Journal of Cellular Biochemistry 85:369–380 (2002)

Interferon Regulatory Factor-1 Mediates Interferon-γ-Induced Apoptosis in Ovarian Carcinoma Cells

Eun-Joo Kim,[1] Joon-Mo Lee,[2] Sung-Eun Namkoong,[2] Soo-Jong Um,[3]* and Jong-Sup Park[1,2]**

[1]Department of Medical Bioscience, Graduate School, Catholic University, Seoul, Korea
[2]Department of Obstetrics and Gynecology, Catholic University Medical College, Seoul, Korea
[3]Department of Bioscience and Biotechnology/Institute of Bioscience, Sejong University, Seoul, 143-747, Korea

Abstract Interferon-γ (IFN-γ), as one of interferon family that regulates antiviral, antiproliferative, and immunomodulatory responses, has been implicated for the growth regulation of ovarian cancer cells. However, the molecular mechanisms are not yet fully defined. To analyze detailed mechanisms, the ovarian cancer cell lines (2774, PA-1, OVCAR-3, and SKOV-3) were treated with IFN-γ. The growth of 2774 was most effectively suppressed than that of other cells in both time-course and dose-dependent experiments. The order of sensitivity in other cells was PA-1 ≫ OVCAR-3 > SKOV-3 (not responded at all). The DNA fragmentation and DAPI staining assays suggested that the IFN-γ-mediated cytotoxicity could be triggered by apoptosis. The treatment induced IFN regulatory factor-1 (IRF-1) in two IFN-γ-sensitive cells (2774, PA-1), whereas IRF-1 was not induced in two IFN-γ-resistant cells (OVCAR-3, SKOV-3). The levels of p53 and p21WAF1 were not strikingly changed in all four cells. Interestingly, the expression of interleukin-converting enzyme (ICE, or caspase-1) was increased by the treatment in a kinetically consistent manner to the induction of IRF-1. However, CD95 (Fas/APO-1) was not changed. Apoptosis was greatly induced, when IRF-1 was transiently expressed in PA-1 without the treatment of IFN-γ. However, it was repressed when IRF-1 together with IRF-2, an antagonist of IRF-1, were coexpressed. In addition, the effect of IFN-γ was reduced in the 2774 and PA-1 cells stably expressing either *IRF-1* antisense or *IRF-2* sense, as shown by the cytotoxicity and FACS analysis. Furthermore, the IFN-γ-induced apoptosis was greatly reduced, when inhibitors of ICE were treated into PA-1 cells. Taken together, these results suggest that IRF-1 directly mediates the IFN-γ-induced apoptosis via the activation of *caspase-1* gene expression in IFN-γ-sensitive ovarian cancer cells. J. Cell. Biochem. 85: 369–380, 2002. © 2002 Wiley-Liss, Inc.

Key words: IFN-γ; apoptosis; IRF-1; IRF-2; caspase-1; ovarian cancer cells

Despite an aggressive surgical debulking followed by cisplatin-containing chemotherapy, only 20–30% of patients with stage III epithelial ovarian carcinoma are alive after 5 years [Ozols and Young, 1991]. Resistance to chemotherapy is an important factor in the failure to cure many patients with ovarian cancer. Several strategies to overcome resistance are still investigated and the high-dose chemotherapy, which is supported with hematopoietic growth factors and progenitor cells, would be one of the approaches in the treatment of high-risk group of ovarian cancer [Kotz and Schilder, 1995]. Prolongation of disease-free survival may emerge in use of interferon (IFN) as an adjuvant to high-dose chemotherapy for high-risk patients of ovarian cancer.

Epithelial ovarian carcinomas very frequently involve a mutation in the *p53* tumor suppressor gene. It has been widely accepted that p53 plays an important role in protecting against ovarian tumor formation [Aunoble et al., 2000]. Recently, p53 and interferon regulatory factor-1 (IRF-1) proteins induced by the treatment of IFN-γ in HPV-positive cervical cancer cells [Um et al., 2000], or activated in response to DNA damage caused by radiation [Tanaka et al., 1996], have

Grant sponsor: Ministry of Health and Welfare, Republic of Korea; Grant number: HMP-01-M-2-0020.

*Correspondence to: Soo-Jong Um, Department of Bioscience and Biotechnology, Sejong University, 98 Kunja-dong, Kwangjin-gu, Seoul, 143-747, Korea.
E-mail: umsj@sejong.ac.kr

**Correspondence to: Jong-Sup Park, Department of Obstetrics and Gynecology, Catholic University Medical College, 505 Banpo-dong, Secho-gu, Seoul, 137-040, Korea.
E-mail: jspark@cmc.cuk.ac.kr

Received 19 November 2001; Accepted 17 January 2002
DOI 10.1002/jcb.10142

© 2002 Wiley-Liss, Inc.

been shown to mediate G1 arrest via the cooperative induction of p21WAF1, a cell cycle inhibitor. IRF-1, originally identified as an *IFN-β* promoter binding transcription factor, has been implicated for a mediator of IFN signaling, when induced by various stimuli such as the viral infection, IFNs, retinoic acids, prolactin, and TNF-α [Mamane et al., 1999]. Moreover, it has been reported that IRF-1 inhibits *c-myc* or *fosB*-induced cell transformation [Tanaka et al., 1994a], and that introduction of activated c-Haras alone is sufficient to transform embryo fibroblasts from $IRF\text{-}1^{-/-}$ mice [Tanaka et al., 1994b]. These data would suggest that the *IRF-1* is a tumor suppressor gene that may be associated with the growth regulatory function of IFNs.

Of two IFN types, type II (IFN-γ) has been known to increase cytotoxic sensitivity to TNF-α and CD95/Fas antibody in various cancer cell lines [Fransen et al., 1986; Yonehara et al., 1989]. The IFN-γ treatment of ovarian cancer xenograft in vivo induced growth arrest and apoptosis of tumor cells, and increased mouse survival [Burke et al., 1997]. Recently, the maximal induction of both *p21WAF1* and *IRF-1* mRNA was observed after 2–3 days of IFN-γ exposure as cells became committed to cell death [Burke et al., 1999], but the downstream regulation of cell death in ovarian carcinoma cells is still unclear. Caspases are aspartate-specific cysteine proteases that are activated sequentially in a hierachical cascade and play a central role in the execution phase of apoptosis [Salvesen and Dixit, 1997]. A different pathway could activate the apoptosis of the various target cells by different agents. One of them is related to the activation of interleukin-1β converting enzyme (ICE)/caspase-1 that utilizes the Fas/APO-1 pathway in the apoptosis of T lymphoid cells [Enari et al., 1995]. IRF-1 has been initially known to mediate the radiation-induced apoptosis through the transcriptional induction of the *ICE* gene in T lymphocytes [Tamura et al., 1995]. Afterwards, it has been further reported that IRF-1, induced either by the IFN-γ treatment, DNA damage, or serum depletion, could mediate apoptosis via induction of ICE in U937 leukemia cells, primary hepatocytes, vascular smooth muscle cells, and pancreatic islets [Tamura et al., 1996; Horiuchi et al., 1999; Kano et al., 1999; Karlsen et al., 2000]. However, the evidence was not clearly provided that IRF-1 plays a direct role in apoptosis in those cells.

