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Type I interferons activate apoptosis in a Jurkat cell variant by caspase-dependent and independent mechanisms

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Abstract

Although the antiviral actions of interferons (IFNs) are observed in most types of cells, the antiproliferative effects of IFN α/β are variable as are the mechanisms of growth inhibition that may or may not be due to the induction of apoptosis. To understand more about the mechanisms that are responsible for IFN α/β -stimulated apoptosis, we have characterized a new human Jurkat T cell variant named H123 where IFN α activates programmed cell death (PCD). No differences in IFN α -stimulated, Stat-dependent gene expression were detected between H123 cells and the parental Jurkat cells, which are growth inhibited, but do not undergo apoptosis with IFN α . Although IFN α stimulates the activity of both caspase 3 and 9 in H123 cells, the general caspase inhibitor Z-VAD only partially reverses the apoptotic actions of IFN α . Induction of apoptosis by IFN α occurs through a mitochondrial-dependent pathway in H123 cells, as demonstrated by the release of cytochrome C from the mitochondria. Furthermore, IFN α treatment of H123 cells stimulates the release of the serine protease HtrA2/Omi from the mitochondria, suggesting that it plays a role in the apoptotic actions of this cytokine. These results provide evidence for a novel type 1 IFN-mediated pathway that regulates apoptosis of T cells through a mitochondrial-dependent and caspase-dependent and independent pathway.

Keywords

Type; One; Interferone; Signaling; Jak/Skat; Apoptosis

1. Introduction

The antiviral, antigrowth, and immunomodulatory activities of interferons (IFNs) are controlled, in part, by a set of cellular genes which are rapidly induced upon type 1 (IFN α/β) or type 2 (IFN γ) binding to specific cell surface receptors. Type 1 IFNs consist of several subtypes of IFN α , a single IFN β as well as IFN τ and IFN ω . In most cellular systems, the actions of IFN α and IFN β are generally the same although IFN β has been shown to be more potent and elicit IFN β -dependent gene activation at lower concentrations than IFN α in several cell types. Interferon stimulated gene factors (ISGFs) mediate gene induction by binding to enhancers within the promoters of both IFN α/β - and IFN γ -induced genes [1,2]. Isolation of

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the promoters of several IFN α/β -stimulated genes permitted the mapping of an enhancer termed the interferon-stimulated response element (ISRE), which was demonstrated to be necessary and sufficient for activation of these genes [3,4]. Several genes have also been characterized whose expression is rapidly increased after incubation of cells with IFN γ . The promoter regions of these genes contain an element referred to as the gamma activation sequence (GAS) or gamma response elements (GRR) [5,6]. Latent cytoplasmic transcription factors called Stats are required for the expression of early response genes by IFNs. Stats are covalently modified by tyrosine phosphorylation performed by the Jak family of tyrosine kinases [7], and subsequently translocate to the nucleus where they interact with enhancers needed for interferon-stimulated gene expression. The information gained concerning IFN signaling has now been extended to include a broad network of cytokine-regulated signaling systems that use tyrosine phosphorylation of a family of structurally related Stat proteins to activate transcription of early response genes.

Type 1 IFNs are inhibitors of cell growth, and are presently used for the treatment of chronic myelogenous leukemia and other malignancies such as melanomas and renal cell carcinomas. The variety of mechanisms by which IFN α/β inhibits cell growth has not, for the most part, been correlated with activation of the Jak/Stat pathway. Although it is clear that IFN α/β activation of the Jak/Stat pathway is required for its antiproliferative effects [8], the mechanisms by which these cytokines inhibit cell proliferation vary. For instance, treatment of the Daudi Burkitt lymphoma B cell line with IFN α/β results in cell cycle arrest at the G0/G1 phase, while in the Jurkat T cell line, IFN α induces a slowing of the cell cycle without inducing cell cycle arrest or apoptosis [9]. Daudi cells also do not undergo apoptosis after treatment with IFN α/β ; however, they show a suppression of the DNA-binding activity of the E2F transcription factor as well as decreased levels of phosphorylation of the retinoblastoma protein [10,11].

A variety of hematopoietic derived T and B cell lines, certain melanomas, and other cell lines derived from solid tumors have been reported to undergo apoptosis when incubated with type 1 IFNs [12–18]. The best described system to examine IFN α/β -stimulated apoptosis of primary cells is with murine, IL-7-dependent bone marrow derived proB cells. These cells exhibit inhibition of cell growth as well as apoptosis when incubated with IFN α/β . Similar apoptotic effects have been seen in several IL-7-dependent murine pro-B cell lines treated with IFN α/β [18]. Two reports using animal models confirm in vitro studies concerning the ability of IFN α/β to stimulate apoptosis in pro-B cells. IFN α/β treatment of newborn mice inhibits the development of both T and B cell populations, and infection of mice with lymphocytic choriomeningitis virus (LCMV) causes a transient bone marrow aplasia due to the production of type 1 IFNs [19,20]. In both of these reports, the actions of either IFN α/β or infection of mice with LCMV did not occur in mice lacking the IFNAR1 subunit of the type 1 IFN receptor. It remains to be determined whether under these conditions type 1 IFNs inhibit cell proliferation by inducing apoptosis. The inhibition of cell growth in primary murine B cells does not require the expression of Stat1 but appears to require IFN α/β -stimulated expression and translocation of Daxx [21].

