

Ectopic and IFN-induced expression of Fas overcomes resistance to Fas-mediated apoptosis in multiple myeloma cells

Lina Y. Dimberg, Anna I. Dimberg, Karolina Ivarsson, Thomas Strömberg, Anders Österborg, Kenneth Nilsson, Fredrik Öberg, and Helena Jernberg Wiklund

Multiple myeloma (MM) is an as-yet incurable B-cell malignancy. Increased survival in vitro is a hallmark of MM cells, implying that a therapeutic potential may lie in circumventing antiapoptotic signals. We have previously reported that interferons (IFNs) sensitize MM cells to Fas/CD95-mediated apoptosis. In the present study, we explore the mechanism underlying this effect. In a wide screening of apoptosis-related genes, Apo2L/TRAIL (tumor necrosis factor [TNF]-related apoptosis inducing ligand) and Fas were identified as IFN targets. Sensitization to Fas-

mediated apoptosis by IFNs was not affected by blocking Apo2L/TRAIL, suggesting that Apo2L/TRAIL is not a key mediator in this process. In contrast, we found that an elevated Fas expression was functionally linked to increased susceptibility to Fas-mediated apoptosis. This was further supported by the finding that IFN treatment enhanced Fas-mediated caspase-8 activation, one of the earliest signaling events downstream receptor activation. In addition, IFN treatment attenuated the interleukin 6 (IL-6)-dependent activation of signal transducer and activa-

tor of transcription 3 (Stat3), interfering with a known survival pathway in MM that has previously been linked with resistance to Fas-mediated apoptosis. Taken together, our results show that IFN-induced up-regulation of Fas sensitizes MM cells to Fas-mediated apoptosis and suggest that attenuation of Stat3 activation may be a potentially important event in this process. (Blood. 2005;106:1346-1354)

© 2005 by The American Society of Hematology

Introduction

Multiple myeloma (MM) is a B-cell malignancy characterized by the accumulation of malignant plasmablasts/plasma cells in the bone marrow. Although recent therapeutic advancements have resulted in improved clinical outcomes and prolonged survival of patients with MM,¹ complete remission is almost never achieved, and the disease is still considered to be incurable. New therapeutic strategies, as well as new insights regarding existing treatments, are therefore imperative.

Because MM can be characterized as a tumor composed of long-surviving rather than fast-growing malignant cells, the therapy efficacy is highly dependent on the possibility of circumventing antiapoptosis signals. It is therefore important to delineate the pathways involved in the control of MM survival and death to identify possible molecular targets of intervention. We have previously reported that MM cells are highly resistant to Fas-mediated apoptosis, but that they may be resensitized to such induction by pretreatment with interferon- α (IFN- α) or IFN- γ .² The benefit of IFNs in MM treatment is under debate due to the diverse response to therapy in different patients,³ as well as reports on adverse effects.⁴⁻⁶ Nevertheless, the greatest therapeutic potential of the IFNs may be achieved in combination with other therapeutic agents. An elucidation of the mechanisms underlying the ability of IFNs to sensitize MM cells for apoptosis may lead to an increased understanding of the nature of the resistance of MM

cells to apoptosis and ultimately contribute to the design of improved strategies in the treatment of MM.

Apoptosis may be triggered by 2 converging pathways (for a review, see Hengartner⁷). Binding of death receptors of the tumor necrosis factor receptor family, including Fas, by the corresponding ligand induces trimerization and receptor activation. This activation enables recruitment of caspase-8 to Fas-associating proteins with death domain (FADD), which induces cleavage and activation of caspase-8, ultimately leading to activation of caspase-3. A parallel pathway, the mitochondrial pathway, involves the activation and deactivation of proapoptotic and antiapoptotic B-cell lymphoma (Bcl-2) family members and converges with the death receptor pathway at the level of caspase activation. Potentially, IFN treatment may target the function or expression of components of these apoptotic pathways.

Both IFNs and interleukin 6 (IL-6), an important survival factor in MM, activate signal transducer and activator of transcription (Stat) proteins, a family of transcription factors that have been associated with regulation of growth and survival of hematopoietic cells.⁸ On ligand binding, Stat proteins are tyrosine phosphorylated by Janus-activated kinases associated with the receptor, heterodimerize or homodimerize, translocate to the nucleus, and activate transcription of specific genes.⁹ Additional transcriptional control is mediated by phosphorylation of a conserved serine residue

From the Department of Genetics and Pathology, Rudbeck Laboratory, Uppsala University, Uppsala, Sweden; and Department of Hematology and Oncology, Karolinska Hospital, Stockholm, Sweden.

Submitted April 8, 2004; accepted April 6, 2005. Prepublished online as *Blood* First Edition Paper, April 28, 2005; DOI 10.1182/blood-2004-04-1322.

Supported by grants from the Swedish Cancer Society, the Multiple Myeloma Research Foundation (MMRF), von Kantzows stiftelse, Magnus Bergwalls stiftelse, and Göran Gustafssons stiftelse.

A.I.D. and L.Y.D. contributed equally to this work as first authors.

The online version of the article contains a data supplement.

Reprints: Helena Jernberg Wiklund, Department of Genetics and Pathology, Rudbeck Laboratory, S-751 85 Uppsala, Sweden; e-mail: helena.jernberg_wiklund@genpat.uu.se.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 U.S.C. section 1734.

© 2005 by The American Society of Hematology

present in Stat1, Stat3, Stat4, Stat5a, and Stat5b.¹⁰ Stat3 is constitutively activated in a number of human cancers¹¹ and has been implicated as a crucial mediator of the prosurvival function of IL-6 in MM cell lines, targeting the antiapoptotic protein Bcl-X_L.^{12,13} In contrast, Stat1, which is activated by IFNs, has been attributed an important role in promoting apoptosis.¹⁴⁻¹⁷ Intriguingly, several reports suggest that Stat3 and Stat1 may counteract the effects of each other.¹⁸⁻²⁰ Of particular interest for MM, Stat1 and Stat3 were shown to exert opposing effects on the transcription of Bcl-2 family members Bcl-2 and Bcl-X_L.²⁰

To determine the mechanism of IFN-induced sensitization to Fas-mediated apoptosis in MM, we investigated the effect of IFNs on the expression of candidate apoptosis-related genes. In a screening to identify IFN-regulated target genes, Apo2L/TRAIL and Fas were found to be transcriptionally regulated by IFNs. Blocking Apo2L/TRAIL had no apparent effect on IFN-induced sensitization of Fas-mediated apoptosis, suggesting that the Apo2L/TRAIL up-regulation may not be directly involved in this process. In contrast, although U-266-1970 cells already express a considerable basal level of Fas, we found that an increased level of Fas expression per se sensitizes U-266-1970 cells to Fas-mediated apoptosis. We also investigated the activity of Stat3 and found that IFNs inhibited the IL-6-induced activation of Stat3, indicating an additional level at which IFNs can affect the survival of MM cells.

Patients, materials, and methods

Cell lines and primary cells

The IL-6-dependent MM cell lines U-266-1970^{21,22} and U-1958²³ were maintained at 37°C in 5% CO₂ in RPMI 1640 medium (Sigma Biosciences, St Louis, MO) supplemented with 10% heat-inactivated fetal bovine serum (Gibco, Grand Island, NY), 2 mM glutamine, and antibiotics (penicillin 100 U/mL, streptomycin 50 µg/mL; Sigma Biosciences). Cells were routinely grown on a layer of the IL-6-producing human fibroblast line AG1523 (Human Mutant Genetic Cell Repository, Camden, NJ) or in medium supplemented with 20 U/mL IL-6 (R&D Systems Europe, Abington, United Kingdom). Experiments were performed on exponentially growing cells, induced by IL-6 (R&D Systems Europe) alone or in combination with IFN-γ (a kind gift from Dr Adolf, Bender, Vienna, Austria) or IFN-α2b (Schering-Plough, Madrid, Spain). Heparinized bone marrow samples were obtained from patients with newly diagnosed MM or relapsed disease. Mononuclear cells were isolated by Ficoll-Paque Plus Density Sedimentation (Amersham Biosciences, Uppsala, Sweden) and subjected to positive selection using magnetic-activated cell sorting (MACS) CD138 MicroBeads and MACS LS separation columns (Miltenyi Biotec, Paris, France). Purified tumor cells (>95% plasma cells as determined by May-Grünwald-Gimsa staining) were seeded at 0.5 to 1 × 10⁶ cells/mL followed by addition of indicated reagents. Analysis of the number of viable cells was performed using the resazurin assay as previously described.²⁴ The study was conducted in accordance with the Helsinki protocol and approved by the local ethics committee. Approval was obtained from the regional ethics committee Dnr 2004:M-332 for these studies. Informed consent was obtained from all patients.