In this study, we analyzed the growth suppressive effects of the IFN-γ on the ovarian carcinoma cell lines, which carry a different genetic condition of *p53*, by focusing on the ability of IFN-γ to the induction of *IRF-1* expression and its direct role in apoptosis. Our investigation provides direct evidence that IRF-1 is a critical mediator of the IFN-γ-induced apoptosis in ovarian carcinoma cells, which could be a molecular basis for the use of IFN-γ in the chemotherapeutic treatment of ovarian cancers.

MATERIALS AND METHODS

Cell Culture and Treatment of IFN-γ

In this study, we used four kinds of ovarian carcinoma cell lines (ATCC, Rockville, MD), which carry a different condition of p53 (Table I). These cells were routinely maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (HyClone Laboratories, Logan, UT), previously inactivated at 56°C for 20 min. Human recombinant IFN-γ (Gibco BRL, Gaithersburg, MD) was reconstituted in sterile water to a concentration of 0.1 mg/ml ($\sim 10^6$ IU/ml), and added to the medium to a final concentration of 1 ng/ml, 5 ng/ml, and 10 ng/ml ($\sim$ 100 IU/ml) depending on the experimental conditions.

Cell Proliferation Assay

The effects of IFN-γ on the cell proliferation were determined by direct cell counting using hemocytometer. Cells were grown in six-well culture plates starting at an initial density of 10^4 cells/2 ml medium/well. One day after seeding, different concentrations of IFN-γ (0, 1, 5, and 10 ng/ml) were added to the medium and cells were grown for 4 days. The results were expressed as mean values of cell numbers of at least four wells.

DNA Fragmentation Assay

Pretreated ovarian carcinoma cells (2×10^5) with IFN-γ at a concentration of 10 ng/ml were

TABLE I. Ovarian Carcinoma Cell Lines Used

Cell line	Origin (human)	*p53* status
PA-1	Teratocarcinoma	CCC → CCT (Pro316Pro)
2774	Adenocarcinoma	CGT → CAT (Arg273His)
OVCAR-3	Adenocarcinoma	CGG → CAG (Arg248Gln)
SKOV-3	Adenocarcinoma	Deletion (no p53 expression)

washed twice with ice-cold PBS. The cells were lysed with 0.5 ml of lysis buffer (100 mM NaCl, 10 mM Tris-HCl, pH 8.0, 25 mM EDTA, pH 8.0, 0.5% SDS, 0.1 mg/ml proteinase K), incubated overnight at 37°C, and extracted with a phenol-chloroform-isoamyl alcohol (25:24:1) mixture. After separation of phase by centrifugation at 8,000*g* for 10 min twice, the DNA was precipitated with 2.5 vol ethanol and pelleted at 10,000*g* for 30 min. The pellet was dissolved in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0). Solubilized DNA was treated with DNase-free RNase (Sigma, St. Louis, MO) and electrophoresed in a 2% agarose gel at 2 volts/cm for 5–6 h. A 100 bp DNA ladder (Amersham Pharmacia Biotech, UK) was used as a molecular size marker standard. The gel was stained, visualized by UV transilluminator, and photographed.

DAPI Staining

IFN-γ-treated cells were washed with 1 × PBS, fixed with 70% ethanol for 20 min at room temperature, and washed again with 1 × PBS. The cells were then treated with DAPI (1 mg/ml) (Sigma) at the 1:1,000 dilution, left for 12 min, and washed again with 1 × PBS for 5 min. After being treated with 50 μl VectaShield (Vector Laboratories, Burlingame, CA), cells were observed under a fluorescence microscope (Provis AX70: Olympus Optical Co, Japan).

Western Blot Analysis

The procedure was performed as described previously [Um et al., 2000]. Depending on the experimental conditions, ovarian carcinoma cells were seeded in 10 cm dishes and grown with different concentrations of IFN-γ (0, 1, 5, 10 ng/ml) for 4 days. Primary antibodies and the dilutions used for immunoblotting were: p53 (1:500), mouse monoclonal, Ab-6 (Calbiochem, La Jolla, CA); IRF-1 (1:250), rabbit polyclonal, C-20 (Santa Cruz Biotechnolgy, Santa Cruz, CA); IRF-2 (1:200), rabbit polyclonal, C-19 (Santa Cruz Biotechnolgy); p21WAF1 (1:100), mouse monoclonal, Ab-1 (Calbiochem); Fas (1:200) rabbit polyclonal, C-20 (Santa Cruz Biotechnology), ICE (1:200), rabbit polyclonal, C-20 (Santa Cruz Biotechnology). Either peroxidase-conjugated mouse or rabbit IgG secondary antibodies were purchased from Amersham Pharmacia Biotech. All antibodies were diluted in PBS containing 5% skim milk and 0.1% Tween 20. Protein bands were detected by ECL system (Amersham Pharmacia).

Transient Transfection and In Situ β-Gal Staining

PA-1 cells (2×10^5/100 mm dish) were co-transfected with SV40-driven β-gal reporter plasmid [Um et al., 2000], and 4 μg of pCDNA3 (Invitrogen, Carlsbad, CA), pCDNA3-IRF-1, and/or pCDNA3-IRF-2 plasmid mixtures by LipofectAMINE (Gibco BRL). After 48 h, transfected cells were washed with 1 × PBS and fixed lightly in 5 ml fixative (2% formaldehyde + 0.2% glutaraldehyde in PBS) for 5 min. The cells were washed twice with 10 ml PBS and stained with 5ml staining solution (5mM K ferricyanide, 2 mM $MgCl_2$, 1 mg/ml X-gal) for 2 h at 37°C. Stained cells were rinsed with 10 ml PBS, counted, and photographed.

Construction of Stable Cells Expressing IRF-1 Antisense or IRF-2 Sense

IRF-1 antisense [IRF-1(L)] and IRF-2 sense [IRF-2(R)] expression vectors were constructed by subcloning reversely oriented IRF-1 cDNA and rightly oriented IRF-2 cDNA, both amplified by PCR and verified by sequencing, into neomycin-resistant pCDNA3. 2774 and PA-1 cells were transfected with either IRF-1 antisense or IRF-2 sense using LipofectAMINE (Gibco BRL) for 48 h. Cells were treated with Geneticin (Gibco BRL) at 500 μg/ml for 2774 and 400 μg/ml for PA-1. After 5–7 days, cells were incubated with fresh culture medium containing Geneticin, and resistant colonies were selected for 2 months.

FACS Analysis

After IFN-γ treatment for 72 h, adherent and detached cells were combined and fixed overnight with 70% ethanol in washing buffer (PBS containing 5 mM EDTA) at 4°C. After centrifugation at 3,000*g* for 1 min, cell pellets were incubated for 30 min with 500 μl washing buffer and 50 μl RNase A (10 mg/ml). Cells were then stained in 500 μl of PBS containing 100 μg/ml of propidium iodide. A total of 1×10^6 cells were analyzed in a flow cytometer (FACS-vantage: Becton Dickinson Biosciences, San Jose, CA).

