells showed distinct morphological changes such as rounding up and detachment from the plate, a finding consistent with apoptosis. 4′,6-diamidino-2-phenylindole staining was performed to support this contention. This identified 20–30% of cells with fragmented or condensed nuclei subsequent to IFN-β treatment.

Induction of Apoptosis in Cell Lines by IFN-β and IFN-α2. On the basis of the hypothesis that part of the antigrowth effects of IFNs might result from apoptosis, WM9 cells were treated with IFN-β and assessed by Annexin V staining. No significant increase in the percentage of cells staining with Annexin V occurred after 24 h of IFN-β treatment (500 units/ml). However, >12% of cells were Annexin V-positive at 48 h. This increased to 19% at 72 h and 25% at 96 h. Studies were not carried beyond 96 h, because >80% of the cells were dead by 120 h. Dose-response studies at 72 h resulted in greater numbers of Annexin V-positive cells with increasing doses of IFN-β (Fig. 2, *A–E*). IFN-α2 did not induce apoptosis at any concentration up to 1000 units/ml (Fig. 2, *F* and *G*).

Three other melanoma cell lines were treated with different doses of IFN-α2 and IFN-β for 72 h and stained with Annexin V. Cell lines more sensitive to the antiproliferative effects of

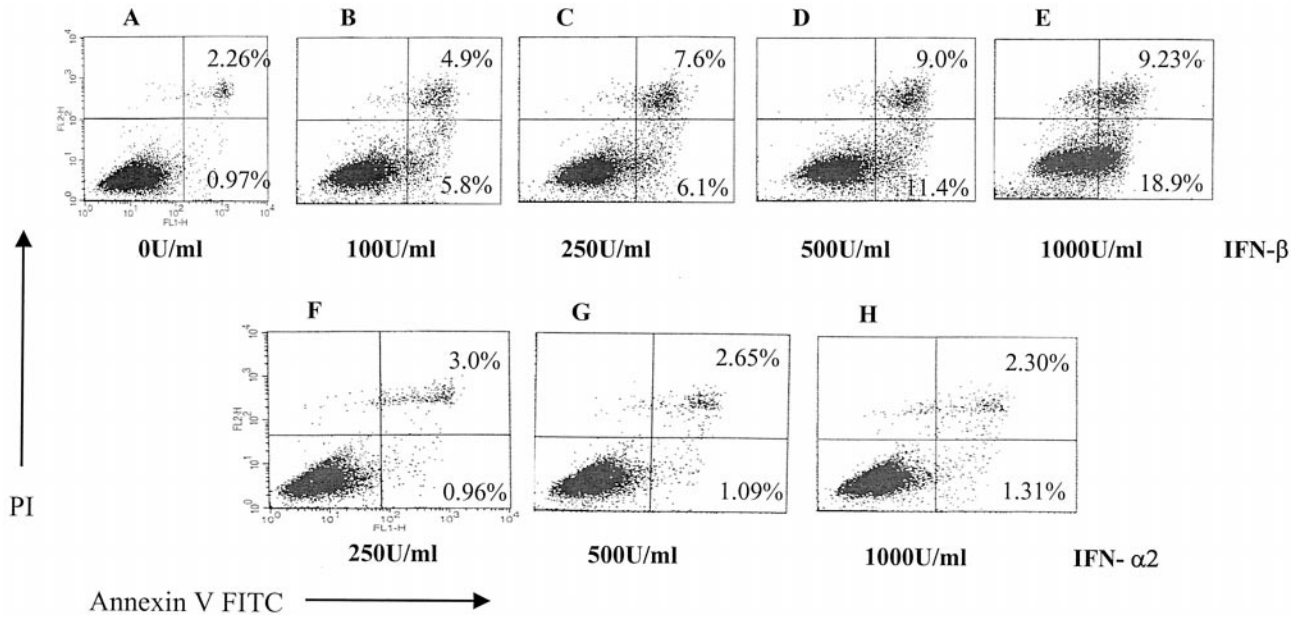

Fig. 2 IFN-β, but not IFN-α2, induces Annexin V-positivity in WM9 cells. Expression of phosphatidylserine on the plasma membrane was measured by staining cells with FITC-conjugated Annexin V and PI to assess cell membrane permeability. WM9 cells treated with increasing doses of IFN-β or IFN-α2 for 72 h were stained with Annexin V and PI and subjected to bivariate FACs analysis. The percentage of Annexin V-positive cells and the percentage of Annexin V- and PI-positive are shown in the *bottom right* and *top right* panels, respectively. *A*, untreated cells; *B–D*, cells treated with different doses of IFN-β; and *E–F*, cells treated with different doses of IFN-α2.

IFN-β, such as FemX, had greater induction of apoptosis at higher doses; for example 100 units/ml of IFN-β resulted in 9.1% Annexin V-positive cells compared with 5.1% in control. This increased to 17.5% positive cells with 1000 units of IFN-β. No increase in Annexin V-positive cells occurred with IFN-α2 even at 1000 units/ml The partially resistant cell line, Guilliams, had a 2-fold increase in the percentage of apoptotic cells, and the relatively IFN-resistant cell line, A375, had no increase in Annexin V staining after IFN-β or IFN-α2 treatment.