RPA

Cells were harvested and total RNA (5 µg/sample) was isolated using the RNeasy Total RNA Isolation System (Promega, Madison, WI). Ribonuclease protection assays (RPAs) were performed according to the manufacturer of RiboQuant Multi-Probe RNase Protection Assay System (PharMingen, San Diego, CA) using α-[³²P]UTP-labeled (Sigma) probes. Multiprobe template human (h) hAPO-2c, hAPO-3b, hAPO-3c, and hAPO-6 (PharMingen) were used to analyze expression of apoptosis-related genes. Four independent experiments were performed with

U-266-1970 cells and template hAPO-3c and 3 with U-1958. For each template, hAPO-2c, hAPO-6, and hAPO-3b, 2 independent experiments with U-266-1970 were performed. Quantifications were performed on representative RPAs using glyceraldehyde phosphate dehydrogenase (GAPDH) and L32 as internal standards in a Fujix Bio-imaging Analyzer Base 2000 (Fuji, Stockholm, Sweden).

Quantitative real-time PCR

Primary CD138⁺ plasma cells (0.5-1 × 10⁶) from patients with MM were treated as indicated for 24 hours immediately following purification. Total cellular RNA was isolated using TRIzol reagent (Life Technologies, Paisley, United Kingdom). RNA quantity and quality was analyzed using the RNA 6000 Nano Assay, (Agilent Technologies, Waldbronn, Germany). cDNA was prepared by First-Strand cDNA Synthesis using SuperScript III for reverse transcription-polymerase chain reaction (RT-PCR; Invitrogen, Carlsbad, CA). The mRNA expression levels of Fas/Apo1, TRAIL/Apo2L, and β-actin were quantified using TaqMan gene expression "Assays on Demand" (ID Hs00531110 m1, Hs00234355 m1). Quantitative real-time PCR was performed using TaqMan Universal Master Mix. Thermal cycling conditions were 95°C for 10 minutes, 40 times (95°C for 15 seconds, 60°C for 60 seconds). Relative mRNA expression was calculated using the comparative cycle time method.²⁵

Western blotting

Cells were harvested and washed in phosphate-buffered saline (PBS); lysates were prepared and analyzed by Western blot as previously described.²⁴ Primary antibodies used were α-TRAIL (66251A; PharMingen), α-Bcl-X_L (H-62), α-Bcl-2 (C-21), α-actin (I-19), α-Stat1 (C-111), α-Stat3 (C-20; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit polyclonal α-pStat1, rabbit polyclonal α-pStat3 (New England Biolabs, Beverly, MA), α-FLIP (cellular FLICE inhibitory protein) (804-127-C100; Alexis, Laufelfingen, Switzerland), and α-caspase-8 (1C12; Cell Signaling Technology, Beverly, MA).

Analysis of apoptosis and Fas protein expression by flow cytometry

Cells were incubated with 20 U/mL IL-6 alone or in combination with 1000 U/mL IFN-γ or 1000 U/mL IFN-α, agonistic anti-Fas antibody CH-11 (100 ng/mL; Immunotech, Marseilles, France) recombinant human (rh) TRAIL-R1:Fc (Fc-DR4; 0.5 mg/mL; Alexis), isotype-specific control immunoglobulin (Ig) M (X-0942, 100 ng/mL), or recombinant Apo2L/TRAIL²⁶⁻²⁸ (50 ng/mL, a kind gift from Genentech, San Francisco, CA and Immunex, Seattle, WA) as indicated. The percentage of annexin V⁺/propidium iodine-negative (PI⁻) apoptotic cells was measured on a FACScan flow cytometer and analyzed with CellQuest software (Becton Dickinson, San José, CA), using the annexin V, Alexa fluor 647 kit (Molecular Probes, Eugene, OR). Fas expression was determined by flow cytometry, using a primary antibody against Fas (UB2, 10 µg/mL; Immunotech), together with a secondary, fluorescein isothiocyanate (FITC)-conjugated antibody (F-0479, 30 µg/mL; Dako, Glostrup, Denmark) or an allophycocyanin (APC)-labeled secondary antibody (BD Biosciences, Palo Alto, CA).

Caspase-8 activity assay

U-266-1970 cells were incubated with 20 U/mL IL-6 alone or in combination with 1000 U/mL IFN-γ or 1000 U/mL IFN-α for 96 hours and were then either treated with CH-11 (100 ng/mL) or with isotype-specific control IgM (100 ng/mL) for 6 hours. Then, 2.5 × 10⁶ trypan blue-excluding cells from each sample were collected and lysed. Caspase-8 activity was determined using the BD ApoAlert caspase colorimetric assay kit (BD Biosciences). As controls, samples without substrate and samples incubated with a caspase-8 inhibitor (BD Biosciences) were included (data not shown).

Determination of Fas expression in living cells treated with agonistic anti-Fas antibody CH-11

U-266-1970 cells were incubated with IL-6 (20 U/mL) alone or in combination with IFN- γ (1000 U/mL) for 96 hours, followed by a 24-hour incubation with CH-11 (100 ng/mL) or isotype-specific control IgM (100 ng/mL). The Fas expression of annexin V⁻/PI⁻ cells remaining after IFN preincubation followed by CH-11 treatment was determined by flow cytometry as described (see "Analysis of apoptosis and Fas protein expression by flow cytometry").

Sorting of cells according to Fas expression

U-266-1970 cells were preincubated with IL-6 and stained with UB-2 anti-Fas antibody and FITC-conjugated secondary antibody as described. The cells were sorted by flow cytometry according to Fas expression and divided into 3 groups designated "high Fas" (comprising the upper 25%), "low Fas" (the lower 25%), and "all Fas" (excluding only PI⁺ cells). After 96 hours, the cells were treated with CH-11 or IgM overnight. The proportion of apoptotic cells and the Fas expression were determined by flow cytometry as described (see "Analysis of apoptosis and Fas protein expression by flow cytometry").

Plasmids

The pcDNA3.1-CD95 plasmid (designated pcDNA3-Fas) was a kind gift from Dr T. H. Landowski.²⁹ The empty pcDNA3.1 vector was used as a negative control, and pcDNA3.1.EGFP expressing green fluorescent protein (GFP) was used as a marker for transfection.

Transfections

The cells were transfected using the Amaxa Nucleofector apparatus (Amaxa, Cologne, Germany). Briefly, 2×10^6 U-266-1970 cells were grown without antibiotics for 24 hours, washed once in cold PBS, and resuspended in 100 μ L electroporation buffer R with 3 μ g pcDNA3-Fas or empty vector, together with 1 μ g pcDNA3.1.EGFP. The cell suspension was transfected using program U-05. In a separate experiment, pcDNA3-Fas was substituted for pDsRed2-C1 expressing red fluorescent protein (BD Biosciences), enabling us to determine that under these conditions, 65% of the enhanced GFP (EGFP⁺) cells coexpress both the transfected vectors (data not shown). At 24 hours after transfection, the cells were treated with CH-11 or control IgM for the indicated time and subjected to flow cytometry analysis of apoptosis as described (see "Analysis of apoptosis and Fas protein expression by flow cytometry"), gating for EGFP⁺ cells. Fas expression in EGFP⁺ cells was determined by flow cytometry 24 hours after transfection, using the UB2 antibody as described together with an APC-labeled goat antimouse secondary antibody (BD Biosciences).