In most cells where type 1 IFNs induce apoptosis, it has not been elucidated whether the actions of IFN α/β are mediated through the intrinsic or extrinsic apoptotic pathways. Some reports suggest that both pathways contribute to IFN α/β -induced programmed cell death [12,13]. It remains unclear as to the role caspase activation plays in the apoptotic actions of these cytokines. To gain a better understanding of the molecular events that govern the apoptotic effects of IFN α/β , we have taken advantage of a Jurkat cell variant called H123. These cells undergo massive apoptosis when incubated with IFN α/β while the parental Jurkat cells exhibit an antigrowth response, but do not undergo apoptosis under the same conditions. Comparison

of the H123 and parental Jurkat cells has allowed us to define a previously uncharacterized set of events that play an important role in the apoptotic actions of these cytokines.

2. Materials and methods

2.1. Cell culture, transfection and reagents

The human leukemic T-cell line Jurkat, (subclone E6) and the H123 Jurkat variant line were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), containing 2 mM L-glutamine, penicillin and streptomycin (Invitrogen Corp.) at 37 °C and 5% CO₂. H123 cells were obtained from parental Jurkat mutagenized with ICR-191 and stable cell lines deficient in Ca²⁺ signaling were isolated as described [22]. Stable lines of H123 cells expressing CrmA and dominant negative FADD or overexpressing Bcl-2 were generated by transfection with Superfect reagent (Qiagen). Cells were maintained in the presence of 500 µg/ml of G418. Transient transfections with H123 cells were performed by electroporation according to the manufacturer's recommendations (BIORAD). A mixture of 5 µg of pEGFP-C1 plasmid (Clontech) with either 20 µg of pCEFL empty or HA-tagged cIAP1 plasmid cDNA, (a gift from Charles Tannenbaum, Cleveland Clinic Foundation, OH) was added to a total volume of 150 µl of cells followed by electroporation at 140 V, 1000 µF. Murine IL-7-dependent bone marrow derived B cells were isolated from 129 Sv mice as described [23]. Recombinant human IFNα-2a was a gift from Hoffman-LaRoche, N.J. Murine IFNβ was obtained from BiogenIdec [24].

2.2. Antibodies and chemical reagents

Anti-cytochrome C and anti-Fas monoclonal antibody were purchased from Pharmingen and used at 1:500 dilution and 100 ng/ml, respectively. Rabbit antiserum to tubulin was obtained from Santa Cruz Biotechnology and monoclonal actin antibody was purchased from Abcam. These antibodies were used at 1:1000 and 1:5000 dilution, respectively. Anti-Sp1-2/CrMA monoclonal ascites was kindly provided by Richard Moyer and used at 1:500 dilution. Anti-Flag monoclonal antibody and staurosporine were obtained from Sigma. Anti-PARP and anti-HA monoclonal antibodies were purchased from Biomol and Covance, respectively. The HtrA2 inhibitor, ucf101, was generously provided by Dr. Antonis Zervos (University of Central Florida).

2.3. Preparation of whole cell and cytoplasmic extracts

Following treatment of cells with IFNα, cytosolic fractions were generated using a digitonin-based subcellular fractionation technique as described previously [25]. Briefly, 1 × 10⁷ cells were harvested by centrifugation at 1000 rpm and washed in PBS pH 7.2, and re-pelleted. Cells were digitonin-permeabilized for 5 min on ice at a density of 1 × 10⁷/ml in cytosolic extraction buffer (250 mM sucrose, 70 mM KCl, 137 mM NaCl, 4.3 mM Na₂HPO₄, 1.4 mM KH₂PO₄, pH 7.2, 100 µM PMSF, 200 µg/ml digitonin, 10 µg/ml leupeptin, and 2 µg/ml aprotinin). Plasma membrane permeabilization of cells was verified by staining the sample with 0.2% trypan blue. Cells were centrifuged at 1000 rpm for min at 4 °C. The supernatant fractions were collected and saved as cytosolic extracts. For whole-cell extracts, cells were lysed in whole-cell extraction buffer (20 mM Hepes, pH 7.5, 300 mM NaCl, 10 mM KCl, 1 mM MgCl₂, 20% glycerol, 1% NP-40, 10 mM β-glycerophosphate, 1 mM sodium orthovanadate, 25 mM NaF, 200 µM PMSF, 0.5 mM dithiothreitol). After centrifugation at 4 °C for 10 min, supernatants were collected. Protein concentration was measured by the Bio-Rad/Bradford protein assay.

