Effects of type I interferons on IGF-mediated autocrine/paracrine growth of human neuroendocrine tumor cells

Giovanni Vitale,1 Peter M. van Koetsveld,1 Wouter W. de Herder,1 Katy van der Wansem,1 Joop A. M. J. L. Janssen,1 Annamaria Colao,2 Gaetano Lombardi,2 Steven W. J. Lamberts,1 and Leo J. Hofland1

1Division of Endocrinology, Department of Internal Medicine, Erasmus Medical Center, Rotterdam, The Netherlands; and 2Department of Molecular and Clinical Endocrinology and Oncology, “Federico II” University of Naples, Naples, Italy

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Vitale G, van Koetsveld PM, de Herder WW, van der Wansem K, Janssen JA, Colao A, Lombardi G, Lamberts SW, Hofland LJ. Effects of type I interferons on IGF-mediated autocrine/paracrine growth of human neuroendocrine tumor cells. Am J Physiol Endocrinol Metab 296: E559–E566, 2009. First published January 13, 2009; doi:10.1152/ajpendo.90770.2008.—We recently demonstrated that interferon (IFN)-β has a more potent antitumor activity than IFN-α in BON cells, a neuroendocrine tumor (NET) cell line. The present study showed the role of type I IFNs in the modulation of the insulin-like growth factor (IGF) system in NETs. BON cells expressed IGF-I, IGF-II, IGF-I receptor, and insulin receptor mRNA. In addition, IGF-I and IGF-II stimulated the proliferation of BON cells and induced an inhibition of DNA fragmentation (apoptosis). As evaluated by quantitative RT-PCR, treatment with IFN-α (100 IU/ml) or IFN-β (100 IU/ml) inhibited the expression of IGF-II mRNA (~42% and ~65%, respectively, both P < 0.001), whereas IGF-I receptor mRNA was significantly upregulated by IFN-β (+28%, P < 0.001) and downregulated by IFN-β (~47%, P < 0.001). Immunoreactive IGF-II concentration decreased in the conditioned medium during IFN-α (~16%, P < 0.05) and IFN-β (~69%, P < 0.001) treatment. Additionally, IGF-I receptor bioactivity was reduced (~54%) after IFN-β treatment. Scatchard analysis of 125I-labeled IGF-I binding to cell membrane of BON cells revealed a dramatic suppression of maximum binding capacity only in the presence of IFN-β. Finally, the proapoptotic activity of IFN-β was partially counteracted by the coadministration of IGF-I and IGF-II (both at 50 nM). In conclusion, these data demonstrate that the IGF system has an important role in autocrine/paracrine growth of BON cells. The more potent antitumor activity of IFN-β compared with IFN-α could be explained by several effects on this system: 1) both IFNs inhibit the transcription of IGF-II, but the suppression is significantly higher after IFN-β than IFN-α and 2) only IFN-β inhibits the expression of IGF-I receptor.

neuroendocrine tumors; type I interferons; insulin-like growth factor-II; insulin-like growth factor-I receptor

THE INSULIN-LIKE GROWTH FACTOR (IGF) signaling system plays a pivotal role in the tumorigenesis, proliferation, and spread of many tumors by controlling cell cycle progression and preventing apoptosis (18, 26). The IGF system consists of three peptide hormones (IGF-I, IGF-II, and insulin); three cell surface receptors, including the IGF-I receptor (IGF-IR), the IGF-II receptor (IGF-IIR), and the insulin receptor; and a family of IGF-binding proteins and acid-labile subunit. Whereas insulin is mainly involved in controlling metabolic homeosta-
sis, IGF-I and IGF-II are essential for normal growth and development. IGF-I and IGF-II interact with the IGF-IR, a trans-membrane tyrosine kinase, whereas only IGF-II has a high affinity for the isoform A of insulin receptor. Both IGF-IR and the insulin receptor isoform A mediate predominantly proliferative effects. IGF-II can also bind to the IGF-IIR with high affinity, which acts as a clearance receptor for IGF-II. IGF-IIR binds and internalizes IGF-II, resulting in the lysosomal degradation of this ligand and influencing the extracellular levels of IGF-II (10, 18, 26). Therefore, the stimulation of tumor growth by IGF-I is primarily modulated by the activation of IGF-IR, whereas IGF-II can act through either the IGF-IR or the insulin receptor isoform A. In the circulation, the ligands are complexed to a family of high-affinity binding proteins (IGF-binding proteins and acid-labile subunit) that serve to protect IGF-I and IGF-II from degradation by proteases (10, 18, 26).

