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The Effect of Interferon- β on Mouse Neural Progenitor Cell Survival and Differentiation

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Abstract

Interferon- β (IFN- β) is a mainstay therapy for relapse-remitting multiple sclerosis (MS). However, the direct effects of IFN- β on the central nervous system (CNS) are not well understood. To determine whether IFN- β has direct neuroprotective effects on CNS cells, we treated adult mouse neural progenitor cells (NPCs) *in vitro* with IFN- β and examined the effects on proliferation, apoptosis, and differentiation. We found that mouse NPCs express high levels of IFN α / β receptor (IFNAR). In response to IFN- β treatment, no effect was observed on differentiation or proliferation. However, IFN- β treated mouse NPCs demonstrated decreased apoptosis upon growth factor withdrawal. Pathway-specific polymerase chain reaction (PCR) arrays demonstrated that IFN- β treatment upregulated the STAT 1 and 2 signaling pathway, as well as GFRA2, NOD1, Caspases 1 and 12, and TNFSF10. These results suggest that IFN- β can directly affect NPC survival, possibly playing a neuroprotective role in the CNS by modulating neurotrophic factors.

Keywords

multiple sclerosis; mouse neural progenitor cells; interferon- β ; IFN α / β receptor; neuroprotection; STAT signaling

Introduction

Multiple sclerosis (MS) is a chronic autoimmune disease characterized by multifocal inflammation and demyelination in the central nervous system (CNS). Permanent neurological impairment is thought to be due to axonal loss or dysfunction resulting from chronic episodes of immune-mediated demyelination. Neurotrophic factors that regulate oligodendrocyte development may be deficient in MS, or inflammation may inhibit the repair and/or regeneration of the lesion [1]. Over time, the neurodegenerative process continues, with mounting damage to axons and oligodendrocytes resulting in cumulative disease progression and disability [2,3]. Because of the progressive neurodegenerative nature of MS, there is a need for developing treatments that exhibit direct neuroprotective effects.

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Current therapies function indirectly by modulating the immune system. Although these treatments are effective at inhibiting immune cell-driven inflammation and reducing the relapse rate, they are ineffective in controlling the predominantly neurodegenerative processes that occur later in the disease course [4].

Interferon β -1b (Betaseron) and interferon β -1a (Avonex or Rebif) were among the first drugs that obtained FDA approval for treating MS, and they remain first-line therapies for this disease. IFN- β is able to down-regulate the T-cell inflammatory response, and these immunomodulatory functions have been well described [5]. Treatment of MS with IFN- β has been shown to reduce clinical relapse and to decrease MRI lesion load [6,7]. Recently, it has been shown that IFN β -1a may reduce the development rate of brain atrophy in MS [8]. In addition, IFN- β partially reverses the loss of N-acetylaspartate (NAA) signal [9], a marker of axonal integrity. These results suggest that IFN- β exerts a neuroprotective role and slows the neurodegenerative process in MS. However, the precise mechanisms relating IFN- β to these neuroprotective effects are unknown.

Dysregulation of endogenous adult NPC survival is believed to contribute to progression of MS [10]. Very little is known about the factors that regulate the proliferation, survival, migration, and differentiation of these cells into oligodendrocytes, which may contribute to myelin repair and neuroprotection. We hypothesize that IFN- β could play a direct neuroprotective role by promoting NPC survival or differentiation into oligodendrocytes. To test this hypothesis, we treated adult mouse NPCs with IFN- β and consequently examined its effects on apoptosis, proliferation, and differentiation *in vitro*. Our results demonstrate that withdrawal of epidermal growth factor (EGF) and fibroblast growth factor (FGF) results in extensive death of mouse NPCs, which can be significantly reduced with IFN- β treatment. Pathway-specific PCR array showed that IFN- β treatment activated the STAT1 and 2 signal pathways, in addition to upregulating GFRA 1, NOD 1, and Caspases 1 and 12. These results suggest that IFN- β directly affects NPC apoptosis, which may serve a neuroprotective role in the CNS. Additionally, the JAK/STAT pathway in NPCs may be an important therapeutic target.

Methods

Reagents

Penicillin, streptomycin, B-27 Supplement, L-glutamine, epidermal growth factor (EGF), fibroblastic growth factor (FGF), BD Pharmingen FITC BrdU Flow Kit, streptavidin fluorescein isothiocyanate (Strep FITC), biotinylated dUTP, terminal deoxynucleotidyl transferase (Promega), reverse transcriptase (BioLabs), *Taq* polymerase Master Mix and Gene Expression Assay Primers (Applied Biosystems), SABiosciences PCR arrays.