One of the later steps in apoptosis is DNA fragmentation, a process that results from the activation of endonucleases that degrade chromatin into smaller fragments with free 3′-OH ends. Tdt was used to catalyze the addition of bromolated deoxyuridine triphosphates to the free 3′-OH ends of double and single stranded DNA (TUNEL assay). A time course study in WM9 cells with IFN-β (500 units/ml) identified few changes until 72 h; however, at 96 h, >30% of cells were TUNEL-positive (data not shown). Again this effect was greater with increasing doses of IFN-β; IFN-α2 did not result in any positive staining (Fig. 3, *A–F*). Other melanoma cell lines revealed similar patterns of labeling. For example, 20–25% of FemX cells were TUNEL-positive with IFN-β after 96 h (Table 1). The effect in FemX was also dose-dependent; at 250 units/ml, there were only 2.12% TUNEL-positive cells, which increased to 9.53% with 500 units/ml IFN-β. In contrast, no significant changes were observed, in IFN-α2-treated cells (Table 1). Guilliams, a partially sensitive cell line, had 11–12% apoptotic cells after IFN-β, whereas A375, a relatively resistant cell line, were unaffected by IFN-α2 or IFNβ even at higher doses (up to 1000 units/ml).

To determine whether apoptosis occurred in cell lines other than melanoma, Annexin V and TUNEL staining were performed in various cell lines treated with IFN-α2 and IFN-β (1000 units/ml). Cells of several different histologies were TUNEL-positive after 96 h of treatment with IFN-β (Table 1). IFN-α2 did not induce apoptosis in any cell type. TUNEL assay results were confirmed by Annexin V assay. A rough, although not absolute, correlation existed between antiproliferative and apoptotic effects in these cells (data not shown). Cell lines that were relatively resistant to the cell growth-inhibitory effects of IFNs (IC_{50}, >250 units/ml), including U937, HeLa, and T47D, did not become TUNEL-positive in response to IFN-α2 or IFN-β. Other cell lines, including ACHN (renal cell carcinoma), Minors (melanoma), NIHOVCAR3 (ovarian carcinoma) and MCF-7 (breast carcinoma), which were more sensitive to IFN-β (IC_{50}, <250 units/ml), had an increase in apoptotic cells in response to IFN-β but not IFN-α2. However, two cell lines, Daudi and Jurkat, did not undergo apoptosis in response to either IFN-α2 or IFN-β despite their response to the growth inhibitory effects of IFNs (IC_{50}, <20 units/ml).

IFN-β Induces cyt *c* Release from Mitochondria to Cytoplasm. To evaluate intracellular changes associated with apoptosis, studies were focused on WM9 melanoma cells. Release of cyt *c* from mitochondria into the cytoplasm is a critical signal for cell death by either a rapid apoptotic or a slow necrotic process. Cytoplasmic cyt *c* forms an essential part of the vertebrate apoptosome that is composed of cyto *c*, Apaf1, and procaspase 9 (13). Distribution of cyt *c* in cytosolic and mitochondrial cellular fractions was assessed in IFN-β-treated WM9 cells. Cyt *c* was undetectable in the cytosolic fraction of untreated cells. However, after 24 h of IFN-β treatment, cyt *c* was detectable in the cytosolic fraction, and even greater

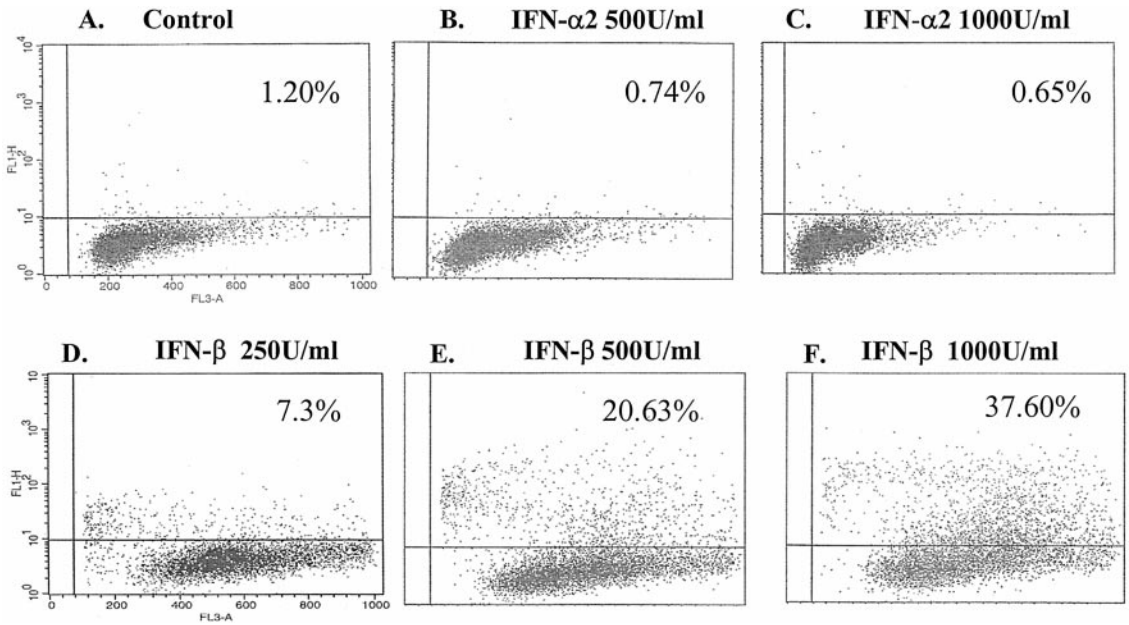

Fig. 3 IFN-β, but not IFN-α2, induces increased TUNEL positivity in WM9 cells. DNA fragmentation, a late-stage apoptosis marker, was detected by TUNEL analysis. After 96 h of treatment with increasing doses of IFN-α2 and IFN-β, cells were fixed, labeled with Br-dUTP by the enzyme Tdt, and then stained with FITC-labeled anti-BrdUrd mAb. The percentage of FITC-positive cells was analyzed by FACS (*upper panel*). *A*, percentage of TUNEL-positive cells in control, untreated cells; *B–F*, cells treated with 250,500 or 1000 units/ml of IFN-α2 and IFN-β.

