Synergistic antitumor effect of interferon-ß with gemcitabine in interferon-α-non-responsive pancreatic cancer cells

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Abstract. Interferon (IFN)-ß is reported to have more potent antitumor effects than IFN-α. The aim of this study was to compare the synergistic antitumor activity of both IFNs when combined with gemcitabine on cultured pancreatic cancer cells expressing various levels of IFN receptor. The growth-inhibitory effects of IFN-α and IFN-ß in combination with gemcitabine on three human pancreatic cancer cell lines (BxPC-3, MIAPaCa-2, Panc-1) were evaluated by MTT assay and isobolographic analysis. We also correlated their growth-inhibitory effects with the expression status of type I IFN receptor type 2 (IFNAR2). Western blot analysis indicated strong expression of IFNAR2 in BxPC-3 and MIAPaCa-2, but weak expression in Panc-1. The growth-inhibitory effect of gemcitabine was enhanced synergistically by IFN-α in BxPC-3 and MIAPaCa-2, but not in Panc-1. IFN-ß exhibited more potent synergistic growth-inhibitory effects with gemcitabine in BxPC-3 and MIAPaCa-2 compared to IFN-α, and also synergistic enhancement in Panc-1. In conclusion, our results indicated that the growth-inhibitory effect of IFN-ß with gemcitabine was synergistic not only in pancreatic cancer cells with strong expression of IFNAR2, but also in those with weak expression of IFNAR2.

Introduction

Pancreatic cancer is one of the leading causes of cancer-related mortalities (1). The prognosis of patients with pancreatic cancer is especially poor even after curative resection, and more than 50% of patients develop tumor recurrence at distant or locoregional sites, with an estimated 5-year survival of only 20% (2-5). Therefore, chemotherapy and/or radiotherapy play an important role in the treatment of pancreatic cancer. Gemcitabine (GEM), a cell-cycle specific inhibitor of DNA synthesis and ribonucleotide reductase, has become the golden standard chemotherapeutic agent (6,7). However, the response rate to GEM is also less than 20%, suggesting that the outcome remains unsatisfactory (6).

To date, many investigators have reported that interferon (IFN) has antitumor properties when combined with other chemotherapeutic agents. For example, our group has reported the antitumor effect of IFN-α and 5-fluorouracil on hepatocellular carcinoma (HCC) (8-10), while others have also documented the clinical effect of IFN-α in pancreatic cancer (11-15). In this regard, a 5-year survival of 55% following IFN-α-based adjuvant chemoradiotherapy for patients with resectable pancreatic cancer it has been reported (16). However, as we showed previously, type I IFN receptor type 2 (IFNAR2), which plays an important role in IFN-induced signal transduction pathways and is useful for predicting the effectiveness of IFN-α-based combination chemotherapy, is expressed only in only 25% of immunohistochemically stained resected specimens of pancreatic cancer (17-20). Considering such expression rate, it is conceivable that the anticipated clinical outcome of IFN-α-based combination therapy is poor in pancreatic cancer.

IFN-ß belongs to the same type of IFNs as IFN-α, and interacts with the same IFN receptor as IFN-α (21-24). The reported antitumor activity of IFN-ß exceeds that of IFN-α in several kinds of cancers (25-29). We compared previously the antitumor of IFN-ß to IFN-α on HCC and reported that IFN-ß had synergistic antitumor effect with anticancer drugs even in HCC cells with weak IFNAR2 expression level (30).

The tested hypothesis in the present study was that IFN-ß has a synergistic antitumor effect with GEM in pancreatic cancer cells, which are known to weakly express IFNAR2. For this purpose, we compared the antitumor effects of IFN-α and IFN-ß on pancreatic cancer cells with various expression levels of IFNAR2, and investigated whether IFN-ß has synergistic antitumor effect with GEM not only in pancreatic cancer cells with strong expression of IFNAR2 but also in those with weak expression of IFNAR2.

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Materials and methods

Pancreatic cancer cell lines. Three human pancreatic carcinoma cell lines were used in the present study. MIAPaCa-2 cell line was obtained from the Japanese Collection of Research Bioresources (JCRB, Tokyo, Japan). BxPC-3 and Panc-1 cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). These cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 100 U/ml penicillin and 100 mg/ml streptomycin at 37˚C in a humidified incubator with 5% CO_2 in air.