Isolation of mouse fetal neural progenitor cells

Mouse NPCs were isolated from whole brains of individual post-natal day 4 C57/Bl6 mice using a Neurocult Enzymatic Dissociation Kit (Stemcell Technologies, Inc.). NPCs were isolated based on neurosphere formation and confirmed through staining with the NPC marker nestin.

Cell culture

Undifferentiated mouse NPCs and neurospheres were cultured in Nunclon delta-coated 75cm² filter flasks (Nunc) in mouse NPC complete medium: [1x Neurobasal-A (Invitrogen), 1x B27 supplement (Invitrogen), 20 ng/ml epidermal growth factor (BDBiosciences), 10 ng/ml basic fibroblast growth factor (BDBiosciences), 2 mM L-glutamine (Mediatech Inc.), 100 units/ml penicillin, and 100 µg/ml streptomycin (Mediatech Inc.)]. To differentiate NPCs, cells within four to five passages were cultured in poly-D-lysine and laminin coated plates, upon reaching 70–80% confluency (approximately 3 days), EGF and FGF were withdrawn and the cells were treated with IFN-β at 100pg/ml, 1ng/ml, 10ng/ml, and 100ng/ml in minimal media [1x Neurobasal-A (Invitrogen), 1x B27 supplement (Invitrogen), 2 mM L-glutamine (Mediatech Inc.), 100 units/ml penicillin, and 100 µg/ml streptomycin (Mediatech Inc.)] for 2–3 days.

Immunocytochemistry

Cells were plated on coated (poly-D and laminin) coverslips in 12-well plates. After the treatment period, cells/coverslips were fixed Zamboni's Fixative (4% paraformaldehyde; 15% picric acid). Cells were incubated overnight at 4°C in anti-mouse nestin (Novus Biologicals, 1:250) and IFNAR1 (Affinity BioReagents, 1:1000) primary antibodies. After rinsing twice in phosphate buffered saline, cells were incubated for 2 hours at room temperature in both anti-chicken Cy2 (1:250) and anti-rabbit Cy3 (1:500) secondary antibodies (both Jackson ImmunoResearch).

NPC BrdU proliferation assay

Cell proliferation in the presence of IFN-β was characterized by bromodeoxyuridine (BrdU) assay (BD Pharmingen). Upon reaching approximately 80% confluency, NPCs were pulsed overnight with 1 mM bromodeoxyuridine (a thymidine analog used during DNA synthesis). Cells were then lifted from their wells, washed in staining buffer, and then resuspended in Cytofix/Cytoperm buffer for 20 minutes on ice (according to manufacturer protocol).

TUNEL assay

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was performed to quantify cell death. Biotinylated dUTP (Roche) was incorporated into late stage apoptotic DNA using terminal deoxynucleotidyl transferase (Promega). Cells were stained with streptavidin fluorescein isothiocyanate (1:1000; Jackson ImmunoResearch), a biotin ligand. Cell nuclei were labeled with Hoechst dye (1:2000) and visualized with UV microscopy. Cell counts were conducted using Neurolucida Stereoinvestigator software by selecting fields of roughly 1000 Hoechst-positive cells at a magnification of 20X and counted by hand comparing total Hoechst signal (491nm) versus apoptotic nuclei positive for Strep-FITC (515–565nm).

RT-qPCR

Total RNA was isolated from both IFN-β treated and minimal media replicate control NPCs using the RNeasy mini kit (Quiagen). NPC RNA was quantified by NanodropTM, and synthesis of cDNA was completed using BioLabs Reverse Transcription Assay. NPC cell

fate analysis was completed using Applied Biosystems 7500 Fast Software real time-quantitative polymerase chain reaction (RT-qPCR). Amplification consisted of 40 cycles (95°C for 15 seconds and 60°C for 1 minute) with specific primer pairs for β -tubulinIII, doublecortin, GFAP, and GLAST, using *TaqMan* Master Mix (Applied Biosystems). Neuronal markers β -tubulinIII and doublecortin along with astrocyte markers GFAP and GLAST were analyzed. Cycle threshold amplifications for NPC cDNA were normalized against endogenous control markers β -actin and subsequently expressed as fold differences relative to the lineage marker and the treatment condition.