The IGF-I autocrine loop seems to be important in growth control of neuroendocrine tumors (NETs), particularly in functionally active histotypes (22, 36). Von Wichert et al. (35) described the presence of an IGF-I autocrine loop in BON cells, a functioning human gastroenteropancreatic NET cell line. In these cells, IGF-I is expressed and active, promoting both anchorage-dependent and -independent growth, regulating levels of cell cycle proteins, and stimulating the cellular secretion of chromogranin A (34, 35). Therefore, inhibition of the IGF system may be a promising approach for novel treatment strategies of NETs (15). It is unknown if there is a role for IGF-I in the modulation of NET cell proliferation. However, IGF-II and IGF-IR are expressed in ~30 and 70% of NETs, respectively (36).

In a recent study, we have compared the antitumor effects of interferon (IFN)-α and IFN-β in BON cells (32). The antiproliferative activity of IFN-β is considerably more potent than IFN-α. On the basis of these findings, the aim of the present study was to evaluate the role of IGF-II on tumor growth regulation of BON NET cells and the potential effects of type I IFNs on the IGF system.

MATERIALS AND METHODS

Cell lines and culture conditions. BON cells were obtained from Dr. C. M. Townsend (The University of Texas Medical Branch, Galveston, TX). The cells were cultured in a humidified incubator containing 5% CO2 at 37°C. The culture medium consisted of a 1:1

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mixture of DMEM and F-12K medium, supplemented with 10% FCS, 1 × 10^5 U/l penicillin, 0.5 mg/l fungizone, and 2 mmol/l l-glutamine. Periodically, cells were confirmed as Mycoplasma free. Cells were harvested with trypsin/EDTA (0.05%/0.53 mM) 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%. Supplements were obtained from Gibco Bio-cult Europe (Invitrogen, Breda, The Netherlands).

All experiments have been performed under serum-starved conditions.

**Drugs and reagents.** Human recombinant IFN-α-2b (Intron-A) was purchased from Schering-Plough, and human recombinant IFN-β-1a was from Serono (Re bif, Rockland, MA).

All compounds were stored at −20°C, and the stock solution was constituted in distilled water according to the manufacturer instructions. IGF-I and IGF-II were purchased from Biosource (Invitrogen). Neutralizing polyclonal IGF-II antibody (Ab-IGF-II) was obtained from R&D systems (Oxon, UK).

**Quantitative RT-PCR.** The expression of IGF-I, IGF-II, IGF-IR, and insulin receptor mRNA on BON cells was evaluated by quantitative RT-PCR. Total RNA was isolated using a commercially available kit (high pure RNA isolation kit; Roche, Almere, The Netherlands). cDNA synthesis and quantitative PCR using the TaqMan Gold nucleic acid assay and the ABI PRISM 7700 sequence detection system (Perkin-Elmer Applied Biosystems, Groningen, The Netherlands) were performed as described in detail previously (13). The primer and probe sequences were purchased from Biosource (Nivelles, Belgium). The sequence of the primers for IGF-II and the concentrations of primers and probes used in the assay have been described previously by van Koetsveld et al. (31). The other primer and probe sequences were as follows: IGF-I forward, 5'-TTGATTTCTCAGAACTGGAAGGATC-3'; IGF-I reverse, 5'-CGTTGGCAAGCTGTGCAAGG-3'; IGF-I probe, 5'-FAM-TAATCCGCTTGTCCTTGCTCA-TAMRA-3'; IGF-IR forward, 5'-CACAACATGAGCAGCAGGAG-3'; IGF-IR reverse, 5'-GGCTCCGGTATGTGTTAGGT-3'; IGF-IR probe, 5'-FAM-AAGCGAGAACACCAAGCCCTG-TAMRA-3'; insulin receptor forward, 5'-GCTCAGCGAGACTGCAAGG-3'; insulin receptor reverse, 5'-ACAAATCTTCTGGCAGTTA-TAMRA-3'. The primer set for the insulin receptor recognizes both insulin A and B. Dilution curves were constructed for calculating the PCR efficiency (E) for every primer set. E values were 1.92 for IGF-I, 1.98 for IGF-II, 1.85 for IGF-IR, and 1.82 for the insulin receptor. The estimated copy numbers were calculated using the comparative threshold method with efficiency correction (19). The amount of mRNA was normalized to the total amount of RNA. To exclude contamination of the PCR mixtures, the reactions were also performed in the absence of cDNA template in parallel with cDNA samples.

