Interferon-γ Modulates a p53-independent Apoptotic Pathway and Apoptosis-related Gene Expression

(Received for publication, March 12, 1997)

Natalya K. Ossina, Angela Cannas, Virginia C. Powers, Paul A. Fitzpatrick, John D. Knight, James R. Gilbert, Eugene M. Shekhtman, L. David Tomei, Samuil R. Umansky, and Michael C. Kiefer†

From LXR Biotechnology Inc., Richmond, California 94804

Interferon (IFN)-γ increases the sensitivity of tumor cell lines, many of which are p53 mutants, to tumor necrosis factor-α-mediated and anti-Fas antibody-mediated cell death. To better understand the mechanism of IFN-γ action in modulating the cell death response independently of p53 function, we analyzed the death of the human colon adenocarcinoma cell line, HT-29, following treatment with IFN-γ and various cytotoxic agents. Here we show that IFN-γ modulates cell death by sensitizing the cells to killing by numerous pro-apoptotic stimuli but not pro-necrotic stimuli. Furthermore, we show that select genes from several important apoptosis-related gene families are induced by IFN-γ, including the apoptosis-signaling receptors CD95 (Fas/Apo-1) and TNFR 1 and interleukin-1β-converting enzyme (Ice) family members Ice, CPP32 (Yama, apopain), ICEα/β (TX, Ioch-2), Mch-3 (ICE-LAP3, CMH-1), Mch-4, and Mch-5 (MACH, FLICE). Of the bcl-2 family members, IFN-γ directly induced bak but notably not bax, which is activated by p53. The IFN-responsive transcriptional activator interferon regulatory factor-1 was also strongly induced and translocated into the nucleus following IFN-γ treatment. We propose that IFN-γ modulates a p53-independent apoptotic pathway by both directly and indirectly inducing select apoptosis-related genes.

Cell death is an essential event in the development and function of multicellular organisms. Two forms of cell death have been described, necrosis and apoptosis (1), which differ in the nature of their induction stimulus, underlying signaling and effector mechanisms, morphological features, and the degree to which they are subject to therapeutic intervention (for review, see Refs. 1–5). Necrosis is usually the result of catastrophic cell damage that leads to metabolic failure, early loss of membrane integrity, and often to inflammation and further tissue damage. Apoptosis, or physiological cell death, is a genetically programmed active process that occurs in all multicellular organisms and plays a major role in development, tissue homeostasis, defense, and, when dysregulated, many diseases. It is generally considered that apoptosis is marked by cell shrinkage, chromatin condensation, internucleosomal DNA cleavage, membrane blebbing, and the formation of apoptotic bodies that are phagocytosed by other cells.

Recent evidence indicates that apoptosis can be regulated through p53-dependent and -independent pathways (6, 7). This has been most clearly demonstrated in T lymphocytes, which are particularly susceptible to DNA damage-induced apoptosis. Normal thymocytes readily undergo apoptosis following treatment with ionizing radiation, etoposide, glucocorticoids, or calcium ionophores. Thymocytes lacking p53 are resistant to apoptotic cell death following treatment with ionizing radiation and etoposide, but retain normal sensitivity to glucocorticoids and calcium ionophores. However, mitogen-activated mature T lymphocytes lacking p53 do undergo apoptosis following irradiation or genotoxic treatments (8). Taken together, these data indicate the existence of p53-dependent and -independent apoptotic pathways. More recently, the p53-independent apoptotic pathway utilized by the mitogen-activated mature T lymphocytes was shown to require transcription factor interferon regulatory factor (IRF)1-1, because activated splenocytes lacking IRF-1 were resistant to apoptotic cell death after treatment with irradiation and genotoxic drugs (9). IRF-1 is a transcription factor that activates type I interferon (IFN) and IFN-inducible genes, has tumor-suppressive activities and when inactivated, may be linked to the development of hematopoietic malignancies (for review, see Ref. 10). Thus, there are at least two different anti-oncogenic transcription factors, p53 and IRF-1, required for distinct apoptotic pathways.

