IFN-β Is a Highly Potent Inhibitor of Gastroenteropancreatic Neuroendocrine Tumor Cell Growth In vitro

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Abstract

IFN-α controls hormone secretion and symptoms in human gastroenteropancreatic neuroendocrine tumors (GEP-NET) but it rarely induces a measurable tumor size reduction. The effect of other type I IFNs, e.g., IFN-β, has not been evaluated. We compared the antitumor effects of IFN-α and IFN-β in BON cells, a functioning human GEP-NET cell line. As determined by quantitative reverse transcription-PCR analysis and immunocytochemistry, BON cells expressed the active type I IFN receptor mRNA and protein (IFNAR-1 and IFNAR-2c subunits). After 3 and 6 days of treatment, IFN-β significantly inhibited BON cell growth in a time- and dose-dependent manner. IC50 and maximal inhibitory effect on day 6 were 8 IU/mL and 98%, respectively. In contrast, the effect of IFN-α resulted significantly in a less potent effect (IC50: 44 IU/mL, maximal inhibition: 26%). IFN-α induced only cell cycle arrest, with an accumulation of the cells in S phase. IFN-β, apart from a more potent delay in S-G2-M phase transit of the cell cycle, also induced a strong stimulation of apoptosis, evaluated by flow cytometry (Annexin V and 7-AAD) and measurement of the DNA fragmentation. Besides, only IFN-β severely suppressed chromogranin A levels in the medium from BON cells after 6 days of treatment. In conclusion, IFN-β is much more potent, compared with IFN-α, in its inhibitory effect on GEP-NET cell proliferation in vitro through the induction of apoptosis and cell cycle arrest. Further studies are required to establish whether IFN-β has comparable potent tumor growth inhibitory effects in vivo. (Cancer Res 2006; 66(1): 554-62)

Introduction

Gastroenteropancreatic-neuroendocrine tumors (GEP-NET) are a heterogeneous category of tumors and represent the largest group of all NETs (1). GEP-NETs frequently synthesize several biologically active substances, including bioamines and neuropeptides, leading to disease characterized by diarrhea, abdominal cramps and pain, cardiac valve disease, bronchoconstriction, flushing, telangietasias, and pellagra-like dermatitis, often debilitating for patients (1–3). In the liver, monoamine oxidases can detoxify the amines released by the tumor and prevent the occurrence of the tumors associated hypersecretion syndrome. However, the presence of hepatic and occasionally large retroperitoneal and ovarian metastases, where secreted products by-pass the liver detoxification, is commonly associated with this syndrome (4).

In addition to the impaired quality of life, patients with advanced GEP-NETs have a dismal prognosis with a 5-year survival of ~20% to 30% (5). In these cases, there are relatively few therapeutic options and most of them are palliative. Surgery is indicated only for conservative resections of the intestine, mesenteric tumors, and fibrotic areas to improve symptoms and quality of life (6). Hepatic resection in selected patients with metastatic functioning GEP-NETs is safe, provides temporary relief of symptoms, and may prolong survival (6, 7). As an alternative to hepatic resection in patients with liver metastases, local ablative procedures may also be considered, including chemoembolization, laser, and radiofrequency ablation, as palliative support to control symptoms of hypersecretion syndrome (6). The use of chemotherapeutic regimens is limited to fast growing or undifferentiated tumors (8). Few studies suggest the use of targeted radiotherapy with somatostatin analogues coupled to β- or Auger electron emitters in the management of GEP-NETs, but its definitive role has yet to be defined (9–11). The most common medical treatment in advanced GEP-NETs includes the use of somatostatin analogues and IFN-α, alone or in combination. Somatostatin analogues are used in the treatment of clinical symptoms, but tachyphyaxis is frequently recorded and these drugs are unable to control tumor progression (12, 13). IFN-α induces a 42% biochemical and 14% tumor response in carcinoid tumors, and a 51% biochemical and 12% tumor response in neuroendocrine pancreatic tumors (14, 15).

Therefore, therapeutic options to inhibit the growth of metastatic GEP-NET tumors are still unsatisfactory and many patients become refractory to the conventional palliative therapy. For all these reasons, novel treatment strategies seem mandatory. Few authors reported that IFN-β has greater antitumor effects than IFN-α on melanoma, squamous carcinoma, glioma, breast, and hepatocellular cancer (17–21). On these bases, IFN-β seems to be of potential interest in the treatment of tumors.

To further explore the possibilities of new medical therapies in GEP-NETs, we compared in this study the role and mechanism of action of IFN-α and IFN-β in the regulation of cell growth in BON cells, a functioning human NET cell line, derived from a lymph node metastasis of a pancreatic carcinoid.

