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Interferon-γ Induces *Ice* Gene Expression and Enhances Cellular Susceptibility to Apoptosis in the U937 Leukemia Cell Line

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The roles of interferons (IFNs) in apoptosis are not fully understood. In this study we show that in the U937 monoblastic leukemia cell line, pretreatment with IFN-γ enhanced sensitivity to apoptosis triggered by γ-irradiation or antitumor agents (etoposide or adriamycin), as well as by anti-Fas antibody. In addition, IFN-γ caused an increased expression of the interleukin-1β-converting enzyme (*Ice*) gene, following strong induction of the interferon regulatory factor-1 (*IRF-1*) gene, the product of which is a transcriptional activator of the *Ice* gene. An inhibitor of ICE/Ced-3 family proteases, Z-Asp-CH_2-DCB, blocked apoptosis in control cells as well as in IFN-γ-pretreated cells. These results suggest that enhanced susceptibility of IFN-γ-pretreated cells to apoptosis is mediated through the induction of *Ice* by IRF-1. This pathway is not affected by interleukin-1β (IL-1β) since neutralizing antibody against IL-1β failed to suppress the IFN-γ-mediated enhancement of cell death, and IL-1β itself did not mimic the effect of IFN-γ.

Interferons (IFNs) exhibit antiproliferative and antiviral activities against a variety of normal and malignant cells. In addition, several studies have reported that IFNs can induce or modulate apoptosis (1-5). IFNs are used in the therapy of human malignancies as a single agent as well as in combination with other chemotherapeutic agents (reviewed in Ref. 6). However, the effects of IFNs on cellular susceptibility to anticancer agents- or radiation-induced apoptosis have not been elucidated.

Recently, the positive regulator of the IFN system, the tumor suppressor interferon regulatory factor-1 (IRF-1), has been shown to play an essential role in apoptosis, including DNA damage-induced apoptosis (7, 8). Furthermore, IRF-1 has been shown to be a transcriptional activator of the interleukin-1β-converting enzyme (*Ice*) gene, the first member of mammalian homologs of the *Caenorhabditis elegans* cell death gene *ced-3* (8).

In this study, we demonstrate that IFN-γ enhances susceptibility to apoptosis triggered by γ-irradiation, antitumor agents, or anti-Fas antibody in the U937 human leukemia cell line. Furthermore, in an attempt to characterize the pathway responsible for this enhanced apoptosis, we show that IFN-γ induces expression of *Ice* gene following *IRF-1* expression.

MATERIALS AND METHODS

Cell culture and materials. A human monoblastic leukemia cell line U937 was supplied by the Japanese Cancer Research Resources Bank and cultured in RPMI-1640 medium (Life Technologies Inc., Grand Island, NY, USA)

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Abbreviations used: IFN, interferon; IRF-1, interferon regulatory factor-1; ICE, interleukin-1β-converting enzyme; IL-1β, interleukin-1β; Asp, asparatic acid; Z-Asp-CH_2-DCB, benzyloxycarbonyl-Asp-CH_2OC(O)-2,6,-dichlorobenzamine.

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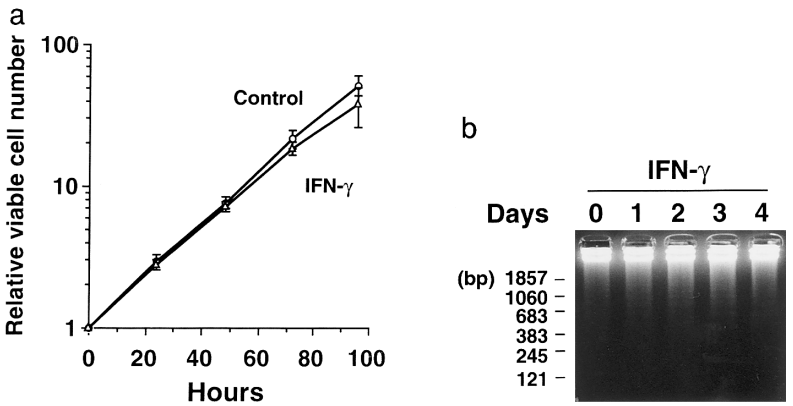