Table 1 Percent TUNEL-positive cells in IFN-α2 and IFN-β treated cell lines of different histopathologies

All cell lines except Daudi and Jurkat were treated with IFN-α2 and IFN-β (1000 units/ml) for 96 h. Daudi cells and Jurkat, being most sensitive to antigrowth effects of IFNs (IC_{50} > 20 units/ml), were treated with 500 units/ml IFNs. Cells were fixed in paraformaldehyde and stained for TUNEL assay as described in "Materials and Methods." Untreated cells were processed in parallel as controls.

Histology	Cell line	Control	IFN-α2	IFN-β
Melanoma	A375	1.2	0.6	1.5
Melanoma	WM9	1.2	0.7	33.5
Melanoma	Guilliams	0.8	0.3	11.0
Melanoma	FemX	0.5	2.6	22.6
Melanoma	Minors	1.8	2.6	16
Breast carcinoma	MCF7	1.7	5.2	9.6
Breast carcinoma	MDA-MB231	3.1	2.0	11.0
Breast carcinoma	T47D	2.2	2.5	5.4
Hematopoietic	Daudi	1.4	5.8	1.9
Hematopoietic	Jurkat	2.6	1.6	1.6
Hematopoietic	U937	0.8	1.7	4.0
Ovarian carcinoma	NIHovcar3	9.0	11.4	26.5
Cervical carcinoma	Hela	1.2	1.1	1.3
Renal cell carcinoma	ACHN	0.9	1.4	37.5

amounts of cyt *c* were detected at later times. In contrast, no cyt *c* release was observed in cells treated with IFN-α2 (Fig. 4). The mitochondrial fraction had constant levels of cyt *c* in both treated and untreated cells. Results suggested that IFN-β might be inducing apoptosis through cyt *c* release from mitochondria.

Activation of Caspases by IFN-β in WM9 Cell Line. Release of cyt *c* from mitochondria to cytosolic fractions in IFN-β-treated cells suggested that IFN-β might induce apoptosis via caspase-dependent pathways. To test this, WM9 and A375 cells were treated with IFN-β for different time periods, after which caspase 3 activation was examined using a fluorogenic caspase tetrapeptide substrate, Ac-DEVD-AMC. Consistent with time-dependent, Annexin V positivity, WM9 cells treated with IFN-β for 24 h had a 3-fold increase in DEVD-AMC cleavage activity that increased at 48 h and 72 h to >15-fold (Fig. 5*A*). After 96 h, total caspase 3 activity decreased, perhaps because of low cell viability. To confirm that AMC release was attributable to activated caspase 3 in WM9 cells, IFN-β-treated WM9 cell extracts were incubated with the caspase 3 inhibitor Ac-DEVD-CHO before the addition of the substrate. Under these conditions, >80% of DEVD-AMC cleavage was inhibited (Fig. 5*A*). Furthermore, when WM9 cells were coincubated with the caspase 3 inhibitor, z-VAD-fmk, and then treated with IFN-β for 48 h, cleavage of caspase 3 substrate did not occur (not shown). In contrast, relatively resistant A375 cells had no significant changes in DEVD-AMC cleavage activity even after 96 h of treatment (Fig. 5*B*).

To identify the upstream regulatory caspases, cleavage of the caspase 8 (Ac-IETD-AMC) and caspase 9 (Ac-LEHD-AMC) substrates was examined. Only a 2–3-fold increase in cleavage of caspase 9 substrate was observed, but cleavage of caspase 8 substrate increased by 3–6-fold in IFN-β-treated WM9 cells (Fig. 5*C*). Treating WM9 cells with caspase 8 or 9 inhibitors decreased caspase 3 activity >70% compared with IFN-β treated cells, confirming the possible upstream roles of caspase 8 and 9 in activating caspase 3.

IFN-β-dependent changes in WM9 cell plasma membrane structure, as measured by Annexin V staining, were inhibited (>70%) by coincubation of cells with IFN-β and various caspase inhibitors (200 μM) such as z-VAD-fmk (general), z-DEVD-fmk

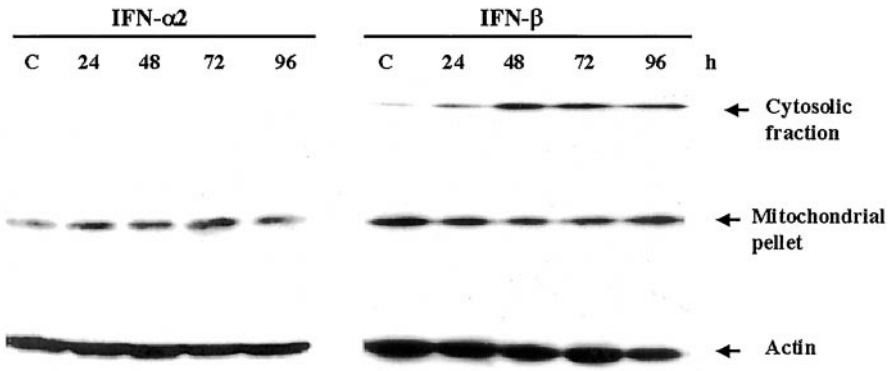

Fig. 4 IFN-β induces cyt *c* release from mitochondria to cytoplasm. WM9 cells were treated with IFN-α2 and IFN-β (500 units/ml) for 0–96 h, and cyt *c* levels were determined in the cellular fractions isolated by differential centrifugation from control- or IFN-treated cells. Immunoblotting with anti-cyt *c* mAb determined the levels of cyt *c* in the *cytosolic* (S100) or the *mitochondrial* (P10) fractions. The S100 blot was reprobed with β-actin mAb to confirm equal protein loading.