EMSA

For electrophoretic mobility shift assay (EMSA), nuclear lysates were prepared as described by Andrews and Faller³⁰ and 5 μ g/lane was incubated with ³²P-labeled double stranded m67SIE probe in a DNA-binding reaction (20 mM HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid], pH 7.9, 40 mM KCl, 1 mM MgCl₂, 0.1 mM EGTA [ethylene glycol tetraacetic acid], 10% glycerol, 0.5 mM dithiothreitol, 2 μ g poly(dIdC)) for 30 minutes on ice. To identify specific DNA-binding proteins, nuclear extracts were preincubated with 2 μ g α -Stat3 (C-20X) or α -Stat1 (E-23X) antibodies (Santa Cruz Biotechnology), and the specificity of binding was corroborated by cold probe competition (data not shown). DNA protein complexes were separated by migration on a 6% polyacrylamide gel, and the DNA-binding complexes were visualized by autoradiography.

Statistical analysis

Statistical analysis was done by analysis of variance (ANOVA) followed by multiple comparison by the Fisher method using StatView software (Cary, NC).

Results

Bcl-2 and Bcl-X_L levels are not down-regulated by IFNs in U-266-1970

Expression of antiapoptotic Bcl-2 family proteins, such as Bcl-2 and Bcl-X_L, has previously been associated with apoptosis resistance in MM.^{12,31,32} Interestingly, IFN-induced apoptosis of MM cells has been correlated with Bcl-X_L down-regulation and Bcl-2 cleavage.³¹ To investigate the possibility that IFN treatment sensitizes MM cells to Fas-mediated apoptosis through regulation of Bcl-2 family proteins, we performed an RPA using RNA prepared from IFN-induced U-266-1970 cells and the hAPO-2c template set (Figure 1, Figure S1A). A slight up-regulation of proapoptotic Bax and Bak and antiapoptotic Bcl-X and Mcl-1 mRNA was noted in response to IFN- γ or IFN- α treatment in U-266-1970 cells. The quantification of Bcl-X, Mcl-1, Bak, and Bcl-2 expression in U-266-1970 cells is shown in Figure 1G. No other Bcl-2-related gene included in the template was appreciably (> 2 times) affected (Figure 1A). To further explore this finding, we analyzed the levels of Bcl-X_L and Bcl-2 protein after IFN-stimulation (Figure 2A). Although a slight increase in Bcl-X_L mRNA was noted by RPA analysis, the protein levels of Bcl-X_L and Bcl-2 remained unchanged during IFN treatment in U-266-1970 cells. Additionally, no Bcl-2 cleavage was detected by Western blot.

Turning to other potential targets of IFN, we performed RPAs using multiprobe template sets covering a variety of apoptosis-related genes. Using the hAPO-6 template set, containing among others Harakiri, apoptosis signal-regulating kinase 1, death associated protein (DAP), and DAP kinase, quantification did not detect any substantial (> 2 times) changes in gene expression during IFN-treatment in U-266-1970 cells (Figure 1B, Figure S1B, and data not shown).

A strong up-regulation of TRAIL and a modest up-regulation of Fas are detected after IFN-treatment in U-266-1970 cells and in primary MM cells

Next, we examined the transcriptional regulation of a number of genes that are more directly associated with death-receptor signaling. The template sets hAPO-3b (Figure 1C and Figure S1C) and hAPO-3c (Figure 1D) both contain caspase-8, receptor-interacting protein (RIP), Fas, and FasL. RIP is a proapoptotic protein implicated in caspase-independent pathways during Fas-mediated apoptosis.³³ RIP mRNA expression was transiently increased at early times in response to IFN treatment, but the expression had returned to basal level before 96 hours after induction, when sensitization to Fas-mediated apoptosis is most pronounced (Figure 1G). A moderate up-regulation of Fas mRNA, up to 4 times, was seen in response to both IFN- α and IFN- γ , consistent with our previous observations.² The relative levels of Fas mRNA in U-266-1970 cells as compared to the internal standard mRNA are shown in Figure 1G. This increase was sustained for 96 hours after induction and mirrored by an elevated Fas protein expression on the surface of IFN-treated cells, detected by fluorescence-activated cell sorting (FACS) analysis (Figure 2B). The levels of caspase-8 and FasL mRNA remained unchanged during IFN treatment.

Transcription of c-FLIP, encoding a caspase-8 binding inhibitor of death receptor-induced apoptosis, was slightly up-regulated by IFN- α treatment (Figure 1C,G). The c-FLIP protein is present in 2 splice forms, the long form, FLIP_L, and the short form, FLIP_S. FLIP_S has been suggested to be a more potent apoptosis inhibitor.³⁴

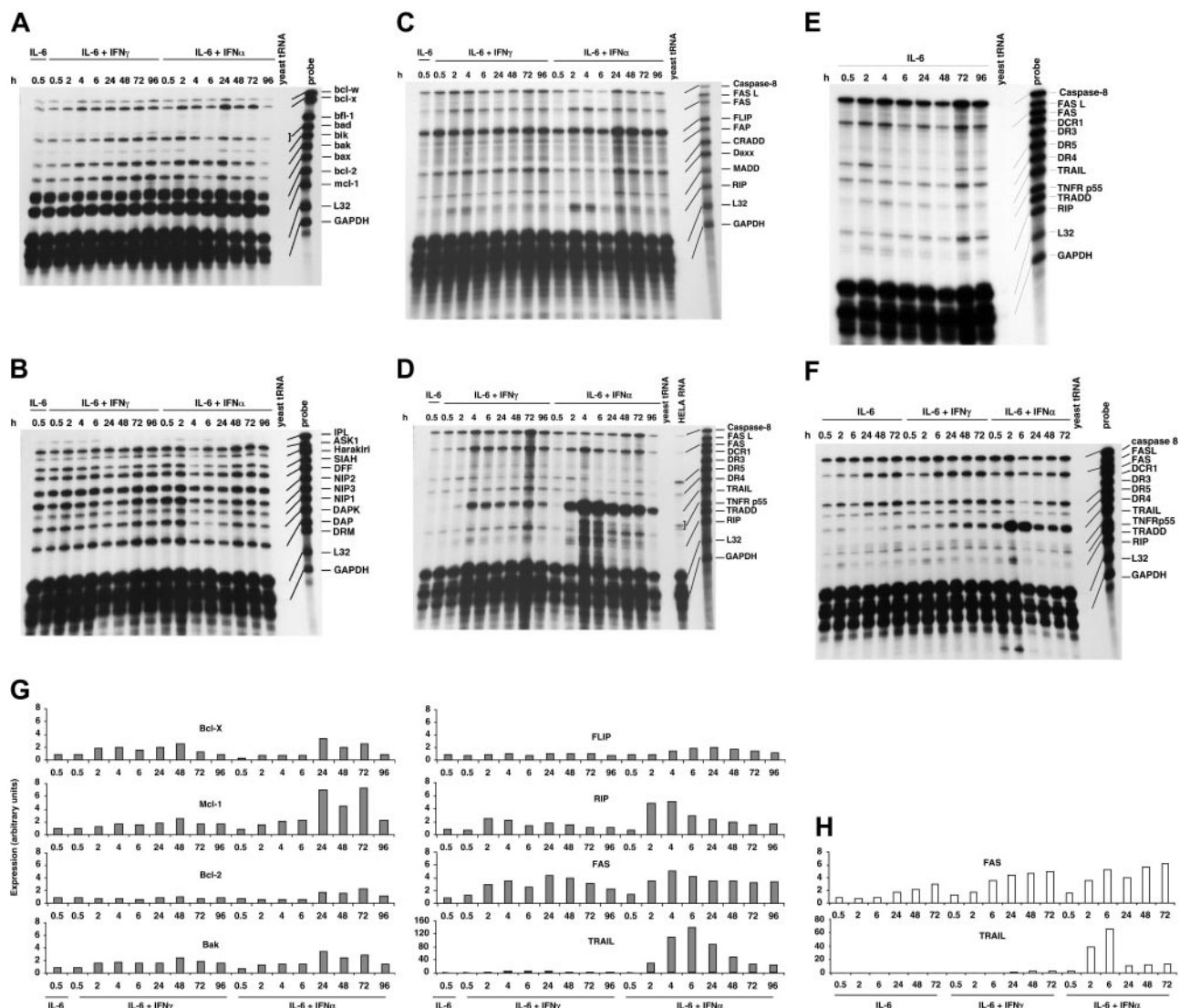


Figure 1. TRAIL and Fas are induced by IFN- α and IFN- γ in MM, whereas Bcl-2 family members remain unchanged. U-266-1970 cells (A-E) or U-1958 cells (F) were treated with IL-6 (20 U/mL) alone or in combination with IFN- α (1000 U/mL) or IFN- γ (5000 U/mL) and were harvested at the indicated time points. Total RNA was isolated and RPA was performed using PharMingen multiprobe templates (A) hApo-2c, (B) hApo-6, (C) hApo-3b, (D) hApo-3c, and (E-F) hApo-3c. Representative experiments are shown, and quantifications using the internal standards GAPDH and L32 are shown in panels G (U-266-1970) and H (U-1958).