Apoptosis may be induced by a variety of physiological signals such as TNF-α and CD95L (for review, see Refs. 11–13), as well as exposure to genotoxic and cytotoxic agents. Various cell lines display an increased sensitivity to cytotoxic signaling through TNFR 1 or CD95 following treatment with IFN-γ (14–16). The human colon adenocarcinoma cell line, HT-29, is particularly responsive to IFN-γ, which markedly increases its sensitivity to TNF-α-mediated as well as anti-Fas antibody (Ab)-mediated cytotoxicity. Additionally, HT-29, like many other tumor cell lines, has a mutated p53 gene that results in a non-functional protein (17).

The biological effects of IFN-γ are mediated by the expression of new gene products (for review, see Ref. 18), and the IFN-γ-induced sensitivity of HT-29 cells to TNF-α-mediated or anti-Fas Ab-mediated cell death may be partly attributed to the up-regulation of CD95 and TNFR 1. However, it is possible that other apoptosis-related gene products are regulated by IFN-γ, and when induced, could participate in different apoptotic pathways following treatment with various cytotoxic agents. Two gene families that play critical roles in apoptosis are ced-9/bcl-2 and ced-3/Ice. The Bcl-2 family of proteins are important intracellular modulators of apoptosis and can be divided into two groups based on their ability to either suppress...
(Bcl-2, Bcl-xL, Mcl-1) or enhance (Bax, Bak, Bad, Bcl-xL) cell death following an apoptotic signal (for review, see Refs. 19–22). ICE family members are cysteine proteases that participate in both the signaling and effector functions of the apoptotic pathway presumably through a hierarchical proteolytic cascade (for review, see Refs. 23 and 24).

In an attempt to understand mechanisms of p53-independent cell death pathways, we analyzed the type of cell death induced by different cytotoxic agents and the apoptosis-related genes that were expressed in HT-29 cells following IFN-γ treatment.

EXPERIMENTAL PROCEDURES

HT-29 Cell Culture and IFN-γ Treatment—HT-29 cells (ATCC HTB-38) were grown in McCoY's 5a medium supplemented with 10% fetal bovine serum at 37 °C in a humidified 5% CO₂ atmosphere. Cells were seeded (1 × 10⁵/ml) in six-well plates (Corning) for the cell death assay or in flasks for DNA, RNA, or protein analysis and grown to 60–80% confluence. IFN-γ (Boehringer Mannheim) was then added (200 units/ml) and the cells were analyzed by luminescence microscopy.

In an attempt to understand mechanisms of p53-independent cell death pathways, we analyzed the type of cell death induced by different cytotoxic agents and the apoptosis-related genes that were expressed in HT-29 cells following IFN-γ treatment.

RNA Isolation and Northern Blot Analysis—Total RNA was isolated from HT-29 cells by the single-step acid guanidinium thiocyanate-phenol/chloroform extraction method (26). RNA samples (20 µg) were fractionated by electrophoresis on 1% agarose, 2.2 M formaldehyde gels, transferred to nitrocellulose membranes, and then prehybridized and hybridized at 42 °C in a standard solution containing 40% formamide (25). Standard high stringency washing conditions were used (0.1 × SSC, 0.1% SDS at 65 °C). The cDNA probes (see below) were excised from their respective vectors, gel-purified, and ³²P-labeled (27). Following hybridization and autoradiography, the blots were stripped by washing for 10 min at 100 °C in 0.5% SDS followed by an additional 10 min at 25 °C (in the same preheated solution) with shaking. The blots were rehybridized with ³²P-labeled β-actin cDNA to account for the variation in RNA loading and transfer to nitrocellulose. Quantitation of Northern blot hybridization was performed using a flatbed scanner (HP Scan-Jet 4c) to digitize the autoradiographic signals and Adobe Photoshop 3.0 to measure the pixel intensities. The relative intensity of each signal was normalized to the β-actin signal to give a normalized value (NV) and the fold activation for each gene or protein was then calculated (NV_{Target}/NV_{β-actin}).