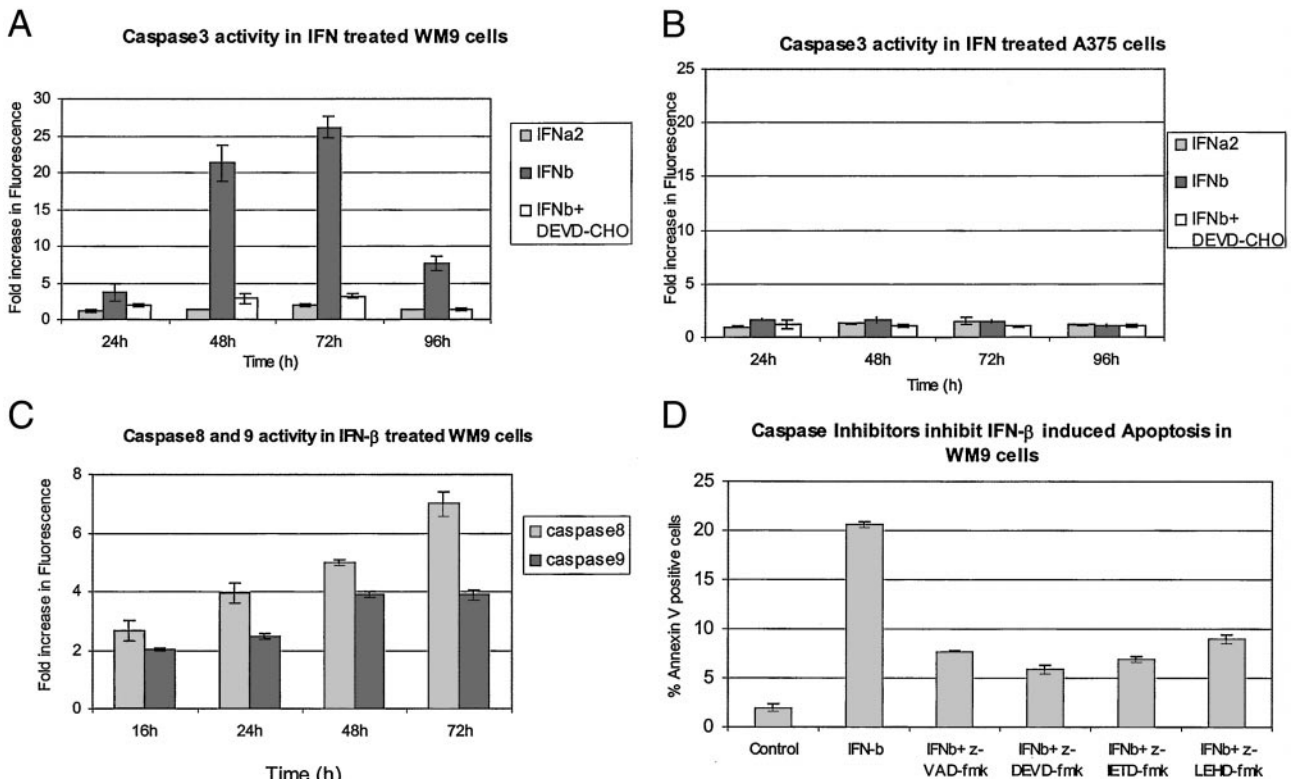

Fig. 5 IFN-β mediates apoptosis by caspase activation. WM9 and A375 cells were treated with IFN-α2 and IFN-β (500 units/ml) for the indicated time periods. Cell lysates containing equivalent amounts of protein were assayed for cleavage of the caspase 3, 8, or 9 peptide substrates, Ac-DEVD-AMC, Ac-IETD-AMC, and Ac-LEHD-AMC, respectively. To confirm that AMC liberation is caspase 3-mediated, cell lysates were incubated with the caspase 3 inhibitor Ac-DEVD-CHO before assay. The caspase enzymatic activity is expressed as the fold increase in fluorescence attributable to AMC liberation, as compared with the untreated cells. Values are the means of three independent experiments with SD <10%. *A*, a time-dependent increase in caspase 3 activity in IFN-β-treated WM9 cells. No changes were observed in IFN-α2-treated cells. *B*, no changes were observed in A375 after IFN-α2 or IFN-β treatments. *C*, time-dependent increase in caspase 8 and 9 activities in IFN-β-treated WM9 cells. *D*, inhibition of caspase-mediated apoptosis in WM9 cells by caspase inhibitors. WM9 cells were either left untreated or treated with IFN-β alone or in combination with various caspase inhibitors [z-*VAD-fmk*, z-*DEVD-fmk*, z-*IETD-fmk*, or z-*LEHD-fmk* (200 μM)] for 72 h with subsequent Annexin V staining. The percentage of Annexin V-positive cells represents the sum of Annexin V-positive, PI-negative, and AnnexinV/PI-double-positive cells.