2.4. Immunoblot analysis

Proteins were separated on 8% SDS-PAGE gels and transferred to PVDF membranes (Millipore). Membranes were immunoblotted with the indicated antibodies. Immunoblots were

developed using horseradish peroxidase conjugated secondary antibodies (Zymed) and visualized with ECL reagents (Amersham-Pharmacia).

2.5. RNase protection assay

Cells were left untreated or stimulated with IFN α for the indicated times. Total cellular RNA was isolated using RNazol B (TEL-TEST Inc.). Antisense RNA probes were synthesized by in vitro transcription using T7 or SP6 RNA polymerase (GIBCO-BRL) and [α - 32 P]-UTP (ICN) [26]. 10 μ g of RNA and 32 P-labeled riboprobes were incubated overnight in hybridization buffer containing 80% formamide, 40 mM PIPES pH 6.7, 400 mM NaCl and 1 mM EDTA at 56 °C, followed by digestion with T1 RNase (GIBCO-BRL) for 1 h at 37 °C, phenol extraction and ethanol precipitation. Protected RNA fragments were solubilized in RNA loading buffer (98% formamide, 10 mM EDTA (pH 8.0), bromophenol blue and xylene cyanole), boiled for 2 min, and resolved by electrophoresis on 4.5% polyacrylamideurea gels.

2.6. Measurement of apoptosis by annexin V staining

Cells were left untreated or stimulated with IFN α for various times at 37 °C. Cells were collected, washed in PBS and resuspended in 100 μ l of binding buffer (10 mM HEPES pH 7.4, 140 mM NaCl and 2.5 mM CaCl $_2$). Cells were then incubated with 5 μ l of annexin V-FITC (Pharmingen, Palo Alto, CA) and 5 μ l of propidium iodide (50 μ g/ml) (Sigma) for 10 min at room temperature in the dark, followed by addition of 400 μ l of binding buffer. Cells were collected ungated (10,000 events) and analyzed by two-color flow cytometry using a FACScanTM (Becton-Dickinson). Measurement of apoptosis in H123 cells transiently expressing cIAP1 was performed by staining cells with annexin V-PE and gating cells based on their GFP expression. Data were further analyzed using CellQuestTM (Becton-Dickinson) to discriminate between apoptotic versus necrotic cells.

2.7. Measurement of mitochondrial transmembrane potential ($\Delta\psi_m$)

Mitochondrial transmembrane potential was measured using the fluorescent dye JC-1 (Molecular Probes Inc., Eugene, OR). JC-1 was used because it provides more accurate estimates of $\Delta\psi_m$ than other fluorescent dyes. Briefly, cells were treated with IFN α as indicated and cells were stained with 2.5 μ M of JC-1 resuspended in PBS for 20 min at 37 °C, washed, and immediately analyzed by flow cytometry. A loss in membrane mitochondrial potential is indicated by a decrease in red/green fluorescence intensity ratio leading to an increase in FL1 (green) fluorescence.

2.8. Measurement of caspase activity

Following stimulation with IFN α , cell extracts were prepared as described above. Caspase-3 activity was assayed in a 150 μ l reaction mixture with a caspase-3 specific fluorogenic substrate peptide (Ac-DEVD-AMC) from Pharmingen, or with a caspase-9 fluorogenic substrate peptide (Ac-LEHD-AMC) from Biomol. The substrate peptide (100 μ M) was incubated with 20 μ g of cell extract in either caspase-3 buffer (20 mM Hepes pH 7.5, 10% glycerol, 2 mM DTT) or caspase 9-buffer (100 mM Hepes, pH 7.5, 20% sucrose, 1% Triton X-100 and 10 mM DTT,) at 37 °C for 2 h. Fluorescence was measured using a Cytofluor 2350 spectrofluorometer plate reader with an excitation of 380 nm and an emission wavelength of 460 nm (Millipore).

3. Results

As a consequence of our previous observation that the antigrowth actions of IFN α in Jurkat cells required the expression of ZAP-70, Lck, and CD45 [9], we screened a series of Jurkat cell variants that were mutagenized with the frameshift mutagen ICR191 and isolated cells displaying a defect in T-cell receptor signaling. To our surprise, one of the variants (H123)

showed a dramatic decrease in cell growth when incubated in the presence of IFN α (Fig. 1A). In this experiment, cells were treated with 1000 U/ml of IFN α for 72 h and then counted to determine percent growth inhibition. The parental Jurkat cells showed approximately 35% fewer cells after incubation with IFN α compared to untreated cells. In contrast, IFN α caused a dramatic reduction of 90% with H123 cells. Similar results were obtained when we assayed the antigrowth effect of IFN α by measuring ^3H thymidine incorporation in these cells (data not shown).