RESULTS

Effect of IFN-γ on the Proliferation of Ovarian Carcinoma Cells

Direct cell counting by hemocytometer was employed to determine the IFN-γ-dependent

cytotoxicity of ovarian carcinoma cells. As shown by a decreased number of cells, which is indicative of cell viability, IFN-γ treatment greatly inhibited the proliferation of both PA-1 and 2774 cells in a time-dependent manner (Fig. 1A). The effect of IFN-γ in both PA-1 and 2774 cells was dose-dependent, and was the highest at a concentration of 10 ng/ml (Fig. 1B). In contrast to the growth-suppressive effect of IFN-γ in PA-1 and 2774 cells, the treatment weakly suppressed the growth of OVCAR-3 cells and did not suppress the growth of SKOV-3 cells carrying deleted form of *p53* gene, of which product is not expressed (Fig. 1 and Table I). At this point, it is not clear whether the expression of wild-type p53 is critical for the IFN-γ response to ovarian cancer cells. Differential modulation mechanism of cell growth by IFN-γ in different ovarian carcinoma cells will be further described in the next.

Detection of Apoptosis by DNA Fragmentation Assay and DAPI Staining

During cell apoptosis, an early biochemical event is the activation of an endogenous nuclear endonuclease, leading to the degradation of genomic DNA, which can be revealed as a characteristic DNA ladder pattern by gel electrophoresis. When OVCAR-3 and SKOV-3 cells were treated with IFN-γ, the gel electrophoresis revealed no observable DNA fragmentation pattern (data not shown). However, the DNA ladder pattern became evident in 2774 and PA-1 cells after the treatment of IFN-γ starting at 1 ng/ml (for 2774) and 5 ng/ml of IFN-γ (for PA-1), indicating the degradation of nucleosomal DNA, a hallmark of apoptosis, in cells (Fig. 2A). The DNA ladder formation was more pronounced at the concentration of 10 ng/ml.

DAPI nuclear staining was performed to confirm the apoptotic changes shown by the DNA fragmentation. The control culture contained normal, healthy tumor cells. After 4 days of IFN-γ treatment in 2774 and PA-1 cells, while the viable cells were reduced, the number of apoptotic cells increased. The shrunk nucleus and apoptotic bodies were characteristic for apoptosis in DAPI staining (Fig. 2B). These data indicate that the cytotoxicity of IFN-γ is likely due to the induction of apoptosis in IFN-sensitive 2774 and PA-1 ovarian carcinoma cells.

IFN-γ Response Analyzed by Western Blotting

To determine which protein is involved in the IFN-γ-induced apoptosis, we first analyzed the expression of *IRF-1*, one of IFN-inducible genes. The ovarian carcinoma cells were treated with different amounts of IFN-γ, collected at Day 4, and steady-state IRF-1 protein level was analyzed by Western blotting. When equal amounts of total protein were loaded as determined by Western blotting of β-actin (data not shown), IRF-1 proteins were begun to be detected at the concentration of 1 ng/ml (for 2774) and 5 ng/ml IFN-γ (for PA-1), and the protein were steadily elevated by increasing the concentration of IFN-γ (Fig. 3). However, right-sized IRF-1 was not expressed in OVCAR-3 and SKOV-3 cells regardless of IFN-γ, whereas a faster migrating, than the induced wild-type IRF-1, band was detected. We excluded this band to be a dominant-negative IRF-1, because we also observed in several other cell lines including mouse NIH3T3 cells, and more clearly we could not clone by several RT–PCR approaches (data not shown). The expression pattern of IRF-1 was correlated to the cytotoxic and apoptotic responses of the IFN-sensitive ovarian cancer cells to IFN-γ. These overall results suggest that the IFN-γ signaling might be operating through the expression of IRF-1 in 2774 and PA-1 cells, but not in OVCAR-3 and SKOV-3 cells.

Since wild-type p53 protein is critical to initiate growth arrest and apoptosis, we have investigated the changes of p53 protein by the treatment of IFN-γ in the ovarian carcinoma cells that carry different *p53* conditions (Table I). As shown in Figure 3, the p53 level was not changed by the treatment, suggesting that the growth suppression in IFN-γ-sensitive 2774 (mutant *p53*) and PA-1 (wild-type *p53*) cells is not likely mediated by p53-dependent pathway. Furthermore, faintly weak expression of p21WAF1 indicates that the IFN response might not be affected by the p21WAF1 induction, and thus not be related to G1 arrest (data not shown).

In order to clarify the mechanism of IFN-γ induced apoptosis in 2774 and PA-1 ovarian carcinoma cell lines, Western blotting assays for apoptosis-related proteins (CD95: Fas/APO-1, interleukin-1β-converting enzyme: ICE or caspase-1) were performed. As described above, we observed an increase in cell death and IRF-1 levels following IFN-γ treatment in 2774 and