Drugs and reagents. Purified human IFN-α and IFN-ß was kindly supplied by Otsuka Pharmaceutical Co. (Tokyo, Japan) and Daiichi Sankyo Co. (Tokyo, Japan), respectively. GEM was purchased from Eli Lilly and Co. (Indianapolis, IN, USA). Rabbit polyclonal anti-human IFNAR2 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) was used as the primary antibody in Western blot analysis and immunofluorescence. The antibody targets IFNAR2 long-form, which is considered important for IFN binding and signal transduction (17,18). Specific rabbit anti-human signal transducer and activator of transcription factor (STAT) 1, phosphorylated STAT (pSTAT) 1 (Tyr701) (Cell Signaling Technology, Beverly, MA, USA), STAT2, pSTAT2 (Tyr689) (Upstate Biotechnology, Lake Placid, NY, USA), STAT3, pSTAT3 (Tyr705) (Cell Signaling Technology), and actin (Sigma-Aldrich Co. St. Louis, USA) were used in Western blot analysis.

Real-time quantitative reverse transcription-polymerase chain reaction. Real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was performed using the designed oligonucleotide primers and the Light Cycler (Roche Diagnostics, Mannheim, Germany). The expression of the target gene was normalized relative to the expression of *porphobilinogen deaminase* (*PBGD*), which was used as an internal control. The designed PCR primers were as follows; *IFNAR2* forward primer 5'-AGTCCACTCCAGGA CCCTTT-3', *IFNAR2* reverse primer 5'-TCCTCTGGGTCA ACCATCTC-3', *PBGD* forward primer; 5'-TGTCTGGTAA CGGCAATGCGGCTGCAAC-3'; *PBGD* reverse primer; 5'-TCAATGTTGCCACCACACTGTCCGTCT-3'.

Western blot analysis. Cells grown to semiconfluence were washed with phosphate-buffered saline (PBS) and collected with a rubber scraper. After centrifugation, the cell pellets were resuspended in RIPA buffer [25 mM Tris (pH 7.5), 50 mM NaCl, 0.5% sodium deoxycholate, 2% Nonidet P-40, 0.2% sodium dodecyl sulfate, 1 mM phenylmethylsulphonyl fluoride and 500 KIE/ml Trasylol, proteinase inhibitor, (Bayer LeverKusen, Germany)] with phosphatase inhibitor (Sigma-Aldrich Co.). The extracts were centrifuged and the supernatant fraction was collected. Western blot analysis was carried out as described previously (31). The intensity of each protein band was determined with a densitometer and expressed relative to that of actin.

Growth-inhibitory assay. Cell growth was assessed by the 3-(4-,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma-Aldrich Co.) assay as described previously (32). Cells were incubated with various concentrations of the IFNs and/or GEM for 72 h. After treatment, the MTT solution was added to each well. Non-reacted MTT was then removed, leaving the resultant formazan crystals at the bottom of the well. Then, acid-isopropanol was added to dissolve the crystal. Absorbance was measured in a microplate reader at a wavelength of 570 nm with a 650 nm reference, and the results were expressed as the percentage of absorbance relative to untreated controls.

Immunofluorescence. Cells were fixed with PBS containing 4% paraformaldehyde at room temperature. After washing with PBS, the cells were treated with PBS containing 5% normal rabbit serum, 1% bovine serum albumin, and 0.1% Triton X-100 at room temperature. The cells were then blocked with normal rabbit serum in PBS. Cells were incubated overnight at 4˚C with anti-IFNAR2 antibody as the primary antibody. After washing in PBS, the sections were incubated with secondary antibody [Texas Red-conjugated mouse anti-rabbit IgG (Santa Cruz Biotechnology)]. Finally, the nuclei were counterstained with 4', 6'-diamidino-2-phenylindole (DAPI) solution (Sigma-Aldrich Co.). The cells were visualized with a Biozero digital microscope (Keyence, Osaka, Japan).

Annexin V assay. The binding of annexin V was used as a sensitive method for assessment of apoptosis, using the method described previously (33). Twenty-four hours after treatment with 10 ng/ml GEM and/or 1000 IU/ml IFN-α/ß, the cancer cells were stained by Annexin V-FITC and propidium iodide (PI) (BioVision Research Products, Mountain View, CA, USA), and analyzed on a FACS Calibur (BD Biosciences, Franklin Lakes, NJ). Annexin V-positive and PI-negative cells considered as early apoptotic cells were used for the assessment of apoptosis in the study (34).