Analysis of the doubling time of parental and H123 Jurkat cells showed that the rate of growth of both lines was approximately the same (data not shown). However, it was evident by visual microscopic examination that the number of viable H123 cells was significantly decreased after incubation with IFN α . To determine whether IFN α treatment of H123 cells was stimulating programmed cell death (PCD) or apoptosis, cells were analyzed for annexin V staining. Both untreated parental and H123 Jurkat cells showed low levels (1–4%) of annexin V positive cells (Fig. 1B and C). No significant increase in the numbers of apoptotic cells were detected in parental Jurkat cells incubated with IFN α (Fig 1B). However, IFN α induced apoptosis in H123 cells as early as 18 h after the addition of the cytokine (data not shown). Moreover, prolonged treatment with IFN α for 24, 48, or 72 h showed a time-dependent increase in apoptotic cells (Fig. 1C). Following 72 h of IFN α treatment, greater than 95% of the H123 cells were apoptotic. These results were confirmed by TUNEL assays (data not shown). The half maximal dose of IFN α required for apoptosis in H123 cells was 100 units/ml (data not shown). It should be noted that IFN β also induced PCD in H123 cells. IFN γ , which activates the Jak/Stat pathway, did not inhibit cell growth or induced apoptosis in either parental or H123 cells, while cyclic AMP inhibited the growth of both cell lines in the absence of programmed cell death (data not shown). Interestingly, both parental and H123 Jurkat cells underwent apoptosis when anti-CD3 crosslinking was used to activate TCR signaling. We do not presently know whether the defect in TCR signaling in the H123 cells is linked with the mutation that permits IFN α to stimulate apoptosis in these cells. The fact that several components critical for TCR signaling are also required for the antiproliferative actions of IFN α [9] makes it possible that the defect is in common.

3.1. IFN α activation of the Jak/Stat pathway is not altered in H123 cells

IFN α stimulation of early response genes is regulated by tyrosine phosphorylation of the transcription factors Stat1 and Stat2 which permit these proteins to translocate to the nucleus and bind to either interferon-stimulated response elements (ISREs) or gamma response elements (GRR or GAS) located in the promoters of IFN-responsive genes [27,28]. We determined whether the induction of apoptosis in H123 cells was due to an alteration in the IFN α -dependent activation of the Jak/Stat pathway compared to the parental Jurkat cells. Incubation of either parental or H123 Jurkat cells with IFN α induced similar activation of Stat1 and Stat2 as assayed by the formation of the ISGF3 complex or tyrosine phosphorylation of Stat1 as assayed by formation of a GRR binding complex (data not shown). Maximal activation of IFN-induced gene expression requires phosphorylation of serine 727 of Stat1 [29,30]. Since serine phosphorylation of Stat1 does not alter its ability to bind ISRE or GRR elements, or to be tyrosine phosphorylated [29], we next determined whether there were any differences in IFN α induced RNAs between these Jurkat lines. Total RNA was isolated from cells, which had been incubated with (1000 units/ml), or without IFN α for various times and RNase protection assays were performed to determine the expression of RNAs whose activation requires an ISRE (ISG15, ISG54, and ISG56) or a GAS element (GBP) (Fig. 2). IFN α induction of all of these RNAs in H123 Jurkat cells was not different when compared to parental cells. Using gene chip experiments we also did not detect any differences in IFN α -stimulated RNAs in parental cells compared to H123 cells (data not shown). Therefore, in these lines the Jak/

Stat pathway is intact from activation of tyrosine phosphorylation of the Stat proteins, through induced expression of ISRE- and GRR-dependent RNAs.

3.2. The role of caspases in IFN α -stimulated programmed cell death

Activation of the caspase pathway triggers apoptosis of cells. Based on our above observations, we determined whether IFN α -stimulated apoptosis of H123 cells resulted in the activation of caspases (Fig. 3). Parental or H123 Jurkat cells were incubated with IFN α for various times prior to preparing cell extracts. Extracts were assayed for caspase-3 activity using Ac-DEVD-AMC, a fluorogenic substrate that is specifically cleaved by this enzyme. Incubation of H123 cells with IFN α for as little as 6 h induced an increase in caspase-3 activity compared to untreated cells (Fig. 3A). The activity of the enzyme continued to increase up to 24 h, and correlated with cleavage of PARP, a caspase-3 endogenous substrate (Fig. 3B). As expected from the annexin V staining of parental Jurkat cells (see Fig. 1), there was no detectable IFN α -stimulated increase in caspase-3 activity in these cells. Using a specific fluorogenic substrate for caspase-9, IFN α also stimulated its activity with similar kinetics to that observed for caspase-3 in H123 cells (Fig. 3C).