Western blot analysis

NPC protein samples were obtained by lysing cells with 0.5% NP-40 in buffer (Tris HCl, pH 7.4 2mM sodium orthovanadate, 10% glycerol, Roche protease inhibitor tablet). Proteins were fractionated on 4–10% SDS page gels and then transferred onto a PDVF membrane. The blot was stripped (100mM 2-mercaptoethanol, 2% SDS, 62.5mM Tris-HCl, pH 6.7) between primary antibody incubations. Blots were incubated with Horse-raddish peroxidase (HRP)-conjugated primary antibodies (against β -tubulinIII, GFAP, or β -actin) for 2hrs. Bands were detected using enhanced chemiluminescence and exposure to autoradiographic film.

Pathway-specific PCR array

Total RNA from both the minimal media control samples (n=4) and from the 1ng/mL IFN- β treated samples (n=4) was isolated and harvested using column separation (Quiagen). DNA digestion (Quiagen DNase) was performed on the columns for approximately 15 minutes prior to eluting RNA. RNA concentrations were quantified using Nanodrop and RNA integrity was assessed with using Agilent 2100 Bioanalyzer. Absence of genomic DNA contamination was confirmed by RT-qPCR. 200ng of RNA were reversed transcribed to cDNA using the SuperArray RT² First-Strand kit. cDNA samples were then run on PCR array plates (SABiosciences) using Applied Biosystems 7900HT. The following arrays were used: “Neurotrophins and Receptors” (PAMM-031), “Interferon and Receptor” (PAMM-064) and “Apoptosis” (PAMM-012).

Results

Mouse NPCs express IFN α / β receptor

IFN- β belongs to the type I IFN family and exerts its action after binding to the IFN α / β receptor (IFNAR) [11]. Gene-array analysis has shown IFNAR expression on mouse adult spinal cord-derived NPCs [12]. To confirm the presence of IFNAR on our NPCs, both surface receptor and mRNA expression were investigated. Expression of IFNAR in NPC minimal media cultures treated and untreated with ligand IFN- β were determined using RT-qPCR. C_t values from both the “Mouse Interferon and Receptor” SuperArray plate analysis and RT-qPCR indicated IFNAR was expressed in NPCs. C_t values from IFN- β treated and untreated NPCs were 24.41 and 23.72, respectively. NPCs were stained for both nestin and IFNAR, confirming surface expression with immunocytochemistry. Confocal microscopy demonstrates co-localization for both antibodies (Figure 1).

Effects of IFN- β on cell proliferation

We observed that IFN- β mildly enhanced the survival of NPCs in the absence of EGF and FGF, suggesting that IFN- β may function as a trophic factor to promote cell proliferation. To test this, IFN- β treatment was applied to NPCs deprived of EGF and FGF. Using BrdU assay for flow cytometry, the IFN- β treated NPCs showed no significant increase in proliferation as compared to the minimal media control (data not shown).

Effects of IFN- β on NPC apoptosis

To investigate whether the pro-survival effects we observed were due to a decrease in cell death, TUNEL assay was performed. As apoptotic cells are characterized by genomic DNA fragmentation, the 3'-hydroxyl termini of DNA can be fluorescently labeled and classified as TUNEL-positive cells. When compared to minimal media controls, IFN- β treated NPCs displayed a significant reduction in TUNEL positive cells (Figure 2a). The minimal media control displayed 35% of apoptotic cells after 1 day without EGF and FGF, whereas IFN- β concentrations of 100pg/ml, 1ng/ml, 10ng/ml and 100ng/ml treated NPCs showed 29%, 16%, 12% and 32% respectively (Figure 2b). This observation was the most prominent at the IFN- β concentration of 1ng/ml (ANOVA, $P < 0.05$; approximately 23% reduction from the control); however, 10ng/ml IFN- β treatment also showed a significant decrease in apoptosis as compared to the minimal media control.

Effects of IFN- β on differentiation

To determine the regulatory effects of IFN- β on the differentiation of NPCs, RT-qPCR results of IFN- β treated NPCs were compared to untreated controls. When normalized to the endogenous control β -actin and GAPDH, NPCs treated with either 1ng/mL or 100pg/mL IFN- β showed no difference in the expression of the neuronal lineage markers β -tubulinIII and doublecortin, and oligodendrocyte markers NG2 and PDGFR α , as compared to the minimal medium controls (Figure 3a and b). IFN- β did not affect expression levels of the astrocyte markers GLAST and GFAP. These results indicate that IFN- β has no effect on both neuronal and oligodendrocyte cell lineage differentiation. Lack of differentiation was confirmed using Western blot analysis, from which no changes in β -tubulinIII or GFAP protein levels were detected.