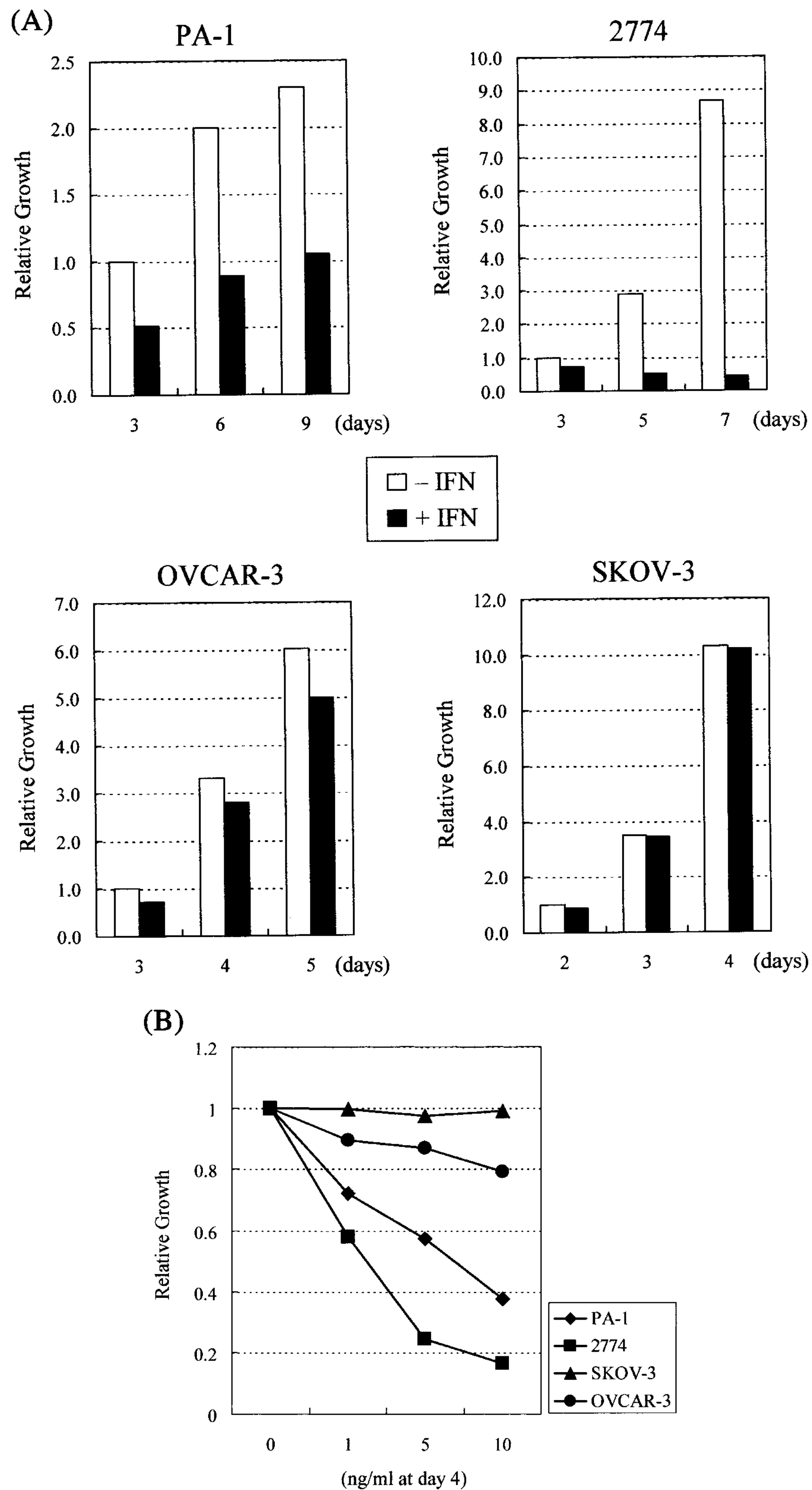

Fig. 1. Cytotoxic effects of IFN-γ. Relative growth responses of four ovarian cancer cells were measured. For time-course experiment (**A**), cells were cultured in the presence of 1 × PBS (□) or 10 ng/ml of IFN-γ (■) for various days. For dose-dependent experiment (**B**), cells were grown with different concentrations of IFN-γ (0, 1, 5, 10 ng/ml) for 4 days. The numbers of viable cells were counted at each condition using hemocytometer and expressed as a relative average growth of at least three wells.

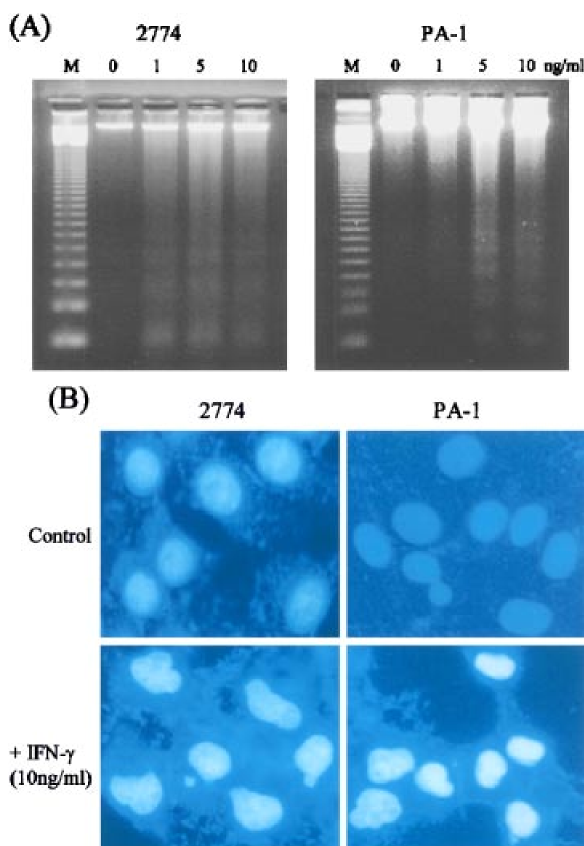

Fig. 2. IFN-γ induces apoptosis in two ovarian carcinoma cells cytotoxic to IFN-γ. Apoptosis was analyzed by DNA fragmentation (**A**) and DAPI staining (**B**) in 2774 and PA-1 cells. Either cell line (2×10^5) was treated with 0, 1, 5, and 10 ng/ml of IFN-γ. After 4 days, DNA was purified and ladder formation was examined. M, molecular weight marker; DAPI staining was described in Materials and Methods. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

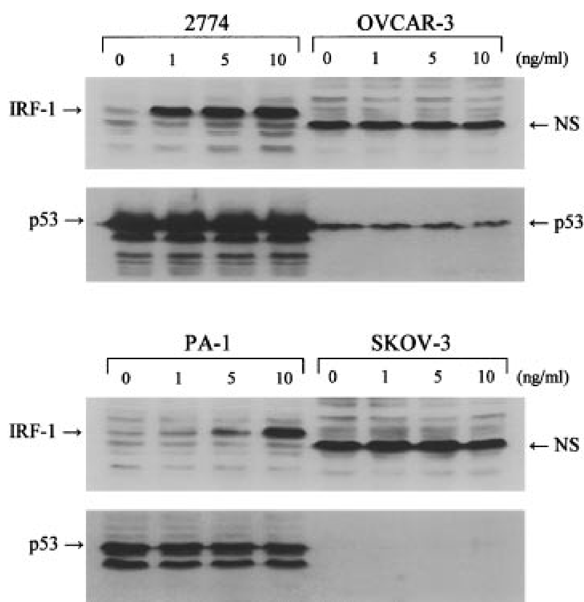

Fig. 3. IFN-γ induces IRF-1 only in the apoptosis-sensitive ovarian carcinoma cells. Four ovarian carcinoma cells were seeded and grown with different concentrations of IFN-γ (0, 1, 5, 10 ng/ml) for 4 days. After cell extract was prepared, the amounts of total protein were measured by the Bio-Rad protein assay. An equal amount (35 μg) of protein was withdrawn, fractionated on a 12% polyacrylamide/SDS gel, transferred to Hybond ECL nitrocellulose membrane, and probed with rabbit polyclonal IRF-1 or mouse p53 antibody, and then peroxidase-conjugated rabbit anti-IgG antibody.

PA-1 cells. Our analysis revealed that ICE protein was induced markedly by increasing the concentration of IFN-γ, but the protein levels of Fas were not changed (Fig. 4). Again, the expression pattern of ICE was correlated to the cytotoxicity, induction of apoptosis, and expression of IRF-1 in IFN-sensitive ovarian cancer cells. In SKOV-3 and OVCAR-3 cells, no expression of ICE protein was observed in response to IFN-γ treatment (data not shown). These results suggest that IFN-γ-induced IRF-1 may directly mediate the apoptosis of IFN-sensitive 2774 and PA-1 ovarian carcinoma cells through the induction of ICE, one of target genes of IRF-1 transcription factor.

IRF-1 Directly Mediates the Induction of Apoptosis

To determine whether IRF-1 is directly involved in the induction of apoptosis, we performed several experiments as followed. First, either expression vector of IRF-1 or IRF-1 plus IRF-2 was transiently transfected together with SV40-NLS-β-gal (designed for expressing in nucleus) into IFN-sensitive PA-1 cells. After stained with X-gal, blue cells expressing NLS-β-gal in nucleus were selected, counted and photographed as shown in Figure 5. When IRF-1 was transiently expressed, the number of stained cells was dramatically decreased. When IRF-1, together with IRF-2, an antagonist of IRF-1 [Yamamoto et al., 1994], was

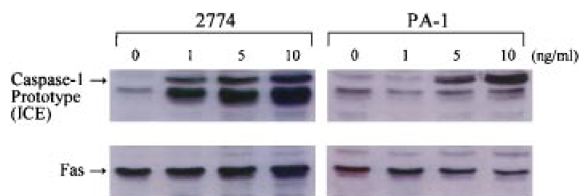

Fig. 4. IFN-γ induces ICE in two ovarian carcinoma cells cytotoxic to IFN-γ. Two ovarian carcinoma cells (2774 or PA-1) were seeded and grown with different concentrations of IFN-γ (0, 1, 5, 10 ng/ml) for 4 days. Details for Western blotting were described in Materials and Methods and above.

