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Biochemical and Biophysical Research Communications 344 (2006) 1334–1341
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Novel interferon-λs induce antiproliferative effects in neuroendocrine tumor cells

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Received 4 April 2006
Available online 24 April 2006

Abstract

Interferon-α (IFN-α) is used for biotherapy of neuroendocrine carcinomas. The interferon-λs (IL-28A/B and IL-29) are a novel group of interferons. In this study, we investigated the effects of the IFN-λs IL-28A and IL-29 on human neuroendocrine BON1 tumor cells. Similar to IFN-α, incubation of BON1 cells with IL-28A (10 ng/ml) and IL-29 (10 ng/ml) induced phosphorylation of STAT1, STAT2, and STAT3, significantly decreased cell numbers in a proliferation assay, and induced apoptosis as demonstrated by poly(ADP-ribose) polymerase (PARP)-cleavage, caspase-3-cleavage, and DNA-fragmentation. Stable overexpression of suppressor of cytokine signaling proteins (SOCS1 and SOCS3) completely abolished the aforementioned effects indicating that SOCS proteins act as negative regulators of IFN-λ signaling in BON1 cells. In conclusion, the novel IFN-λs IL-28A and IL-29 potently induce STAT signaling and antiproliferative effects in neuroendocrine BON1 tumor cells. Thus, IFN-λs may hint a promising new approach in the antiproliferative therapy of neuroendocrine tumors.

Keywords: Interferon-λs; Interleukins; IL-28; IL-29; STAT; SOCS; Apoptosis; Neuroendocrine carcinoma; BON1 cells

Efficient antiproliferative strategies against neuroendocrine tumors (NET) of the gastroenteropancreatic (GEP) system are still lacking. So far, interferon-α (IFN-α) or somatostatin analogues are utilized for so-called biotherapy [1]. Since treatment with IFN-α is often inefficient or accompanied by severe hematopoietic side effects, new strategies need to be developed. Recently, several interleukin (IL)-10-related cytokines have been discovered. Among these are IL-22, IL-26, and interferon-λs (IFN-λs). Because of their ability to activate IFN-stimulated response elements, IFN-λ proteins have been considered to be a novel group of "interferons" [2] with potential better antiproliferative actions against NET.

The IFN-λ gene family comprises three members: IL-29 (IFN-λ1), IL-28A (IFN-λ2), and IL-28B (IFN-λ3) [2,3]. IFN-λs exhibit limited sequence similarity to IL-10. However, IL-10 and IFN-λs use the same receptor subunit (IL-10R2) as a component of their respective receptor complexes. The IFN-λ receptor complex consists of the unique ligand-binding chain IL-28R1 (also known as IFN-λR) and the accessory chain IL-10R2.

IFN-α inhibits proliferation and cellular differentiation of various cell types by transducing regulatory signals through the Janus tyrosine kinase/signal transducers and activators of transcription (Jak/STAT) pathway. This mechanism is negatively regulated by suppressor of cytokine signaling (SOCS) proteins [4–6]. Although their receptor subunits display no detectable homology, IFN-λs and type I IFNs (IFNα/β) activate similar transduction pathways. Signaling through their respective receptor

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0006-291X/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2006.04.043

complexes results predominantly in the phosphorylation of STAT1 and STAT2 but can additionally involve STAT3 and STAT5 [2,3,7]. The STAT proteins homo- or heterodimerize and, together with the accessory factor IFN regulatory factor 9 (IRF-9; p48), form a transcription factor complex which is known as IFN-stimulated gene factor 3 (ISGF3). Activation of ISGF3 is characteristically associated with the induction of type I IFN-responsive genes. Signaling through IFN-λR results in the induction of many of the same genes that are induced by signaling through type I IFNRs [2]. Thus, it can be expected that the novel λ-IFNs share a variety of biological activities ascribed to type I IFNs. Because of limited receptor expression in hematopoietic cells, IFN-λ cytokines may have therapeutic advantage over the conventional therapy with IFN-α, which is often accompanied by myelosuppression [8].