Materials and Methods

Cell lines and culture conditions. Human pancreatic carcinoid BON cells were obtained from Dr. Townsend (The University of Texas Medical
The cells were cultured in a humidified incubator containing 5% CO2 at 37°C. The culture medium consisted of a 1:1 mixture of DMEM and F12K medium, supplemented with 10% FCS, penicillin (1 \times 10^4 units/L), fungizone (0.5 mg/L), and l-glutamine (2 mmol/L). Periodically, cells were confirmed as M yc o p l as ma - free. Cells were harvested with 10% trypsin EDTA and resuspended in medium. Before plating, cells were counted microscopically using a standard hemocytometer. Trypan blue staining was used to assess cell viability, which always exceeded 95%. Media and supplements were obtained from Life Technologies Bio-culture Europe (Invitrogen, Breda, the Netherlands).

Drugs and reagents. Human recombinant IFN-α-2b (Referon-A) was obtained from Roche (Mijdrecht, the Netherlands). Human recombinant IFN-β-1a was obtained from two preparations: Serono, Inc. (Reibif, Rockland, MA) and Life Technologies (Paisley, United Kingdom). All compounds were stored at –20°C and the stock solution was constituted in distilled water according to the instructions of the manufacturer. Anti-IFN-β-neutralizing antibody was purchased from Sigma-Aldrich (St. Louis, MO).

Quantitative reverse transcription-PCR. The expression of type I IFN receptors (IFNAR-1, IFNAR-2) total, the short form IFNAR-2b, and the long form IFNAR-2c) and housekeeping gene hypoxanthine phosphoribosyltransferase (HPRT) mRNA was evaluated by quantitative reverse transcription-PCR (RT-PCR) in BON cells. Polyadenylated mRNA [poly(A)+ mRNA] was isolated using Dynabeads Oligo (dT)\(_{25}\) (Dynal AS, Oslo, Norway) from cell pellets containing ~5 x 10\(^6\) cells. The cells were lysed for 2 minutes on ice in a buffer containing 100 mmol/L Tris-HCl (pH 8), 500 mmol/L LICL, 10 mmol/L EDTA (pH 8), 1% LiDS, 5 mmol/L DTT, and 5 units/100 mL of RNase inhibitor (HT Biotec Ltd, Cambridge, United Kingdom). To the lysate, 30 μL prewashed Dynabeads Oligo (dT)\(_{25}\) were added, and the mixture was incubated for 10 minutes on ice. Thereafter, the beads were collected with a magnet, washed thrice with 10 mmol/L Tris-HCl (pH 8), 0.15 mol/L LECI, 1 mmol/L EDTA, 0.1% LiDS, and twice with a similar buffer from which LiDS was omitted. mRNA was eluted from the beads in 2 x 20 μL of H\(_2\)O for 2 minutes at 65°C. cDNA was synthesized using the poly(A)+ mRNA in a Tris buffer [50 mmol/L Tris-HCl (pH 8.3), 100 mmol/L KCL, 4 mmol/L DTT, and 10 mmol/L MgCl\(_2\)] together with 1 mmol/L of each deoxynucleotide triphosphate, 10 units RNase inhibitor, and 2 units avian myeloblastosis virus Super Reverse Transcriptase (HT Biotec Technology) in a final volume of 40 μL. This mixture was incubated for 1 hour at 42°C.

One tenth of the cDNA library was used for quantification of IFN receptors using a real-time PCR assay. A quantitative PCR assay was designed using AmpliTag Gold DNA Polymerase and the ABI PRISM 7700 sequence detection system (Perkin-Elmer Applied Biosystems, Gröningen, the Netherlands) for real-time amplifications according to the protocol of the manufacturer. The assay was composed of 15 μL TaqMan Universal PCR Master Mix (Applied Biosystems, Capelle aan de IJssel, the Netherlands), forward primer (500 nmol HPRT and IFN-AR-2c; 300 nmol IFNAR-1, IFNAR-2 total, and IFNAR-2b), reverse primer (500 nmol HPRT and IFNAR-2c; 300 nmol IFNAR-1, IFNAR-2otal, and IFNAR-2b), probe (100 nmol HPRT, IFNAR-1 and IFNAR-2otal; 200 nmol IFNAR-2b and IFNAR-2c), and 10 μL cDNA template in a total reaction volume of 25 μL. PCR amplification started with a first step for 2 minutes at 50°C, followed by an initial heating at 95°C for 10 minutes; samples were then subjected to 40 cycles of denaturation at 95°C for 15 seconds and annealing for 1 minute at 60°C. The primer and probe sequences were purchased from Biosource (Nivelles, Belgium) and included the following: IFN-AR-1 forward, 5’-CCCAGTTG-GTCTTCTCCTCAGA-3’; IFN-AR-1 reverse, 5’-AAGAAGTGGAAGTGAAGGA-3’; IFNAR-1 probe, 5’-FAM-CCGCGTACAGCTGATGGA-TAMRA-3’; IFNAR-2 (total form) forward, 5’-AGGAAATGGGAAAGTGAATTGAAA-3’; IFNAR-2 (total form) reverse, 5’-TTCCTGATTGCTCCTCCAAA-3’; IFNAR2b reverse, 5’-CCTGAACTGAGTTCCC-3’; IFNAR2b forward, 5’-FAM-TCTTGGAGG-AAGTGGCTGATGGAATTGAA-3’; IFNAR-2c forward, 5’-CCTGAAATGGGCAAGC-3’; IFNAR-2c probe, 5’-FAM-TGGGTTGTTTTATATGCTTAA-GGATAGCTCCCTCCCA-TAMRA-3’; HPRT forward, 5’-TGGGTCTCCTTGG-GTACAGAT-3’; HPRT reverse, 5’-TCAATCCAAAGAAAGTCTGGCTTTA-TATC-3’; and HPRT probe, 5’-FAM-CAAGGTTGGACACGTCATTGG-AACATTTGGA-TAMRA-3’.