FIG. 1. Effect of IFN-γ on U937 cells. a. U937 cells were cultured in the absence (○) or presence (△) of 1000 U/ml IFN-γ. Cells were inoculated at 2×10^5 cells/ml and diluted to 1.5 to 2.0×10^5 cells/ml on a daily basis. Values are means ± S.D. from three independent samples. b. Cellular DNA (3 μg) was analyzed by electrophoresis on a 2% agarose gel.

containing 10% heat-inactivated fetal bovine serum (Hyclone Laboratories Inc., Logan, UT, USA), 10 mM hepes, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C under humidified 5% CO_2 atmosphere. Viable cell number was determined by erythrosin B (Sigma Chemical Co., St. Louis, MO, USA) dye exclusion. Recombinant human IFN-γ (Shionogi & Co., Osaka, Japan), human purified IFN-α (Sumitomo Pharmaceutical Co., Tokyo, Japan), and recombinant human IL-1β (Genzyme, Boston, MA, USA) were used at 1000 U/ml. Etoposide (Nippon Kayaku Co., Tokyo, Japan) was prepared as a stock solution in dimethylsulfoxide (DMSO). The final concentration of DMSO in the culture was 0.1%, which did not induce apoptosis. Adriamycin (Kyowa Hakko Co., Tokyo, Japan) was dissolved in normal saline and stored at −20°C. γ-irradiation was performed using a Gammacell 40 irradiator equipped with a ^{137}Cs source. Benzyloxycarbonyl-Asp-CH_2OC(O)-2,6,-dichlorobenzamine (Z-Asp-CH_2-DCB) was kindly provided by Kirin Brewery Co., Gunma, Japan. An monoclonal mouse anti-human IL-1β antibody was purchased from Genzyme. Anti-Fas antibody was purchased from MBL, Nagoya, Japan.

DNA fragmentation assay. Cells were suspended in lysis buffer (10 mM Tris-HCl, 200 mM NaCl, 10 mM EDTA, 0.2% Triton-X, 0.1 mg/ml proteinase K, pH 7.5) and incubated at 50°C for at least 10 hours. After a phenol extraction followed by a chloroform extraction, the aqueous phase was treated with 20 μg/ml RNase at 37°C for 1 hour. The DNA was precipitated with ethanol, resuspended in 10 mM Tris, pH 7.5, electrophoresed on a 2% agarose gel in 0.5 μg/ml ethidium bromide, and visualized under ultraviolet light.

Northern blot analysis. Total cellular RNA was prepared by denaturation in guanidium thiocyanate followed by pelleting through a cesium chloride cushion. For Northern blot analysis, 10 μg of total RNA were electrophoresed on a 1% agarose formaldehyde gel and transferred onto a nylon membrane. To prepare the probes, the following DNAs were labeled with [α-^{32}P] dCTP using a Multiprime labeling kit (Amersham, Buckinghamshire, UK) and hybridized as previously described (7): *IRF-1*, a 748-base-pair (bp) KpnI/SacI fragment of the human *IRF-1* cDNA (a generous gift from Dr. M. Ishihara, Department of Immunology, University of Tokyo, Tokyo, Japan); *Ice*, a 674-bp fragment generated by polymerase chain reaction from a plasmid containing human *Ice* cDNA (a generous gift from Dr. M. J. Tocci, Department of Molecular Immunology, Merck Research Laboratories, Rahway, NJ, USA) using the primers GCAAGACTCTCAAGGAGTAC and CCTTCTCTATGTGGGCTTTC. The blots were visualized by autoradiography following exposure for 7 days.