Polymerase Chain Reaction—cDNAs containing the coding regions of the bcl-2 and Ice family members, TNFR 1, and soluble CD95 (Fas/ΔTM) were generated by RT-PCR using the Hot Start/AmpliWax method described by the supplier (Perkin Elmer). The template DNAs for RT-PCR were either previously cloned cDNAs (bah, ICEv-I, and Fas/ΔTM) or cDNA that was reverse transcribed from various tissue or cell line total RNAs using SuperScript II Reverse Transcriptase as described by supplier (Life Technologies, Inc.). PCR primers were designed based on the published sequences for bcl-2 (28), bcl-xL (30), bax (31), mcl-1 (32), bad (33), Ice (34), ICEv-I (35), CPP32 (36), Ich-1 (37), Mch2 (38), Mch3 (39), Mch4 (40), Mch5 (41), TNFR 1 (41), Fas/ΔTM (42), and IRF-1 (43) and included restriction site extensions for subcloning into pC5 DNA or pBlueBac III (Invitrogen, San Diego, CA). For the bcl-xL/bcl-xL PCR, 50 µg of HT-29 total RNA (plus or minus IFN-γ treatment) was reverse transcribed as above. RT-PCR was performed as above using ~25 ng of cDNA as template and the following primers: FP (5'-AGATCTGAAATTCTGAAAAGCAAGCGCTGAGG-3') and RV (5'-CTTCAACTGTTCCCTTGCGCTC-3').

DNA Isolation and Electrophoresis—DNA was isolated from adherent and non-adherent cells separately by proteinase K-phenol method as described (25). DNA samples were treated with 100 µg/ml of DNase-free RNase, extracted twice with phenol/chloroform, precipitated with ethanol, and dissolved in 10 mM Tris-HCl, pH 7.6, 1 mM EDTA. DNA samples (10 µg) were fractionated by electrophoresis on 1.2% agarose gels (25) and visualized by staining with ethidium bromide (0.5 µg/ml).

Dose dependence of HT-29 cell death induced by pro-apoptotic (A) or pretreated (closed symbols) with IFN-γ were incubated with the various cytotoxic agents shown for 24 h (A) or 2 h (B). The percentage of dead cells was determined as described under “Experimental Procedures.”
RP (5'-AGATCTAAGCTTGAAGTGCAGGCACGAG-3'). The oligonucleotides were synthesized by the phosphoramidite method with an Applied Biosystems model 394 DNA/RNA synthesizer, purified by polyacrylamide gel electrophoresis, and desalted on Sep-Pak C18 cartridges (Waters Associates, Milford, MA).

Western Blot Analysis—Cell extracts for Bak, Bax, Bel-2, and Bel-x analysis were prepared by lysing 1 × 10^7 HT-29 cells in 200 μl of 1 × phosphate-buffered saline containing 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 1 μg/ml aprotinin for 10 min at 4 °C. Lysates were centrifuged for 10 min at 10,000 × g, and supernatants were saved. Cytosol and nuclei-containing cell extracts for IRF-1 analysis were prepared by lysing 1 × 10^7 HT-29 cells in 200 μl of 1% Nonidet P-40, 20 mM Tris (pH 7.5), 150 mM NaCl, 3 mM MgCl2, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 1 μg/ml aprotinin for 15 min at 4 °C. Lysates were centrifuged for 10 min at 10,000 × g, and the cytosol-containing supernatants were separated from the nuclei-containing pellet. The pellet was resuspended in 200 μl of the above buffer and sonicated. The samples were quantitated by the Bradford assay (44) and fractionated (25–100 μg of protein/lane) by 15% SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and incubated for 1 h in blocking buffer (phosphate-buffered saline containing 5% milk powder and 0.05% Tween 20). For Bak analysis, the blot was incubated for 1 h with rabbit anti-Bak antiserum in blocking buffer followed by a 1-h incubation with anti-rabbit IgG-peroxidase conjugate (1:20000) and then developed using the ECL method (Amersham). The anti-Bak antiserum was generated in rabbits using purified recombinant Bak protein expressed in yeast and used at 1:5000 dilution. The anti-Bcl-x, -Bax, -Bcl-2, and -Bcl-xL antibodies were used as recommended by the supplier (Santa Cruz Biotechnology, Inc.).