The detection of HPRT mRNA was used for normalization of IFN receptor mRNA levels. Expression of IFNAR-2a mRNA, the soluble form of IFNAR-2 subunit, was determined indirectly by subtracting IFNAR-2b and IFNAR-2c from IFNAR-2 total. Several controls were included in the RT-PCR experiments. To exclude contamination of the PCR reaction mixtures, the reactions were also done in the absence of DNA template in parallel with cDNA samples. As a positive control for the PCR reactions of HPRT and type I IFN receptors, human cDNA was amplified in parallel with the cDNA samples.

Immunocytochemistry. Cytospin preparations of BON cells were fixed with acetone and subsequently incubated for 30 minutes at room temperature with antibodies to human IFNAR-1 (rabbit polyclonal antibody, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), IFNAR-2b (rabbit polyclonal antibody, Santa Cruz Biotechnology), and IFNAR-2c (monoclonal antibody, Dr E. Croze, Berlex Biosciences, Richmond, CA) subunits. Finally, a peroxidase complex (IL Immunologic, Duiven, the Netherlands) for IFNAR-1 and IFNAR-2b, or standard streptavidin-biotinylated alkaline phosphatase (IL Immunologic) for IFNAR-2c, were used according to the recommendations of the manufacturer to visualize the bound antibodies.

Negative controls for the immunohistochemistry included (a) omission of the primary antibody and (b) preabsorption of the antibody for IFNAR-2b with the respective immunizing peptide receptor peptide.

Cell proliferation assay. After trypsinization, the cells were plated in 1 mL of medium in 24-well plates at a density of 5 x 10^3 per well. The plates were then placed in a 37°C, 5% CO2 incubator. Three days later, the cell culture medium was replaced with 1 mL/well medium containing various concentrations (0, 1, 5, 10, 50, 100, 1000 IU/mL) of IFN-α or IFN-β (Serono). Quadruplicates of each treatment were done. Plates were further incubated at 37°C and 5% CO2. After 3 and 6 days of treatment, cells were harvested for DNA measurement. Plates treated for 6 days were refreshed after 3 days and compounds were added again. Measurement of total DNA contents, representative for the number of cells, was done using the bisbenzimide fluorescent dye ( Hoecht 33258; Boehringer Diagnostics, La Jolla, CA) as previously described (22).

To evaluate the specificity of the effects of IFN-β on cell growth, we exposed type I IFNs with antihuman IFN-β neutralizing antibody. Cells (5 x 10^3 per well) were plated in 24-well plates and after 3 days of incubation at 37°C and 5% CO2, the medium containing IFN-β (0.5, and 50 IU/mL) or IFN-α (100 IU/mL) in the absence or in presence of antihuman IFN-β-neutralizing antibody (4 μg/mL) was changed. Plates were refreshed after 3 days and compounds were added again. After 6 days of treatment, plates were collected for DNA measurement. Both IFNs, alone or in combination with antihuman IFN-β neutralizing antibody, have been incubated for 1 day at 4°C before being added to BON cells to antagonize the IFN-β activity.