Because the RPA probe did not distinguish between these 2 forms, we performed a Western blot to assay alterations in the expression of these forms following IFN treatment (Figure 2A). Whereas FLIP_L levels remained unchanged, FLIP_S was slightly up-regulated by IFNs and, after 72 hours of stimulation, also by IL-6 alone.

Interestingly, the TNF-related apoptosis-inducing ligand (Apo2L/TRAIL) was rapidly and potently up-regulated in response to both IFN- α (> 100 times) and IFN- γ (6 times; Figure 1D,G), while remaining at a fairly constant level in the presence of IL-6 alone (Figure 1E). IFN- α -induced up-regulation of Apo2L/TRAIL was also confirmed on the protein level (Figure 2A). In contrast, the regulation of Apo2L/TRAIL protein in response to IFN- γ was less evident (in agreement with a modest mRNA up-regulation) and could only be detected at longer exposure (data not shown). The expression of Apo2L/TRAIL receptors DR5 and DR4 remained unaffected.

To determine whether the IFN-induced regulation of gene expression in U-266-1970 is a unique property of this particular cell line, we studied the transcriptional response to IFNs in the MM cell line U-1958. These cells share several features with the U-266-1970 cell line, including IL-6 dependence and the ability to be sensitized for Fas-

mediated apoptosis by pretreatment with IFNs.² Using RPA with the multiprobe templates hAPO-2c, hAPO-3b, and hAPO-3c, we found that U-1958 cells respond similarly to IFNs as U-266-1970 cells, exhibiting a modest up-regulation of Fas and a strong up-regulation of Apo2L/TRAIL in response to both IFN- α and IFN- γ , whereas other genes of the apoptotic machinery remained largely unaffected (Figures 1F,H and Figure S2A, B).

To study the regulation of Apo2/TRAIL and Fas mRNA expression in primary MM cells, purified CD138⁺ cells from 11 patients with MM were subjected to treatment with IL-6 alone or in combination with IFN- α for 24 hours. From primary cells of 6 MM patients, total RNA was isolated and quantitative real-time PCR was performed using β -actin as an internal standard. In all primary MM cell samples analyzed, we could confirm the IFN-induced up-regulation of the Apo2L/Trail gene (range 3.5-184 times; Figure 3A) and of the Fas gene (range 2-6.6 times; Figure 3B). Reflecting a previously known heterogeneity of primary MM cells, 4 of 11 responded to IFN- α by a decrease in the number of viable cells as determined using resazurin assay (patients II, IV, V, and VIII; Figure 3C). The sensitizing effect of IFN- α on apoptosis induced by the agonistic anti-Fas antibody CH-11 in the MM

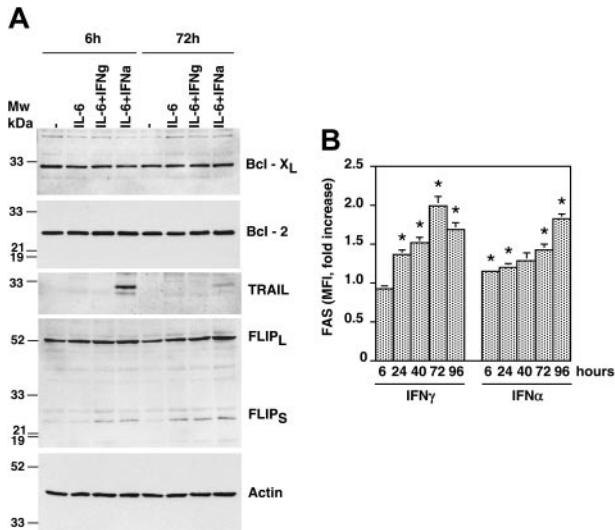


Figure 2. Protein expression analysis of TRAIL, Bcl-2, Bcl-XL, FLIP, and FAS. U-266-1970 cells were treated with IL-6 (20 U/mL) alone or in combination with 1000 U/mL IFN- α or 1000 U/mL IFN- γ for the indicated times. (A) Western blot analysis. The cells were lysed and 20 μ g total protein per lane was separated on NuPAGE 10% Bis-Tris (Bis[2-hydroxyethyl]imino-tris[hydroxymethyl]methane) gel and Western blot analysis was performed using specific antibodies targeting Apo2L/TRAIL, Bcl-2, Bcl-XL, FLIP, and β -actin. Three experiments were performed and one representative Western blot is shown. (B) FACS analysis. FAS surface expression was determined by flow cytometry using a primary antibody against Fas and a FITC-conjugated secondary antibody. The bars represent the fold induction of mean fluorescence intensity (MFI) as compared to cells treated with IL-6 alone. Data are expressed as mean \pm SD, n = 3. The asterisks indicate significant differences to the corresponding control values ($P < .05$).

cell lines could be confirmed in 4 of 11 cases (patients III, V, VII, and VIII; Figure 3C).

The sensitizing effect of IFNs on Fas-mediated apoptosis cannot be blocked by inhibiting TRAIL

IFN- α -induced apoptosis of Fas-resistant MM cells has been suggested to be mediated through induction of Apo2L/TRAIL, followed by an autocrine stimulation of Apo2L/TRAIL receptor signaling.³¹ Therefore, we investigated the functional importance of the up-regulation of Apo2L/TRAIL by IFNs in terms of apoptosis sensitization.

Twenty-four hours of incubation with the agonistic anti-Fas antibody CH-11 resulted in 27% apoptotic cells, whereas pretreatment with IFN- γ for 96 hours before addition of CH-11 increased the percentage of apoptotic cells to 51% (Figure 4). To evaluate if an IFN-induced, autocrine Apo2L/TRAIL loop is involved in IFN-induced sensitization to Fas-mediated apoptosis, we inhibited Apo2L/TRAIL using rhTRAIL-R1:Fc, consisting of the extracellular domain of human TRAIL-R1, which is fused to the Fc portion of human IgG1. Incubation with rhTRAIL-R1:Fc reduced TRAIL-induced apoptosis (26% annexin V⁺/PI⁻) to background levels (11%). In cells pretreated with TRAIL-R1:Fc in combination with IFN- γ , the percentage of apoptotic cells following incubation with CH-11 (53%) was similar to the percentage seen in CH-11-exposed cells pretreated with IFN- γ alone (51%), suggesting that the sensitizing effect of IFNs to Fas-mediated apoptosis is unlikely to be due to Apo2L/TRAIL induction.

IFN-induced sensitization of MM cells to Fas-mediated apoptosis is associated with an increased activation of caspase-8

Apo2L/TRAIL and Fas-mediated signaling pathways converge downstream of caspase-8 activation, eventually leading to apopto-

sis. However, IFN treatment sensitizes MM cells to Fas-mediated apoptosis, but not TRAIL-induced apoptosis (data not shown). Therefore, we reasoned that at least part of the sensitizing effect of IFNs must be associated with Fas-specific signaling events upstream of caspase-8 activation. We analyzed the effect of IFNs on caspase-8 activation by Western blot by comparing the cleavage of caspase-8 in IFN-treated cells following Fas and TRAIL stimulation. Interestingly, we found that IFN treatment enhanced Fas-mediated cleavage of caspase-8, suggesting that IFN-induced sensitization of Fas-mediated apoptosis does indeed occur upstream to caspase-8 activation (Figure 5A). To further monitor caspase-8 activity, we analyzed caspase-8-induced cleavage of Ile-Glu-Thr-Asp-p-nitroanilin (IETD-pNA) using the BD ApoAlert Caspase Colorimetric Assay Kit. In concordance with an increased cleavage of caspase-8, we found that IFN treatment augmented caspase-8 activation following Fas stimulation (Figure 5B). The IFN-induced increase in caspase-8 activation occurred only in response to Fas stimulation, and a similar increase was not evident when cells were treated with IFNs in combination with TRAIL or control IgM. Instead, IFN- α attenuated TRAIL-induced caspase-8 activation (Figure 5A-B).