RESULTS

IFN-γ Treatment Increases the Sensitivity of HT-29 Cells to Pro-apoptotic but Not Pro-necrotic Stimuli—The human colon adenocarcinoma cell line HT-29 is relatively insensitive to killing by TNF-α and anti-Fas Ab but can be killed by these cytotoxic agents after pretreatment with IFN-γ (Fig. 1A; Refs. 14–16). Pretreatment of HT-29 cells with IFN-γ not only increases their sensitivity to killing by TNF-α and anti-Fas Ab, it also increases their sensitivity in a dose-dependent manner to a variety of apoptotic stimuli that act through different mechanisms (Fig. 1A). Staurosporin induces apoptosis by inhibiting protein kinases (45). Cisplatin causes DNA-DNA and DNA-protein cross-linking (46), while adriamycin inhibits topoisomerase II (47). Ceramide, a bioactive sphingolipid, is a second messenger in apoptotic pathways induced by different agents, including TNF-α and anti-Fas Ab (48, 49). Various methods were used to confirm that these agents induced apoptotic cell death. HT-29 cells were treated with IFN-γ and staurosporine and then were stained with PI and Hoechst 33342. Luminescent microscopy revealed cells with condensed chromatin, a nuclear morphology characteristic of apoptosis (Fig. 2). Many of the cells with condensed chromatin were impermeable to PI (white arrows), indicating that nuclear changes preceded the increase in outer membrane permeability. In other cells (black arrow), both chromatin condensation and outer membrane permeability had occurred, indicating a later stage of apoptosis. In addition, agarose gel electrophoresis of DNA isolated from staurosporine- or ceramide-treated cells showed nucleosomal DNA laddering (data not shown). Taken together, these data indicate that HT-29 cells are dying by an apoptotic mechanism.

In the above experiments, we observed an increase of 10–20% in cell death following IFN-γ treatment in the absence of any additional pro-apoptotic stimuli. Thus, it is possible that HT-29 cells pretreated with IFN-γ are more sensitive to any cytotoxic agent. However, we found that the sensitivity of HT-29 cells to the pro-necrotic agents, potassium cyanide and ethacrynic acid, was identical in IFN-γ-treated and untreated cells (Fig. 1B). Ethacrynic acid was shown to induce necrotic cell death in HT-29 cells by luminescent microscopy (Fig. 2), as evidenced by PI staining of the cells (outer membrane permeability) in the absence of chromatin condensation (white triangular arrows). DNA electrophoresis of KCN-treated cells displayed a lack of nucleosomal DNA laddering (data not shown).

Thus, IFN-γ sensitizes HT-29 cells to pro-apoptotic but not pro-necrotic stimuli. This indicates that p53-independent

**Fig. 2. Luminescent microscopy of HT-29 cells treated with various cytotoxic agents.** HT-29 cells were pretreated with IFN-γ and then incubated 9 h with 1 μg/ml staurosporine or 2 h with 1.5 mg/ml ethacrynic acid. Control cells were only pretreated with IFN-γ. Cells were stained with 1 μg/ml propidium iodide and 10 μg/ml Hoechst 33342 and analyzed by luminescent microscopy.

**Fig. 3. Expression of Bel-2 family members in HT-29 cells.** A, Northern blot analysis of bel-2 family members. Total RNA (20 μg) isolated from untreated (−) and IFN-γ-treated (+) HT-29 cells was hybridized with the specific gene probes indicated below each panel. The 18 S rRNA probe (data not shown). B, PCR analysis of bel-xL and bel-xS mRNAs expressed in untreated (−) and IFN-γ-treated (+) HT-29 cells. PCR products corresponding to bel-xL and bel-xS are indicated. Markers (base pairs, bp) are shown on the right. C, Western blot analysis of Bak, Bel-x, and Bel protein expression in HT-29 cells at various times (h) after IFN-γ treatment. Immunoblotting was performed as described under “Experimental Procedures.” Blots were incubated with antibodies specific for the protein shown below each panel. A molecular mass marker (30.5 kDa) is shown by a line (−) on the left of each panel. D, fold activation was calculated as described under “Experimental Procedures.”
apoptotic pathway(s) are being modulated by IFN-γ and suggests the mode of action involves the regulation of apoptosis-related proteins, including those downstream of ceramide. Since the known biological effects of IFN-γ require gene expression, we decided to investigate the effects of IFN-γ on expression of apoptosis-related genes in HT-29 cells.