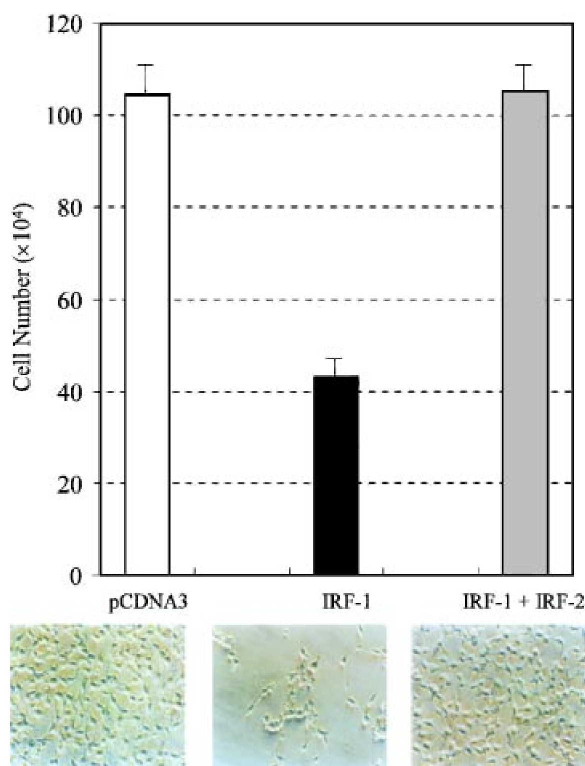

Fig. 5. Transiently expressed IRF-1 reduces, whereas IRF-2 resumes the proliferative potential of PA-1 cells. Transient transfections were performed in 6-well plates with SV40-driven β-gal reporter plasmid, and pCDNA3, pCDNA3-IRF-1, and/or pCDNA3-IRF-2 plasmid mixtures as indicated. After transfection, cells were fixed, washed, and stained with X-gal. The numbers of stained cells were counted and expressed as average cell numbers of at least three wells. Each bar: the mean ± SE of three determinations; $P < 0.001$. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

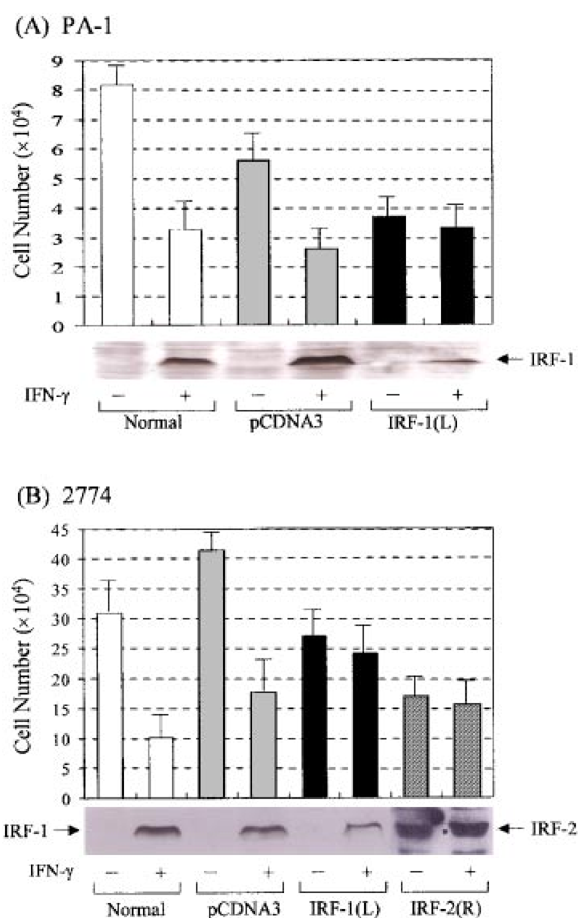

Fig. 6. The cytotoxic effect of IFN-γ was almost abrogated in either stable cells expressing IRF-1 antisense [IRF-1(L)] or IRF-2 sense [IRF-2(R)]. PA-1 cells was constructed to express IRF-1(L). Two kinds of 2774 cells were constructed to express IRF-1(L) or IRF-2(R). Three clones of each stable cell line were pooled for cytotoxicity assay and for Western blotting as described. Each bar: the mean ± SE of three determinations; $P < 0.01$.

expressed, the number of cells was recovered to the number of cells transfected with empty pCDNA3 vector. Thus, it is possible that IRF-1 increases cell death and IRF-2 interferes with the IRF-1-induced cell death, implying that IRF-1 may directly mediate apoptosis of PA-1 cells. Second, we constructed stable cell lines either expressing IRF-1 antisense [IRF-1(L)] or IRF-2 sense [IRF-2(R)]. At least three stable clones from each construction were selected, pooled, and extracted for Western blotting and cytotoxicity analysis. As shown in Figure 6A, the IFN-γ treatment had much less effect on the cytoxicity, when the expression of IRF-1 was reduced by the IRF-1(L) in PA-1 cells. Again, in 2774 cells expressing either IRF-1(L) or IRF-2(R), the IFN response was much reduced than in control cells (Fig. 6B). The expression of IRF-1 and IRF-2 was normalized by Western blotting of β-actin (data not shown). Third, FACS was employed to analyze the IFN effect in these stable cells. As shown in Figure 7, the sub-G1 populations of parental 2774 and PA-1 cells were increased, whereas those of stable cells were not much changed with increasing the concentration of IFN-γ. Again, the kinetics of IFN effect in FACS analysis was quite consistent with those of cytoxocity and apoptosis as shown previously in parental cells. Taken together, it could be suggested that IRF-1, induced in response to IFN-γ, is critical mediator for the IFN-γ-induced cytotoxicity and apoptosis of 2774 and PA-1 cells.