Apoptosis assays. After trypsinization, BON cells were plated in six-well plates at a density of 0.5 x 10^3 to 1 x 10^3 per well. These plates were placed in a 37°C, 5% CO2 incubator. Three days later, the medium was refreshed in the presence and absence (control group) of IFN-α or IFN-β (Serono) at the concentration of 100 IU/mL. After 1, 2, and 3 days of incubation, cells were gently trypsinized and washed with ice-cold PBS. Cells were resuspended in 100 μL of 1% binding buffer (Nexins Research B.V., Hoven, the Netherlands), and stained with 5 μL of FITC-Annexin V (25 μg/mL) and 10 μL of 7-amino-actinomycin D (7-AAD; 1 mg/mL). Cells were incubated for 15 minutes on ice before being added to BON cells to antagonize the IFN-β activity.

Type I IFNs and Carcinoids
Annexin and binding buffer were obtained from Nexins Research (Kattendijke, the Netherlands), whereas 7-AAD was from Becton Dickinson.

Apoptosis was further confirmed by the analysis of the DNA fragmentation. After plating $5 \times 10^4$ cells per well on 24-well plates, cells were incubated at 37°C. Three days later, the cell culture medium was replaced with 1 mL/well medium containing various concentrations of IFN-α or IFN-β (Serono). Quadruplicates of each treatment were done. After an additional incubation of 1 day, apoptosis was assessed using a commercially available ELISA kit (Cell Death Detection ELISA Plus, Roche Diagnostic GmbH, Penzberg, Germany). The standard protocol supplied by the manufacturer was used, as previously described (24).

Cell cycle analysis. Cells ($0.8 \times 10^6$–$2 \times 10^6$) were plated in 75 cm² flasks. After 3 days, medium was changed with fresh medium (control group) and with fresh medium plus IFN-α or IFN-β (Serono) at the concentration of 100 IU/mL. After 1, 2, and 3 days of incubation (with a confluence of 60–70%), cells were harvested by gentle trypsinization, washed with cold PBS (calcium and magnesium free), and collected by centrifugation. For cell cycle analysis, $10^6$ cells were resuspended in 200 µL of PBS and fixed in 70% ice-cold ethanol with an overnight incubation at −20°C. After brief centrifugation, cells were washed once with PBS and incubated for 30 minutes at 37°C in PBS containing 40 µg/mL of propidium iodide (Sigma-Aldrich, Zwijndrecht, the Netherlands) and 10 µg/mL of DNase-free RNase (Sigma-Aldrich). For each tube, 20,000 cells were immediately measured on a FACScalibur flow cytometer (Becton Dickinson) using CellQuest Pro Software.

Chromogranin A determinations. BON cells ($5 \times 10^5$ per well) were cultured in 24-well plates and placed in a 37°C, 5% CO₂ incubator. Three days later, the culture medium was replaced with 1 mL/well medium containing various concentrations of IFN-α or IFN-β (0, 1, 5, 10, 50, and 100 IU/mL; Serono). Plates were further incubated at 37°C and 5% CO₂, refreshed after 3 days, and compounds were added again at escalating concentrations. After 6 days of treatment, the supernatant and the plates were collected for chromogranin A and DNA measurements, respectively.

Human chromogranin A was measured in the conditioned medium by a solid-phase, two-site immunoradiometric assay based on monoclonal antibodies that bind to two distinct contiguous epitopes within the 145 to 245 region of chromogranin A (Cromogranin A-RIA CT, CIS Bio International, Gif-sur-Yvette, France) according to the procedures of the manufacturer (25).

Effects of two different preparations of IFN-β-1a on cell growth and chromogranin A production. After trypsinization, the cells were cultured...
in 24-well plates at a density of \(5 \times 10^3\) per well. The plates were then placed in a 37°C, 5% CO2 incubator. Three days later, the cell culture medium was replaced with 1 mL/well medium containing various concentrations (0, 1, 5, 10, 50, and 100 IU/mL) of IFN-\(\alpha\) from two different preparations (Serono and Life Technologies). Plates were refreshed after 3 days and compounds were added again. After 6 days of treatment, cells and conditioned medium were harvested, as indicated above, for DNA and chromogranin A measurement.

**Statistical analyses.** All experiments were done at least thrice and gave comparable results. For statistical analysis, GraphPad Prism 3.0 (GraphPad Software, San Diego, CA) was used. Relative IC\(_{50}\) values and maximal inhibitory effect were calculated using nonlinear regression curve-fitting program. The comparative statistical evaluation among groups was first done by ANOVA. When significant differences were found, a comparison between groups was made using the Newman-Keuls test. The unpaired Student’s \(t\) test was chosen to analyze the differences in concentration-effect curves (IC\(_{50}\) and maximal inhibitory effect) and effects in cell cycle modulation between different types or preparations of IFNs, and the differences of the growth inhibitory effects of IFNs after 3 and 6 days of treatment. Correlation analyses were done using Pearson’s coefficients. In DNA fragmentation analyses, the means of quadruplicates for each treatment with the same dose of IFN-\(\alpha\) have been correlated to the corresponding means of quadruplicates in the corresponding plates for the cell proliferation assay after 6 days of treatment with IFN-\(\beta\).