IFN-γ Up-regulates the mRNA and Protein Levels of Apoptosis-related Genes from Several Families—mRNA and, in some cases, protein levels of members from several known families of apoptosis modulators were analyzed before and after IFN-γ treatment. Northern blot analysis of bcl-2 family members revealed that bak and mcl-1 mRNA levels were induced 5.4- and 1.4-fold, respectively, after 16 h of IFN-γ treatment, whereas the levels of bax, bcl-x, and bad mRNA were unchanged (Fig. 3, A and D). bcl-2 mRNA was not detectable in HT-29 cells before or after IFN-γ treatment (data not shown). Alternate splicing of the bcl-x transcript results in two distinct mRNAs whose protein products either inhibit (Bcl-xL) or accelerate (Bcl-xS) cell death (29). Since the difference in size between the two transcripts is too small to distinguish by Northern blot analysis, the ratio of bcl-xL to bcl-xS mRNAs was determined by RT-PCR. This analysis indicated that the ratio of the two transcripts was unchanged following IFN-γ treatment (Fig. 3B). Bak protein levels increased 4.5-fold in HT-29 cells following 16 h of IFN-γ treatment, as shown by Western blot analysis (Fig. 3, C and D). The increase in Bak protein is consistent with the corresponding increase in bak mRNA levels. Both Bax and Bcl-x protein levels remain unchanged following IFN-γ treatment, which is consistent with their constant mRNA levels (Fig. 3, C and D).

Analysis of ICE family members by Northern blotting indicated that Ice, Mch3, Mch4, Mch5, CPP32, and ICErel-II mRNA levels increased in HT-29 cells following 16 h of IFN-γ treatment (Fig. 4), whereas Ich-1 and Mch2 mRNA levels remained constant (data not shown). The largest fold induction was seen with Mch4, Mch5, and Mch3 (6.0-, 4.7-, and 4.0-fold, respectively). Ice mRNA levels also increased significantly, whereas CPP32 and Icerel-II mRNA levels were upregulated only 1.9- and 1.6-fold, respectively. The fold activation for Ice gene induction could not be accurately determined in this experiment because of a lack of signal in the uninduced cells (lane 1).

The levels of TNFR 1 and CD95 mRNA also increased in HT-29 cells in response to 16 h of IFN-γ treatment (Fig. 4), which is consistent with the increased levels of cell surface TNFR 1 (16) and CD95 (14). CD95 mRNA levels were dramatically increased (13.5-fold), while TNFR 1 mRNA levels showed a modest induction (2.3-fold).

IFN-γ Induces mRNA Expression of the Apoptosis-related Genes Both Directly and Indirectly—IFN-γ-regulated genes can

---

**FIG. 4. IFN-γ-induced expression of apoptosis-related genes in HT-29 cells and dependence on protein synthesis.** Northern blot analysis was performed with total RNA (20 μg) isolated from HT-29 cells treated for 16 h with IFN-γ and CHX as indicated. Hybridization was performed with the specific gene probes shown to the left of each panel. The amount of RNA in each lane was normalized by hybridization with a β-actin specific probe (data not shown). The values shown under lanes 3 and 4 represent the fold activation by IFN-γ treatment in the absence (lane 3 versus lane 1) or presence (lane 4 versus lane 2) of CHX.