Evaluation of cooperative effects. The synergistic cytotoxicity of GEM and IFNs was determined by isobolographic analysis as described by Berenbaum *et al* (35).

Statistical analysis. Data were expressed as mean ± SD. Continuous variables were compared using the Student's t-test. Statistical analysis was performed using StatView (version 5.0, SAS Institute Inc., Cary, NC, USA). A $p<0.05$ was considered statistically significant.

Results

IFNAR2 expression. The *IFNAR2* mRNA expression level was determined in the three cell lines by real-time qRT-PCR, and that of protein level by Western blot analysis and immunofluorescence. As shown in Fig. 1A, *IFNAR2* mRNA level was significantly higher in BxPC-3 than in the other two cell lines ($p<0.05$), and significantly lower in Panc-1 than in the other two cell lines ($p<0.05$). The same trend was also observed in the IFNAR2 protein level as assessed by Western blot analysis (Fig. 1A). In immunofluorescence, IFNAR2 was homogeneously and strongly expressed in the cell membrane in BxPC-3 and MIAPaCa-2 cells, but was not obvious in

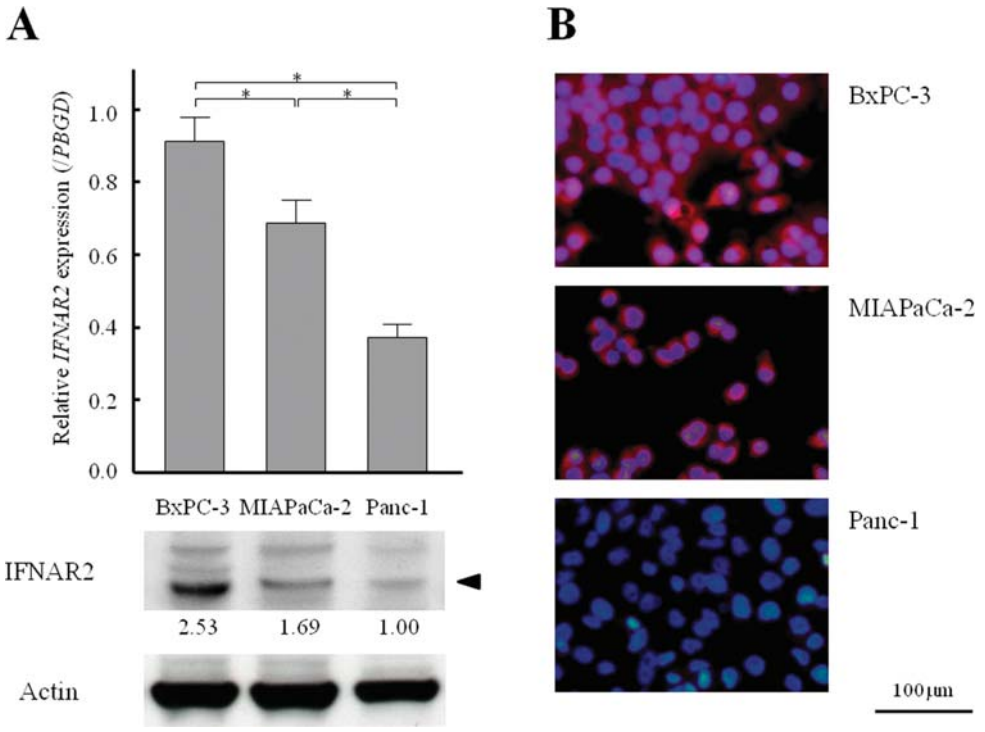

Figure 1. IFNAR2 expression level in pancreatic cancer cell lines. *IFNAR2* mRNA expression level was examined by real-time qRT-PCR (top panel) and IFNAR2 protein expression level by Western blot analysis (bottom panel) (A) and by immunofluorescence (B). The relative intensity of IFNAR2 protein band assessed with a densitometer is shown below the protein bands. Data in (A) represent the mean ± SD of three experiments. *p<0.05. Western blot analysis showed strong expression of IFNAR2 in BxPC-3 and MIAPaCa-2, and weak expression in Panc-1. Immunofluorescence showed that IFNAR2 was not clearly identified in Panc-1, but in BxPC-3 and MIAPaCa-2.