High Fas expression correlates to increased sensitivity to Fas-mediated apoptosis

Given the specific increase in Fas-mediated caspase-8 activity in response to IFN treatment, we wanted to analyze if IFN-induced sensitization occurred at the level of receptor activation. Fas expression is moderately increased by IFN treatment, whereas a substantial basal expression of Fas is detected on untreated cells (Figure 6A top panel). However, after treatment with the agonistic anti-Fas antibody CH-11, the remaining IFN-treated viable cells express approximately equal levels of Fas on their surface as untreated cells (Figure 6A lower panel). This prompted us to investigate if cells expressing high levels of Fas were more prone to

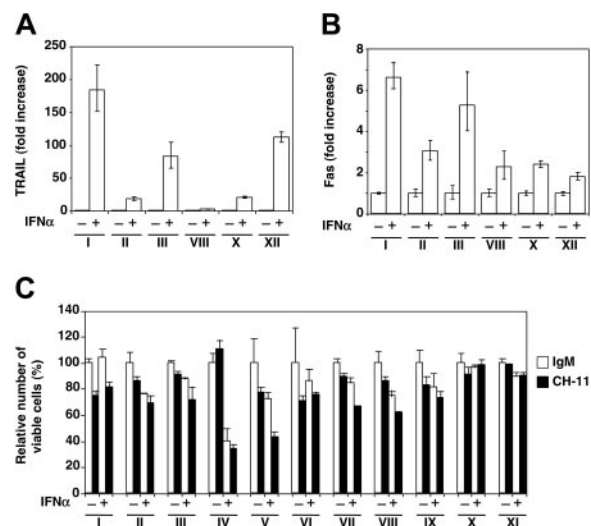


Figure 3. TRAIL and Fas are induced by IFN- α in MM primary cells. Purified CD138⁺ plasma cells from 11 patients with MM were cultured with IL-6 (20 U/mL) alone or in combination with IFN- α (1000 U/mL) for 24 hours. Total RNA was isolated from 6 cases and mRNA expression levels of (A) TRAIL/Apo2L and (B) Fas/Apo1 were quantified using TaqMan Gene Expression "Assays on Demand" (ID Hs00531110 m1, Hs00234355 m1) and β -actin as a reference. (C) Resazurin assay. In parallel cultures preincubation with IFN was followed by a 24-hour incubation with the agonistic anti-Fas antibody CH-11 and subsequent addition of resazurin. After 3 hours of incubation the wells were analyzed using a 96-well microplate fluorometric reader. Results are presented as mean \pm SD (n = 3).

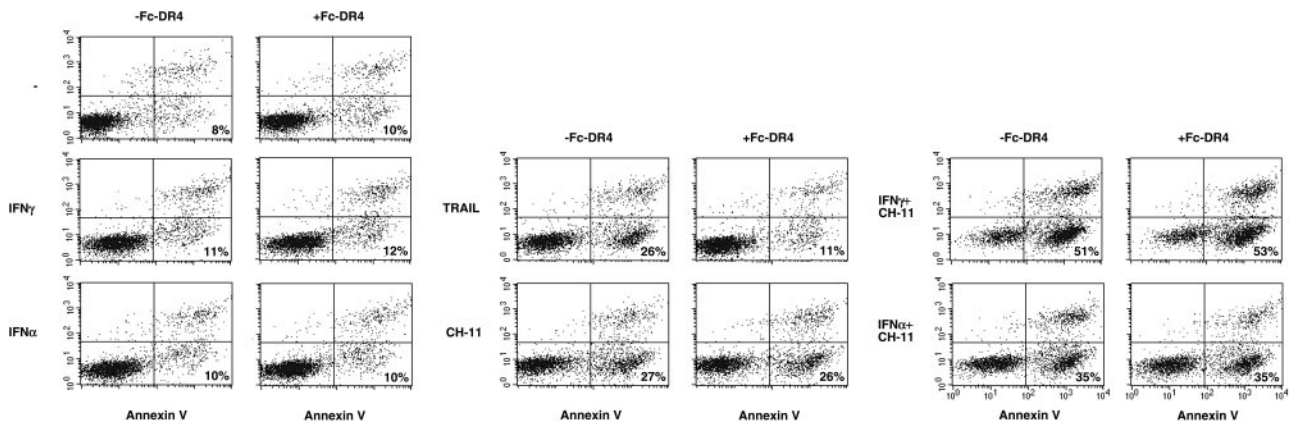


Figure 4. IFN-induced sensitization to Fas-mediated apoptosis is independent of Apo2L/TRAIL. U-266-1970 cells were preincubated with IL-6 (20 U/mL) alone or in combination with IFN- γ (1000 U/mL) or IFN- α (1000 U/mL) for 96 hours, followed by a 24-hour incubation with agonistic anti-Fas antibody CH-11, recombinant soluble Apo2L/TRAIL (TRAIL), or isotype-specific control IgM. In addition, blocking agent rhTRAIL-R1:Fc (Fc-DR4) was continually added during the incubation time in the experiments shown in the right panels. The percentage of annexin V⁺/PI⁻ apoptotic cells was evaluated by flow cytometry as described in "Patients, materials, and methods." Four independent experiments were performed. The percentage number in the lower right quadrants of each graph represents the proportion of annexin V⁺/PI⁻ cells, that is, early apoptotic cells from one representative experiment.

undergo Fas-mediated apoptosis. U-266-1970 cells with a high or low expression of Fas were separated using FACS. After sorting, the cells were allowed to recover for 4 days. The cells were then incubated with CH-11 for 24 hours and an annexin V/PI apoptosis assay was performed (Figure 6B). Interestingly, cells expressing a

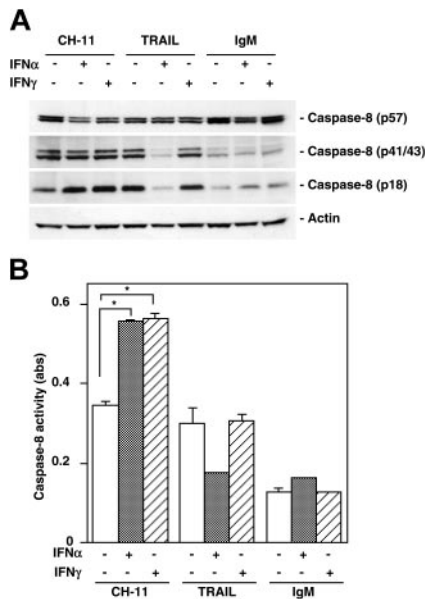


Figure 5. IFN treatment enhances cleavage of caspase-8 induced by the agonistic anti-Fas antibody, but not by TRAIL. (A) Western blot analysis of caspase-8 activity. U-266-1970 cells were treated with IL-6 (20 U/mL) alone or in combination with 1000 U/mL IFN- α or 1000 U/mL IFN- γ for 96 hours, followed by a 6-hour incubation with agonistic anti-Fas antibody CH-11, recombinant soluble Apo2L/TRAIL (TRAIL), or isotype-specific control IgM. Whole cell extracts were prepared and Western blot analysis was performed as described in "Patients, materials, and methods" using specific antibodies against caspase-8 and β -actin. The uncleaved, full-length caspase-8 (p57) and the cleaved and activated forms (p41/p43 and p18) of caspase-8 are indicated. One representative experiment of 3 performed is shown. (B) Colorimetric assay of caspase-8 activity. U-266-1970 cells were treated with IL-6 (20 U/mL) alone or in combination with 1000 U/mL IFN- α or 1000 U/mL IFN- γ for 96 hours, followed by a 6-hour incubation with agonistic anti-Fas antibody CH-11, recombinant soluble Apo2L/TRAIL (TRAIL), or isotype-specific control IgM. The cells were then analyzed using the BD ApoAlert Caspase Colorimetric Assay kit. The bars represent the measured absorbance after 2 hours of incubation with the chromophore-coupled caspase-8 substrate. The graph shows one representative experiment performed in duplicate. Mean \pm SD (n = 2). The asterisks indicate significant differences to the corresponding control values ($P < .05$).