**Cell proliferation assay.** After trypsinization, the cells were plated in 1 ml of complete culture medium in 24-well plates at the density of 1–2 × 10^4 cells/well. The plates were then placed in a 37°C, 5% CO₂ incubator. Later (3 days), the cell culture medium was replaced with 1 ml fresh serum-free medium or medium with IFN-β (100 IU/ml). Plates were further incubated at 37°C and 5% CO₂ for 3 days, and the supernatants were collected for IGF-II determination by a nonextraction enzymatically amplified “two steps” sandwich-type immunoassay (Diagnostic Systems Laboratories, Assendelft, The Netherlands), according to the procedures of the manufacturer. Intra- and interassay coefficients of variation (CVs) were 5.2 and 6.8%, respectively. The data were corrected for the effect of treatment on cell number.

**IGF-I kinase receptor activation assay.** BON cells were plated in 1 ml of complete culture medium in 24-well plates at the density of 2 × 10^4 cells/well and placed in a 37°C, 5% CO₂ incubator. Later (3 days), the culture medium was replaced with 1 ml fresh serum-free medium/well or medium with IFN-β (100 IU/ml). Plates were further incubated at 37°C and 5% CO₂ for 3 days, and the supernatants were collected for the evaluation of IGF-IR bioactivity. The data were corrected for the effect of treatment on cell number. IGF-I bioactivity was measured using an in-house IGF-I kinase receptor activation assay (KIRA), as previously described (2). Briefly, human embryonic renal cells (293 EBNA; Invitrogen) stably transfected with copy DNA of the human IGF-IR gene were used as read-out after stimulation with either recombinant IGF-I standards or with conditioned media from BON cells. IGF-I standards and conditioned media were measured in duplicate on each culture plate. Intra-assay and interassay CV were 6.0 and 10.9%, respectively. To exclude a direct effect of IFN-β on the IGF system of 293 EBNA, we evaluated the bioactivity of conditioned media (BON cell free) with or without IFN-β (100 IU/ml) after 3 days of incubation at 37°C and 5% CO₂.

**IGF-IR binding study.** Membrane preparations of BON cells, incubated with 3 or without IFN-α (100 IU/ml) or IFN-β (100 IU/ml), were prepared as previously described (30). The radioligand used in the binding studies was 3-[125I]iodotyrosyl-IGF-I (GE Healthcare, Brussels, Belgium). Briefly, membrane preparations (corresponding to 50 μg protein) of BON cells were incubated for 60 min with increasing concentrations of 3-[125I]iodotyrosyl-IGF-I in the absence or presence of excess (100 nM) unlabeled IGF-I in HEPES buffer (10 mM HEPES, 5 mM MgCl₂, and 0.2 g/l bacitracin, pH 7.6) containing 0.2% BSA (Sigma, St. Louis, MO). After incubation, 1 ml ice-cold HEPES buffer (pH 7.6) was added to the assay mixture, and membrane-bound radioactivity was separated from unbound by centrifugation during 2 min at 14,000 revolutions/min in an Eppendorf microcentrifuge. The remaining pellet was washed twice with ice-cold HEPES buffer, and the final pellet was counted in a gamma counter. Specific binding was taken to be the total binding minus binding in the presence of 100 nM unlabeled IGF-I (30).