RESULTS

Effect of IFN-γ on U937 Cells

We first examined the ability of IFN-γ alone to induce apoptosis in U937 cells. Cells were cultured in the presence or absence of IFN-γ (1000 U/ml). IFN-γ manifested only a very minor antiproliferative effect (Fig. 1a). Gel electrophoresis of cellular DNA from IFN-γ-treated cells revealed a faint appearance of apoptotic DNA fragmentation (Fig. 1b). Thus, IFN-γ alone causes only a small amount of apoptosis in this cell line.

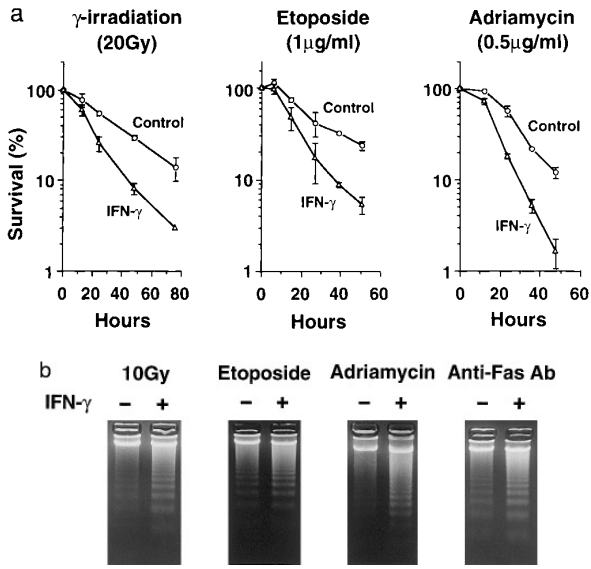

FIG. 2. Effect of IFN-γ on susceptibility to apoptosis. a. Cells were pretreated for 24 hours in the absence (○) or presence (△) of 1000 U/ml IFN-γ, and then treated with γ-irradiation (20 Gy), etoposide (1 μg/ml), or adriamycin (0.5 μg/ml). Survival of cells was calculated as the percentage of viable cells relative to the number of cells prior to apoptotic stimulations. b. Following pretreatment of cells as in Fig. 2a, cells were subjected to apoptotic stimulation with γ-irradiation (10Gy), etoposide (1 μg/ml), adriamycin (0.5 μg/ml), or anti-Fas antibody (50 ng/ml). Cellular DNA was extracted 6 hours later, except in the case of adriamycin treatment when it was extracted 12 hours later. DNA (2 μg) was analyzed by electrophoresis on a 2% agarose gel.

Enhanced Susceptibility to Apoptosis by Pretreatment with IFN-γ

To further assess the possible role of IFN-γ in controlling apoptosis, cells were pretreated with or without IFN-γ (1000 U/ml, 24 hours) and then either γ-irradiated, or treated with antitumor agents (etoposide and adriamycin). Viable cell number was then measured. Pretreatment with IFN-γ resulted in a significantly increased cell death following γ-irradiation, etoposide, or adriamycin treatments (Fig. 2a). Cellular DNA was either extracted 6 hours after γ-irradiation or treatment with etoposide, or 12 hours after treatment with adriamycin. Gel electrophoresis of these DNA demonstrated enhanced DNA fragmentation in IFN-γ-pretreated cells, suggesting that IFN-γ-mediated enhancement of cell death is accompanied by an enhanced susceptibility to apoptosis (Fig. 2b). Although the appearance of DNA fragmentation was already apparent in non-IFN-γ-pretreated cells irradiated with high dose (20 Gy) (data not shown), IFN-γ-mediated enhancement of DNA fragmentation was clearly observed in cells irradiated with lower dose such as 10 Gy. Apoptosis induced by anti-Fas antibody treatment was also enhanced by pretreatment with IFN-γ (Fig. 2b), as reported previously (9).