**FIG. 5. Kinetics of induction of the IFN-γ-induced apoptosis-related genes in HT-29 cells.** Northern blot analysis was performed with total RNA (20 μg) that was isolated from HT-29 cells treated with IFN-γ for 0, 1, 4, 8, and 16 h. Blots were hybridized with the specific gene probes indicated at the left of each panel. The amount of RNA in each lane (20 μg) was normalized by hybridization with a β-actin specific probe (data not shown), and the fold activation was determined as described under "Experimental Procedures."
be either directly or indirectly induced (18). Direct gene induction requires no protein synthesis, whereas indirect gene activation requires the synthesis of additional proteins such as transcriptional activators. To test whether the IFN-γ-inducible apoptosis-related genes were directly or indirectly induced, Northern blot analysis was performed with HT-29 RNA following IFN-γ treatment of the cells in the absence or presence of protein synthesis inhibitor, cycloheximide (CHX), respectively. Mch3 gene expression was increased 4.3- and 4.7-fold in the presence and absence of CHX, respectively. Mch4 mRNA levels were increased 2.0-fold in the presence and absence of CHX, respectively. CHX treatment alone resulted in increased levels of most of the IFN-γ-regulated genes (10) and has been shown to be required for at least one p53-independent apoptotic pathway (9). Fig. 6A shows that IFN-γ mRNA is also induced in HT-29 cells after 16 h of IFN-γ treatment (lane 3 versus lane 1) and that its gene activation is direct, not requiring additional protein synthesis (lane 4 versus lane 2). The kinetics of IFN-γ mRNA expression indicate that the transcript is strongly induced and is near a maximum by 1 h of IFN-γ treatment (Fig. 6B). A 23.4-fold increase of IFN-γ mRNA is observed after 1 h of IFN-γ treatment with only a slight increase occurring by 16 h. Additionally, DNA analysis of the HT-29 IFN-γ cDNA that was isolated by RT-PCR showed no mutations in the coding region (data not shown).

Western analysis of fractionated HT-29 lysates indicates that IFN-γ protein levels have increased in the cytosol-containing fraction (Fig. 6C, lane 2 versus lane 1) and in the nuclei-containing fraction (lane 4 versus lane 3) following IFN-γ treatment. Additionally, a significant percentage of the protein has shifted from the cytosol- to the nuclei-containing fraction. Before IFN-γ treatment, all of the detectable IRF-1 is found in the cytosol-containing but none in the nuclei-containing fraction (lane 1 versus lane 3). However, following IFN-γ treatment approximately 50% of the total IRF-1 is found in both the cytosol- and nuclei-containing fractions (lanes 2 versus lane 4).

**DISCUSSION**

We have shown that HT-29 cells, which contain a non-functional p53 protein, become more sensitive to pro-apoptotic but not pro-necrotic agents following treatment with IFN-γ. IFN-γ treatment significantly increased the levels of cell death induced by six different pro-apoptotic stimuli that induce cell death through different or overlapping pathways. This suggests that IFN-γ primes cells for p53-independent apoptotic pathways induced by diverse stimuli. A pro-apoptotic role for IFN-γ is consistent with, and most likely contributes significantly to, the general activities described for the interferons, i.e. establishment of the antiviral state, growth inhibition, and immunomodulation (18). Clearly, increasing the sensitivity of virally infected cells or tumor cells to pro-apoptotic stimuli would decrease viral replication and tumor cell growth.

It is axiomatic that the diverse biological effects of IFN are mediated by their binding to specific cell surface receptors, activation of signal-transducing molecules, and the consequent modulation of gene expression. It is also known that the transcriptional activator, IRF-1, is involved in mediating transcription of IFN-inducible genes (10) and is required for DNA damage-induced apoptosis in mitogen-activated T lymphocytes (9),
a process that does not require p53 (8). To further investigate the mechanism of IFN-γ-induced sensitivity of HT-29 cells to pro-apoptotic agents, we analyzed its effect on gene and protein expression of members from three important families involved in apoptosis: TNF/nerve growth factor receptor, Bel-2, and ICE. We also analyzed the effect of IFN-γ in regulating IRF-1 expression and subcellular distribution. We found that mRNA levels of various members from all three families and the transcriptional activator IRF-1 were inducible by IFN-γ, bak, Mch5, and IRF-1 mRNAs were directly induced, i.e., had no requirement for additional protein products such as transcriptional activators for maximal induction. Mch4, ICE_αII, CD95, and TNFR1 mRNAs were weakly to moderately induced by IFN-γ in the absence of protein synthesis but were more strongly induced if protein synthesis occurred. The mcl-1, Ice, Mch3, and CPP32 genes were indirectly induced and had an absolute requirement for additional protein factors for their induction. These findings are consistent with the regulation of other known IFN-γ-induced genes that can either be directly induced or which require the expression of an IFN-γ-inducible transcriptional activator such as IRF-1. Interestingly, IRF-1 mRNA was induced earlier and more strongly by IFN-γ than the apoptosis-related genes and therefore probably participates in activating the indirectly INF-γ-induced genes such as Ice. Indeed, Tamura et al. (9) have shown that IRF-1 is required for mitogen induction of Ice mRNA and have further pointed out that the Ice gene promoter contains an IRF-1 binding site. Additionally, IRF-1 may play a role in the direct activation of bak and Mch5. We have shown that the bak gene promoter also contains an IRF-1 binding site that can bind IRF-1 from nuclear extracts of HT-29 cells following IFN-γ treatment.