**Results**

**Expression of type I IFN receptor mRNA and proteins in BON cells.** Because the activity of type I IFNs is modulated by a common receptor, we analyzed the expression of IFNAR-1 and IFNAR-2 (total, short, and long forms) mRNA in BON cells by quantitative RT-PCR (Fig. 1A). Using sequence-specific primers against the type I IFN receptor subunits, we detected the presence of IFNAR-1 and IFNAR-2 (total, short, and long forms) mRNA in BON cells by quantitative RT-PCR (Fig. 1A).

Specific immunoreactivity for IFN receptor subunits (IFNAR-1, IFNAR-2b, and IFNAR-2c) was found in BON cells at immunocytochemistry (Fig. 1B-D). It was strongly positive for IFNAR-1 on the membrane and cytoplasm (Fig. 1B), moderately positive for

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**Figure 2.** A and B, dose-dependent inhibition of cell proliferation after 3 days (A) and 6 days (B) of treatment with IFN-\(\alpha\) and IFN-\(\beta\) in BON cell line. Measurement of total DNA contents was done using Hoechst 33258. The mean DNA contents in controls were as follows: 3,278 ng/well (IFN-\(\alpha\), 3 days), 3,992 ng/well (IFN-\(\beta\), 3 days), 8,941 ng/well (IFN-\(\alpha\), 6 days), and 8,497 ng/well (IFN-\(\beta\), 6 days). A, IFN-\(\alpha\); B, IFN-\(\beta\). * \(P < 0.001\), ** \(P < 0.01\), *** \(P < 0.05\) versus control. C and D, effects of IFN-\(\beta\) (C) and IFN-\(\alpha\) (D) on cell growth, alone and following preabsorption with neutralizing antibodies against IFN-\(\beta\) (IFN-betaAb), * \(P < 0.001\), ** \(P < 0.05\) versus relative controls. Measurement of total DNA contents was done using Hoechst 33258. Values are expressed as the percentage of control (untreated cells). Columns, mean of at least three independent experiments in quadruplicate; bars, SE.
IFN-α and IFN-β significantly suppressed the growth of BON cells in a dose-dependent manner, with an IC$_{50}$ of 44 and 8 IU/mL, respectively (Fig. 2). The growth inhibitory effect of IFN-β was significantly more potent than that of IFN-α, as shown by the higher maximal inhibition of proliferation induced by IFN-β, compared with IFN-α after 3 days (74.8 ± 2% and 163 ± 1.3%, respectively, P < 0.0001; Fig. 2A) and 6 days (97.6 ± 4.3% and 25.8 ± 2.6%, respectively, P < 0.0001; Fig. 2B) of treatment, as well as by the lower log IC$_{50}$ of IFN-β compared with IFN-α after 3 days (1.1 ± 0.1 and 1.6 ± 0.1, respectively, P < 0.05) and 6 days (0.9 ± 0.1 and 1.6 ± 0.2, respectively, P < 0.0001). Note that after 6 days of incubation, IFN-β induced a statistically significant cell growth inhibition already at very low concentrations (1 IU/mL).

The antiproliferative effects of IFN-α and IFN-β were time dependent. In fact, the maximal inhibition of cell proliferation, induced by both cytokines, resulted to be higher after 6 days compared with 3 days of incubation (IFN-α: P < 0.05; IFN-β: P < 0.0001). No statistically significant difference was observed between the values of IC$_{50}$ of inhibition after 3 and 6 days for either IFN-α or IFN-β.

To clarify the specificity of action of IFN-β, we evaluated the effects on cell growth after a preadsorption of IFN-α and IFN-β with neutralizing antibodies against IFN-β. As shown in Fig. 2C, the antitumor activity of 50 IU/mL IFN-β was significantly weakened by immunoneutralization, whereas the effect of 5 IU/mL IFN-β was completely abolished. Moreover, these antibodies were unable to counteract the inhibitory effects of IFN-α on cell growth (Fig. 2D).

**Effects of type I IFNs on apoptosis.** The effects of IFN-α (100 IU/mL) and IFN-β (100 IU/mL) on the induction of apoptosis in BON cells were examined by flow cytometry. Using Annexin V and 7-AAD, two-color flow cytometric analysis detected three populations: viable, early apoptotic, and both late apoptotic and necrotic cells. Percentages of these populations compared with the untreated control after 1, 2, and 3 days of treatment with IFN-α or IFN-β are shown in Fig. 3A to C.