In this study, we demonstrate that neuroendocrine BON1 tumor cells express the IFN-λ receptor complex and that the IFN-λ cytokines IL-28A and IL-29 are new potential biotherapeutic agents in the treatment of neuroendocrine tumors. Our data suggest SOCS protein expression to be a cause and therapeutic target of IFN-resistance in biotherapy.

Materials and methods

Materials. DMEM/F12 (1:1) media, penicillin/streptomycin, and geneticin were from Gibco/Invitrogen (Karlsruhe, Germany). Amphotericin B and fetal calf serum were purchased from Biochrom (Berlin, Germany). Plasticware for cell culture was from Becton–Dickinson (Heidelberg, Germany). Recombinant human (rh) IL-28A and IL-29 were from R&D Systems (Minneapolis, MN) and human IFN-α was purchased from Roche (Mannheim, Germany). Jak Inhibitor I, a pan Jak inhibitor, was from Calbiochem (Schwalbach, Germany). The general caspase inhibitor Z-VAD-FMK was from Bachem (Heidelberg, Germany). Antibodies against pSTAT1 were from BD Transduction Laboratories (Franklin Lakes, NY) and antibodies against pSTAT2, pSTAT3, and STAT2 were from Upstate Biotechnology (Lake Placid, NY). Antibodies against STAT1 and STAT3 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against PARP and caspase-3 were from Cell Signaling (Beverly, MA). Horseradish peroxidase-conjugated secondary antibodies to mouse or rabbit IgG and chemiluminescent substrate SuperSignal West Dura Extended Duration Substrate were from Pierce (Rockford, IL).

Cell culture and stable transfection. Human pancreatic neuroendocrine BON1 tumor cells were kindly provided by R. Göke (Marburg, Germany). BON1 cells were cultured in DMEM/F12 (1:1) medium supplemented with 10% fetal calf serum, 1% penicillin/streptomycin, and 0.4% amphotericin B in a 5% CO_2 atmosphere. Stable clones were established as described previously [9]. Briefly, a human SOCS3 cDNA clone, spanning the entire coding sequence (nt 104–808 GenBank Accession No. AB004904), was cloned in TOPO-TA-cloning Vector (Invitrogen, Karlsruhe, Germany) and subcloned in pCR3.1 Vector (Invitrogen, Karlsruhe, Germany). A human SOCS1 cDNA clone, spanning the entire coding sequence (GenBank Accession No. U88326; a friendly gift from Dr. T. Willson, The Walter and Eliza Hall Institute of Medical Research, Victoria, Australia), was subcloned in pCR3.1. All inserts were verified by full-length sequencing. Stable transfections of BON1 cells with pCR3.1 alone (pCR3.1/mock) and pCR3.1/SOCS1 or pCR3.1/SOCS3 inserts were performed with FuGENE 6 (Roche, Mannheim, Germany) according to the manufacturer's instructions. Subsequently, cells were cultured in selection medium containing 1 mg/ml geneticin for 4 weeks before single pCR3.1/mock, pCR3.1/SOCS1, and pCR3.1/SOCS3 clones were isolated. All clones were further expanded in selection medium containing geneticin (1 mg/ml). For further experiments, two different cell clones of each type were selected, based on maximal overexpression of the respective construct, confirmed by Northern-blot analysis.

Reverse transcriptase polymerase chain reaction (RT-PCR). Total RNA was isolated using Trizol reagent (Gibco-BRL/Life Technologies, Gaithersburg, MD). For RT-PCR, RNA was treated with ribonuclease (RNase)-free deoxyribonuclease (TURBO DNA-free™-Kit, Ambion) to remove contaminating genomic DNA. One microgram of total RNA was reverse transcribed using M-MLV Reverse Transcriptase (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions. To exclude contamination with genomic DNA, an identical parallel PCR was performed containing starting material that had not been reverse transcribed. The following conditions were used for PCRs: 35 cycles of denaturing at 94 °C for 45 s, annealing at 60 °C for 45 s, and extension at 72 °C for 45 s. The primers used for amplification were as follows: IL-10R2 forward and reverse: 5′-GGC TGA ATT TGC AGA TGA GCA-3′ and 5′-GAA GAC CGA GGC CAT GAG G-3′; IL-28R1 forward and reverse: 5′-ACC TAT TTT GTG GCC TAT CAG AGC T-3′ and 5′-CGG CTC CAC TTC AAA AAG GTA AT-3′. The PCR products were cloned into pCR 2.1 vector (Invitrogen) and sequenced.

