ABSTRACT

The mechanism by which IFN-γ inhibits tumor cell growth has not been fully understood. Here we report that IFN-γ up-regulated the expression of Fas and Fas ligand (FasL) on HT29 cells, a human colon adenocarcinoma cell line, and subsequently induced apoptosis of these cells. The kinetics of cell death in IFN-γ-treated HT29 cells paralleled the increase in the levels of Fas and FasL expression. We further show that IFN-γ up-regulated the expression of Fas and FasL in STAT1-transfected U3A cells but not in STAT1-deficient U3A cells. Correspondingly, IFN-γ-induced cell death in STAT1-transfected U3A cells but not in STAT1-deficient U3A cells. IFN-γ-induced cell death was inhibited by caspase-1 inhibitors. Our results suggest that cell growth inhibition by IFN-γ is due to apoptosis mediated by Fas and FasL interaction.

INTRODUCTION

The IFNs were initially recognized by their ability to interfere with viral replication and were later demonstrated to affect many cell functions, such as inhibition of cell growth, induction of cell differentiation, and modulation of the immune response (1, 2). IFNs exert their pleiotropic biological effects through transcriptional regulation of gene expression. The intracellular signaling pathway triggered through IFN receptors has been elucidated recently. It involves the activation and tyrosine phosphorylation of JAKs (3) and STAT (3).

Numerous inducible gene products of IFN-γ have been identified, such as the MHC class I and FcγRI (2, 3). Recent studies have demonstrated that IFN-γ is able to stimulate tumor and immune cells to express Fas (4–7), a type I transmembrane protein that triggers apoptosis when stimulated by its ligand or by agonist mAb (7, 8). Fas is constitutively expressed in many types of cells but is most abundant in hepatocytes and in epithelia (5–7, 9–11). The ligand of Fas (FasL) has been identified as a Mr 38,000 type II integral protein, which belongs to the TNF family. In contrast to Fas, FasL is expressed only on the activated T cells and natural killer cells as well as in some immune-privileged sites, such as the Sertoli cells of testis and the anterior chamber of cornea (8). Upon binding to FasL, Fas transduces a cell death signal that involves the recruitment of the FADD (Fas-associated protein with death domain) protein to the intracellular death domain of Fas and the activation of caspase-8 and -1, two cysteine proteases belonging to the interleukin-1β-converting enzyme superfamily (8). Activated caspase-8 induces the proteolysis of death substrates, such as laminins, actin, and poly(ADP) ribose polymerase, subsequently leading to the morphological changes in the cell cytoplasm and nuclei as well as the degradation of chromosomal DNA (8).

The mechanism by which IFN-γ induces growth inhibition of tumor cells has not been fully elucidated. Recently, Chin et al. (12) reported that IFN-γ induces the expression of p21^{WAF1/CIP1}, an inhibitor of cyclin-dependent kinase, suggesting that IFN-γ may arrest cell growth by blocking the cell cycle. Nevertheless, several recent studies have demonstrated that Fas and FasL are expressed at high levels in a variety of human tumors, including gliomas (4, 13), breast cancer (14), prostate cancer (15), and hepatomas (16). In the present study, we report that IFN-γ not only up-regulates the expression of Fas but also FasL in two tumor cell lines, subsequently resulting in apoptosis. We further show that up-regulation of Fas and FasL required the expression and activation of STAT1 protein. Therefore, our data provide a novel explanation for the mechanism of IFN-γ-induced cell growth inhibition.