We observed (Fig. 3A) a significant decrease in the fraction of viable cells during the incubation with IFN-β (1, 2, and 3 days: P < 0.01). The percentage of early apoptotic cells significantly increased after 1, 2, and 3 days (all P < 0.01) of treatment with IFN-β (Fig. 3B). In the same way, IFN-β induced a significant increase (P < 0.01) in the fraction of late apoptotic/necrotic cells (Fig. 3C). It is noteworthy that, after 2 days of treatment with IFN-β (100 IU/mL), ~20% of cells showed signs of apoptosis or necrosis, which is 2.3 times higher than the control. In contrast, IFN-α did not significantly modify the fractions of vital cells, early apoptotic cells, and late apoptotic/necrotic cells compared with the untreated group.

During treatment with IFN-α and IFN-β, the measurement of the DNA fragmentation was further used to investigate the effect on the apoptosis (Fig. 4A-B). After 1 day of incubation, IFN-α had no remarkable stimulatory effects on DNA fragmentation at any concentration up to 1,000 IU/mL in BON cell line. On the other hand, a dose-dependent induction of apoptosis was observed after IFN-β treatment, with a maximal increase of DNA fragmentation of about thrice compared with the untreated control in BON cells. This cytokine stimulated apoptosis already at very low concentrations (1 IU/mL). These data were also confirmed by morphologic observations. Only the treatment with IFN-β induced morphologic alterations consistent with apoptosis, such as cell shrinkage, picnotic nucleus, and detachment from the plate after 1 to 3 days (not shown).

The inhibitory effects of IFN-β on the cell growth of BON cells seemed to be mainly due to an early proapoptotic activity as shown by the highly significant positive correlation between the cell proliferation inhibition after 6 days of treatment and DNA fragmentation variation after 1 day of incubation ($r^2 = 0.82$, P < 0.001; Fig. 4C).

**Effects of type I IFNs on the cell cycle.** We also evaluated the effect of treatment with IFN-α (100 IU/mL) and IFN-β (100 IU/mL) on cell cycle phase distribution after 1, 2, and 3 days of incubation (Fig. 5).

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**Figure 3.** A to C, effects of IFN-α (100 IU/mL) and IFN-β (100 IU/mL) on the induction of apoptosis in BON cells, evaluated by flow cytometry for Annexin V and 7-AAD. Values of viable cells, early apoptotic cells, and late apoptotic/necrotic cells are expressed as percentage compared with the untreated control. Points, mean; bars, SE. Control values have been set to 100%. The mean percentages ± SE of viable cells in controls were as follows: 89.2 ± 1.3% (1 day), 88.1 ± 0.9% (2 days), and 85.9 ± 1.3% (3 days). The mean percentages ± SE of early apoptotic cells in controls were as follows: 4.5 ± 0.2% (1 day), 4.7 ± 0.9% (2 days), and 4.2 ± 0.4% (3 days). The mean percentages ± SE of late apoptotic/necrotic cells in controls were as follows: 3.7 ± 0.2% (1 day), 3.3 ± 0.5% (2 days), 7.4 ± 1.7% (3 days). C, IFN-α; ■, IFN-β (Serono). *, P < 0.01 versus control.
IFN-α treatment induced a slight but significant accumulation of cells in S phase after 2 days (P < 0.05) and 3 days (P < 0.001) of treatment compared with the control. In the same way, the incubation with IFN-β increased the fraction of BON cells in the S phase of the cell cycle already after 1 day (P < 0.001 after 1, 2, and 3 days), whereas the proportion of cells in G1 phase decreased in comparison with the control (P < 0.001 after 24, 48, and 72 hours). These data suggested that BON cells in S phase failed to transit into G2 and M phases efficiently and exhibited a prolonged stay in S phase after treatment with type I IFNs.

Of note, the cell cycle arrest induced by IFN-β was more potent than that of IFN-α as shown by the statistically significant difference in percentage of cells in S phase compared with the control after 1, 2, and 3 days of incubation with IFN-β and IFN-α (all P < 0.001).

Biochemical effects of type I IFNs. The chromogranin A levels in the medium, collected from BON cells during the interval 3 to 6 days of incubation with IFN-β, were significantly suppressed, also using low dose (Fig. 6B). This effect was dose dependent, with IC50 values of 6 IU/mL (comparable with the IC50 of IFN-β on cell proliferation inhibition). On the other hand, IFN-α (Fig. 6A) induced only a slight suppression of chromogranin A even at high dose (100 IU/mL). It is interesting to observe that chromogranin A values, detected after 6 days of treatment with IFN-β, highly correlated with the relative values of DNA quantification (r² = 0.98, P < 0.0001).