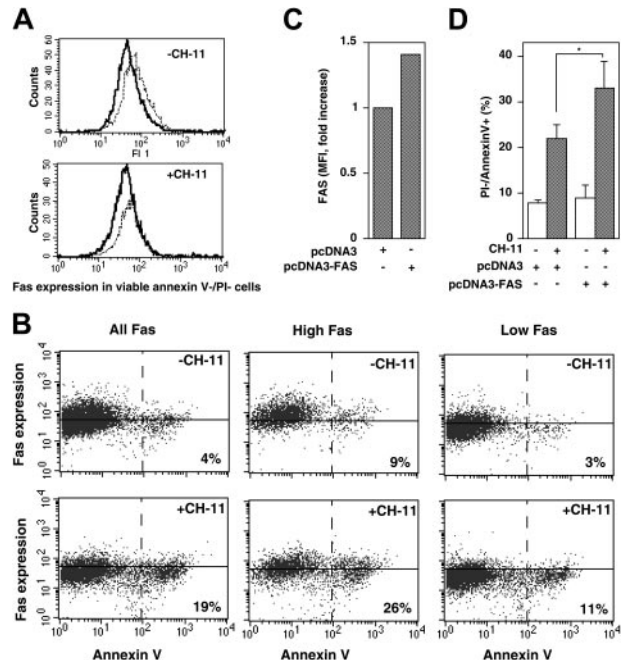


Figure 6. U-266-1970 cells expressing high levels of Fas are more sensitive to Fas-mediated apoptosis. (A) FACS analysis of Fas expression in living cells. U-266-1970 cells were preincubated with IL-6 (20 U/mL) alone (solid line) or in combination with IFN- γ (1000 U/mL; dotted line) for 96 hours as indicated, followed by a 24-hour incubation with agonistic anti-Fas antibody CH-11 or isotype-specific control IgM. The level of Fas expression in annexin V⁻/PI⁻ cells was analyzed in 4 independent experiments using flow cytometry. One representative analysis is shown. (B) Analysis of Fas-mediated apoptosis in cells sorted according to Fas expression. U-266-1970 cells expressing high or low levels of Fas were separated using FACS and allowed to recover for 4 days. The sorted cells were incubated for 24 hours with agonistic anti-Fas antibody CH-11 or isotype-specific control IgM. The graph shows Fas-expression and annexin V expression in PI⁻ cells, evaluated by flow cytometry as described. The horizontal line indicates the MFI of the total population of U-266-1970 cells prior to CH-11 stimulation. Four experiments were performed. The percentage of Fas⁺ cells from one representative experiment is shown. (C) Expression of Fas in transfected cells. U-266-1970 cells were cotransfected with EGFP and either empty pcDNA3 vector or a vector encoding CD95 (pcDNA3-FAS), allowed to recover for 24 hours, and then subjected to FACS analysis of Fas expression as described in "Patients, materials, and methods." The diagram shows the relative expression of Fas in EGFP⁺ transfected cells as fold induction of MFI. (D) Fas-mediated apoptosis in transfected cells. At 24 hours after transfection, cells were stimulated with agonistic anti-Fas antibody CH-11 or isotype-specific control IgM for 24 hours. The proportion of annexin V⁺/PI⁻ cells was determined by flow cytometry as described in "Patients, materials, and methods." The graph shows the percentage of apoptotic cells. Mean values \pm SD (n = 3). The asterisk indicates significant differences to the corresponding control values ($P < .05$).

higher level of Fas were more sensitive to Fas-mediated apoptosis (26% apoptotic cells) than cells expressing a lower level of Fas (11% apoptotic cells).

Increased Fas-expression per se sensitizes MM cells to Fas-mediated apoptosis

These results encouraged us to directly address if a moderate increase in Fas expression, comparable to what is detected after IFN treatment, would sensitize cells to Fas-mediated apoptosis. We transfected U-266-1970 cells with a Fas expression vector or empty vector, together with an EGFP expression vector as a marker for transfection, and compared the ability of transfected cells to undergo Fas-mediated apoptosis. At 24 hours after transfection, the cells were analyzed for Fas expression, left untreated, or incubated with CH-11 for 24 hours. An annexin V/PI apoptosis assay was performed on untreated or CH-11-induced cells, gating for EGFP⁺ cells. Interestingly, the moderate increase in Fas expression in cells cotransfected with pcDNA3-Fas (Figure 6C) was coupled to a significantly increased sensitivity to Fas-mediated apoptosis (33% apoptotic cells) as compared to cells transfected with empty vector (22% apoptotic cells; Figure 6D).

IFN- γ and IFN- α induce Stat1 activation and attenuate IL-6-mediated Stat3 activation in U-266-1970 cells

It has previously been suggested that Stat3, induced by the MM survival factor IL-6, is a crucial mediator of MM survival, including resistance to Fas-mediated apoptosis.¹² Interestingly, Stat3, in a complex with c-Jun, has been shown to negatively regulate Fas expression, whereas Stat1 activation has been associated with increased transcriptional activation of the Fas promoter.^{35,36} To examine the possible role of STAT regulation in IFN-induced sensitization to Fas-mediated apoptosis in MM, we determined the effect of IFN treatment on the expression and activity of Stat1 and Stat3 transcription factors. We stimulated U-266-1970 cells with IFNs for different time periods and analyzed the levels of tyrosine phosphorylated Stat1 and Stat3 proteins. Because U-266-1970 cells are IL-6 dependent, well representing the in vivo situation, we also stimulated the cells with IL-6. As

expected, IFN- γ or IFN- α treatment caused a strong up-regulation of phosphorylated Stat1, and at later times total Stat1, whereas IL-6 treatment resulted in Stat3 phosphorylation (Figure 7A-B). Surprisingly, IL-6-induced Stat3 phosphorylation was markedly attenuated by the simultaneous addition of IFN- γ or IFN- α , evident at later time points (Figure 7A-B). To determine if the changes in Stat phosphorylation were reflected by a change in DNA binding of Stat1 and Stat3 homodimers and heterodimers, we performed an EMSA using an m67SIE probe, containing a STAT-binding site (GAS, γ -activated site). As Figure 7C shows, IL-6 induced formation of Stat3 homodimers, Stat1/Stat3 heterodimers, and, to a lesser extent, Stat1 homodimers. When IL-6 and IFN- γ or IFN- α were administered in combination, the predominance of Stat3 homodimers was attenuated in favor of Stat1/Stat3 and Stat1/Stat1 DNA-binding complexes. An increased ratio of Stat1/Stat3 DNA-binding complexes was maintained by IFN- γ treatment for 24 hours after induction, whereas IFN- α treatment had a less pronounced effect.