MATERIALS AND METHODS

Materials. Anti-Fas mAb, CH-11 (IgM), was purchased from United Biomedical, Inc. (Uniondale, NY) and used for Fas-mediated cell death assay. Anti-Fas polyclonal antibodies were purchased from Santa Cruz Biotechnology (San Diego, CA). Anti-FasL mAbs, clone 33, was purchased from Transduction Laboratories (Lexington, KY) and used for Western blotting assay. Hygromycin B and G418 were purchased from Sigma Chemical Co. (St. Louis, MO). YVAD-cmk and ZVAD-fmk, two specific inhibitors of caspase-1, were purchased from Calbiochem (San Diego, CA). Both inhibitors were dissolved in DMSO at the stock concentration of 10 mM. RNase A, proteinase K, IFN-γ, and TNF-α were purchased from Boehringer Mannheim (Indianapolis, IN). EGF, PGDF, and FGF were purchased from United Biomedical, Inc. Horse-radish peroxidase-conjugated goat anti-mouse IgG and goat anti-rabbit IgG were purchased from Southern Biotechnology, Inc. (Birmingham, AL). ECL detection solution and nitrocellulose membrane were purchased from Amer sham Corp. (Arlington, IL).

Cells. HT29 cells, a human adenocarcinoma cell line, and Jurkat cells, a human leukemia T cell line, were obtained from American Type Cell Collection. HT29 cells were grown in complete McCoy’s medium containing 10% heat-activated FBS. Jurkat cells were grown in complete RPMI 1640 containing 10% FBS. U3A and STAT1-transfected U3A cell lines have been described previously (12, 17, 18). U3A cells were grown in complete DMEM medium with 10% FBS and 250 μg/ml hygromycin B; STAT1-transfected U3A cells were grown in the same medium in the presence of 500 μg/ml G418.

DNA Fragmentation Assay. Cells were grown in six-well plates in the complete medium. Upon 80% confluence, cells were washed twice with HBSS and then maintained in medium containing 0.5% FBS in the presence of variou s stimuli for 48 h. Cells were washed twice with PBS (pH 7.4) and then lifted off with 2.5 mM EDTA/PBS. Cells were pelleted and resuspended in lysis buffer (10 mM EDTA, 50 mM Tris-HCl, pH 8.0) containing 0.5% sodium sarcosinate and 0.5 mg/ml proteinase K. The reaction was maintained at 50°C for 1 h, 5 μl RNase A (1 mg/ml) was added to each sample, and incubation at 50°C was continued for another hour. The reaction was terminated by the addition of 8 μl of sample buffer (100 mM EDTA, 0.1% bromphenol blue, and 25% Ficoll), and 7 μl of 1% low melting agarose were added to each sample, then inactivated at 65°C for 5 min. The samples were then added into dry wells of 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: JAK, Janus kinase; STAT, signal transducer and activators of transcription; mAb, monoclonal antibody; FasL, Fas ligand; TNF, tumor necrosis factor; YVAD-cmk, Ac-Tyr-Val-Ala-Asp-cmk; ZVAD-fmk, Z-Val-Ala-Asp(Ome)-CH2F; ECL, enhanced chemiluminescence; EGF, epidermal growth factor; PGDF, platelet-derived growth factor; FGF, fibroblast growth factor; FBS, fetal bovine serum; IIF, IFN-γ-regulatory factor; JNK, Jun NH2-terminal kinase; MAP, mitogen-activated protein; IFN-γ, interferon-γ; STAT1, signal transducer and activator of transcription 1; U3A, a human adenocarcinoma cell line; HT29, a human colon adenocarcinoma cell line; TNF-α, tumor necrosis factor-α; FasL, Fas ligand; JNK, Jun NH2-terminal kinase.
Specific Induction of Fas Expression by IFN-γ. Previous studies using flow cytometric analysis and Northern blot have demonstrated that IFN-γ and TNF-α induces the expression of Fas protein in HT29 cells, a human colon adenocarcinoma cell line (6). Using Western blot analysis, we examined the induction of Fas expression in IFN-γ-treated HT29 cells. In addition, we tested whether growth factors, PDGF, EGF, and FGF, were able to induce Fas expression. As shown in Fig. 1A, IFN-γ at the concentration of 100 units/ml dramatically enhanced the Fas expression; FGF and TNF-α only slightly upregulated expression of Fas protein, whereas EGF and PDGF had no effect on the expression of Fas. In addition, our Western blot analysis revealed that Fas protein existed in two major forms; this phenomenon has been described previously (7) and is due to the differential glycosylation of Fas proteins. Using anti-Fas mAb (CH-11) as a primary antibody in Western blot analysis, similar results were obtained (data not shown). Additional experiments show that the induction of Fas expression by IFN-γ was both time and dose dependent (Fig. 1, B and C, upper panel). Fas expression was slightly decreased when IFN-γ was used at high concentrations (>50–100 units/ml), probably due to the increasing cell death as described below.