Effects of two different preparations of IFN-β-1a on cell growth and chromogranin A production. The potencies of IFN-β-1a preparations from two different sources (Serono and Life Technologies) on cell proliferation and chromogranin A production were superimposable (IC50 on cell growth: 6 IU/mL for both preparations; IC50 on chromogranin A: 4 and 3 IU/mL, respectively). For IFN-β from Serono and IFN-β from Life Technologies, no statistically significant difference was observed between the values of log IC50 (on cell growth: 0.79 ± 0.04 and 0.75 ± 0.06, respectively; on chromogranin A: 0.65 ± 0.08 and 0.44 ± 0.12, respectively) and maximal inhibition of proliferation (on cell growth: 98% ± 1.6 and 99% ± 2.7, respectively; on chromogranin A: 86 ± 2.8 and 85 ± 3.4, respectively).

Figure 4. A and B, DNA fragmentation after 1 day of treatment of BON cell line with IFN-α (A) and IFN-β (B). Values are absorbance units (OD) and are expressed as percentage control. Columns, mean; bars, SE. *, P < 0.001; **, P < 0.01; ***, P < 0.05 versus control. C, correlation between DNA fragmentation and cell proliferation inhibition after IFN-β treatment in BON cell line. Points, means of four wells for each treatment point with IFN-β in DNA fragmentation analysis after 1 day (on the abscissa) and means of quadruplicate for each treatment point in the respective plates for cell proliferation assay after 6 days of treatment with IFN-β (on the ordinate).

Figure 5. Cell cycle distribution after 1, 2, and 3 days of incubation with 100 IU/mL IFN-α and 100 IU/mL IFN-β in BON cells. Points, mean of the percentage of cells in the different phases, compared with untreated control cells; bars, SE. Control values have been set to 100%. The mean percentages of control cells in G1 phase were as follows: 60 ± 0.8% (1 day), 64.8 ± 0.9% (2 days), and 65.4 ± 0.6% (3 days). The mean percentages of control cells in S phase were as follows: 23.9 ± 0.9% (1 day), 20.8 ± 0.9% (2 days), and 21 ± 0.6% (3 days). □, IFN-α; ■, IFN-β. *, P < 0.001; **, P < 0.05 versus control.
**Discussion**

IFN-α is currently used as a therapeutic agent in several malignancies, including GEP-NETs (14–16, 26–29). This cytokine belongs to the class of type I IFNs (e.g., IFN-α, IFN-β, IFN-κ, and IFN-τ), which modulate a variety of biological responses through the activation of a common receptor, composed by two subunits: IFNAR-1 and IFNAR-2 (30, 31). IFNAR-1 is considered the signaling subunit and it exists as the full chain. The IFNAR-2 is the subunit responsible for the interaction with the ligand. There are three forms of IFNAR-2, which are differentially spliced products of the same gene, e.g., the soluble (IFNAR-2α), short (IFNAR-2b), and long (IFNAR-2c) forms (26, 31, 32). The chain IFNAR-2c with its long cytoplasmic domain and the IFNAR-1 subunit constitute the predominantly active form of the type I IFN receptor complex. IFNAR-2c is capable of binding ligand, but with a lower affinity (20-fold less) than the dimeric IFN receptor complex itself (33). Therefore, both receptor chains are required to form a high-affinity binding site and initiate signal transduction. The short form is able to bind type I IFNs but does not couple to signal transduction because it lacks the signal-transducing tail of IFNAR-2c (34). The soluble form may act as a regulator of free IFNs and, depending on concentration, lead to the neutralization or enhancement of IFN bioactivity (35, 36).

Whereas the role of IFN-α has been extensively studied, the effect of other type I IFNs on neuroendocrine cell proliferation has not been evaluated. IFN-β is a multifunctional cytokine binding to the same receptor of IFN-α, but with higher affinity (37). It seems to be an essential mediator not only for the host defense in the innate immune responses against microbial infections but also for a host defense system against oncogenesis (28). Few studies showed that IFN-β has greater antitumor effects than IFN-α via the activation of apoptosis (17–21). Therefore, IFN-β represents a promising drug in the treatment of cancer.

In the present study, we compared the antitumor effects of IFN-β-1a and IFN-α-2b in human GEP-NETs, as well as the mechanisms that are involved in the growth inhibition. The expression of mRNA and protein for the active subunits of type I IFN receptor (IFNAR-1 and IFNAR-2c) was detected by quantitative RT-PCR and immuno-cytochemistry in the human BON cell line. Both IFNs showed an inhibitory effect on the cell proliferation of these cells but the antitumor activity of IFN-β-1a was significantly higher than that of IFN-α-2b. This difference could be explained by the potent proapoptotic activity of IFN-β-1a, as shown by the increase in apoptotic cells, evaluated by flow cytometry and the increase in DNA fragmentation. On the other hand, IFN-α-2b did not stimulate apoptosis in BON cells but it was able to induce a cell cycle arrest with an accumulation of the cells in S phase. These results are consistent with previous studies, which showed a delay in S-G2-M phase transit of the cell cycle after treatment with IFN-α in the human GEP-NETs cells (38, 39). The present study shows that apart from the potent induction of apoptosis, IFN-β-1a can induce a cell cycle arrest as well, resulting in increasing the fraction of cells in the S phase. Moreover, this latter effect was significantly more potent and earlier than that of IFN-α-2b. The specificity of the effects of IFN-β on BON cell growth is shown by the abolishment of the inhibitory effect of IFN-β after incubation with neutralizing antibodies against IFN-β in BON cells.