(caspase3), z-IETD-fmk (caspase 8), and z-LEHD-fmk (caspase 9). Caspase 3 and caspase 8 inhibitors most effectively inhibited IFN-β-mediated Annexin V-positive cells (Fig. 5*D*).

Caspase 3 cleavage also was confirmed by Western blotting of IFN-β-treated cell lysates with an antibody against caspase 3. In WM9 cells, the M_r 32,000 procaspase 3 was partially cleaved to a M_r 17,000 active form as early as 24 h after IFN-β treatment and increased through 48 h (Fig. 6*A*). However, IFN-α2 did not promote caspase 3 cleavage in WM9 cells. In A375 cells, which did not undergo apoptosis, no caspase 3 cleavage

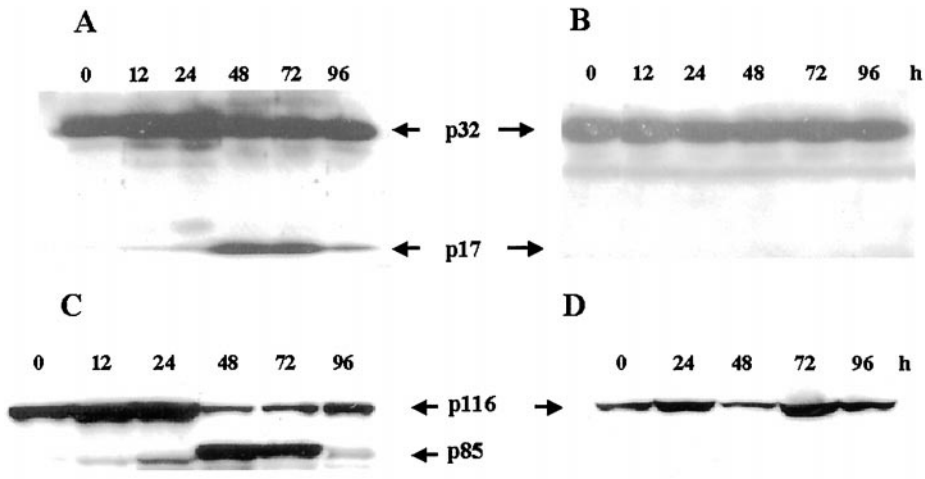

Fig. 6 IFN-β induces cleavage of caspase 3 and PARP. *A*, WM9 and A375 cells were treated with IFN-β (500 units/ml) for increasing periods of time. Whole cell lysates were prepared and subjected to Western blot analysis using caspase 3 polyclonal antibody and PARP mAb and then HRP-conjugated secondary antibodies. Immunoreactive bands were visualized by chemiluminescence. IFN-β-treated WM9 cells show cleavage of caspase 3 (M_r 32,000) to smaller active fragment (M_r 17,000). *B*, absence of caspase 3 cleavage in A375. *C*, IFN-β-induced cleavage of the active PARP (M_r 116,000) to inactive form (M_r 85,000) in WM9 cells after 24, 48, and 72 h. *D*, absence of PARP cleavage in IFN-β-treated A375 cells.

was observed with IFN-β (Fig. 6*B*). Cleavage of the precursor caspase 8 to the active fragments in IFN-β-treated WM9 cells was confirmed by Western blot analysis (data not shown).

Induction of PARP Cleavage by IFN-β. PARP, an enzyme involved in surveillance of genome integrity and DNA repair, is proteolytically cleaved from a M_r 116,000 active form to M_r 85,000 and 25,000 inactive fragments by activated caspase 3. IFN-β-treated A375 and WM9 cell extracts were immunoblotted with antibody against PARP. In WM9 cells, the M_r 85,000 cleaved form could be detected starting at 48 h and peaking at 72 h after treatment (Fig. 6*C*). No PARP cleavage was observed in A375 cells (Fig. 6*D*). These data were consistent with TUNEL results where increased TUNEL-positive cells occurred after 96 h of treatment with IFN-β in WM9 but not in A375 cells.

Induction of TRAIL/Apo2L by IFN-β in Apoptosis-sensitive Melanoma Cells. Once it was established that IFN-β could induce apoptosis, we sought to understand the mechanism by which this process is activated. During these studies, an Affymetrix-based oligonucleotide analysis in WM9 cells revealed preferential induction of TRAIL/Apo2L by IFN-β as compared with IFN-α2.[4] To confirm these results, RNase protection assays were performed using the multiprobe set (hAPO3c; PharMingen) with probes against *caspase 8*, *Fas*, *FasL*, *DcR1*, *DR3*, *DR4*, *TRAIL*, *TNFRp55*, *TRADD*, *RIP*, and two housekeeping genes, *L32* and *GAPDH*. Caspase 8, FasL, TRAIL receptors DR5 and DR4, TNFRp55, and TRADD mRNA were expressed constitutively in both WM9 and A375 melanoma cells. WM9 cells displayed induced expression of TRAIL in response to IFN-β as early as 8 h. Transcript levels were maintained through 60 h in the presence of IFN-β. In contrast, TRAIL was not induced in response to IFN-β in A375 cells at any time (Fig. 7*A*). This correlated with activation of caspase 3 and 8 in WM9 but not in A375 cells.

Time course studies in WM9 cells by Northern blotting, revealed no induction of TRAIL by IFN-α2. However, RT-PCR with subsequent Southern blotting confirmed preferential induction of TRAIL by IFN-β in WM9 cells. Both IFN-α2 and IFN-β induced TRAIL at 2 h and 8 h. However, beyond 16 h, TRAIL mRNA induction was sustained by IFN-β but returned to basal levels in IFN-α2-treated cells (Fig. 7*B*). The *TRAIL* gene is a primary ISG and does not require *de novo* protein synthesis, as confirmed by transcript induction within 2 h of stimulation.