Gene expression arrays

"Neurotrophins and Receptors", "Interferon and Receptor", and "Apoptosis" PCR arrays were used to screen for genes regulated by IFN- β treatment. Of the >200 genes examined, 6 exhibited a statistically significant fold change in 1ng/ml IFN- β treated NPCs compared to minimal media controls. These were STAT1/2, NOD1, Caspases 1 and 12, TNFSF10, and GFRA2 (Table 1).

Discussion

IFN- β is a cytokine whose main function is to promote innate immunity during infections [13]. MS lesions are marked by an inflammatory response, resulting in the recruitment of activated T-cells to the CNS. One way in which IFN- β ameliorates MS symptomology is by modifying T-cells peripherally outside of the CNS. IFN- β has been shown to downregulate

the level of expression of MHC class II on antigen presenting cells, resulting in decreased activation of T-cells [14]. Thus, IFN- β is thought to act primarily through an indirect mechanism by modulating inflammatory infiltrates and preventing their recruitment to the CNS.

Treatment of MS with IFN- β has also been shown to reduce the progression of disability and hinder brain atrophy [8], suggesting that IFN- β also acts directly on the CNS. MS is a demyelinating disease and disease progression may be influenced by the dysregulation of NPC development. Promoting NPC survival and/or differentiation may be helpful in slowing disease progression. We tested the hypothesis that IFN- β acts directly on the CNS by promoting NPC survival and/or proliferation. Our results suggest that IFN- β is capable of providing a direct neuroprotective effect by promoting the survival of NPCs at low concentrations *in vitro* (1ng/ml and 10ng/ml), while demonstrating no influence on activating the oligodendrocyte differentiation pathway.

Promoting cell survival can be accomplished through two mechanisms: increasing proliferation to expand the population, or decreasing apoptosis to promote the survival of existing cells. IFN- β is not a mitogen and we did not expect to see treatment to have any effect on proliferation by acting as a growth factor. Both the BrdU Assay and PCR array analysis for various proliferation markers (FGF2, Myc, Stat4, Trp53) confirmed our hypothesis. Indeed, our experiments suggest that IFN- β decreases apoptosis in mouse NPCs. Figure 2 clearly demonstrates a decreased percentage of cells undergoing apoptosis upon growth factor withdrawal in populations treated with IFN- β at two different concentrations. The concentrations of IFN- β used span the physiological dose given to MS patients, suggesting that NPCs may be able to survive longer in response to IFN- β treatment. This finding demonstrates that IFN- β may have a direct affect the CNS in addition to the peripheral immunomodulatory effect.

The neurotrophin and apoptosis mRNA expression profiles for NPCs treated with IFN- β provide clues into how this mechanism may work. Significant fold increases in expression were seen for the proteins STAT 1 and 2, NOD 1, Caspase 1 and 12, TNFSF 10, and GFRA 2. Binding to an IFNAR activates the Jak/Stat signaling pathways (for recent reviews see [15–17]); upregulated expression of the Jak/Stat signaling proteins STAT 1 and STAT 2 confirm that IFN- β is directly binding and influencing NPCs. Upon activation, STAT 1 and STAT 2 are phosphorylated and dimerize, resulting in the formation of a transcription complex than travels to the nucleus of the cell [18]. Here it binds to an interferon stimulating response element (ISRE) [19], leading to the transcription of various anti-apoptotic genes, including those that induce the anti-apoptotic NF- κ B pathway [20]. Additionally, STAT 1 has been shown to activate Caspase 1, a protein participating in membrane repair and cell survival via unknown mechanisms [21].

Other mRNA expression patterns also converge on the NF- κ B pathway. NOD 1, a protein found in the cytoplasm and plasma membrane of cells [22], participates in the adaptive immune response [23]. Upon recognizing a foreign molecule, NOD 1 forms a transcription complex, travels to the nucleus, and activates transcription of genes involved with the NF- κ B pathway [24]. The NOD 1 transcription complex can also bind to the ISRE [25]. TNFSF

10 (also called TRAIL) belongs to the TNF superfamily and also activates the NF- κ B pathway [26]. Additionally in experimental autoimmune encephalitis (EAE), the animal model of multiple sclerosis, inhibition of the TRAIL receptor exacerbates the condition. TRAIL has been shown to inhibit functions of autoimmune T-cells, which would decrease the number of active T-cells [27].