Although IFN-α and IFN-β interact with the same receptor, the induction of differential pathways can be explained by the differences in the structure between both cytokines (40, 41), generating differential interactions with the same receptor. In fact, although both IFNs induce tyrosine phosphorylation of the receptor subunits, IFN-β, but not IFN-α, induces the association of IFNAR-1 and IFNAR-2c chains, indicating that the specificity of signaling for distinct type I IFN subtypes could be established by differential conformation of the receptor complex (34, 42). Moreover, it was shown that IFN-α can trigger survival pathways in human tumor cells that could, at least in part, explain its poor effects on apoptosis onset if compared with that one caused by IFN-β (43). On the basis of these results, studies on the pathways activated by the two IFN isoforms are warranted. However, we cannot exclude that apoptosis induced by IFN-β was likely due to the strong lack of transition of BON cells from S to G2-M phase of cell cycle. In fact, apoptosis could arise as a consequence of inefficient cell cycle progression, culminating in apoptosis (44).

In addition, we evaluated the effects of IFN-α and IFN-β on chromogranin A production. Chromogranin A is the most sensitive and specific marker in GEP-NETs (45, 46) and BON cells are able to release this substance. In patients, circulating chromogranin A levels are dependent on several factors: tumor mass, secretory activity, and granule density of tumor cells (45, 46). This may render the use of chromogranin A in monitoring the response of tumor mass during medical treatment difficult. In fact, several drugs (e.g., somatostatin analogues) induced a decrease in plasma chromogranin A levels, through an inhibitory effect on synthesis and secretion without any effect on tumor growth (46). We detected

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**Figure 6.** Effect of IFN-α (A) and IFN-β (B) on the production of chromogranin A in human BON cells after 6 days of incubation. Plates were refreshed after 3 days of treatment and compounds were added again. Values are expressed as the percentage of control chromogranin A release. Columns, mean; bars, SE. The mean chromogranin A levels in controls were as follows: 3,554 ng/well (A) and 3,759 ng/well (B). *P < 0.001; **, P < 0.01 versus control.
a very potent dose-dependent suppression of the chromogranin A levels in the conditioned medium collected from BON cells in the interval 3 to 6 days of incubation with IFN-α, whereas the biochemical effect of IFN-α was minor and statistically present only at high dose. On the basis of a strong positive correlation between chromogranin A concentrations and DNA quantification after 6 days of treatment with IFN-α, we hypothesize that the suppression of this tumor marker results from the decrease in the number of the cells rather than from an inhibition in synthesis and secretion. This observation suggests that, in patients, the determinations of plasma chromogranin A could give important information about the effect on tumor growth during the treatment of GEP-NETs with IFN-α, also considering that the estimation of the total tumor mass in disseminated GEP-NETs is not easy (46).

Finally, our findings support the clinical attractiveness to use IFN-α in the treatment of GEP-NETs, considering that IFN-α inhibited cell proliferation and stimulated apoptosis already at very low concentrations (1–10 IU/mL). These concentrations can be achieved in vivo because 12.3 IU/mL is the maximal IFN-α serum concentration reported in healthy subjects after s.c. administration of this cytokine (47). Translation of these findings into a clinical application is, however, not easy, considering the short half-life of IFN-α. But new promising strategies (PEGylated form, carrier proteins like IFNAR-2a, or gene therapy) can improve pharmacokinetic and pharmacodynamic properties of IFN-α, bringing its real clinical application in cancer closer (36, 49, 50).

In conclusion, this is the first study showing that the antitumor activity of IFN-α in GEP-NETs is considerably more potent than IFN-α. This seems to be related to the fact that IFN-α induced cell cycle arrest only, whereas both apoptosis and cell cycle arrest were responsible for IFN-α-mediated growth suppression even at very low doses. These data provide rationales for future preclinical and clinical trials using IFN-α in GEP-NETs.

Acknowledgments

Received 8/26/2005; revised 10/14/2005; accepted 10/20/2005.

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We thank Diana M. Moosj for technical assistance.

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