Neutralization of IFN-β-mediated Apoptosis by TRAIL-specific Antibody. To ascertain the role of TRAIL in IFN-β-mediated apoptosis, neutralization experiments were done using human TRAIL mAb (clone M180; Immunex Corp). WM9 cells were treated with IFN-β (500 units/ml) alone, IFN-β + TRAIL mAb (1 μg/ml), or IFN-β + Isotype control mouse IgG (1 μg/ml) for 72 h. Cells were stained with Annexin V/PI and FACS analyzed. IFN-β alone induced Annexin V positivity in 28.9% of cells, compared with 5.8% in untreated control cells. Cotreatment by IFN-β and TRAIL mAb revealed only 6.9% Annexin V-positive cells, whereas isotype control mouse IgG in combination with IFN-β induced apoptosis comparable with IFN-β alone (Fig. 8). Neutralization of IFN-β-mediated apoptosis was also confirmed with DR5-Fc peptide (Alexis Biochemicals). Cotreatment of cells with IFN-β and DR5-Fc resulted in 70% fewer Annexin V-positive cells (7.6%) compared with IFN-β alone (22%; data not shown).

DISCUSSION

Many examples of cell growth arrest by IFNs-α or IFN-β exist, but a limited number of studies have examined induction of apoptosis by IFN-α or IFN-β as single agents. In B cell lines derived from lymphomas and myelomas, IFN-α2 induced apoptosis (14–17).[5] Clinical efficacy of IFN-α2 in chronic myelogenous leukemia may result in part from amplification of

[4] D. W. Leaman, M. Chawla-Sarkar, K. Vyas, A. Ozdemir, and E. C. Borden. Identification of novel interferon (IFN)-stimulated genes in melanoma cell line WM9 by oligonucleotide microarray, manuscript in preparation.

[5] Q. Chen, B. Gong, A. Zhou, E. His, M. Hussein, and A. Almasan. Induction of Apo2L and modulation of Bcl-2-related proteins regulate type I interferon-induced apoptosis in multiple myeloma, submitted for publication.

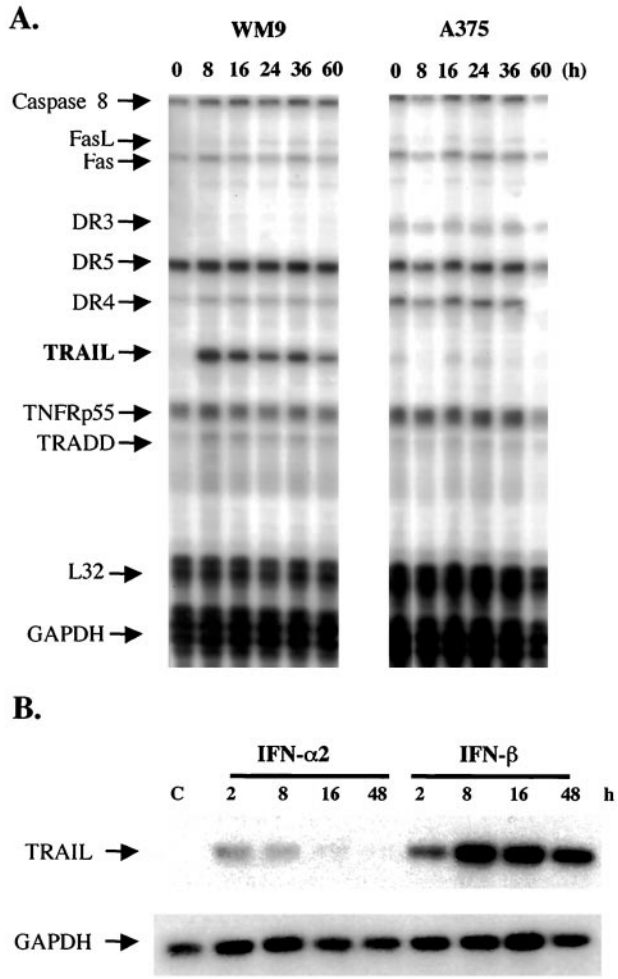

Fig. 7 IFN-β induces TRAIL/Apo2L expression in melanoma cells. Expression of caspase 8-related genes by IFN-β was analyzed by RNase protection assay. *A*, IFN-β induces TRAIL/Apo2L expression in WM9 cells but not in A375. Cells were treated with IFN-β for the indicated time periods, and total RNA was isolated using Trizol (Life Technologies, Inc.). The [^{32}P]UTP-labeled hAPO3c (PharMingen) multiprobe set representing *caspase 8*, *FasL*, TRAIL receptors *DR5* and *DR4*, *TRAIL*, *TNFRp55*, and *TRADD* genes was hybridized with 10 μg of total RNA from each treatment and subjected to a denaturing 6% acrylamide gel. Housekeeping genes *L32* and *GAPDH* were included to demonstrate equal RNA loading. *B*, preferential induction of TRAIL/Apo2L by IFN-β compared with IFN-α. WM9 cells were treated with IFN-α2 and IFN-β for indicated time periods, and total RNA was isolated using Trizol (Life Technologies, Inc.). An equal concentration of RNA was subjected to RT-PCR with TRAIL and GAPDH primers with subsequent Southern blotting. The membrane was hybridized using [^{32}P]CTP-labeled TRAIL and GAPDH-specific probe.

the FasL/FasR-induced apoptosis mechanism (16). The FasL/FasR system may also mediate effects of IFN-α2 in basal cell carcinoma (17). After injection of IFN-α2 into basal cell carcinomas, FasR and apoptosis were induced, and tumors regressed. IFN-α induced apoptosis in different hepatic carcinoma cell lines (18). IFN-β has been implicated in inducing apoptosis in melanoma, colorectal, and non-small cell carcinoma cell lines (19–21). Although mechanistic similarities and differences have not been defined, IFN-α and IFN-β induce apoptosis, possibly in a cell type-selective manner.

