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Bimodal effect of interferon-β on astrocyte proliferation and survival: Importance of nuclear factor-κB

Olga Barca, Pablo Devesa-Peleteiro, Marcos Seoane, Rosa Mª Señarís, Víctor M. Arce *

Departamento de Fisioloxía, Facultade de Medicina, Universidade de Santiago de Compostela, 15782 Santiago de Compostela, Spain

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ABSTRACT

Accumulating evidence indicates that interferon-β (IFN-β) can modify the complex immunopathogenic scenario causing clinical relapse activity and disease progression in MS. However, the beneficial effects of IFN-β in MS patients may also depend on non-immune mechanisms, including the modulation of astrocyte function. In the present report, we have shown that, depending on the dose, IFN-β treatment can either promote astrocyte proliferation and survival, or result astrocyte death. These actions depend, at least in part, on the regulation of nuclear factor-kappa B (NF-κB), an inducible transcription factor present in neurons and glia. This bimodal effect of IFN-β adds a new layer of complexity in the actions of IFN-β within the CNS.

1. Introduction

Interferon-β (IFN-β) is a type I interferon with diverse biological functions which include the modulation of immune and antiviral responses, and the regulation of cell proliferation, differentiation and survival. All these actions are mediated through the induction of related but distinct signalling pathways, among which the Janus kinase 1 (JAK1)-signal transducer and activator of transcription (STAT) pathway in the best characterized (Stark, et al., 1998). Binding of IFN-β to its high-affinity cell surface receptor facilitates the activation of the receptor-associated JAK1 and Tyk2 tyrosine kinases, which interact with and activate the SH2-containing cytoplasmic STAT proteins. IFN-β-activated STATs bind to the IFN gene regulatory factor 9 (IRF9/p48/ISGF3γ) and the resulting complex translocates to the nucleus and binds to the IFN-stimulated regulatory elements (ISREs) to induce the expression of a set of early response genes denominated IFN-stimulated genes (Darnell et al., 1994; Stark et al., 1998; Aaronson and Horvath, 2002). However, accumulating evidence indicates that this central pathway does not account for the complete repertoire of IFN-β-mediated responses. Multiple other signalling cascades are activated by the type I IFNs, including p38 and p42/p44 mitogen-activated protein kinases (MAPKs), insulin receptor substrate-1, phosphatidylinositol-3 kinase (PI-3K) and nuclear factor-κB (Rani and Ransohoff, 2005).

Evidence gathered over the last years, has shown that IFN-β reduces the rate of clinical relapse and the accumulation of active lesions detected by magnetic resonance imaging in multiple sclerosis (MS) patients (Noseworthy et al., 2000; Bayas and Gold, 2003; Markowitz, 2007). Although the exact cause of MS is still unknown, its pathogenesis comprises the activation of immune cells and break-down of the blood-brain barrier, leading to selective demyelination and eventually neurodegeneration (Markowitz, 2007; Kieseier and Hartung, 2007). Most of the therapeutic benefit of IFN-β in MS patients is attributed to its ability to modify this complex immuno-pathogenic scenario ought to its immunomodulatory effects (Markowitz, 2007; Billiau, 2006). However, accumulating evidence indicates that non-immune actions of IFN-β may also contribute to its efficacy. Thus, IFN-β enhances neuronal survival (Sättler et al., 2006; Jin et al., 2007) and promotes the endothelial barrier function (Kraus et al., 2004; Biernacki et al., 2005; Niemelä et al., 2008). In addition, IFN-β influences several aspects of astrocyte physiology that may have implications in the pathogenesis of the disease including the production of neurotrophic factors (McLaurin et al., 1995; Boutros et al., 1997; Okada et al., 2005), the prevention of the release of neurotoxic factors (Stewart et al., 1998; Guthikonda et al., 1998; Jin et al., 2007), and the regulation of astrocyte proliferation and survival (Garrison et al., 1996; Satoh et al., 1996; Malik et al., 1998; Barca et al., 2003, 2007, 2008).