To determine the requirement of caspase activation in IFN α -stimulated apoptosis in H123 cells, experiments were performed to see whether the pan-caspase inhibitor peptides Z-Asp, or Z-VAD or the caspase 3-specific inhibitor Z-DEVD, blocked the apoptotic actions of IFN α (Fig. 4A). Cells were incubated in the presence or absence of IFN α for 18 h prior to staining for annexin V. IFN α induced apoptosis in approximately 25% of the H123 cells, which was inhibited if the cells were pretreated with Z-VAD (50 μ M), Z-Asp (5 μ M) or Z-DEVD (5 μ M) (lanes 3, 4, and 5, respectively). The inactive analogue of Z-FA, did not significantly inhibit the apoptotic actions of IFN α (lane 6). These results indicated that IFN α -stimulated apoptosis of H123 cells that was observed at 18 h required one or several activated caspases since the general caspase inhibitor Z-VAD or Z-Asp, or Z-DEVD, a more specific inhibitor of the caspase-3 family of proteases, blocked IFN α -stimulated apoptosis. To our surprise, when H123 cells were examined after 42 h of incubation with IFN α , Z-VAD was less effective in preventing PCD triggered by this cytokine (Fig. 4B). One possible explanation for this observation might be that Z-VAD was inactivated after 42 h in culture. However, this appeared not to be the case since incubation of H123 cells with Z-VAD for 36 h followed by the addition of anti-Fas antibody for 6 h completely prevented the apoptotic action of the antibody. Addition of Z-VAD inhibitor every 12 h did not augment protection of H123 cells from undergoing apoptosis with IFN α treatment (data not shown) while anti-Fas-induced apoptosis of these cells was prevented in the presence of Z-VAD for up to 48 h (Fig. 4C). Furthermore, we could detect a significant reduction in caspase-3 activity in extracts prepared from H123 cells incubated with IFN α for 48 h (data not shown) even though caspase-3 activity was maximally detected 24 h following addition of IFN α (Fig. 3A). These results suggest that IFN α -stimulated apoptosis in H123 cells is initially caspase-mediated but after extended periods of incubation with IFN α , activation of caspases is not required for apoptosis. It is interesting that IFN β -stimulated apoptosis of primary murine IL-7-dependent pro B cells also is insensitive to addition of Z-VAD even though caspase-3 is activated (Fig. 4D and data not shown). These results indicate that the ability of these cytokines to induce PCD in several systems does not rely solely on the activation of caspases even though they are activated.

3.3. IFN α -stimulated apoptosis is driven through a mitochondrial-dependent pathway

We have been able to detect IFN α -stimulated activation of caspase-9, but not caspase-8 in H123 cells suggesting that a mitochondrial-dependent (intrinsic) pathway is being used by type 1 IFNs to exert their apoptotic effects. To study this possibility in more detail, we generated three different H123 cell lines that express either CrmA, a dominant negative FADD protein, or overexpress Bcl-2. The expression of either CrmA or dominant negative FADD has been

documented to inhibit death receptor pathways that require activation of caspase-8. We examined both IFN α and Fas receptor stimulated apoptosis in these cell lines by annexin V staining (Fig. 5A). While the ability of anti-Fas to mediate apoptosis in H123 cells expressing CrmA or DN FADD was inhibited, IFN α -stimulated apoptosis was not prevented in any of the cell lines (Fig. 5A). These results confirm that caspase-8 activation is not essential for IFN α -stimulated PCD in H123 cells, and validates our inability to demonstrate IFN α -dependent activation of caspase-8 in these cells.

We also measured apoptosis in individual H123 clones overexpressing Bcl-2 after treatment with IFN α for 72 h (Fig. 5B). Approximately 85% of H123 cells or those transfected with a vector only control, were apoptotic in the presence of IFN α . In contrast, the four clones that express elevated amounts of Bcl-2 (see immunoblot), showed no IFN α -stimulated PCD (Fig. 5B) or anti-Fas-induced PCD (Fig. 5C). To examine whether clones that overexpress Bcl-2 displayed differences with regard to the growth inhibitory effects of IFN α , cells were directly counted after being cultured 72 h in the presence or absence of this cytokine (Fig. 5D). As expected, parental Jurkat cells contained about 35% fewer cells, and the H123 variant had about 85% fewer cells after being exposed to IFN α . Those H123 lines that overexpressed Bcl-2 responded to IFN α in a similar manner to the parental Jurkat cells showing a 30–45% decrease in cell number when exposed to IFN α .

To directly measure mitochondrial transmembrane potential we incubated both parental and H123 cells in the presence or absence of IFN α for 24 or 48 h followed by incubation with the fluorescent dye JC-1 (Fig. 5E). Fluorescence was analyzed by flow cytometry. A loss of mitochondrial membrane potential results in an increase in green fluorescence. Treatment of H123 cells but not parental Jurkat cells with IFN α for as little as 24 h results in an increase in green fluorescence. These findings highlight the importance of the mitochondrial-dependent pathway in IFN α -stimulated PCD in H123 cells. They also confirm that the antigrowth effects of IFN α can be uncoupled from its apoptotic effects.