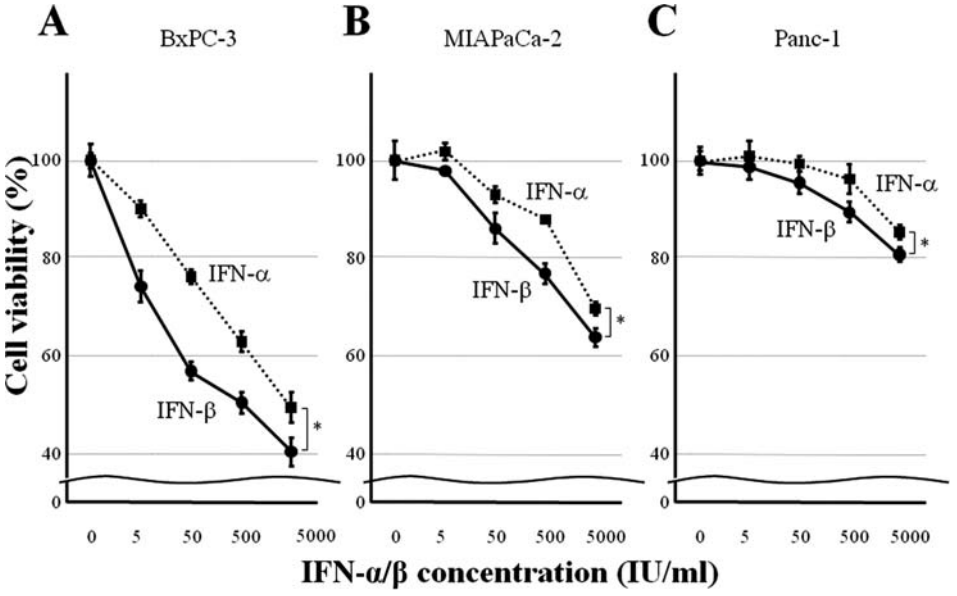

Figure 2. Growth-inhibitory effects of IFN-α (dotted lines) and -ß (thick lines) on pancreatic cancer cell lines (A) BxPC-3, (B) MIAPaCa-2 and (C) Panc-1. After incubation for 72 h, cell viability was determined by the MTT assay. The growth-inhibitory effect of IFN-ß was significantly more potent than that of IFN-α in all three cell lines. Data are mean ± SD of three experiments. *p<0.05.

Panc-1 (Fig. 1B). Thus, BxPC-3 and MIAPaCa-2 expressed IFNAR2 protein as confirmed by immunofluorescence and Western blot analysis, while Panc-1 was judged not to express IFNAR2 protein in immunofluorescence and weakly expressed IFNAR2.

Antiproliferative effects of IFNs. The effects of IFN-α and -ß on cell growth were compared in three human pancreatic cancer cell lines (BxPC-3, MIAPaCa-2 and Panc-1) (Fig. 2). The sensitivity of cells to both IFNs was considerably different among the cell lines. The growth-inhibitory effect of IFNs