**Apoptosis assays.** After trypsinization, BON cells were plated in 24-well plates at a density of 2 × 10⁴ cells/well. Later (3 days), the cell complete culture medium was replaced with 1 ml/well serum-free medium with or without IFN-β (100 IU/ml), IGF-I (50 nM), IGF-II (50 nM), IFN-β (100 IU/ml) + IGF-II (50 nM), or IFN-β (100 IU/ml) + IGF-II (50 nM) for an additional 3 days.

In another experiment, BON cells were preincubated with Ab-IGF-II (1 μg/ml) for 1 day, followed by combined treatment with IFN-β (100 IU/ml) and Ab-IGF-II (1 μg/ml) for an additional 3 days, or incubated for 3 days with IFN-β (100 IU/ml) alone.

Quadruplicates of each treatment were performed, and DNA fragmentation (as a measure for apoptosis) was assessed using a commercially available ELISA kit (Cell Death Detection ELISAPlus; Roche Diagnostic, Penzberg, Germany). The standard protocol supplied by the manufacturer was used, as previously described (11). The data were corrected for the effect of treatment on cell number.

**Statistical analyses.** All experiments were carried out at least three times and gave comparable results. For statistical analysis, GraphPad Prism 3.0 (GraphPad Software, San Diego, CA) was used. The comparative statistical evaluation among groups was first performed by ANOVA test. When significant differences were found, a comparison between groups was made using the Newman-Keul’s test. The unpaired Student’s t-test was chosen to analyze the differences in IGF bioactivity.
In all analyses, values of $P < 0.05$ were considered statistically significant. Data are reported as means ± SE.

**RESULTS**

**Characterization of the IGF system in BON cells.** Using sequence-specific primers, we detected the presence of IGF-I, IGF-II, IGF-I receptor, and insulin receptor mRNA, normalized for the total amount of RNA, in BON cells (Fig. 1).

Among all evaluated components of the IGF system, IGF-II mRNA was the form expressed at the highest level.

This system is active in BON cells, as shown by the significant stimulation of cell proliferation observed after 6 days of incubation with IGF-I (135.9 ± 3.1% vs. control, $P < 0.001$) or IGF-II (138.1 ± 3% vs. control, $P < 0.001$), both at the concentration of 50 nM.

**Effect of type I IFNs on IGF-I, IGF-II, IGF-IR, and insulin receptor mRNAs.** As evaluated by quantitative RT-PCR, after 1 day of treatment, only IFN-α (100 IU/ml) induced a moderate increase in the expression of IGF-II mRNA (+49%, $P < 0.05$) compared with untreated control (Fig. 2A), whereas, after 3 days of incubation, both IFN-α (100 IU/ml) and IFN-β (100 IU/ml) inhibited the expression of IGF-II mRNA (−42%, −65%, respectively, both $P < 0.001$) in BON cells (Fig. 2B). Moreover, the inhibitory effect on IGF-II transcription was significantly higher after IFN-β than IFN-α ($P < 0.01$). No significant effect was observed on IGF-I (Fig. 2, C and D) and insulin receptor (Fig. 3, A and B) mRNAs after treatment with both IFNs. Interestingly, IGF-IR mRNA was upregulated by IFN-α (+28%, $P < 0.001$) and downregulated by IFN-β (−47%, $P < 0.001$) after 3 days of incubation (Fig. 3D). An inhibitory activity of IFN-β on IGF-IR mRNA (−27%, $P < 0.05$) was already present after 1 day of treatment, suggesting an early effect (Fig. 3C).