In order to gain further insight into the non-immune actions of IFN-β that may help to understand its beneficial effects in MS, in the present report we have investigated the outcome of different IFN-β doses on astrocyte proliferation and survival. Our findings show that while low doses of IFN-β stimulate astrocyte proliferation and survival, higher

* Corresponding author. Departamento de Fisioloxía, Facultade de Medicina, Universidade de Santiago de Compostela, 15705 Santiago de Compostela, Spain. Tel.: +34 981582658; fax: +34 981574145.
E-mail address: victor.arce@usc.es (V.M. Arce).

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doses of the cytokine result in a cytotoxic effect. This bimodal effect of IFN-β may be of interest in order to address some of the dosing issues related with IFN-β treatment in MS patients (Markowitz, 2007), and may also help to understand the possible roles of the cytokine in the pathogenesis of the disease.

2. Materials and methods

2.1. Cell cultures and treatments

Primary monolayer cultures of cortical astrocytes were established from fetal (embryonic day 15) Sprague–Dawley rat cerebral cortices. The care and use of all experimental animals was in accordance with institutional guidelines. Cells were dissociated by an enzymatic method that employs protease treatment with 2 mg/mL dispase II (Boehringer-Mannheim Gmbh, Mannheim, Germany) and 25 μg/mL DNAse I (Sigma-Aldrich, St. Louis, MO), for 60 min at 37 °C, with additional mechanical shearing. Cells were plated in Falcon polystyrene culture dishes (BD Biosciences, San Jose, CA) and grown in Dulbecco's modified Eagle's medium (Sigma-Aldrich) supplemented with 5% fetal bovine serum (FBS) (Invitrogen Corporation, Carlsbad, CA), 2 mM glutamine, 2.5 U/mL penicillin, 2.5 μg/mL and streptomycin (all from Sigma-Aldrich). The cultures were maintained at 37 °C in a humidified atmosphere of 5% CO2 for one week and thereafter cells were trypsinized and subcultured for the different experiments. These astrocyte-enriched cultures contained more than 96% astrocytes by immunofluorescence, using a monoclonal antibody directed against glial fibrillary acidic protein (Dako, Glostrup, Denmark; dilution 1:40).

Human recombinant IFN-β was provided by Schering Plough (Kenilworth, NJ). The selective phosphatidylinositol 3-kinase (PI-3K) inhibitors (LY 294002 and wortmannin) were purchased from Sigma-Aldrich. The caspase inhibitor z-VAD-fmk, the p38 MAPK inhibitor SB203580, and the inhibitor of the nuclear translocation of NF-κB (SN50) were purchased from Calbiochem (San Diego, CA).

2.2. Assessment of apoptosis

Apoptotic cell death was assessed by fluorescence microscopic analysis of cell DNA staining patterns with Hoechst 33258. Cells were incubated with 4 μg/mL of Hoechst 33258 (Sigma-Aldrich) for 40 min at 37 °C and, after washing, cell morphology was examined under an Olympus fluorescence microscope (IX70, Olympus Optical Co, Tokyo, Japan) with the appropriate filter combination. Cells were scored for apoptosis by their nuclear morphology, (shrinkage, condensation, and fragmentation) and the higher intensity of blue fluorescence, and photographed at a 40x magnification with a DP10 microscope digital camera (Olympus Optical Co).