Specific Induction of FasL Expression by IFN-γ. Regulation of Fas expression by IFN-γ in both malignant and normal cells has been reported previously (4–7); however, little is known about how FasL is regulated. Here we tested whether FasL was regulated by PDGF, EGF, FGF, IFN-γ, and TNF-α. As shown in Fig. 1A (bottom panel), IFN-γ and TNF-α increased the expression of FasL by two to four times as determined by densitometric analysis. In contrast, PDGF, EGF, and FGF had no effect on the expression of FasL. We further demonstrated that IFN-γ induced FasL expression in HT29 cells in a dose- and time-dependent manner (Fig. 1, B and C, bottom panel). We attempted to quantitate FasL expression by using reverse transcription-PCR and flow cytometric analysis; however, FasL was undetectable. This is consistent with previously published data showing that FasL was undetectable (19) or very weakly detectable in HT29 cells (20).
A specificity of IFN-γ-induced cell death and enhancement by anti-Fas mAb. HT29 cells were incubated with growth factors (20 ng/ml each of PDGF, EGF, and FGF). Treatment of HT29 cells with anti-Fas mAb alone induced moderate cell death (Fig. 2A), suggesting that PDGF or EGF synergizes with anti-Fas mAb to induce apoptosis. Finally, our results show that IFN-γ (100 units/ml) induced cell death of HT29 in a time-dependent manner (Fig. 2C).

IFN-γ-induced DNA Fragmentation in HT29 Cells. To confirm that trypan blue-stained dead cells in IFN-γ-treated HT29 cells had undergone apoptosis, we performed DNA fragmentation analysis. The results in Fig. 3 (left panel) show that IFN-γ (100 units/ml), but not EGF (20 ng/ml) alone, induced DNA fragmentation in HT29 cells. Treatment of HT29 cells with anti-Fas mAb alone induced moderate DNA fragmentation; pretreatment with IFN-γ followed by anti-Fas mAb did not induce more DNA fragmentation because IFN-γ alone already induced intensive DNA fragmentation. Interestingly, EGF treatment, which by itself did not induce apoptosis, sensitized these cells to anti-Fas-mediated apoptosis. These results are consistent with the data of percentage of cell death as determined in a trypan blue exclusion assay (Fig. 2A). DNA fragmentation was also observed in a positive control of Jurkat cells incubated with anti-Fas mAb for 20 h (Fig. 3A, Lane 3) but not in Jurkat cells incubated with mouse IgM (Fig. 3A, Lane 2).

We then tested whether DNA fragmentation induced by IFN-γ was dose dependent and whether low concentrations of IFN-γ could sensitize HT29 cells to anti-Fas mAb-induced DNA fragmentation. The results in Fig. 3B (right panel) show that IFN-γ (0.1 and 1 unit/ml) did not induce significant apoptosis in HT29 cells, although IFN-γ at these concentrations induced 35 and 45% cell death in trypan blue exclusion analysis, respectively (Fig. 2B). IFN-γ at the concentrations of 5 and 10 units was able to significantly induce DNA fragmentation in these cells and further enhanced anti-Fas-mediated apoptosis.