![Fig. 1. Expression of insulin-like growth factor (IGF)-I, IGF-II, IGF-I receptor, and insulin receptor in BON cells. Bars represent the no. of copies (mean ± SE) of IGF-I, IGF-II, IGF-I receptor, and insulin receptor mRNA/100 ng of total RNA in the RT reaction. The vertical axis represents a log scale.](http://ajpendo.physiology.org/)

![Fig. 2. Expression of IGF-II (A and B) and IGF-I (C and D) mRNA, corrected for total RNA, after 1 day (A and C) and 3 days (B and D) of treatment with interferon (IFN)-α and IFN-β (both at the concentration of 100 IU/ml). Data are means ± SE and are expressed as %control. *$P < 0.05$ and **$P < 0.001$ vs. control.](http://ajpendo.physiology.org/)
Effect of type I IFNs on IGF-II secretion. IGF-II concentrations in BON conditioned media were decreased significantly during IFN-α/H9251 (16%, *P* < 0.05) and IFN-β/H11002 (69%, **P* < 0.001) treatment (Fig. 4A). The inhibition of IGF-II secretion by IFN-β was statistically more potent than by IFN-α (*P* < 0.001).

Effect of type I IFNs on IGF-IR bioactivity. The development of the KIRA allows the determination of IGF system bioactivity by measuring in vitro ligand-induced receptor tyrosine kinase activation for IGF-IR in terms of receptor phosphorylation.

The bioactivity for IGF-IR (Fig. 4B), evaluated in BON conditioned media, significantly decreased (−54%, *P* < 0.005) after 3 days of treatment with IFN-β (100 IU/ml) compared with the untreated control. This effect seems to be mainly related to the modulation of the IGF system by IFN-β and not to a direct effect of IFN-β on human embryonic renal cells (293 EBNA), since IGF-IR bioactivity of unconditioned (cell free) media with IFN-β (100 IU/ml) did not differ from the control (unconditioned media without IFN-β), as shown in Fig. 4C.

IGF-IR binding study. To investigate whether type I IFNs exert a regulatory role on IGF-IR at the protein level as well, membrane ligand-binding studies were performed.

BON cells were cultured in the presence or absence of IFN-α (100 IU/ml) or IFN-β (100 IU/ml) for 3 days and subsequently collected for cell membrane-binding assays with $^{125}$I-labeled IGF-I. Saturation binding data and Scatchard analysis are shown in Fig. 5. Maximum IGF-I-binding capacity ($B_{max}$) was dramatically suppressed by 65% compared with the control group after treatment with IFN-β ($B_{max}$ from 33 to 11.6 fmol/mg in the absence and presence of IFN-β, respectively). In contrast, IFN-α did not induce a change in $B_{max}$ (34.2 fmol/mg in IFN-α-treated cells).

Effects of IFN-β, IGF-I, IGF-II, and Ab-IGF-II on DNA fragmentation. IFN-β (100 IU/ml) is a potent inducer of apoptosis in BON cells (Fig. 6A), as shown by the threefold increase in DNA fragmentation compared with the control (*P* < 0.001). The stimulatory effects of IGF-I and IGF-II (both at 50 nM) on proliferation are accompanied by a moderate inhibition of DNA fragmentation (both *P* < 0.05 vs. control; Fig. 6A). It is interesting to observe that coincubation of IFN-β with IGF-I or IGF-II significantly reduced the proapoptotic activity of IFN-β alone (both *P* < 0.01).

Treatment with neutralizing Ab-IGF-II (1 μg/ml for 4 days) significantly increased the DNA fragmentation in BON cells compared with the control (*P* < 0.001; Fig. 6B), thereby further underlining the importance of an autocrine/paracrine IGF-II growth loop in these cells. After preincubation with Ab-IGF-II (1 μg/ml) for 1 day, followed by combined treatment with IFN-β (100 IU/ml) and Ab-IGF-II (1 μg/ml) for an additional 3 days, we observed a significant increase in the...
instances as well (24). Type I IFNs exert their activity via a common receptor, and interaction with this receptor results in phosphorylation of receptor-associated tyrosine kinases (Jak1 and Tyk2), which in turn phosphorylate and activate a family of proteins described as signal transducers and activators of transcription (STAT). Following the activation, STATs dimerize and rapidly translocate to the nucleus and interact with specific regulatory elements inducing target gene transcription. Most of these genes encode proteins that control the cell cycle and apoptosis (3, 4).