2.3. Western blotting

For phospho-Akt and phospho-p38 MAPK determination, cells were maintained in 1% FBS for 48 h followed by serum starvation for 2 h. IκB immunoreactivity was determined in cells growing in the presence of 5% FBS. In all cases, cells were collected by centrifugation, and the pellet was then lysated by heating at 95 °C for 5 min in 1% SDS, and immediately cooled at 4 °C for 15 min with ice-cold lysis buffer (50 mM Hepes, pH 7.5; 150 mM NaCl; 10% Glycerol; 1% Triton X-100; 5 mM EGTA; 1.5 mM MgCl2; 20 mM Na4P2O7; 20 mM Na3VO4; 50 μg/mL aprotinin and 4 mM phenylmethylsulfonyl fluoride). After centrifugation (15,000×*g*, 15 min, 4 °C) to separate cellular debris, the lysates were resolved in a 12% SDS-PAGE, and electrotransferred onto a nitrocellulose paper (Protran; Schleicher and Schuell, Dassel, Germany). For phospho-Akt determination, membranes were probed with a commercial kit (Phosphoplus Akt Ser473 Antibody Kit, New England Biolabs, USA) that allows specific recognition of both non-phosphorylated and serin-phosphorylated Akt. The status of p38 MAPK phosphorylation was determined with an antibody that recognizes Thr180/Tyr182 phosphorylated p38 MAPK (Cell Signalling Technology Inc, Danver, MA). WIP-1 immunoreactivity was detected with a specific polyclonal antibody (Santa Cruz Biotech, Heidelberg, Germany). Membranes were reprobed with antibodies against α-tubulin (Santa Cruz Biotech) as loading control. IκB-α immunoreactivity was detected with a specific polyclonal antibody (Santa Cruz Biotech), using α-tubulin or β-actin (Santa Cruz Biotech) as a loading control. In all cases, immunoreactive bands were detected with a western-light chemiluminiscence detection system (ECL, GE Healthcare Bio-Sciences AB, Uppsala, Sweden) and photographed (HyperfilmECL, GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Immunoreactive bands were scanned with a GelDoc system (Bio-Rad, Hercules, CA).

2.4. Cell proliferation assays

In order to evaluate the effect of IFN-β on astrocyte proliferation, 4×10^4 cells were seeded in Falcon 24-well plates, and treated with increasing amounts of IFN-β for 48 h. Cell number was determined with a Neubauer counting chamber. To assess the level of cell proliferation, astrocytes were labelled with bromodeoxyuridine (BrdU) using the FITC-BrdU Flow Kit (BD Biosciences) as described in the manufacturer's protocol. Briefly, BrdU was used at a final concentration of 10M, for the last 6 h. After washing with the BD perm/wash buffer, cells were fixed and permeabilized with the BD cytofix/cytoperm buffer. Astrocytes were then treated with DNase for 1 h at 37 °C and incubated with a fluoresceinated anti-BrdU antibody and with 4 μg/mL of Hoechst 33258, for 20 min at room temperature. Cells were examined under an Olympus fluorescence microscope (IX70, Olympus Optical Co, Tokyo, Japan) with the appropriate filter combination and photographed at a 40x magnification with a DP10 microscope digital camera (Olympus Optical Co). Four random fields were photographed for each replicate.

2.5. Statistical analysis

Statistical analysis was performed with the non-parametric Mann–Whitney test. Statistical significance was established at $p<0.05$.

3. Results

As we have already described elsewhere (Barca et al., 2003, 2007, 2008) serum starvation of astrocyte-enriched cultures resulted in a

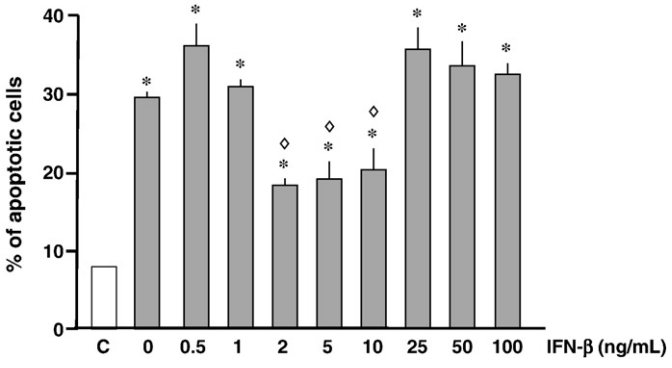

Fig. 1. Effect of IFN-β treatment on cell death induced by serum starvation. Primary astrocytes were serum-starved and treated with increasing amounts of IFN-β or vehicle. Apoptosis was determined after 24 h by fluorescent microscopic analysis of cell DNA staining patterns with Hoechst 33258. Cells were scored for apoptosis by their nuclear morphology and the higher intensity of blue fluorescence. Cells growing in the presence of 5% FBS were used as control (C). Each bar represents the mean ± S.E.M. of three independent experiments in triplicate. * = $p<0.05$ *vs.* control cells; ◊ = $p<0.05$ *vs.* serum-starved cells.