ICE Is Involved in IRF-1-Mediated Apoptosis

To determine that ICE (caspase-1) is one of downstream target of IRF-1 and critical for

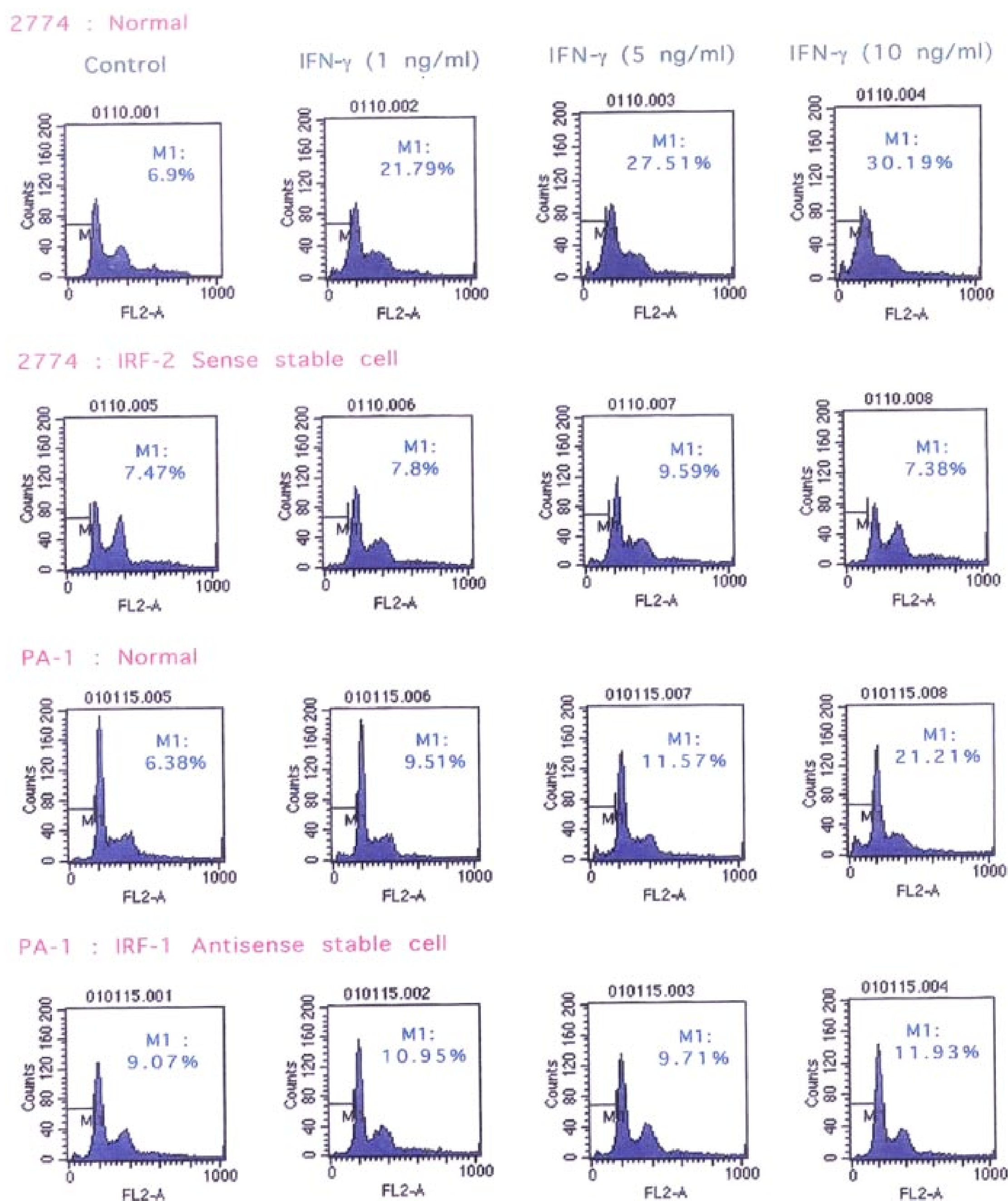

Fig. 7. Inhibition of IRF-1 expression or activity blocks the IFN-γ-induced cytotoxicity. 2774[IRF-2(R)] or PA-1[IRF-1(L)] stable cells treated with IFN-γ. 96 h after treatment, sub-G1 populations were evaluated by FACS analysis. M1 (%) represents portion of sub-G1 (hypodiploid DNA). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

IRF-1-medated apoptosis, we first analyzed the expression of ICE in response to IFN-γ as shown in Figure 4. Second, the IFN response was analyzed by the treatment of caspase-1 inhibitor (#1: AC-YVAD-CHO) or caspase-1 like inhibitor (#2: Z-VAD-FMK). When either inhibitor was treated together with IFN-γ in PA-1 or 2774 cells, the cytotoxic effect of IFN was almost abrogated (Fig. 8). This and previous Western blotting data suggest that the IFN-induced IRF-1 may mediate apoptosis of IFN-sensitive ovarian cancer cells via induction of ICE.

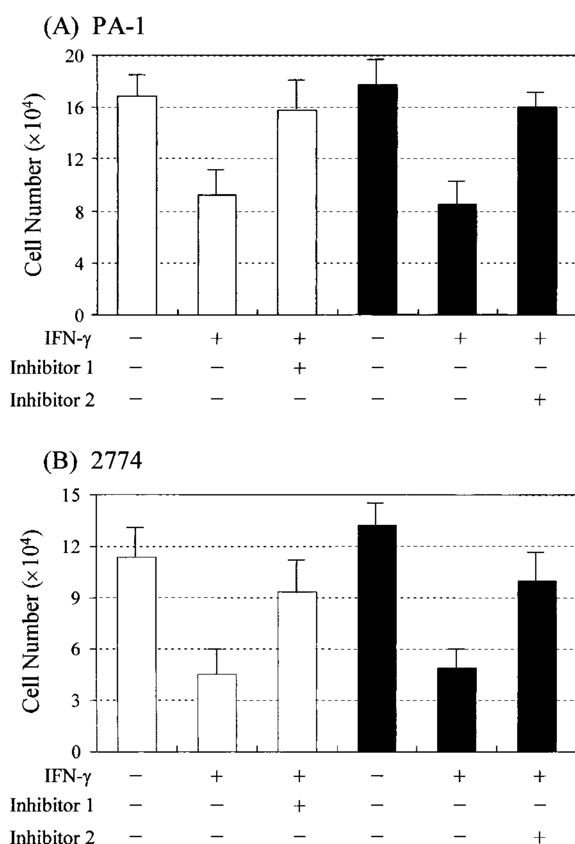

Fig. 8. ICE is involved in the IFN-γ-induced apoptosis. The effect of ICE (caspase-1) inhibitor was measured by direct counting after being treated together with IFN-γ for 4 days. The numbers of cells were expressed as average cell numbers of at least three wells. Inhibitor (10 μM) used were #1: caspase-1 inhibitor (AC-YVAD-CHO) and #2: caspase-1 like inhibitor (Z-VAD-FMK). Each bar: the mean ± SE of three determinations; $P < 0.001$.