Few studies reported that, at least in part, the antitumor activity of IFN-α involves transcriptional modulation of the IGF system. Thulasi et al. (29) have shown that IFN-α induces growth arrest and enhancement of myogenic differentiation in Rh30, an alveolar rhabdomyosarcoma cell line, through the downregulation of the IGF-IR expression. Type I IFNs and poly(I:C), a double-stranded RNA and potent activator of type I IFNs expression, downregulated (about −70%) IGF-I mRNA expression in C6 rat glioma cells (5). IFN-α inhibited cell proliferation and induced apoptosis in Calu-6, a human non–small lung cancer cell line. A significant decrease of IGF-I and IGF-II (−95% and −55%, respectively) and an increase of IGF-binding protein-3 (+110%) levels have been observed in conditioned media after treatment with IFN-α (10 IU/ml), whereas the IGF-IR and IGF-IIR expression increased in Calu-6 cells during incubation with the cytokine (9). In addition, gene expression analyses showed a mutual exclusivity between IGF-II expression and IFN-induced genes in breast cancer and in hepatocellular carcinoma, suggesting an inverse relationship between both pathways (1, 23).

IFN-β binds to the same receptor of IFN-α, but with higher affinity and differential conformation of the receptor complex (7, 25). Notwithstanding IFN-β half-life (1 h) is shorter than that of IFN-α (4–7 h), several studies showed that IFN-β has more potent antitumor effects than IFN-α, via the activation of apoptosis (6, 8, 12, 16, 27, 31–33). In addition, new strategies could improve in future the pharmacokinetic and pharmacodynamic profile of IFN-β, like the pegylated formulation (21). The potential effects of IFN-β on the modulation of the IGF system are still poorly investigated. In a recent paper (31), we showed a potent antitumor activity of type I IFNs in human adrenocortical cancer cell lines H295 and SW-13. The antiproliferative effect of IFN-β was significantly higher compared with that of IFN-α in both cell lines. This appeared to be associated with an induction of apoptosis in H295 cells only and accumulation of cells in the S phase in both cell lines. H295, but not SW-13, cells expressed IGF-II. This cytokine is considered to be an important growth factor in adrenocortical cancer and in hepatocellular carcinoma. Therefore, we also evaluated the effects of both IFNs on IGF-II mRNA expression. In H295 cells, IGF-II mRNA was inhibited in a dose-dependent manner by IFN-β (21).

Type I IFNs are a family of cytokines (IFN-α, IFN-β, IFN-ε, IFN-κ, IFN-ω, IFN-δ, and IFN-τ) that elicit potent antiviral and antiproliferative cellular responses (24). The mechanisms of the antitumor activity appear to be multiple and complex. Whereas in some instances IFNs may induce or enhance host mechanisms including immune-mediated effects (20, 24), they appear to act directly on the tumor itself in many other

DNA fragmentation higher than those observed after IFN-β or Ab-IGF-II alone (both P < 0.01; Fig. 6B).

**DISCUSSION**

![Fig. 4. Effects of type I IFNs on IGF-II secretion and IGF-I receptor (IGF-IR) ligand bioactivity.](#)

**A** Immunoreactive IGF-II concentration (corrected for DNA) in conditioned medium after 3 days of incubation with IFN-α and IFN-β (both at the concentration of 100 IU/ml). *B* and **C**: IGF-IR ligand bioactivity, evaluated by IGF-I kinase receptor activation assay (KIRA), in conditioned media of BON cells (**B**) and in unconditioned (cell free) media (**C**) after 3 days of incubation with or without IFN-β (100 IU/ml). Data are means ± SE. **P < 0.05, **P < 0.005, and ***P < 0.001 vs. control.
cells, as shown by: 1) the expression of IGF-IR and the high IGF-II synthesis and secretion in BON cells; 2) the growth stimulating effect of exogenous IGF-II on BON cells, probably through the inhibition of DNA fragmentation; and 3) the proapoptotic activity of neutralizing antibody against IGF-II. It is interesting to observe that incubation with exogenous IGF-II induces only a moderate increase in cell proliferation (38%) and inhibition in DNA fragmentation (28%). This could be explained by the fact that, in BON cells, the very high amount of endogenous IGF-II may blunt the in vitro proliferative response of exogenous IGF-II. However, the IGF-II autocrine/paracrine growth loop seems to have a crucial role in the modulation of BON proliferation. This is evidenced by a 2.5-fold increase in apoptosis during incubation of BON cells with the neutralizing antibody against IGF-II, comparable with that observed during IFN-β treatment.