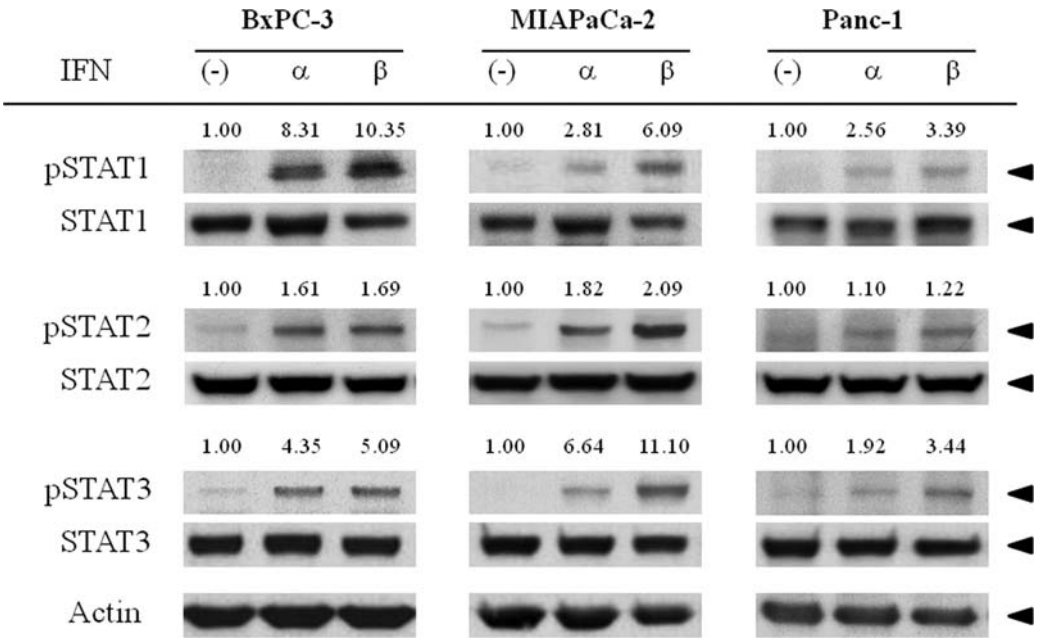

Figure 3. Activation of STATs by IFNs in pancreatic cancer cell lines. Activation of STATs was assessed by phosphorylation of STAT1, STAT2 and STAT3. The relative intensity of pSTAT1, pSTAT2 and pSTAT3 assessed with a densitometer is shown below the protein bands. Activation of STATs by IFN-ß was stronger in all three cell lines compared with IFN-α. Representative examples of three experiments with similar results.

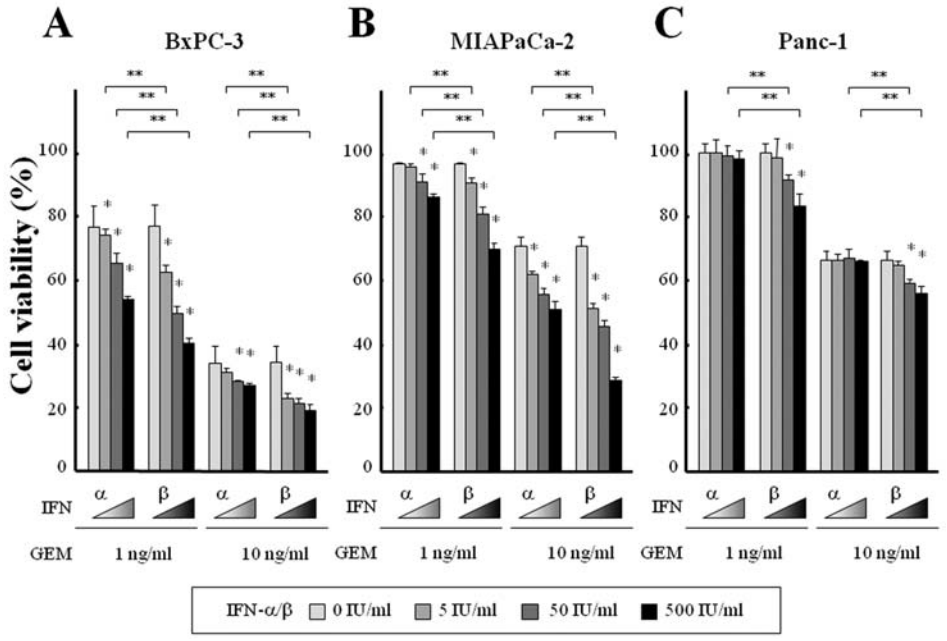

Figure 4. Growth-inhibitory effects of GEM and IFNs on (A) BxPC-3, (B) MIAPaCa-2, (C) Panc-1 pancreatic cancer cell lines. After incubation for 72 h, cell viability was determined by MTT assay. In BxPC-3 and MIAPaCa-2, both types of IFNs also enhanced the growth-inhibitory effects of GEM ($^{*}p<0.05$), but the enhancement was more remarkable in IFN-ß than IFN-α ($^{**}p<0.05$). In Panc-1, IFN-ß, but not IFN-α, enhanced the growth-inhibitory effect of GEM. Data are mean ± SD of three experiments.

was weak in Panc-1 and moderate in MIAPaCa-2, whereas BxPC-3 cells were most sensitive to both IFNs compared to the other two cell types. These findings indicate that BxPC-3 and MIAPaCa-2 with strong IFNAR2 expression are sensitive to IFNs, while Panc-1 with weak IFNAR2 expression was resistant to IFNs, suggesting significant correlation between IFNAR2 status and the growth-inhibitory effect of IFNs.