DISCUSSION

Several chemotherapeutic drugs are routinely used in treating ovarian carcinoma, but the clinical effectiveness is limited by the emergence of drug resistance in tumor cell population. The main problems of chemotherapy are toxicity to normal immune cells and failure to kill cancer cells. As an adjunctive method, IFN-γ was additionally supplemented to the conventional treatment of ovarian cancer. IFN-γ showed growth inhibitory effect in the ovarian carcinoma cells, and thus might be of therapeutic benefit to some patients of ovarian cancer [Pujade-Lauraine et al., 1996]. IFN-γ is known to induce cell cycle arrest or apoptosis in a model of ovarian cancer by enhanced effects of p53-independent, intermediate molecules [Burke et al., 1997]. Maximal induction of both *p21WAF1* and *IRF-1* mRNA was observed after 2–3 days of IFN-γ exposure as cells became committed to cell death by G1 arrest and apoptosis [Burke et al., 1999], but the downstream regulation of cell death in ovarian carcinoma cells is still unclear. In this study, we found that IFN-γ had remarkable growth-suppressive effect in PA-1 and 2774 ovarian carcinoma cells, which carry wild type *p53* and mutant *p53*, respectively. In contrast to other report [Burke et al., 1999], p21WAF1 was very weakly induced in our conditions, suggesting that the IFN-γ-dependent growth-suppression in the ovarian cancer cells mainly resulted from apoptosis rather than G1 arrest. Respective gel electrophoresis and DAPI staining revealed DNA fragmentation and shrinkage of nucleus, a hallmark of apoptosis, in pretreated PA-1 and 2774 cells with IFN-γ. Apoptosis is a cell suicide mechanism that enables to control cell number in tissues and to eliminate individual cells that threaten the host's survival. Apoptosis can be initiated by a various insults that can damage DNA, including ultraviolet and X-irradiation and chemotherapeutic drugs. The central component of apoptosis machinery is a proteolytic system involving a family of proteases called "caspases." These enzymes participate in a cascade that is triggered in response to proapoptotic signals and culminates in cleavage of a set of proteins, resulting in disassembly of the cell [Salvesen and Dixit, 1997]. IFN-γ, when bound to its receptor complex, activates the JAK/STAT (Janus kinase/signal transducer and activator of transcription) pathway [Igarashi et al., 1994]. The IFN-γ-activated JAK phosphorylates STAT-1 protein, which is then translocated to the nucleus, resulting in transcriptional activation of specific target genes, including interferon regulatory factor-1 (IRF-1). Recently, it has been reported that IRF-1 is involved in IFN-γ-induced cell arrest or apoptosis of some tumor and primary cells as a critical mediator [Tamura et al., 1996; Tanaka et al., 1996; Horiuchi et al., 1999; Kano et al., 1999; Karlsen et al., 2000; Um et al., 2000]. When the ovarian carcinoma cells were treated with IFN-γ in our experiments, IRF-1 protein in 2774 and PA-1 cells were expressed starting at the concentration of 1 and 5 ng/ml IFN-γ, respectively, and the protein levels were steadily elevated by increasing the concentration of IFN-γ. Right-sized IRF-1 protein was not induced in

OVCAR-3 and SKOV-3 cells regardless of the presence of IFN-γ. The expression kinetics of IRF-1 was fairly correlated to the cytotoxic and apoptotic responses of the IFN-sensitive ovarian cancer cells to IFN-γ. Thus, it could be suggested that the IFN-γ signaling might be operating through the expression of IRF-1 in 2774 and PA-1 cells, but not in OVCAR-3 and SKOV-3 cells. Given the notion that IRF-1 mediates the antiproliferative function of IFNs, it is possible that elevated IRF-1 in response to IFN-γ may contribute at least partly to the induction of apoptosis of 2774 and PA-1 cells.

From three experiments to determine whether IRF-1 directly mediates the induction of apoptosis, first we demonstrated that transiently expressed IRF-1 increases cell death, whereas IRF-2 interferes with the IRF-1-induced cell death in PA-1 cells. Since an IRF-1 response element (ISRE) is located in *tet* operator [Rang and Will, 2000], and thus IRF-1 inducible cell line could not be constructed using a classical *tet*-on/off system, we detoured to construct stable cells expressing either IRF-1 antisense [IRF-1(L)] or IRF-2 sense [IRF-2(R)]. From the second experiment using the stable cells, we found that the IFN response was greatly abrogated in those cell lines, as shown by cell counting after in situ β-gal staining. However, the IFN response was not totally abolished, probably because the expression of antisense IRF-1 in both cells could not completely block the induction of sense IRF-1 by the IFN treatment. These results suggest that a certain threshold level of IRF-1 is required for the regulation of cell death, which remains to be determined. Third, we confirmed second data by FACS that was used to count the sub-G1 populations. Again, the FACS results were kinetically consistent with those of cytoxocity and apoptosis as shown previously in parental cells. Taken together, it could be suggested that IRF-1 might be necessary and sufficient for the IFN-γ-induced cell death of 2774 and PA-1 cells. However, it should be further determined whether overexpression of IRF-1 leads to apoptosis of the unresponsive cell lines (OVCAR-3 and SKOV-3) for IRF-1 to be necessary and sufficient for the IFN-γ-induced cell death of ovarian cancer cells.

Two different antioncogenic transcriptional factors, p53 and IRF-1, are required for distinct apoptotic pathways in T lymphocyte. When induced by DNA-strand breaking agents, p53 mediates apoptosis in thymocytes [Clarke et al., 1993]. Since it has been proposed that DNA damage also can induce apoptosis in proliferating lymphoid cells via p53-independent mechanisms [Strasser et al., 1994], p53 is not the only mediator of apoptosis provoked by DNA damage. Consistently, other reports showed that DNA damage-induced apoptosis is dependent on IRF-1 in mitogen-activated mature T lymphocytes [Tamura et al., 1995]. In our experiment, the IFN response was not correlated to the genetic status of *p53* gene (compare 2774 and PA-1 cells: Table I) and p53 level was not changed by the treatment, suggesting that growth suppression by IFN-γ is not mediated in the p53-dependent manner. The expression of IFN-inducible genes is regulated by distinct IRF-1-dependent and -independent mechanisms [Mamane et al., 1999]. Besides IRF-1, several factors have been identified in the synergistic activation of type I *IFN* genes. Such factors include NF-κB, Oct-1, and CREB/ATF in the promoter of *IFN-β* gene. IRF-1 mediates diverse functions, including tumor suppression, myeloid differentiation, macrophage activation, antigen presentation, and T- and B-cell differentiation [Taniguchi et al., 1999]. IRF-1 is also considered to be the transcriptional activator of *IL-1β* converting enzyme (ICE or caspase-1) gene, a product of which can induce apoptosis in conjunction with other apoptosis related proteins [Salvesen and Dixit, 1997]. It has been shown that IFN-γ upregulates Fas antigen on the cell surface, and it was firstly proposed that this effect accounted for augmented Fas-mediated apoptosis [Yonehara et al., 1989]. Furthermore, it has been shown that Fas-mediated apoptosis depends on ICE and the overexpression of ICE enhances Fas-mediated apoptosis [Enari et al., 1995; Los et al., 1995]. In our study, we have shown that the IFN-γ treatment induced a transcriptional factor IRF-1 and ICE protein in a kinetically correlated manner in IFN-sensitive ovarian carcinoma cells. In addition, Fas protein was not induced by the treatment. Therefore, it could be postulated that the IFN-γ-induced, ICE-mediated apoptosis might not be related to Fas signaling, but rather ICE alone might be an important control point in the proteolytic cascade leading to apoptosis in ovarian carcinoma cells. This postulation could further be supported by our finding that ICE inhibitors almost prevents the cytotoxicity effect of IFN-γ in 2774 or PA-1 cells, confirming that ICE is involved in

the IFN-γ-induced apoptosis of two ovarian cancer cells.