Since a mitochondrial-dependent pathway involving activation of caspase-9 is required for the initial apoptotic response in H123 cells, one might predict that IFN α would also stimulate the release of cytochrome *c* from mitochondria in these cells. To examine this possibility, H123 and parental cells were incubated with IFN α for various times, and cytosolic fractions were analyzed for the presence of cytochrome *c* by Western blot analysis. Jurkat cells incubated with staurosporine (Sts) were included as a positive control for the release of cytochrome *c*. Incubation of H123, but not parental Jurkat cells, with IFN α stimulated the accumulation of cytosolic cytochrome *c* that was detectable within 18 h (Fig. 6A). The blots were probed for α -tubulin to ensure equivalent protein loading (lower panel). Cytochrome *c* release can either occur via caspase-8-mediated cleavage of BID or by a caspase-8-independent, mitochondrial-dependent mechanism. Caspase inhibitors such as Z-VAD will block the release of cytochrome *c* under conditions where caspase-8 is activated, but not under conditions where caspase-8 is not a component of the cascade. To determine whether IFN α -stimulated release of cytochrome *c* required the activation of caspase-8, H123 cells were incubated with Z-VAD in the presence of IFN α or anti-Fas antibody, a known activator of caspase-8. While Z-VAD inhibited Fas receptor-mediated release of cytochrome *c*, it had no effect on the ability of IFN α to stimulate its release (Fig. 6B) further confirming the lack of a role of caspase-8 in the IFN α -induced apoptotic response.

Although apoptosis of H123 cells after 18 h of IFN α exposure appears to be dependent upon the activation of caspases, at later times, the apoptotic response appears to be caspase-independent. Several proteins have been implicated in mediating apoptosis in a caspase-independent manner including the serine protease HtrA2/Omi [31–34]. We decided to examine this protease since it is located in the mitochondria, released under conditions of stress, and

induces PCD even in the presence of caspase inhibitors. It also appears to function by binding to the inhibitor of apoptosis, XIAP, relieving inhibition of caspase-9 [33,35,36]. H123 cells were incubated with or without IFN α for 24 or 36 h and cytosolic extracts were probed for HtrA2 (Fig. 7A). Incubation with IFN α stimulated the release of HtrA2 into the cytosol of H123 cells. The expression of the protein in the cytosol was detectable after 24 h of IFN α treatment, and very prominent after 36 h. Consistent with its potential role as a mediator of the apoptotic actions of IFN α , the appearance of HtrA2 in the cytosol was not prevented by pretreatment with the caspase inhibitor Z-VAD. Furthermore, we were unable to detect any IFN α induced appearance of HtrA2 in the cytosol of parental Jurkat cells treated with (Fig. 7B), and only very little in H123 cells that overexpress Bcl-2, which do not undergo PCD when incubated with IFN α (Fig. 7C). As shown previously, incubation of cells with anti-Fas antibody also induces the release of HtrA2 from mitochondria [34] and this also occurs in H123 cells, but not when they overexpress Bcl-2. As expected, anti-Fas, but not IFN α stimulated release of HtrA2 is prevented by Z-VAD (Fig. 7B).

To test the potential role of IFN α -stimulated HtrA2 release from the mitochondria of H123 cells in the apoptotic actions of this cytokine, cells were incubated with a specific inhibitor of HtrA2 activity (ucf-101) [31]. IFN α -stimulated apoptosis was measured by annexin V staining 24 h later (Fig. 7D). Incubation of cells with ucf-101 reversed the apoptotic actions of IFN α about 30% compared to cells incubated with only IFN α . Because the effects of the HtrA2 inhibitor were not that dramatic, H123 cells were transiently transfected with cIAP1. cIAP1 is a substrate for HtrA2 and can block its activity when overexpressed in cells [32]. H123 cells were transiently cotransfected with cDNA expression vectors that encode cIAP1 and GFP or with GFP and an empty vector. The next day, cells were incubated with or without IFN α for 30 h and cells that were GFP positive were measured for annexin V by FACS analysis (Fig. 7E). Cells expressing cIAP1 showed about a 40% decrease in IFN α stimulated PCD compared to those only transfected with GFP. These results are similar to those in which the ucf-101 inhibitor was added to H123 cells to block the activity of HtrA2 (Fig. 7D). Taken together, these independent assays are consistent with a role of HtrA2 release from mitochondria in mediating at least part of the apoptotic actions of IFN α .

4. Discussion

IFNs are major regulators of cell growth and antiviral activity. They communicate a variety of cell-specific signals that can result in several outcomes, including cell cycle arrest and apoptosis. To date, it remains unclear what are the molecular mechanisms that dictate whether type I IFNs promote apoptosis in some, but not all tumor cell lines. In order to identify cells where the apoptotic actions of these cytokines are dramatic, we have focused our investigations on H123 cells, a Jurkat cell variant that harbors a defect in the T-cell receptor signaling pathway. To our surprise, these cells show a dramatic decrease in cell growth due to the induction of apoptosis when incubated in the presence of IFN α (Fig. 1). Examination of the mechanisms by which IFN α treatment of H123 cells induces apoptosis has provided several new insights as to how these cytokines may function to induce PCD. Activation of the Jak/Stat pathway is a critical signaling event that may be required for IFN α / β -stimulated apoptosis. We have recently isolated some H123 revertants that do not undergo PCD when incubated with IFN α . These revertants do not express Jak1 or Stat2, suggesting that activation of the Jak/Stat pathway is required but not sufficient for type I IFNs to induce apoptosis [37] and data not shown. Results shown here indicate that expression of all of the IFN α -activated early response genes we examined were not altered in H123 cells compared with parental Jurkat cells, suggesting that this signaling cascade is not sufficient for IFN α -stimulated apoptosis. Alternatively, the protein products of IFN α -stimulated genes may regulate different downstream targets in H123 cells compared with parental Jurkat cells.