Apoptosis results from programmed pathways that eliminate unnecessary, aged, or damaged cells. The process of apoptosis can be subdivided into three different phases: initiation, activation, and protein degradation. The initiation stage depends on the type of apoptotic stimuli, whereas the activation and degradation stages are common among apoptotic processes. These latter stages involve cell shrinkage, chromatin condensation, mitochondrial dysfunction, DNA fragmentation, and selective proteolysis by cysteine proteases called caspases. In probing mechanisms involved in apoptosis with IFNs, this study assessed the stages of activation (or execution) and degradation.

Changes in plasma membrane morphology, evaluated by the increase in exposed phospholipid phosphatidylserine, have been considered to be an early event in apoptosis. Time- and dose-dependent studies in WM9 cells by Annexin V staining confirmed that induction of apoptosis by IFN-β was both a time- and dose-dependent process. An increase in Annexin V-positive cells was apparent after 48 h of treatment, peaking at later time points. Increasing doses of IFN-β (100–1000 units/ml), enhanced the percentage of Annexin V-positive cells. DNA fragmentation, a hallmark for late-stage apoptosis, was analyzed by TUNEL staining. Consistent with Annexin V staining data, WM9 cells had an increased percentage of TUNEL-positive cells after IFN-β treatment. All IFN-β-treated cells (apoptotic or nonapoptotic) shifted toward the right on the *X* axis (increase in total cellular DNA) during FACS analysis compared with control cells. This feature was not observed in IFN-α2 treatments. This shift likely resulted from accumulation of cells with >2 N ploidy DNA content. This endoreduplication property of IFN-β could not be correlated to its apoptotic potential, because both A375 (apoptotic resistant) and Guilliams (weak apoptotic response) exhibited a similar increase in DNA content.

To assess other histologies, different cell lines were treated with IFN-α2 and IFN-β and assessed for apoptosis (Table 1). Cell lines that were more sensitive to antigrowth effects typically underwent apoptosis after IFN-β. However, some cells like Daudi and Jurkats, which were particularly sensitive to the growth inhibitory effects, failed to undergo apoptosis. IFN-α caused G_0/G_1 arrest in Daudi cells (B cell lymphoma) without induction of apoptosis (22). Daudi cells express decoy receptors (DcR1) for TRAIL/Apo2L (data not shown) and may have other defects in the apoptotic cascade.

Once cells are activated or committed into the death pathway, cyt *c* is released from the mitochondria into the cytoplasm where it acts as a cofactor to stimulate the complexing of apoptotic protease activating factor 1 (Apaf1) with caspase 9 (13, 23). This complex initiates activation of the caspase cascade, culminating the proteolytic targeting of key intracellular proteins such as PARP (24). IFN-β, but not IFN-α2, induced the release of cyto *c* from the mitochondria to cytoplasm after 24 h of treatment. This model is supported by activation of caspases 3, 8, and 9 in WM9 cells 24 h after IFN-β treatment. In turn, cyt *c*-activated caspase 9 complex after 48 h of IFN-β may result in >15-fold activation of caspase 3. Thus a feedback loop in IFN-induced apoptosis may exist in WM9 cells. No correlation was observed in melanoma cells between p53 functional status and IFN-mediated apoptosis; WM9 and A375 both had func-

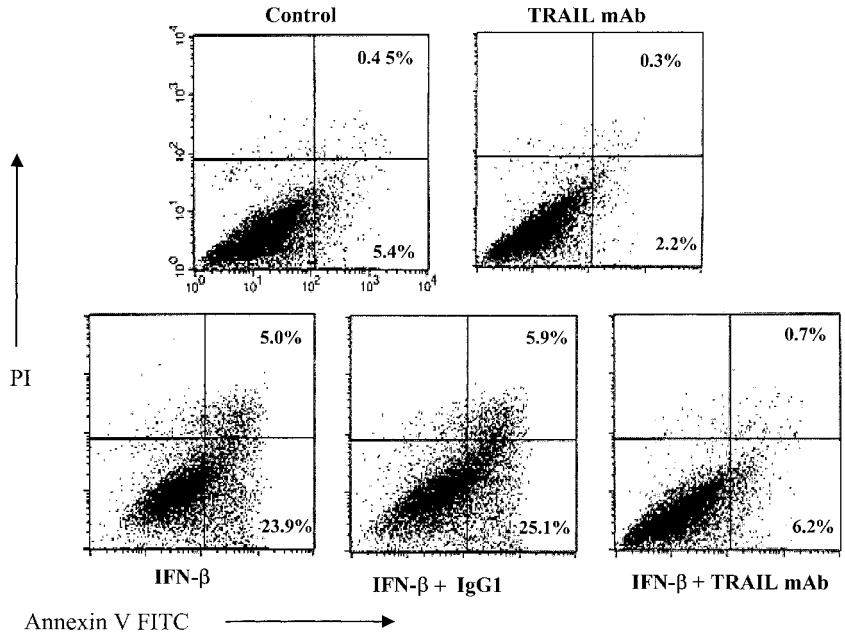

Fig. 8 Neutralization of IFN-β-mediated apoptosis by TRAIL mAb. WM9 cells were left treated or treated with IFN-β (500 units/ml) alone or in combination with TRAIL mAb (1 μg/ml) or Isotype control mouse IgG (1 μg/ml) for 72 h. Cells were stained with Annexin V and PI and subjected to bivariate FACS analysis as described in Fig. 1. The percentage of Annexin V-positive cells and the percentage of Annexin V- and PI-positive are shown in the *bottom right* and *top right* panels, respectively.

tional p53 (data not shown). This is consistent with previous reports on IFN-α-induced apoptosis (14, 18).