We also measured the mRNA and protein levels of the anti-apoptotic genes of the bcl-2 family, since a decrease in their levels of expression could also result in an increase in sensitivity to apoptotic stimuli. We found that the bcl-x mRNA levels and the ratio of the bcl-x<sub>L</sub> to bcl-x<sub>S</sub> remained unchanged and that bcl-2 mRNA was undetectable before or after IFN-γ treatment. This correlated with the protein expression data, which showed that the levels of Bcl-x remained constant and also that Bcl-2 was not detected before or after IFN-γ treatment. mcl-1 mRNA levels increased following IFN-γ treatment. This result was unexpected since Mcl-1 has been shown to protect cells from apoptotic cell death in some cases when overexpressed (51, 52). However, its role as an anti-apoptotic protein is not firmly established since its levels are up-regulated in some tissues that are destined to undergo apoptosis (53, 54).

Of the pro-apoptotic Bcl-2 family members, only Bak was induced by IFN-γ. This suggests that some of the antiviral and antitumor effects of IFN-γ may be realized through the up-regulation of Bak, which would increase the sensitivity of HT-29 cells to apoptotic cell death. This is consistent with the role of Bak as antiviral and antitumor (tumor suppressor) agent. For example, Bak is the target of several viral proteins that not only antagonize its pro-apoptotic activity but convert Bak to an active anti-apoptotic protein, with activity similar to that of Bcl-2 (30, 55). Furthermore, bak mRNA expression is induced in response to poly(I)-poly(C), a synthetic double-stranded RNA that mimics virus infection. Recently, Bak levels were shown to be reduced in primary colorectal cancers (56), suggesting that Bak may play a role in tumor suppression and that Bak down-regulation may define an early event in the pathogenesis of colorectal cancers. It is interesting to note that Bak and Bax are regulated by two different mechanisms. Bak is up-regulated by IFN-γ and most likely requires IRF-1, while Bax is up-regulated by p53. This suggests that Bak may function in p53-independent apoptotic pathways, while Bax may play a more significant role in the p53-dependent apoptotic pathway.

There is growing evidence that the activity of the ICE family of proteases may be regulated through a hierarchical cascade of post-translational proteolysis (24). In our study, we have shown that six of the eight Ice family member transcripts analyzed were induced or up-regulated by IFN-γ treatment. This suggests that the IFN-γ-induced ICE family members may be important control points in the proteolytic cascade leading to apoptosis in HT-29 cells. For instance, Mch5 (MACH, FLICE), whose mRNA levels were up-regulated ~5-fold by IFN-γ treatment, has been shown to bind to CD95 through FADD (MORT1) and therefore is the most upstream ICE family member in the CD95 death-inducing signaling cascade (57, 58).

The coordinate induction of CD95/TNFR1, the pro-apoptotic Bcl-2 member, Bak, and ICE family members by IFN-γ in HT-29 cells suggests a functional relationship between these family members in promoting cell death through p53-independent apoptotic pathways. It will be interesting to determine the relative contribution of Bak and ICE family members in regulating or promoting apoptosis in cells exposed to IFN-γ. It will be important to determine whether IRF-1 is required for IFN-γ-induced sensitivity of HT-29 cells to pro-apoptotic stimuli or if other transcriptional activators such as the STAT or NF-kB family members play a role. Furthermore, it will be of interest to determine whether IFN-γ is capable of priming other cell types such as cardiomyocytes and neurons for apoptotic cell death. If so, this may have important implications in pathophysiological conditions that are associated with infiltrating, IFN-γ-producing leukocytes, such as acute myocardial infarction and stroke.