IFN-γ Induces the Expression of Fas and FasL in STAT1-transfected U3A Cells but not in STAT1-deficient U3A Cells. Previous studies have demonstrated that IFN-γ inhibits the growth of STAT1-transfected U3A cells but not STAT1-deficient U3A cells (12). We postulated that STAT1 activation may play a critical role in IFN-γ-mediated Fas and FasL up-regulation. To test this idea, STAT1-deficient U3A cells and STAT1-transfected U3A cells were treated with IFN-γ or TNF-α; Fas and FasL expression was analyzed by Western blotting. As shown in Fig. 4, IFN-γ (100 units/ml) did not up-regulate the expression of either Fas or FasL in STAT1-deficient U3A cells; in contrast, IFN-γ significantly induced the expression of both Fas and FasL in STAT1-transfected U3A cells. TNF-α (1000 units/ml) had no effect on the Fas and FasL expression in either cell line. These results suggest that STAT1 protein is required for IFN-γ-mediated up-regulation of Fas and FasL expression.

IFN-γ Induces Apoptosis in STAT1-transfected U3A Cells but not in STAT1-deficient U3A Cells. We further tested whether the up-regulation of Fas and FasL in STAT1-transfected U3A cells resulted in apoptosis. The results in Fig. 5A show that IFN-γ (100 units/ml) did not induce STAT1-deficient U3A cell death but induced death in 87% of STAT1-transfected U3A cells. In contrast, TNF-α (1000 units/ml) induced cell death in both cell lines, although STAT1-transfected U3A cells were more sensitive to TNF-α than STAT1-deficient U3A cells. Consistent with these observations, chromosomal DNA integrity analysis (Fig. 5B) shows that IFN-γ induced DNA fragmentation in STAT1-transfected U3A cells but not in STAT1-deficient U3A cells, whereas TNF-α and anti-Fas mAb (CH-11; 0.5 μg/ml) induced DNA fragmentation in both cell lines. These results suggest that STAT1 protein is required for IFN-γ-mediated apoptosis.

Fig. 2. Analysis of IFN-γ-induced cell death. HT29 cells were grown in 24-well plates in the complete McCoy's 5 medium containing 10% FBS. Upon 80% confluence, complete medium was replaced by DMEM containing 0.5% FBS. Cells were then incubated in the presence of the indicated stimuli. The percentage of cell death was determined by trypan blue exclusion assay. A, specificity of IFN-γ-induced cell death and enhancement by anti-Fas mAb. HT29 cells were incubated with growth factors (20 ng/ml each of PDGF, EGF, and FGF), IFN-γ (100 units/ml), or TNF-α (1000 units/ml) for 40 h, mouse IgM, or anti-Fas mAb (CH-11; 0.5 μg/ml) was added to each well. Cells were incubated for another 20 h. Single-cell suspensions were prepared, and cell death was monitored by trypan blue exclusion assay. The data are means of three separate experiments; bars, SD. CTR, control; FBS, fetal bovine serum.

Fig. 3. IFN-γ-induced DNA fragmentation in HT29 cells. HT29 cells were preincubated with IFN-γ (100 units/ml) or EGF (20 ng/ml) for 40 h, then mouse IgM or anti-Fas mAb (CH-11) (0.5 μg/ml) was added. After 20 h of incubation, cells were collected and lysed in buffer containing 10 mM EDTA, 50 mM Tris-HCl (pH 8.0), and 0.5% sodium sarkosinate. Cell lysates were then digested by proteinase K and RNase A (0.5 mg/ml each). DNA integrity was examined by electrophoresis on a 2% gel and stained by ethidium bromide. DNA samples from Jurkat cells treated with mouse IgM (Lane 2) or anti-Fas mAb (Lane 3) were included as controls.
FIG. 4. IFN-γ-induced Fas and FasL expression in STAT1-transfected U3A cells but not in STAT1-deficient cells. U3A and STAT1-transfected U3A cells were grown in 12-well plates. Upon 80% confluence, cells were washed twice and then incubated for 40 h in DMEM medium with 0.5% FBS in the absence or presence of IFN-γ (100 units/ml) or TNF-α (1000 units/ml). Cell lysates were prepared, and proteins were separated on a 10% SDS-PAGE. Fas (upper panel) and FasL (bottom panel) were detected by Western blotting. The data shown are a representative of two separate experiments with similar results.