The growth-inhibitory effect of IFN-ß was more potent than that of IFN-α in all three cell lines ($p<0.05$). In BxPC-3 cells, the most sensitive to IFNs, the 50% growth-inhibition concentrations (IC_{50}) of IFN-α and IFN-ß were 4,432 and 705 IU/ml, respectively, i.e., the growth-inhibitory effect of the former was 6.3-fold stronger than the latter. In MIAPaCa-2 and Panc-1, the growth-inhibitory effect of IFN-ß was 5.8- and

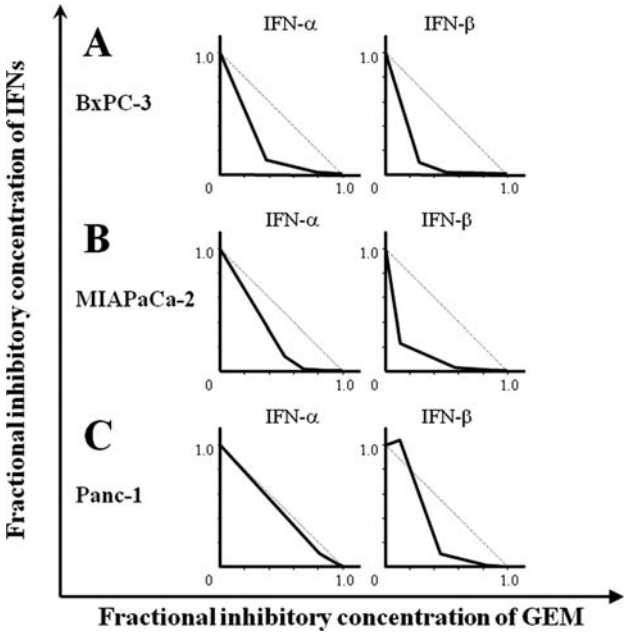

Figure 5. Isobolographic analysis of the cooperative effect of GEM and IFNs on (A) BxPC-3, (B) MIAPaCa-2 and (C) Panc-1 cells. The isobologram analysis was performed based on the results of the growth-inhibitory effects. The cooperative effect was synergistic in BxPC-3 and MIAPaCa-2, and additive or antagonistic in Panc-1.

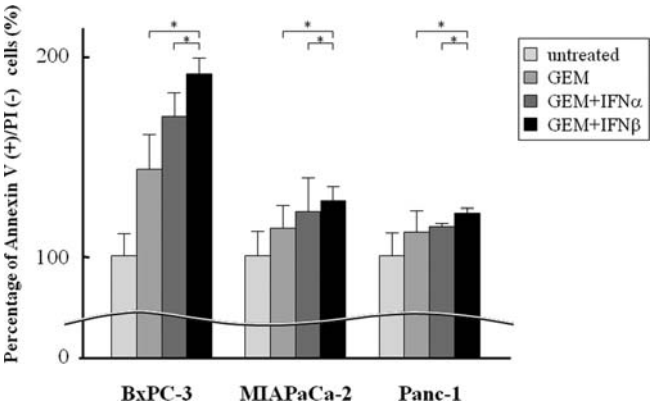

Figure 6. Apoptosis assessed by annexin V assay. In each cell line, the proportion of early apoptotic cells present without any treatment was considered 100%. The proportion of early apoptotic cells in cultures treated with GEM and IFN-ß was significantly higher than those treated with GEM and IFN-α and those treated with GEM alone in all the three cell lines. Data are mean ± SD of three experiments. *p<0.05.

3.5-fold stronger than IFN-α, respectively (the growth inhibition was 20% in MIAPaCa-2 and 10% in Panc-1, compared to the controls).

Activation of STATs by IFNs. To study the IFN signal transduction, we treated the cells with or without 1,000 IU/ml IFNs for 20 min and examined the expression of STAT proteins (STAT1, STAT2 and STAT3). Activation of STATs was assessed by phosphorylation of STAT proteins (pSTAT1, pSTAT2 and pSTAT3), as described previously (36). In the three cell lines, the phosphorylation of STAT1, STAT2, and STAT3 by IFN-ß was greater than by IFN-α (Fig. 3).

Antiproliferative effects of combination therapy of GEM and IFNs. Next, we investigated whether IFNs enhance the antiproliferative effects of GEM on the three cell lines (Fig. 4). In these experiments, the concentrations of GEM were selected based on the IC_{50} of GEM in each cell line (data not shown). The simultaneous addition of both GEM and IFN-α to the cultured cells enhanced the growth-inhibitory effects compared with that of GEM alone in BxPC-3 and MIAPaCa-2, while such enhancement was not observed in Panc-1. IFN-ß also enhanced the growth-inhibitory effects of GEM in a dose-dependent manner in BxPC-3 and MIAPaCa-2 cells. Moreover, such enhancement of the growth-inhibitory effects of GEM by IFN-ß was mild in Panc-1, which did not show IFN-α-induced growth inhibition.