IFN α -stimulated apoptosis in H123 Jurkat cells is accompanied by an increase in caspase-3 and caspase-9 activity. In contrast to other cells reported to undergo IFN α -induced PCD, caspase-8 is not activated in H123 cells and IFN α -induced apoptosis in H123 cells is not abrogated by the expression of CrmA or dominant negative FADD [13]. The fact that overexpressed Bcl-2 inhibits the apoptotic actions of IFN α indicates that this cytokine likely regulates a mitochondrial-dependent pathway to exert its death promoting actions. The fact that H123 cells incubated with IFN α display a decrease in mitochondrial membrane potential confirms the role of the intrinsic pathway in IFN α -stimulated PCD in these cells. Although we have detected activation of caspases in H123 cells, general caspase inhibitors only prevent the apoptotic actions of IFN α when PCD is first detectable at 18 h after the addition of this cytokine. At 24–48 h after the addition of IFN α , caspase inhibitors are ineffective in preventing cell death suggesting the activation of a second death pathway that is caspase-independent and very important for the apoptotic response. It is notable that type 1 IFN-induced apoptosis of primary murine pro-B cells also occurs in the presence of caspase inhibitors (Fig. 4D), indicating that the effects that we observe in H123 cells are also likely to occur in other systems. A similar observation was recently made where cells overexpressing a constitutively activated form of Stat1 were not fully protected from IFN γ -induced apoptosis in the presence of Z-VAD inhibitor [38]. In searching for other caspase-independent proteases that might account for the actions of IFN α in H123 cells, we have focused on the serine protease HtrA2/Omi. This protease has been described as being released from mitochondria when cells are stimulated to apoptose by a number of stimuli including DNA damaging agents and Fas ligand. This protease has several roles. It can catalytically cleave IAPs that leads to their inactivation. It has been shown to directly induce apoptosis both in the absence of caspase activity and to indirectly induce permeabilization of the outer mitochondrial membrane and subsequent release of cytochrome *c* and caspase activation [31,33,34,39–41]. This is the first report indicating that HtrA2 might be also a player in cytokine-mediated death signaling pathways. Our data indicate that HtrA2 is released from the mitochondria of H123 cells, but not from parental Jurkat cells with kinetics consistent with it being responsible for the apoptotic actions of IFN α . Furthermore, the release of HtrA2 is not prevented by pretreatment with the caspase inhibitor Z-VAD and it does not occur in H123 cells that overexpress Bcl-2 and consequently are resistant to the apoptotic actions of IFN α . Thus far, we have not been successful in disrupting the expression of HtrA2 using RNAi or antisense oligonucleotides in H123 cells. This is not surprising since treatment of H123 cells with poly I:C that mimics viral infection of cells induces H123 cells to undergo massive apoptosis within 24 h (data not shown). In addition, it has been recently reported that RNAi can activate the Jak/Stat pathway [42]. However, incubation of H123 cells with the HtrA2-specific inhibitor ucf-101 or overexpression of c-IAP1 both partially prevent IFN α -stimulated PCD to about the same degree (Fig. 7D and E) which is consistent with our other findings that this protease plays a role in IFN α -induced cell death. Together, our observations reveal a distinct early phase apoptotic signaling mechanism invoked by type I IFNs that occurs via a mitochondrial-dependent pathway and requires caspase activity, but later is driven through a caspase-independent death mechanism.

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Abbreviations

Stat	signal transducer and activator of transcription
ISG	interferon-stimulated gene
ISGF3	interferon-stimulated gene factor 3
ISRE	interferon stimulated response element
GRR	gamma response element
EMSA	electrophoretic mobility shift assay
PCD	programmed cell death
IFN	interferon

Fig. 1.

H123 Jurkat cells are more sensitive than parental Jurkat cells to the growth inhibitory effects of IFN α . (A) Parental and H123 Jurkat cells were left untreated or stimulated with 1000 U/ml of IFN α and cells were counted after 72 h. The number of viable cells in the IFN α -treated group was divided by the number of untreated cells to calculate % growth inhibition. Standard error bars were determined from three experiments. Parental (B) and H123 (C), Jurkat cells were either left untreated or stimulated with 1000 U/ml of IFN α for the indicated times. Cells were harvested and double labeled with annexin V-FITC and propidium iodide and analyzed by flow cytometry. Results are represented as percent apoptosis and standard error bars were determined from three individual experiments.

Fig. 2.

Activation of IFN α -stimulated RNAs is the same in H123 and parental Jurkat cells. Total RNA was isolated from parental and H123 cells incubated with IFN α for the indicated times and the expression of ISG15, ISG54 and ISG56 RNAs was determined by RNase protection assay. GAPDH RNA was used as an internal control to ensure for equal loading of total RNA for each hybridization.