In addition, we demonstrated that the more potent antitumor activity of IFN-β compared with IFN-α could be explained by several effects on the IGF system in BON cells. As determined by quantitative RT-PCR, both type I IFNs (at a concentration of 100 IU/ml) suppressed the expression of IGF-II mRNA after 3 days of incubation. However, the inhibitory effect on IGF-II transcription after IFN-β (-65%) was statistically (P < 0.01) higher than IFN-α (-42%). Besides, IGF-IR mRNA was upregulated by IFN-α (+28%) and downregulated by IFN-β (-47%). The paradoxical increase in IGF-IR expression together with the upregulation of IGF-II mRNA after 1 day of incubation with IFN-α may be explained by the activation of survival pathways induced by IFN-α in BON cells. In fact, in several models, the antitumor activity of IFN-α is limited by the activation of mechanisms of tumor resistance (3).

These mRNA expression data were confirmed at the protein level. IGF-II values in the conditioned medium significantly decreased during IFN-α (-16%) and IFN-β (-69%) treatment. However, the suppression of IGF-II in the medium was lower than the effect observed at the mRNA level during IFN-α incubation. This discrepancy may be related to the fact that the production of mRNA is earlier than that of protein; therefore, we cannot exclude a higher and delayed inhibitory effect of IFN-α on IGF-II protein. In addition, we evaluated the effects of type I IFNs on the IGF bioactivity by KIRA in BON cells. Previous studies have used the circulating concentrations of free or total IGF-I as an estimate of IGF bioactivity. However, these dosages do not take into account the effects of
other ligands for IGF-IR, as IGF-II, and the modifying effects of IGF-binding proteins and proteases on the interactions between IGF-I/IGF-II and IGF-IR (17). KIRA is a highly specific and sensitive IGF-I kinase receptor activation assay, able to determine the serum concentration of IGF-IR ligands that can phosphorylate and activate the IGF-IR in vitro (17). Therefore, it is possible to evaluate the effects of the whole IGF system interacting with IGF-IR through this innovative technique. We observed a significant decrease (~54%, P < 0.005) in bioactivity of IGF-IR ligands during IFN-β treatment in BON cells. On the basis of the previous data, showing an IGF-II suppression at the mRNA and protein levels, it is conceivable that the reduction of IGF bioactivity after incubation with IFN-β may be related to the transcriptional inhibition of IGF-II gene. However, we cannot exclude that the antitumor activity of IFN-β is partially mediated by an additional mechanism involving the modulation of IGF-binding proteins or proteases. In fact, IGF-binding proteins can influence apoptosis by direct and IGF-independent effects (28). Finally, the Scatchard analysis of uptake of [125I]-IGF-I binding to cell membrane of BON cells revealed a dramatic suppression of Bmax only in the presence of IFN-β, suggestive of IGF-IR and, eventually, insulin-IGF-IR hybrid receptor downregulation.

Taken together, we hypothesize that the antitumor activity of IFN-β is partially mediated by the modulation of the IGF system. This is further confirmed by the observation that the proapoptotic activity of IFN-β is counteracted by the exogenous addition of IGF-I and IGF-II.

In conclusion, our study provides the first evidence of an autocrine/paracrine loop for IGF-II in BON cells. This factor seems to be essential in the growing of these cells, considering the high amount produced. In addition, we showed that IFN-β potently inhibits the growth of NET cells through a transcriptional inhibition of IGF-II and IGF-IR genes. This opens a new scenario in the use of IFN-β for the treatment of tumors where the IGF system plays a pivotal role in the development and progression of cancer (NETs, colon cancer, lung cancer, prostate cancer, breast cancer, adrenal cancer, endometrium and ovary cancer).

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