Discussion

IFNs have the potential to influence growth and survival in MM, on their own, or in synergy with other agents. Delineating the pathways involved in IFN stimulation may lead to an increased understanding of MM tumor development and to definition of possible targets for therapeutic intervention. We have previously shown that IFNs can sensitize U-266-1970 MM cells for Fas-mediated apoptosis.² In the current study, we explored the mechanism of this effect and the downstream effectors involved. In a wide screening for IFN-induced, apoptosis-associated genes, Apo2L/TRAIL and Fas were identified. These genes were also up-regulated in primary MM cells stimulated with IFN. We could not find any evidence supporting an involvement of Apo2L/TRAIL in the sensitization to apoptosis mediated by Fas. In contrast, an elevated level of Fas expression was associated with an increased susceptibility to Fas-mediated apoptosis. This finding was emphasized by the observation that MM cells could be sensitized to

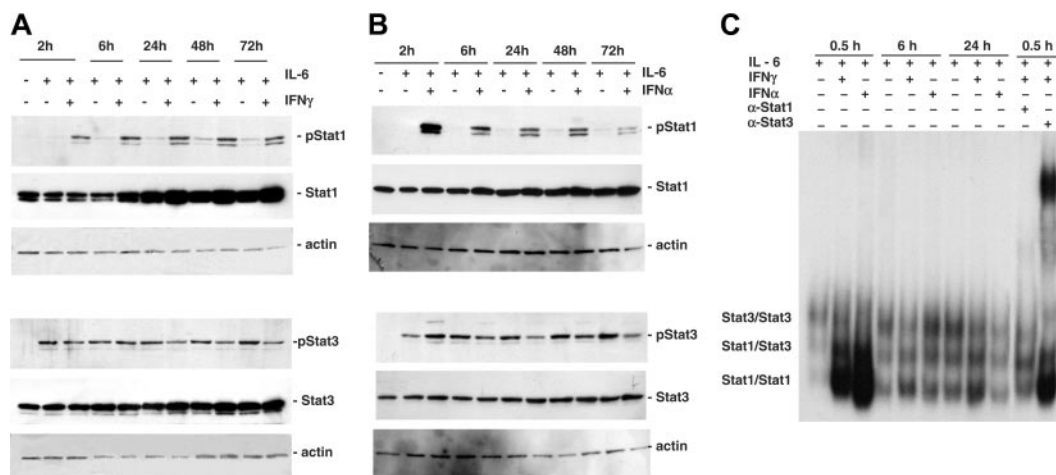


Figure 7. IFNs induce Stat1 phosphorylation, attenuate Stat3 phosphorylation, and repress IL-6-induced DNA binding of Stat3 in favor of Stat1. (A-B) U-266-1970 cells were either treated with IL-6 (20 U/mL) alone or in combination with IFN- γ (1000 U/mL; A) or IFN- α (1000 U/mL; B). At the indicated time points, the cells were harvested and protein extracts were prepared and analyzed by Western blot using antibodies specific for Tyr701 phosphorylated Stat1, Tyr705 phosphorylated Stat3, Stat1, Stat3, and β -actin. (C) U-266-1970 cells were treated with IL-6 (20 U/mL) alone or in combination with IFN- γ (1000 U/mL) or IFN- α (1000 U/mL) for the indicated time points. Nuclear extracts were prepared and DNA-binding complexes were analyzed by EMSA, as described in "Patients, materials, and methods," using a ³²P-labeled m67SIE probe, containing a GAS site. The positions of Stat3/Stat3, Stat1/Stat3 and Stat1/Stat1 DNA-binding complexes are indicated by the supershift (Stat3) and reduction of the complexes (Stat1) as a result of the addition of specific antibodies (lanes 10 and 11). The experiments in 7A-C were performed 3 times.

Fas-mediated apoptosis through Fas transfection alone. However, our data also suggest that IFNs may regulate additional targets regulating survival of these cells and that some of these potential targets may be activated downstream Stat1 or Stat3 signaling events.

Apo2L/TRAIL has been shown to induce apoptosis of MM cell lines and freshly isolated MM cells and has also been suggested to mediate IFN- α -induced apoptosis of U-266-1984 cells.³¹ In addition, Apo2L/TRAIL is highly homologous to the Fas ligand and the apoptosis pathways used by Apo2L/TRAIL and FasL share multiple downstream components, suggesting a plausible basis for synergy between these 2 systems. However, preincubation of the Apo2L/TRAIL-blocking agent rhTRAIL-R1:Fc in combination with IFN had no apparent effect on the susceptibility to Fas-mediated apoptosis, whereas Apo2L/TRAIL-induced apoptosis was efficiently blocked. Although we cannot exclude the possibility that internal loops of Apo2L/TRAIL, not accessible by external blocking, may be active, our data provide no evidence for an involvement of Apo2L/TRAIL in IFN-induced sensitization to Fas-mediated apoptosis. Intriguingly, IFNs had no effect on the susceptibility to Apo2L/TRAIL-induced apoptosis, suggesting that the targets of sensitization may be narrowed down to those uniquely found in Fas-mediated apoptosis. This is further supported by the finding that IFN treatment increases Fas-mediated caspase-8 activation, an early signaling event directly downstream of receptor trimerization.

Our results indicate that an up-regulation of a specific part of the Fas death pathway, namely, the receptor itself, is an underlying mechanism in IFN-induced sensitization to Fas-mediated apoptosis. The observed IFN-induced Fas up-regulation in U-266-1970 cells does not reflect an increased percentage of Fas-expressing cells but rather an increased overall expression.² The relevance of an increased Fas expression in these cells is not obvious. The relatively abundant basal expression of Fas on the surface of U-266-1970 is not reflected in a high degree of sensitivity to Fas-mediated apoptosis, which would imply that the amount of Fas is unlikely to be a limiting factor. Furthermore, many studies have pointed out the lack of correlation between the Fas expression in myeloma cell lines and their respective sensitivity to Fas-mediated apoptosis.³⁷⁻³⁹ Nevertheless, our data show that an increased basal expression of Fas, either in a subpopulation of U-266-1970 cells or in Fas-transfected cells, is indeed associated with a higher sensitivity to Fas-mediated apoptosis in U-266-1970 cells. This suggests that the IFN-induced shift to a more sensitive phenotype is mechanistically linked to an up-regulation of Fas. This is in

accordance with a previous study by Shain et al, showing that clonal variability in Fas expression determines sensitivity to Fas-mediated apoptosis in RPMI 8226 MM cells.⁴⁰ However, the increase in Fas-mediated apoptosis observed when Fas was ectopically expressed was, although clearly significant, less dramatic than that observed after IFN treatment, implying that the sensitization by IFNs may involve additional mechanisms. It should be noted that the cotransfection efficiency obtained under these conditions is 65%. Therefore, the enhanced apoptosis sensitivity due to ectopic Fas expression is likely to be underestimated.

The finding that IFN treatment, in addition to activating Stat1, attenuated Stat3 activation in MM cells is, to our knowledge, completely new. The relevance of this finding is emphasized by the newly established role of Stat3 as a crucial prosurvival mediator in many cancers.¹¹ In MM, IL-6-induced activation of Stat3 has been associated with resistance to apoptosis, including Fas-mediated apoptosis.¹² Conversely, inhibition of Stat3 by the JAK-inhibitor AG490, and, to a lesser extent, expression of dominant-negative Stat3, sensitizes U-266-1984 MM cells to Fas-mediated apoptosis.¹² In contrast, the role of Stat1 in MM survival has not been elucidated. Stat1 activation has been shown to influence apoptosis in many systems by transcriptional regulation of proapoptotic genes, including several caspases.^{14,15,41} Importantly, Fas can be positively regulated by Stat1-dependent mechanisms in response to IFN treatment,³⁵ whereas Stat3, in complex with c-Jun, suppresses Fas transcription.³⁶ Interestingly, it has been suggested that the balance between Stat1 and Stat3 could influence survival by exerting opposing effects on the expression of the very same gene, including the Bcl-2 gene and the Bcl-X_L gene.²⁰ Our results imply that in MM, IFN-induced activation of Stat1 may, directly or indirectly, counteract Stat3 activation and thus overcome resistance to Fas-mediated apoptosis by shifting the balance from activation of prosurvival genes in favor of proapoptotic ones. However, aside from Fas, these target genes have yet to be elucidated. Studies investigating the precise role of STAT regulation in MM apoptosis resistance are currently underway.

Acknowledgments

We thank Pernilla Martinsson, Charlotta Sandberg, and Sevinc Koyuncu for excellent technical assistance, and Dr T. H. Landowski, Genentech, and Immunex for kindly providing DNA constructs and reagents.