DISCUSSION

Previous studies have demonstrated that Fas is expressed on HT29 cells (6, 7, 10, 19), and that IFN-γ up-regulates Fas expression as detected by flow cytometric analyses (10) and Northern blot (7). In addition, IFN-γ also increases the expression of Fas in several types of tumor cells, including gliomas (4, 13), breast cancer (14), prostate cancer (15), hepatomas (16), and in normal epithelial cells (5, 6, 9). Consistent with these observations, we confirm that IFN-γ and TNF-α up-regulated Fas expression by Western blot analysis. FasL expression has been detected in a number of tumor cell lines as well as in fresh tumor specimens, including human lung carcinomas (22), astrocytomas (23), hepatomas (24), melanomas (25), and colon adenocarcinomas (19). However, the regulation of FasL expression in tumor cells has not been described previously. Our present studies demonstrate that FasL was also up-regulated by IFN-γ in a dose-dependent and a time-dependent manner.

Up-regulated Fas and FasL proteins in IFN-γ-treated tumor cells may interact with each other to induce apoptosis. Using trypan blue exclusion assay and DNA fragmentation analysis, we show that IFN-γ induced apoptosis in two tumor cell lines, HT29 cells, a human colon adenocarcinoma, and U3A cells, a human myeloblastoma. Our unpublished data show that IFN-γ also induced cell death in A431 cells, a human epidermal carcinoma cell line. These results suggest that IFN-γ-mediated growth inhibition in various types of tumors, especially in the FasL-expressing tumors, is at least in part due to the Fas-mediated apoptosis. Several lines of our experimental evidence support this notion: (a) the kinetics of Fas and FasL expression in IFN-γ-treated cells correlated well with that of cell death; (b) the level of Fas in HT29 cells treated with various doses of IFN-γ correlated with the percentage of cell death and levels of DNA fragmentation; (c) Fas and FasL were up-regulated by IFN-γ in STAT1-transfected U3A cells but not in STAT1-deficient U3A cells. Similarly, IFN-γ induced cell death in former but not in later cell line; (d) the inhibitor of interleukin-1β-converting enzyme blocked IFN-γ-induced cell death. These results collectively suggest that up-regulation of Fas and FasL expression by IFN-γ plays a critical role in IFN-γ-induced apoptosis.

STAT1 protein plays a central role in IFN-γ-triggered signal transduction. Further studies have shown that STAT1 induces expression of Fas and FasL. Inhibition of Cell Death by Caspase-1 Inhibitors. Fas-mediated apoptosis involves the activation of a cascade of proteases, such as caspase-8 and caspase-1. We reasoned that if IFN-γ-induced cell death in HT29 and in STAT1-transfected U3A cells involves the Fas-mediated proteolytic pathway, the caspase-1 inhibitor that blocks the Fas-mediated activation of caspase would also inhibit IFN-γ-induced cell death. The results in Fig. 6 show that IFN-γ (10 units/ml) induced 78.9 and 44.2% cell death in STAT1-transfected U3A and HT29 cells, respectively; ZVAD-fmk reduced IFN-γ-induced cell death to 8.1% in STAT1-transfected-U3A cells, whereas YVAD-cmk reduced INF-γ-induced cell death to 16.9% in HT29 cells. Because ZVAD-fmk alone was somewhat toxic to HT29 cells, whereas YVAD-cmk alone was particularly toxic to STAT1-transfected U3A cells (data not shown), the effects of ZVAD-fmk on HT29 cells and the effects of YVAD-cmk on STAT1-transfected U3A cells were not pursued. Nevertheless, inhibition of cell death in IFN-γ-treated HT29 and STAT1-transfected U3A cells by caspase-1-specific inhibitors strongly suggests that IFN-γ induced tumor cell death by Fas-mediated apoptosis.