Treatment with IFN-γ Induces IRF-1 and Ice Gene Expression

The tumor suppressor IRF-1, whose expression is known to be induced by IFNs, has recently been demonstrated to play an essential role in apoptosis and is a transcriptional activator of the *Ice* gene. Thus, we analyzed the expression of these genes which may be responsible for IFN-γ-mediated enhancement of apoptosis. U937 cells were treated with either 1000 U/ml of IFN-γ or IFN-α. At time points ranging zero to 24 hours, total RNA was extracted and subjected to Northern blot analysis (Fig. 3). *IRF-1* mRNA expression was strongly induced after 1 hour of treatment with IFN-γ and remained high. Following this induction of *IRF-1,*

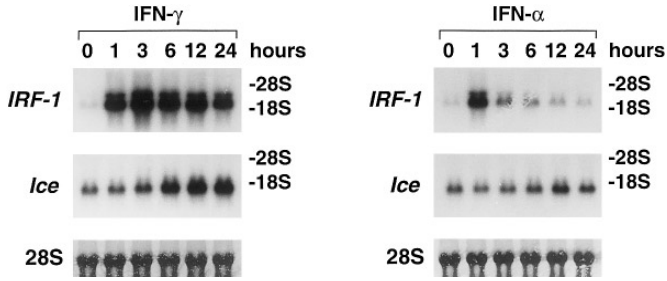

FIG. 3. Induction of *IRF-1* and *Ice* mRNAs by IFNs. Cells were cultured in the presence of 1000 U/ml IFN-γ or IFN-α. Total RNA (10 μg) was analyzed by Northern blot analysis. The 28S rRNA was visualized by staining the filter with methylene blue, and was used to assure equivalent RNA blotting. Blots were hybridized with *IRF-1* and *Ice* specific probes.

expression of the *Ice* mRNA gradually increased and reached its peak at 12 hours. Thus, IFN-γ causes an increase in *Ice* gene expression, possibly through the induction of IRF-1. In contrast, IFN-α induced *IRF-1* expression only transiently and increases in of *Ice* expression were not significant. Additionally, enhanced sensitivity to cell death was lower in IFN-α-pretreated cells than in IFN-γ-pretreated cells (data not shown).

Z-Asp-CH_2-DCB Blocks Apoptosis in Control Cells as well as IFN-γ-Pretreated Cells

An inhibitor of ICE/Ced-3 family proteases, Z-Asp-CH_2-DCB, was shown to block apoptosis in U937 cells triggered by antitumor agents as well as by anti-Fas antibody (10). We examined whether Z-Asp-CH_2-DCB also blocked apoptosis in IFN-γ-pretreated cells. DNA fragmentation induced by γ-irradiation, antitumor agents, or anti-Fas antibody was completely inhibited by Z-Asp-CH_2-DCB, in control cells as well as in IFN-γ-pretreated cells (Fig. 4). These results suggest that the apoptotic pathway in IFN-γ-pretreated cells also involves ICE/Ced-3 family protease(s) and is consistent with the hypothesis that induction of *Ice* by IRF-1 is important in the IFN-γ-mediated enhancement of apoptosis.

Effects of Anti-IL-1β Antibody and IL-1β in IFN-γ-Mediated Enhancement of Cell Death

As ICE is a protease which cleaves inactive proIL-1β to active mature IL-1β, we examined the role of IL-1β in the IFN-γ-mediated enhancement of cell death. However, neutralizing antibody directed against IL-1β did not suppress the IFN-γ-mediated enhancement of cell

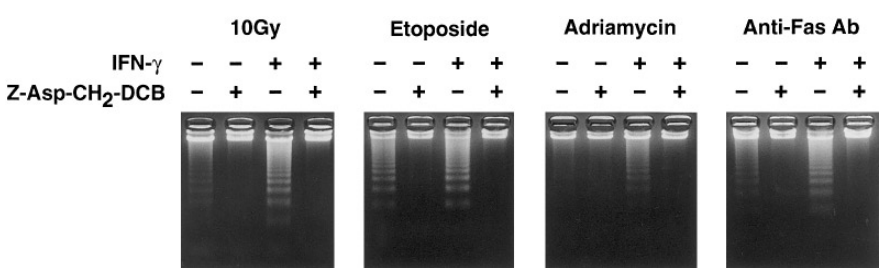

FIG. 4. Prevention of apoptosis by Z-Asp-CH_2-DCB. Cells were either pretreated with or without IFN-γ for 24 hours and apoptotic stimuli were either added with or without 100 μg/ml Z-Asp-CH_2-DCB. Cellular DNA was extracted as in Fig. 2b, and DNA (2 μg) was analyzed by electrophoresis on a 2% agarose gel.