In summary, it is likely that the IFN-γ treatment induces IRF-1 through the IFN-γ signaling pathway, once induced IRF-1 transcriptionally activates the expression of ICE (or caspase-1), a known target of IRF-1 transcription factor, and finally expressed ICE mediates apoptosis of the IFN-sensitive 2774 and PA-1 ovarian cancer cells. The sequential induction of IRF-1 and ICE by IFN-γ leads a functional interplay between these two proteins for a programmed cell death independent of genetic status of *p53* gene in ovarian carcinoma cells. IFN-γ was effective only for the ovarian cancer cells that could express IRF-1 in response to IFN-γ. Therefore, it should be considered that the IFN response is p53-independent and specific for certain cell types that could express IRF-1, i.e., IRF-1-dependent, when IFN-γ is considered as an alternative treatment trial of ovarian cancers.

REFERENCES

Aunoble B, Sanches R, Didier E, Bignon YJ. 2000. Major oncogenes and tumor suppressor genes involved in epithelial ovarian cancer. Int J Oncol 16:567–576.

Burke F, East N, Upyon C, Patel K, Balkwill FR. 1997. Interferon gamma induces cell cycle arrest and apoptosis in a model of ovarian cancer: enhancement of effect by batimastat. Eur J Cancer 33:1114–1121.

Burke F, Smith PD, Crompton MR, Upton C, Balkwill FR. 1999. Cytotoxic response of ovarian cancer cell lines to IFN-γ is associated with sustained induction of IRF-1 and p21 mRNA. Br J Cancer 80:1236–1244.

Clarke AR, Purdie CA, Harrison DJ, Morris RG, Bird CC, Hooper ML, Wyllie AH. 1993. Thymocyte apoptosis induced by p53-dependent and independent pathways. Nature 362:849–852.

Enari M, Hug H, Nagata S. 1995. Involvement of an ICE-like protease in Fas-mediated apoptosis. Nature 375:78–81.

Fransen L, Van der Heyden J, Ruysschaert R, Fiers W. 1986. Recombinant tumor necrosis factor: its effect and its synergism with interferon-gamma on a variety of normal and transformed human cell lines. Eur J Cancer Clin Oncol 22:419–426.

Horiuchi M, Yamada H, Akishita M, Ito M, Tamura K, Dzau VJ. 1999. Interferon regulatory factors regulate interleukin-1beta-converting enzyme expression and apoptosis in vascular smooth muscle cells. Hypertension 33:162–166.

Igarashi K, Garrota G, Ozmen L, Ziemiecki A, Wilks AF, Harper AF, Larner AC, Finbloom DS. 1994. Interferon-γ induced tyrosine phosphorylation of interferon-γ receptor and regulated association of protein kinase, JAK1, and JAK2 with its receptor. J Biol Chem 269:14333–14336.

Kano A, Haruyama T, Akaike T, Watanabe Y. 1999. IRF-1 is an essential mediator in IFN-gamma-induced cell cycle arrest and apoptosis of primary cultured hepatocytes. Biochem Biophys Res Commun 257:672–677.

Karlsen AE, Pavlovic D, Nielsen K, Jensen J, Andersen HU, Pociot F, Mandrup-Poulsen T, Eizirik DL, Nerup J. 2000. Interferon-gamma induces interleukin-1 converting enzyme expression in pancreatic islets by an interferon regulatory factor-1-dependent mechanism. J Clin Endocrinol Metab 85:830–836.

Kotz KW, Schilder RJ. 1995. High-dose chemotherapy and hematopoietic progenitor cell support for patients with epithelial ovarian cancer. Semin Oncol 22:250–262.

Los M, Van de Craen M, Schenk H, Westendorp M, Baeuerle PA, Droge W, Krammer PH, Fiers W, Schulze-Osthoff K. 1995. Requirement of an ICE/CED-3 protease for Fas/Apo-1-mediated apoptosis. Nature 375:81–83.

Mamane Y, Heylbroeck C, Genin P, Algarte M, Servant MJ, LePage C, DeLuca C, Kwon H, Lin R, Hiscott J. 1999. Interferon regulatory factors: the next generation. Gene 237:1–14.

Ozols RF, Young RC. 1991. Chemotherapy of ovarian cancer. Semin Oncol 18:222–232.

Pujade-Lauraine E, Guastalla J-P, Colombo N, Devillier P, Franciois E, Fumoleau P, Monnier A, Nooy M, Mignot L, Bugat R, Margues C, Mousseau M, Netter G, Maloisel F, Larbaoui S, Brandly M. 1996. Intraperitoneal recombinant interferon gamma in ovarian cancer patients with residual disease as second look laparotomy. J Clin Oncol 14:343–350.

Rang A, Will H. 2000. The tetracycline-responsive promoter contains functional interferon-inducible response elements. Nucleic Acids Res 28:1120–1125.

Salvesen GS, Dixit VM. 1997. Caspases: intracellular signaling by proteolysis. Cell 91:443–445.

Strasser A, Harris AW, Jacks T, Cory S. 1994. DNA damage can induce apoptosis in proliferating lymphoid cells via p53-independent mechanisms inhibitable by Bcl-2. Cell 79:329–339.

Tamura T, Ishihara M, Lamphier MS, Tanaka N, Oishi I, Aizawa S, Matsuyama T, Mak TW, Taki S, Taniguchi T. 1995. An IRF-1-dependent pathway of DNA damage-induced apoptosis in mitogen-activated T lymphocytes. Nature 376:596–599.

Tamura T, Ueda S, Yoshida M, Matsuzaki M, Mohri H, Okubo T. 1996. Interferon-gamma induces Ice gene expression and enhances cellular susceptibility to apoptosis in the U937 leukemia cell line. Biochem Biophys Res Commun 229:21–26.

Tanaka N, Ishihara M, Taniquchi T. 1994a. Suppressor of *c-myc* or *fosB*-induced cell transformation by the transcription factor IRF-1. Cancer Lett 83:191–196.

Tanaka N, Ishihara M, Kitagawa M, Harada H, Kimura T, Matsuyama T, Lamphier MS, Aizawa S, Mak TW, Taniguchi T. 1994b. Cellular commitment to oncogene-induced transformation or apoptosis is dependent on the transcription factor IRF-1. Cell 77:829–839.

Tanaka N, Ishihara M, Lamphire MS, Nozawa H, Matsuyama T, Mak TW, Aizawa S, Tokino T, Oren M, Taniguchi T. 1996. Cooperation of the tumor suppressors IRF-1 and p53 in response to DNA damage. Nature 382: 816–818.

Taniguchi T, Tanaka N, Ogasawara K, Taki S, Sato M, Takaoka A. 1999. Transcription factor IRF-1 and its family members in the regulation of host defense. Cold Spring Harb Symp Quant Biol 64:465–472.

Um SJ, Kim EJ, Hwang ES, Kim SJ, Namkoong SE, Park JS. 2000. Antiproliferative effects of retinoid/interferon in cervical carcinoma cell lines: cooperative growth suppression of IRF-1 and p53. Int J Cancer 85:416–423.

Yamamoto H, Lamphier MS, Fujita T, Taniguchi T, Harada H. 1994. The oncogenic transcription factor IRF-2 possesses a transcriptional repression and a latent activation domain. Oncogene 9:1423–1428.

Yonehara S, Ishii A, Yonehara M. 1989. A cell-killing monoclonal antibody (Anti-Fas) to a cell surface antigen co-downregulated with the receptor of tumor necrosis factor. J Exp Med 169:1747–1756.