REFERENCES

1. Kerr, J. F. R., Wyllie, A. R., and Currie, A. R. (1972) Br. J. Cancer 26, 239–257
2. Umansky, S. (1992) J. Theor. Biol. 97, 591–602
3. Tomei, L. D., and Cope, F. D. (eds) (1991) Apoptosis: The Molecular Basis of Cell Death, pp. 1–29, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
4. Barr, P. J., and Tomei, L. D. (1984) Bio/Technology 12, 487–493
5. Thompson, C. B. (1995) Science 267, 1456–1462
6. Lowe, S. W., Schmitt, E. M., Smith, S. W., Osborne, B. A., and Jacks, T. (1993) Nature 362, 847–849
7. Clarke, A. R., Purdie, C. A., Harrison, D. J., Morris, G. R., Bird, C. C., Hooper, M. L., and Wyllie, A. H. (1993) Nature 362, 849–852
8. Strasser, A., Harris, A. W., Jacks, T., and Cory, S. (1994) Cell 79, 329–339
9. Tamura, T., Ishihara, M., Lamberl, M. S., Tanaka, N., Oishi, I., Izawa, S., Matsuyama, T., Mak, T. W., Taki, S., and Taniguchi, T. (1995) Nature 376, 596–599
10. Taniguchi, T., Harada, H., and Lamphier, M. S. (1995) J. Cancer Res. Clin. Oncol. 121, 516–520
11. Nagata, S., and Golstein, P. (1995) Science 267, 1449–1456
12. Cleveland, J. L., and Ihe, J. N. (1995) Cell 81, 479–482
13. Schutte-Osthoff, K. (1994) Trends Cell Biol. 4, 421–426
14. Yonehara, S., Ishii, A., and Yonehara, M. (1989) J. Exp. Med. 169, 1747–1756
15. Fransen, L., Van Der Heyden, J., Ruyschaert, R., and Fiers, W. (1986) Eur. J. Biochemistry 157, 149–155
16. Turk, A., and Harvey, N. L. (1995) Mol. Cell Biol. 15, 133–143
17. Grossman, J. A., and Haber, D. A. (1994) Cell 76, 769–781
18. Greenblatt, M. S., Bennett, W. P., Hollstein, M., and Harris, C. C. (1994) Cancer Res. 54, 4855–4878
19. Schutte-Osthoff, K. (1994) Adv. Virus Res. 42, 57–102
20. Reed, J. C. (1994) J. Cell Biol. 124, 1–6
21. Maley, M., and Clarke, M. P. (1994) Trends Cell Biol. 4, 399–403
22. Korsmeyer, S. J. (1995) Trends Genet. 11, 101–105
23. Cory, S. (1995) Annu. Rev. Immunol. 13, 513–543
24. Henkarta, P. A. (1996) Immunity 4, 195–201
25. Kumar, S., and Harvey, N. L. (1995) FEBS Lett. 375, 109–173
26. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., pp. 7.43–7.52 and 9.14–9.23, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
27. Chomczynski, P., and Sacchi, N. (1987) Anal. Biochem. 162, 156–159
28. Feinberg, A. P., and Vogelstein, B. (1984) Anal. Biochem. 138, 696–697
29. Cleary, M. L., Smith, S. D., and Sklar, J. (1986) J. Cell Biol. 104, 16356–16362
30. A. Cannas, N. K. Ossina, V. C. Powers, and M. C. Kiefer, manuscript in preparation.
31. Kiefer, M. C., unpublished data.
32. N. K. Ossina, A. Cannas, V. C. Powers, J. J. Wu, and M. C. Kiefer, manuscript in preparation.
33. Cannas, A., Ossina, N. K., Powers, V. C., and Kiefer, M. C., unpublished data.