Lastly, GFRA-2 is a receptor for glial cell line-derived neurotrophic factor (GDNF). In other models of brain injury, GDNF has been shown to have neuroprotective effects [28, 29]. Multiple studies have shown that GDNF diminishes axotomy injury in spinal cord motor neurons [30–32], and has also been shown to have an anti-apoptotic effect in dopamine neurons [33–36]. Unfortunately, GDNF cannot pass the blood-brain barrier so its expression can only be upregulated by endogenous mechanisms. Interestingly, vitamin D is one such inducer [28]. Vitamin D deficiency has been shown to increase the progression of MS [37], possibly due to a lack of GDNF. Neurotoxically injured dopamine neurons can be “rescued” with large doses of GDNF [38, 39], so the upregulation of this receptor may be helping NPCs to survive longer.

Taken together, our results describe a novel mechanism in which IFN- β directly acts on the CNS. IFN- β binds to IFNAR on NPCs resulting in an increased survival by decreasing apoptosis. This neuroprotective role may explain the decreased rate of brain atrophy and the slowed rate of disease progression in patients with MS. Our data suggest multiple pathways work together to help directly decrease apoptosis as well as to provide an adaptive immune response, shedding light on the molecular mechanisms involved. Further studies investigating these pathways may be helpful for developing novel neuroprotective therapies for MS.

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Fig. 1. IFN α /b receptor (IFNAR) is expressed in undifferentiated neural stem cells
Undifferentiated, fixed NPCs were immunostained with both Nestin (a neural stem cell marker—*red*) and IFNAR (*green*). (For interpretation of color mentioned in this figure legend the reader is referred to the web version of the article.).

Fig. 2.

IFN treatment reduces Murine Neural Progenitor Cell apoptosis. (A) Fixed MNPCs were stained with Hoechst (blue) to show nuclei and FITC (green) to indicate TUNEL positive cells. Cells were fixed on three slides/treatment, then counted using the Neurolucida Stereoinvestigator software. Four counts of FITC positive cells from fields of 800–1500 Hoechst-positive cells were taken for each condition. The percentage of TUNEL positive cells are shown in (B). IFN concentrations (1 and 10 ng/mL) showed statistically significant neural protection when compared to Minimal Media (* $p < 0.05$). (For interpretation of color mentioned in this figure legend the reader is referred to the web version of the article.).

Fig. 3.

IFN-beta does not influence NPC Differentiation. NPCs were cultured to near confluency before growth factor withdrawal and addition of 1 ng/mL IFN (A) or 100 pg/mL (B). After 72 h, RNA was extracted and prepared for quantitative PCR analysis using Taqman primers and probes. Relative expression is calculated using $2^{-\Delta\Delta C_t}$ equation (β -actin served as endogenous control) and calibrated to minimal media control equaling 1. Shown are results of four separate cultures ($n = 4$). No lineage marker reached statistical significance using ANOVA (C) Parallel NPC cultures were incubated for 48 h before protein extraction and Western blot analysis using HRP-conjugated secondaries and ECL visualization; the blots were stripped between each reprobing step. Shown is a representative blot of three independent cultures for each group. The uneven background staining for the GFAP analysis was due to overexposure to the secondary antibody. GFAP and GLAST = astrocyte markers, Doublecortin and B-III Tubulin = neuronal markers, Olig2 = oligodendrocyte marker.

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Table 1

The effect of Interferon-β treatment (1 ng/ml) on gene expression

RNA from IFN-β treated (1ng/ml) cells were analyzed on “Mouse Neurotrophins and Receptors and “Mouse Apoptosis” superarray plates. NOD 1, Casp1, Casp12, TNFSF 10, STAT 1, STAT 2, and GFRA 2 show an increased fold regulation of statistical significance (p<.005) when treated with IFN-β as compared to the minimal media controls.

Gene	Abbreviation	Fold Regulation	p-value
Nucleotide-binding oligomerization domain containing 1	NOD 1	5.08	0.000022
Caspase 1	Casp1	5.64	0.000514
Caspase 12	Casp12	5.96	0.000806
Tumor necrosis factor (ligand) superfamily, member 10	TNFSF 10	42.51	0.000002
Signal transducers and activators of transcription 1	STAT 1	42.84	0
Signal transducers and activators of transcription 2	STAT 2	37.05	0
GDNF family receptor alpha 2	GFRA 2	5.11	0.000191