Because other known apoptotic agents such as camptothecin, staurosporine, or Fas antibody induce apoptosis within a few hours of treatment, IFN-β may mediate apoptosis by induction of a proapoptotic gene(s) resulting in somewhat slower apoptosis induction. IFNs do induce genes of a wide range of functions that have been associated with apoptosis: *IRF1, 2–5 A synthetase, serine/threonine protein kinase, Mcl-1, FasL, CD95, TNFR1, DAP kinases,* and *TRAIL/Apo2L* (16, 25–28).[5] Several genes that participate in IFN-γ-mediated apoptosis, but which may not be IFN-α2- or -β-induced, have been identified (28–31). TRAIL/Apo2L was increased in IFN-α2- and IFN-β-treated U266 and primary myeloma cells.[5]

Oligonucleotide gene array studies of IFN-α2- and IFN-β-treated WM9 cells showed that TRAIL/Apo2L was induced by both IFN-α2 and IFN-β.[4] In WM9 cells, IFN-β induced TRAIL as early as 2 h, and the transcript persisted through 60 h. In contrast, IFN-β failed to induce TRAIL/Apo2L in apoptotic-resistant A375 cells. Unlike IFN-γ (31), no induction of TRAIL receptor DR5 transcript was observed in both A375 and WM9 cells by IFN-β. When expression of TRAIL was analyzed after IFN-α2 or IFN-β in other sensitive or resistant melanoma cell lines, either early or no induction of TRAIL occurred in response to IFN-α2, whereas IFN-β induced TRAIL in most of the cells tested. These results indicate that TRAIL is an ISG, preferentially induced by IFN-β.

Apoptosis induced by activation of the death receptor ligands is usually rapid (12–24 h). In this study, early induction of TRAIL (8–16 h) by IFN-β was observed, but cells exhibited apoptosis only after 36–40 h of IFN-β treatment. Studies done previously have demonstrated that most TNF family ligands, including TRAIL, are type II transmembrane proteins. They can form active soluble homotrimeric molecules after cleavage by specific proteases (32). Thus, sustained secretion and accumulation of TRAIL protein to biologically relevant concentrations in culture media may explain the delayed apoptotic response by IFN-β.

A functional role for TRAIL in mediating IFN-β-induced apoptosis in WM9 melanoma cells was demonstrated by blocking TRAIL activity. Neutralizing antisera or dominant/negative TRAIL receptor-Fc fusion protein were capable of inhibiting IFN-β-mediated apoptosis. On the basis of this result and the observed kinetics of TRAIL mRNA induction by IFN-β, we postulate that IFN leads to the synthesis of TRAIL protein which, after secretion, acts in an autocrine or paracrine manner to activate death-domain TRAIL receptors (DR4 or DR5) on neighboring melanoma cells, leading to apoptosis. Lack of TRAIL induction by IFN-β in resistant cell lines such as A375, or the transient induction of TRAIL in response to IFN-α2 in all melanoma lines examined, prevents the cells from sustaining an apoptotic response. Other mechanisms of resistance are described below.

Human tumor cells and virus-infected cells have been reported to be sensitive to TRAIL/Apo2L-mediated apoptosis (33–35). Previous studies in melanoma cells showed that melanomas were resistant to apoptosis induced by TNF family members FasL, TNF-α, and CD40L. FasL also was not involved in CD4 T cell-mediated killing of melanoma cells (36). However, TRAIL protein induced apoptosis in various melanoma cell lines, including WM9, within a few hours of treatment. Some melanoma cell lines and also normal melanocytes have been resistant to TRAIL-induced apoptosis (33, 37). Resistance to TRAIL has been attributed to the expression of decoy receptors or to the overexpression of the apoptosis inhibitor Flice (synonym for caspase 8)-inhibitory protein. However no strict correlation between expression of decoy receptors or Flice-inhibitory protein and resistance to TRAIL-induced apoptosis

could be drawn from these studies (33, 37). The $CD4^+$ T cells-mediated killing of melanoma and other target cells was also TRAIL-dependent (27, 36). Additional studies will be needed to elucidate mechanisms for TRAIL sensitivity or resistance in different cells.

Cell growth inhibitory and immunomodulatory effects have received the greatest focus as underlying cellular mechanisms of antitumor action of IFNs (1–3). The antiangiogenic effects of IFNs, presumably resulting in tumor necrosis, have also been identified in studies of preclinical models and clinical regressions of hemangiomas (38, 39). Apoptosis, probably through diverse pathways including FasL and TRAIL, must be added to these potential underlying mechanisms. Specific initiators and executors of apoptosis may be a function of both cell type and the individual IFN family member.

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Clinical Cancer Research

Preferential Induction of Apoptosis by Interferon (IFN)-β Compared with IFN- α2: Correlation with TRAIL/Apo2L Induction in Melanoma Cell Lines

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