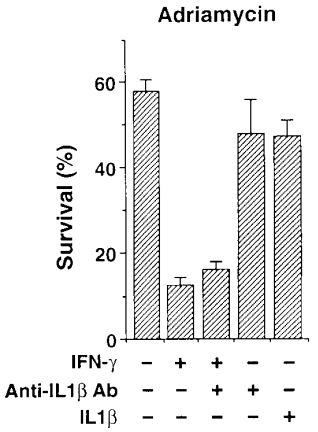

FIG. 5. Role of IL-1β in IFN-γ-mediated enhancement of apoptosis. Cells were pretreated for 24 hours with or without 1000 U/ml IFN-γ or 1000 U/ml IL-1β, and then treated with 0.5 μg/ml adriamycin. Monoclonal antibody against IL-1β was added to a final concentration of 30 μg/ml. Viable cell number was counted 24 hours later. Values are means $\pm$ S.D. from three independent samples.

death caused by adriamycin (Fig. 5). Furthermore, pretreatment with IL-1β rather than with IFN-γ did not result in enhaned cell death (Fig. 5). These results suggest that IL-1β is not a determinant of apoptosis in IFN-γ-mediated enhancement of cell death. Thus, other substrates such as CPP32 may be important in the apoptosis of IFN-γ-pretreated cells.

DISCUSSION

This study demonstrates an IFN-γ-mediated enhancement of sensitivity to apoptosis and the association between IFN-γ and expression of the apoptosis-related genes, *IRF-1* and *Ice*. It is the first demonstration *Ice* gene induction by IFNs.

It has previously been shown that IFN-γ upregulates Fas antigen on the cell surface, and it was proposed that this effect accounted for augmented Fas-mediated apoptosis (9). However, it was recently shown that Fas-mediated apoptosis depends on ICE (11) and that overexpression of ICE enhances Fas-mediated apoptosis (12). Thus, induction of *Ice* by IFN-γ may be another mechanism by which IFN-γ enhances Fas-mediated apoptosis.

The apoptosis induced by γ-irradiation, etoposide, or adriamycin was enhanced by pretreatment with IFN-γ. We also observed that U937 cells undergo apoptosis when cells were cultured at a high cell density and that this apoptosis was also augmented by IFN-γ (data not shown). These observations suggest that IFN-γ may affect a common apoptotic pathway, rather than by simply modulating individual cell death stimuli.

Recent studies using ICE deficient mice have demonstrated that they develop normally and moreover, that γ-irradiation-induced apoptosis in thymocytes was not impaired (11, 13). Mashima et al. have proposed the involvement of an ICE/Ced-3 family protease, rather than ICE itself in etoposide-induced apoptosis in U937 cells (14). These results suggest that ICE is not always required for apoptosis. However, in several cell lines ICE overexpression does induce apoptosis (15-18). In addition, a cascade of cell death pathway that requires ICE(-like) protease(s) for activation of CPP32/Yama(-like) protease(s) has been well established (19, 20). Thus, at present, we speculate that significantly increased levels of ICE in IFN-γ-pretreated cells are important in apoptosis. Of course, other ICE/Ced-3 proteases or more upstream molecules may also mediate the effects of IFN-γ. Further studies are required to establish the role of ICE in IFN-γ-mediated apoptosis.

Our results also provide an approach whereby IFN-γ may render antitumor agents or radiation more efficient in inducing apoptosis in malignancies.

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