References

- Boccadoro M, Palumbo A. New developments and treatment in multiple myeloma: new drugs in the treatment of multiple myeloma. *Ann Oncol*. 2002;13(suppl 4):55-58.
- Spets H, Georgii-Hemming P, Siljason J, Nilsson K, Jernberg-Wiklund H. Fas/APO-1 (CD95)-mediated apoptosis is activated by interferon-gamma and interferon-alpha in interleukin-6 (IL-6)-dependent and IL-6-independent multiple myeloma cell lines. *Blood*. 1998;92:2914-2923.
- Shustik C. Interferon in the treatment of multiple myeloma. *Cancer Control*. 1998;5:226-234.
- Blade J, Lopez-Guillermo A, Tassies D, Montserrat E, Rozman C. Development of aggressive plasma cell leukaemia under interferon-alpha therapy. *Br J Haematol*. 1991;79:523-525.
- Tu KL, Bowyer J, Schofield K, Harding S. Severe interferon associated retinopathy. *Br J Ophthalmol*. 2003;87:247-248.
- Stricker RB, Barlogie B, Kiproff DD. Acquired factor VIII inhibitor associated with chronic interferon-alpha therapy. *J Rheumatol*. 1994;21:350-352.
- Hengartner MO. The biochemistry of apoptosis. *Nature*. 2000;407:770-776.
- Smithgall TE, Briggs SD, Schreiner S, Lerner EC, Cheng H, Wilson MB. Control of myeloid differentiation and survival by Stats. *Oncogene*. 2000;19:2612-2618.
- Schindler C, Darnell JE Jr. Transcriptional responses to polypeptide ligands: the JAK-STAT pathway. *Annu Rev Biochem*. 1995;64:621-651.
- Decker T, Kovarik P. Serine phosphorylation of STATs. *Oncogene*. 2000;19:2628-2637.
- Bowman T, Garcia R, Turkson J, Jove R. STATs in oncogenesis. *Oncogene*. 2000;19:2474-2488.
- Catlett-Falcone R, Landowski TH, Oshiro MM, et al. Constitutive activation of Stat3 signaling confers resistance to apoptosis in human U266 myeloma cells. *Immunity*. 1999;10:105-115.
- Alas S, Bonavida B. Inhibition of constitutive STAT3 activity sensitizes resistant non-Hodgkin's lymphoma and multiple myeloma to chemotherapeutic drug-mediated apoptosis. *Clin Cancer Res*. 2003;9:316-326.
- Chin YE, Kitagawa M, Kuida K, Flavell RA, Fu XY. Activation of the STAT signaling pathway can cause expression of caspase 1 and apoptosis. *Mol Cell Biol*. 1997;17:5328-5337.
- Kumar A, Commame M, Flickinger TW, Horvath CM, Stark GR. Defective TNF-alpha-induced apoptosis in STAT1-null cells due to low constitutive levels of caspases. *Science*. 1997;278:1630-1632.
- Lee KY, Anderson E, Madani K, Rosen GD. Loss of STAT1 expression confers resistance to IFN-gamma-induced apoptosis in ME180 cells. *FEBS Lett*. 1999;459:323-326.

17. Stephanou A, Brar BK, Scarabelli TM, et al. Ischemia-induced STAT-1 expression and activation play a critical role in cardiomyocyte apoptosis. *J Biol Chem*. 2000;275:10002-10008.
18. Shen Y, Devgan G, Darnell JE Jr, Bromberg JF. Constitutively activated Stat3 protects fibroblasts from serum withdrawal and UV-induced apoptosis and antagonizes the proapoptotic effects of activated Stat1. *Proc Natl Acad Sci U S A*. 2001;98:1543-1548.
19. Hong F, Jaruga B, Kim WH, et al. Opposing roles of STAT1 and STAT3 in T cell-mediated hepatitis: regulation by SOCS. *J Clin Invest*. 2002;110:1503-1513.
20. Stephanou A, Brar BK, Knight RA, Latchman DS. Opposing actions of STAT-1 and STAT-3 on the Bcl-2 and Bcl-x promoters. *Cell Death Differ*. 2000;7:329-330.
21. Nilsson K, Bennich H, Johansson SG, Ponten J. Established immunoglobulin producing myeloma (IgE) and lymphoblastoid (IgG) cell lines from an IgE myeloma patient. *Clin Exp Immunol*. 1970;7:477-489.
22. Jernberg-Wiklund H, Pettersson M, Carlsson M, Nilsson K. Increase in interleukin 6 (IL-6) and IL-6 receptor expression in a human multiple myeloma cell line, U-266, during long-term in vitro culture and the development of a possible autocrine IL-6 loop. *Leukemia*. 1992;6:310-318.
23. Jernberg H, Nilsson K, Zech L, Lutz D, Nowotny H, Scheirer W. Establishment and phenotypic characterization of three new human myeloma cell lines (U-1957, U-1958, and U-1996). *Blood*. 1987;69:1605-1612.
24. Stromberg T, Dimberg A, Hammarberg A, et al. Rapamycin sensitizes multiple myeloma cells to apoptosis induced by dexamethasone. *Blood*. 2004;103:3138-3147.
25. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-delta delta C(T)) method. *Methods*. 2001;25:402-408.
26. Wiley SR, Schooley K, Smolak PJ, et al. Identification and characterization of a new member of the TNF family that induces apoptosis. *Immunity*. 1995;3:673-682.
27. Ashkenazi A, Pai RC, Fong S, et al. Safety and antitumor activity of recombinant soluble Apo2 ligand. *J Clin Invest*. 1999;104:155-162.
28. Pitti RM, Marsters SA, Ruppert S, Donahue CJ, Moore A, Ashkenazi A. Induction of apoptosis by Apo-2 ligand, a new member of the tumor necrosis factor cytokine family. *J Biol Chem*. 1996;271:12687-12690.
29. Landowski TH, Shain KH, Oshiro MM, Buyuksal I, Painter JS, Dalton WS. Myeloma cells selected for resistance to CD95-mediated apoptosis are not cross-resistant to cytotoxic drugs: evidence for independent mechanisms of caspase activation. *Blood*. 1999;94:265-274.
30. Andrews NC, Faller DV. A rapid micropreparation technique for extraction of DNA-binding proteins from limiting numbers of mammalian cells. *Nucleic Acids Res*. 1991;19:2499.
31. Chen Q, Gong B, Mahmoud-Ahmed AS, et al. Apo2L/TRAIL and Bcl-2-related proteins regulate type I interferon-induced apoptosis in multiple myeloma. *Blood*. 2001;98:2183-2192.
32. Jourdan M, De Vos J, Mechti N, Klein B. Regulation of Bcl-2-family proteins in myeloma cells by three myeloma survival factors: interleukin-6, interferon-alpha and insulin-like growth factor 1. *Cell Death Differ*. 2000;7:1244-1252.
33. Holler N, Zaru R, Micheau O, et al. Fas triggers an alternative, caspase-8-independent cell death pathway using the kinase RIP as effector molecule. *Nat Immunol*. 2000;1:489-495.
34. Bin L, Li X, Xu LG, Shu HB. The short splice form of Casper/c-FLIP is a major cellular inhibitor of TRAIL-induced apoptosis. *FEBS Lett*. 2002;510:37-40.
35. Xu X, Fu XY, Plate J, Chong AS. IFN-gamma induces cell growth inhibition by Fas-mediated apoptosis: requirement of STAT1 protein for up-regulation of Fas and FasL expression. *Cancer Res*. 1998;58:2832-2837.
36. Ivanov VN, Bhoumik A, Krasilnikov M, et al. Cooperation between STAT3 and c-jun suppresses Fas transcription. *Mol Cell*. 2001;7:517-528.
37. Shima Y, Nishimoto N, Ogata A, Fujii Y, Yoshizaki K, Kishimoto T. Myeloma cells express Fas antigen/APO-1 (CD95) but only some are sensitive to anti-Fas antibody resulting in apoptosis. *Blood*. 1995;85:757-764.
38. Westendorf JJ, Lammert JM, Jelinek DF. Expression and function of Fas (APO-1/CD95) in patient myeloma cells and myeloma cell lines. *Blood*. 1995;85:3566-3576.
39. Hata H, Matsuzaki H, Takeya M, et al. Expression of Fas/Apo-1 (CD95) and apoptosis in tumor cells from patients with plasma cell disorders. *Blood*. 1995;86:1939-1945.
40. Shain KH, Landowski TH, Buyuksal I, Cantor AB, Dalton WS. Clonal variability in CD95 expression is the major determinant in Fas-mediated, but not chemotherapy-mediated apoptosis in the RPMI 8226 multiple myeloma cell line. *Leukemia*. 2000;14:830-840.
41. Fulda S, Debatin KM. IFN-gamma sensitizes for apoptosis by upregulating caspase-8 expression through the Stat1 pathway. *Oncogene*. 2002;21:2295-2308.