FIG. 5. IFN-γ-induced apoptosis in STAT1-transfected U3A cells but not in STAT1-deficient cells. A, IFN-γ-induced cell death in STAT1-transfected U3A cells but not in STAT1-deficient U3A cells. U3A and STAT1-transfected U3A cells were grown in 12-well plates. Upon 80% confluence, cells were washed twice and then incubated for 40 h in DMEM with 0.5% FBS in the absence or presence of IFN-γ (100 units/ml) or TNF-α (1000 units/ml). Cell lysates were prepared, and proteins were separated on a 1.2% agarose gel. Anti-Fas mAb (CH-11; Lane 6 in left panel and Lane 4 in right panel) induced apoptosis in U3A cells (left panel) and STAT1-transfected U3A cells (right panel) are included as positive controls.

Further studies have shown that IFN-γ induces cell death by the interaction of up-regulated Fas and FasL.
counted, and the percentage of cell death was calculated. The results are means of transfected U3A cells were grown in 24-well plates in triplicate. Upon 80% confluence, containing the IFN-stimulated regulatory element (2, 3, 26, 27). The auction pathway. Tyrosine phosphorylation of STATI proteins by 
were lifted off with 2.5 mM EDTA/PBS and ihen stained with trypan blue. Cells were exist in this region (28), suggesting that STATI may indirectly up-regulate of caspase-8, a protease of the caspase family that is involved in the Fas-triggered proteolytic pathway. Up-regulation of caspase-8 results in the sensitization of these tumor cells to anti-Fas-mediated apoptosis. Consistent with these observations, Chin et al. (30) and Kumar et al. (31) more recently reported that the STAT1 signaling pathway is required for the expression of caspase-1 and apoptosis in A431 cells and in STAT1-transfected U3A cells. Taken together, these results suggest that IFN-γ may induce apoptosis of tumor cells by multiple mechanisms. It is interesting to notice that EGF and PDGF, although unable to increase the expression of Fas and FasL in HT29 cells, augmented anti-Fas-induced cell death and DNA fragmentation (Figs. 2A and 3A). EGF and PDGF bind their corresponding receptors, activate receptor tyrosine kinases, and trigger several signal transduction pathways, such as MAP kinase and JNK pathways. Emerging evidence indicates that MAP kinase and JNK signal transduction pathways cooperate with the Fas-mediated proteolytic pathway (32-34). Recent studies by Goillot et al. (33) have demonstrated that activation of MAP kinase and JNK kinase pathways is required for Fas-mediated apoptosis, and that transfection of dominant-negative mutants of JNK and ERK kinases blocks anti-Fas-induced cell death in SHEP cells, a human neuroblastoma cell line. Therefore, sensitization of HT29 cells to anti-Fas mAb-mediated apoptosis by PDGF and EGF may be due to the synergistic effects between activated MAP/JNK kinase pathways and Fas-triggered proteolytic process. Earlier studies by O’Connell et al. (19) show that HT29 cells, although expressing high levels of Fas, are resistant to anti-Fas-mediated apoptosis. We have repeated that experiment; however, we found that anti-Fas IgM mAb (0.5 μg/ml) induced significant DNA fragmentation of HT29 cells when grown in the medium containing either 0.5% serum (Fig. 3A, Lane 7) or 10% serum (data not shown). In addition, anti-Fas mAb also induced the death of 35–40% HT29 cells (Fig. 2, A and B). A plausible explanation for this discrepancy is that the different concentrations of anti-Fas mAb being used; 0.5 μg/ml of anti-Fas mAb (CH-11) was used in our experiments, whereas only 0.1 μg/ml of same antibody was used in theirs. Our results suggest that Fas expressed on HT29 cells is functional and capable of transducing a death signal and inducing apoptosis. In conclusion, our present studies demonstrate that IFN-γ not only up-regulates the expression of Fas but also of FasL in two tumor cell lines. Up-regulation and interaction of Fas and FasL result in tumor cell apoptosis, which is prevented by blocking the STAT1-dependent signal transduction pathway or by the caspase inhibitor. Our results suggest that IFN-γ may inhibit cell growth by Fas-mediated apoptosis, thereby providing a cellular explanation for IFN-γ-mediated antitumor activity.

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IFN-γ Induces Cell Growth Inhibition by Fas-mediated Apoptosis: Requirement of STAT1 Protein for Up-Regulation of Fas and FasL Expression

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