Cell proliferation assay. For proliferation assays, BON1 cells were seeded into 96-well plates at a density of 3000 cells/well and grown for 24 h. The cells were than incubated with 10 ng/ml IL-28A, 10 ng/ml IL-29 or IFN-α (1000 U/ml) in complete medium containing 10% FCS. Cells were preincubated with Jak Inhibitor I (1 μM) and Z-VAD-FMK (40 μM) for 15 min before stimulation with IL-28A, IL-29, and IFN-α. The cell proliferation rate was measured with Cell Titer 96 kit (Promega, Madison, WI) 24 and 72 h after stimulation according to the manufacturer's instructions. Following 3 h of incubation with Cell Titer 96 solution, absorbance at 492 nm was determined using an ELISA plate reader.

Quantification of DNA-fragmentation and cell cycle analysis. The rate of apoptotic cell death was quantified by determining DNA-fragmentation according to Nicoletti et al. [10]. Briefly, cells were incubated for 24 h in a hypotonic buffer (1% sodium citrate, 0.1% Triton X-100, and 50 mg/ml propidium iodide) and analyzed by flow cytometry on a FACScalibur (Becton–Dickinson) using CellQuest software. Nuclei to the left of the 'G1-peak' containing hypodiploid DNA were considered apoptotic.

Protein extraction and Western blotting. Protein extraction and Western blotting were performed as described recently [9,11]. Briefly, cells were lysed in 500 μl lysis buffer. The lysates were centrifuged for 10 min at 4 °C and 13,000*g*, and supernatants were diluted 1:1 with SDS sample buffer. Samples were boiled for 5 min and separated on a SDS–polyacrylamide gel. Proteins were electrotransferred in 60 min onto PVDF membranes (Immobilone; Millipore, Eschboon, Germany) using a semi-dry Western-blot technique. After blocking in 2% non-fat dried milk, the membranes were incubated overnight in appropriate dilutions of antibodies against pSTAT1 (1:20,000), pSTAT2 (1:10,000), pSTAT3 (1:20,000), PARP (1:1000), and caspase-3 (1:1000). After washing with PBS, the membranes were incubated with peroxidase-conjugated secondary antibody (1:25,000) for 2 h. The blots were washed and immersed in the chemiluminescent substrate for 30 min and exposed to XOMAT-AR film (Eastman Kodak, Rochester, NY). Afterwards, the membranes were stripped and incubated with antibodies against STAT1 (1:20,000), STAT2 (1:10,000), and STAT3 (1:20,000) as described above.

Luciferase assay. Transient transfection of BON1 cells with a −970 nt human 2′,5′-OAS promoter-luciferase construct in pGL2-BV vector (a friendly gift from Dr. G. Floyd-Smith, Arizona State University, Tempe, Arizona, USA) was performed with FuGENE 6 (Roche, Mannheim, Germany), as described previously [9]. Following transfection, cells were grown for another 24 h before IFN-α (1000 U/ml), IL-28A (10 ng/ml) or IL-29 (10 ng/ml) was added in serum-depleted medium for 6 h. Following stimulation, cells were then lysed and luciferase activities were measured with Luciferase Assay System (Promega, Madison, WI). Baseline reporter gene activity in the unstimulated control group was set as 1. Induction of 2′,5′-OAS reporter gene activity by stimulation with IFN-α, IL-28A, and IL-29 was calculated as fold increase in comparison to the control. In all experiments, transfection efficiency was verified by β-galactosidase assay.