Cooperative effect of IFNs and GEM. The isobologram analysis indicated that the cooperative effect was synergistic in BxPC-3 and MIAPaCa-2, and that the extent of the synergistic effect for IFN-ß was larger than IFN-α (Fig. 5). These two cancer cell lines also strongly expressed IFNAR2. On the other hand, the combination of IFN-α and GEM exhibited additive or antagonistic cooperative effects in Panc-1 cells with weak IFNAR2 expression, whereas the combination of IFN-ß with GEM showed synergistic effect.

Apoptosis induced by combination therapy of GEM and IFNs. The apoptosis induced at 24 h after the administration of agents was assessed by annexin V assay. In all the three cell lines, the proportion of early apoptotic cells was significantly higher in cultures containing GEM and IFN-ß than those containing GEM and IFN-α or GEM alone (Fig. 6).

Discussion

In the present study, we focused on the antitumor effect of IFNs on pancreatic cancer cells. Although the clinical efficacy of IFN-α for pancreatic cancer has been already reported, most of these studies have reported the effect of IFN-α-based combination therapy with other drugs including 5-fluorouracil, cisplatin retinoic acid, or leucovorin (11-16). Considering that GEM is the golden standard chemotherapeutic agent today, in order to develop more effective combination chemotherapy for pancreatic cancer, we thought it was important to evaluate the antitumor effect of the combination therapy of IFNs and GEM.

Among the various forms of IFNs, we focused on IFN-ß in the present study, based on our previous report that IFN-ß exhibits synergistic antitumor properties when combined with other anticancer drugs even in HCC cells with weak expression of IFNAR2 (30). The stronger antitumor effect of IFN-ß is speculated to be due to tighter affinity for the receptor than IFN-α (37-39). We reported previously a stronger and persistent induction of pSTATs by IFN-ß compared with IFN-α in HCC (36). Also, in the present study, we examined the pSTATs induced by IFNs, and confirmed the difference in the signal transduction.

The results of this study demonstrated that both IFN-α synergistically enhanced the growth-inhibitory effects of

GEM in BxPC-3 and MIAPaCa-2, whereas no such effect was noted in Panc-1 cells. Furthermore, immunofluorescence studies showed that the pattern of the cooperative growth-inhibitory effect of IFN-α and GEM correlated significantly with IFNAR2 expression level, in agreement with the results reported by Saidi *et al* (40). The present study also showed that the cooperative antitumor effect of IFN-ß and GEM was synergistic not only in BxPC-3 and MIAPaCa-2, but also in Panc-1, suggesting that the synergistic effect was not limited to pancreatic cancer cells with strong IFNAR2 expression identified in immunofluorescence studies but also those with weak IFNAR2 expression determined by Western blot analysis. In addition, it was also confirmed that pSTATs, which were not expressed in the absence of IFNs, were detected in the presence of IFNs in all three pancreatic cancer cell lines, indicating IFN signal transduction even in cells with weak IFNAR2 as judged by Western blot analysis. Taken together, the results suggest that IFN-ß exhibits synergistic antitumor effect with GEM through IFN signal transduction even in pancreatic cancer cells with weak IFNAR2 confirmed by Western blot analysis. Clinical extrapolation of these results could provide hope for the combination therapy of IFN-ß and GEM in patients with pancreatic cancer resistant to GEM therapy and IFN-ß and GEM combination therapy. Analysis of the outcome of such treatment could perhaps include determination of tissue IFNAR2 expression by qRT-PCR, Western blot, and immunostaining. However, the present study was limited to *in vitro* analysis, and further *in vivo* studies are required to examine the efficacy of IFN-ß combined with GEM in patients with pancreatic cancer.

In summary, the results of the present study indicated that IFN-ß acts synergistically when combined with GEM on the growth of pancreatic cancer cells with weak expression of IFNAR2. These results suggest that the combination of IFN-ß and GEM might be an effective alternative treatment for patients with pancreatic cancer. While clinical application of IFN-ß for pancreatic cancer is expected in the near future, more studies are needed to determine the optimal dosage and management of potential adverse effects of this combination therapy.

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