Fig. 3.

IFN α stimulates caspase-3 and caspase-9 activities only in H123 cells. Cells were stimulated with IFN α (1000 U/ml) for the indicated times. Cell extracts were prepared and (A) caspase-3 and (C) caspase-9 activities were measured using Ac-DEVD-AMC or Ac-LEHD-AMC peptides as substrates, respectively. This is a representative experiment of three that were performed. Results are presented as mean $\pm$ S.D. of triplicate samples. (B) Cell extracts prepared from cells treated with IFN α as above were resolved by SDS-PAGE and analyzed for cleavage of PARP by immunoblot analysis.

Fig. 4.

IFN α -induced activation of apoptosis in H123 cells is partially reversed by caspase inhibitors. (A) H123 cells were pretreated with the general caspase inhibitors Z-VAD or Z-asp or with the caspase-3 specific inhibitor Z-DEVD for 2 h prior to stimulation with medium or with IFN α (1000 U/ml) for 18 h. Z-FA peptide was included as a negative control. Cells were then doubly labeled with annexin V and propidium iodide and the percent of apoptotic cells was determined by flow cytometry analysis. (B and C) Same as in (A) except cells were pretreated with Z-VAD inhibitor and apoptosis was measured following stimulation with IFN α or anti-Fas antibody for 42 h. (D) The conditions for this assay are the same as those in (A) except

that primary IL-7-dependent murine B cells were incubated with or without murine IFN β This is a representative experiment of three that were performed that gave similar results.

Fig. 5.

Expression of CrmA or dominant negative (DN) FADD does not rescue H123 cells from IFN α -mediated apoptosis, but overexpression of Bcl-2 inhibits apoptosis. (A) Individual H123 clones expressing either CrmA or a pooled population of H123 cells expressing DN FADD were left untreated or stimulated with 1000 U/ml of IFN α for 40 h or with anti-Fas antibody 100 ng/ml for 5 h. Percent apoptosis was assessed by double staining of cells with annexin V and propidium iodide followed by flow cytometry analysis. A representative experiment of three is shown. Individual H123 clones stably expressing an empty vector or Bcl-2 were left untreated or stimulated with either (B) IFN α (1000 U/ml) for 42 h or with (C) anti-Fas antibody (100 ng/ml) for 3 h. Data are represented as percent apoptosis compared with untreated cells. Overexpression of Bcl2 in H123 cells was assessed by immunoblot analysis. (D) Same as in (B) except viable cells were counted following stimulation with IFN α for 72 h. Standard error bars were determined from three experiments. (E) H123 and parental Jurkat cells were

stimulated with or without IFN α for 24 or 48 h followed by staining with 2.5 μ M JC-1. Fluorescence was analyzed by flow cytometry. An increase in fluorescence correlates with decreased mitochondrial membrane potential.

Fig. 6.

IFN α -treated H123 cells show release of cytochrome *c* into the cytosol. (A) Cells were either left untreated or stimulated with IFN α for the indicated times. Cytosolic extracts were prepared and detection of cytochrome *c* was assessed by immunoblot analysis. (B) Same as in (A), except that H123 cells were pretreated with Z-VAD inhibitor (50 μ M) for 2 h followed by stimulation with IFN α . Cells stimulated with 100 nM staurosporine (Sts) or 100 ng/ml of anti-Fas antibody were included as positive controls to measure the presence of cytochrome C in the cytosol. Membranes were reprobed with antibodies Against tubulin to verify equal protein loading (lower panel).

Fig. 7.

(A-C) IFN α activates the release of HtrA2 into the cytosol of H123 cells. (A) H123 or (B) parental Jurkat cells were either left untreated or stimulated with IFN α for the indicated times. In (A), H123 cells were also pretreated with Z-VAD inhibitor (50 μ M) followed by stimulation with IFN α . Cytosolic extracts were prepared and detection of HtrA2 was assessed by immunoblot analysis. Stimulation of cells with 100 nM staurosporine (Sts) was included as a positive control to measure the presence of HtrA2 in the cytosol. (C) Same as described in (A); except HtrA2 release in the cytosol was compared between H123 cells (left panel) and H123 cells stably expressing Bcl-2 (right panel). Membranes were reprobed for actin to verify equal loading of protein (lower panels). (D-E) Inhibitors of HtrA2 partially protect H123 cells from

IFN α stimulated apoptosis. (D) H123 cells were incubated with IFN α in the presence or absence of 5 μ M ucf-101 (an inhibitor of HtrA2) for 24 h. Apoptotic cells were analyzed by annexin V staining. $p < .05$ between cells incubated with or without ucf-101. (E) H123 cells were transfected with cDNA expression vectors encoding GFP with or without an cDNA encoding cIAP1. 18 h later cells were incubated with or without IFN- α for 30 h. Percent apoptosis was assessed by measuring annexin V-PE staining on gated GFP positive cells. Standard error bars were determined from three experiments. Expression of transfected cIAP1 in H123 cells was assessed by immunoblot analysis (lower panel).