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Interferon-beta treatment normalises the inhibitory effect of serum from multiple sclerosis patients on oligodendrocyte progenitor proliferation

Alexandra Kotsiari[a], Elke Verena Voss[a], Refik Pul[a], Thomas Skripuletz[a], Daniela Ragancokova[a,b], Corinna Trebst[a], Martin Stangel[a,b,*]

[a] Department of Neurology, Hannover Medical School, Carl-Neuberg-Str. 1, 30625 Hannover, Germany
[b] Center for Systems Neuroscience, Hannover, Germany

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ABSTRACT

Interferon-beta (IFN-β) is an established therapy for relapsing-remitting multiple sclerosis (MS). However, the mode of action and the effect on oligodendrocytes are not yet clear. In this study, we examined the influence of an IFN-β therapy on the proliferation and differentiation of primary oligodendrocyte precursor cells (OPC) in mixed glial cultures. Mixed glial cultures were incubated for 5 days in medium supplemented with 10% of sera from healthy controls, untreated MS patients and IFN-β treated MS patients. Proliferation and differentiation of OPC were determined by immunocytochemistry. Proliferation of OPC was significantly inhibited by sera from untreated MS patients compared to healthy controls, while this effect was almost completely reversed by serum from IFN-β treated MS patients. No effect on OPC differentiation was observed. A prospective and longitudinal analysis of a second cohort of MS patients treated with IFN-β showed that the reversal of inhibition of OPC proliferation was evident after 12 months of treatment but not during the first 6 months. Thus, our results suggest that IFN-β treatment has the capacity to revert the inhibitory effect of serum from MS patients on OPC proliferation. It is currently not clear what this means for regenerative processes.

Multiple sclerosis (MS) is a demyelinating disease of the central nervous system (CNS). The exact cause of MS remains unknown, but an interplay between environmental factors and susceptibility genes that initiate an autoimmunological process is suggested [7,26]. Interferon-beta (IFN-β) treatment is licensed for relapsing-remitting MS [10,11]. Its mechanism of action is thought to include downregulation of the MHC class II expression on dendritic cells, suppression of proinflammatory cytokine production, reduction of proliferation of T cells, restoration of T suppressor functions, limitation of immune cell trafficking and matrix metalloproteinase production, and promotion of integrity of endothelial cell barrier between the blood and the CNS [15,28]. Further mechanisms are the induction of the B cell survival factor BAFF and Th 1 associated marker [12,27]. However, the exact mode of action is not completely understood and little is known about direct or indirect effects of IFN-β on glial cells in the CNS. IFN-β is also suggested to induce neurotrophic molecules such as nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) that potentially could support neural and glial cell survival [4,5,14]. Cytokines such as interleukin-6 (IL-6) are known to promote oligodendrocyte survival and are upregulated during IFN-β treatment in serum of MS patients [18]. Earlier studies described an inhibition of glial proliferation in vitro by serum from MS patients [8,23]. Oligodendrocyte precursor cells (OPC) are the source of CNS remyelination that is known to occur in MS lesions, but is often incomplete [1,19]. Changes in serum neurotrophic factors and cytokine levels may represent an indirect function of IFN-β on remyelination by influencing OPC functions. The aim of our present study was to investigate effects of IFN-β treatment on proliferation and differentiation of primary rat OPC in mixed glial cultures.

20 untreated and 20 IFN-β treated patients with relapsing-remitting MS according to McDonald criteria [17,20] were included. All treated patients received interferon-beta 1b (Betaferon®, Bayer Schering Pharma, Germany). Sera from a second cohort of 15 untreated MS patients that were subsequently started on IFN-β treatment were collected longitudinally before, 6 and ≥12 months after treatment initiation. None of the untreated patients had received an immunomodulatory or immunosuppressive treatment within 6 months prior to inclusion. None of the venipunctures were performed within 1 month after a relapse and/or treatment with steroids. The clinical disease course was monitored by recording clinical exacerbations and measurement of clinical symptoms

* Corresponding author at: Department of Neurology, Hannover Medical School, Carl-Neuberg-Str. 1, 30625 Hannover, Germany. Tel.: +49 511 5326677; fax: +49 511 5323115.
E-mail address: Stangel.Martin@MH-Hannover.de (M. Stangel).

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Table 1
Biometric data of patients included.

	n	m/f	Age in years	Duration of MS in years	Relapse rate		Duration of IFN-β treatment in months
					Before treatment	Under treatment	
(A) Biometric data of first cohort of patients (cross-sectional study)							
Healthy donors	20	9/11	36.2 ± 10.8				
Untreated MS patients	20	5/15	36.2 ± 13.1	3.1 ± 4.4	1.3 ± 1.1		
Treated MS patients	20	8/12	37.6 ± 8.6	5.2 ± 5.2	1.5 ± 0.9	0.9 ± 1.2	31.4 ± 29.0
(B) Biometric data of second cohort of patients (longitudinal study)							
MS patients	15	5/10	35.6 ± 10.6	6.0 ± 6.2	1.5 ± 1.0	0.7 ± 1.0	16.2 ± 5.0

m = male, f = female.
Mean values ± SEM are shown.

on Kurtzke's Expanded Disability Status Scale (EDSS) [13]. Samples from 20 healthy individuals served as controls (Table 1A and B). Blood samples were taken on the day after IFN-β administration approximately 14–18 h after the last injection. The study was reviewed and approved by the Ethics Committee of the Hannover Medical School. Informed consent was obtained from each participant.

Blood samples were collected in serum separator tubes, centrifuged at 1400 × *g* for 10 min at 4 °C and the supernatant was separated into aliquots and stored immediately at −80 °C until use.

Primary cultures of OPC were prepared from newborn Sprague–Dawley rat cerebra as described [16,24]. Briefly, brains were dissociated using mechanical shearing before enzymatic digestion with trypsin (Biochrom, Berlin, Germany) and DNase (Roche, Mannheim, Germany). Cells were homogenised, plated on poly-L-lysine (Sigma–Aldrich, Steinheim, Germany) coated culture flasks (1–2 brains per T75 flask, Sarstedt, Germany) and cultured in DMEM (Gibco, Karlsruhe, Germany) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Biochrom, Berlin, Germany), 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma–Aldrich).

On day 7 of the mixed glial cultures, microglia were harvested by shaking on an orbital shaker-incubator (Edmund Bühler KS-15 control and TH15, Johanna Otto GmbH, Hechingen, Germany) at 180 rpm for 2 h. Cells were centrifuged at 1000 rpm for 5 min, the pellet was resuspended, and cells were plated at a density of approximately 5×10^2 cells per poly-L-lysine coated glass coverslip.

Loosely attached primary OPC were detached by shaking the mixed glial cell cultures on an orbital shaker-incubator at 150 rpm for 16–18 h at 37 °C. Contaminating microglia were removed by adherence to untreated tissue culture plastic and the enriched OPC in the supernatant were plated on poly-L-lysine coated glass coverslips at a density of 2×10^4 cells. Medium conditioned by the neuroblastoma cell line B104 (B104 CM) was used as a positive control for proliferation [25]. For differentiation assays the cells were cultured in N2B3 medium [DMEM supplemented with 10 ng/μl biotin, 74 ng/μl progesterone, 16 μg/μl putrescine, 400 ng/ml T3, 400 ng/ml T4 (all from Sigma–Aldrich) and 1% ITS (BD Biosciences, Heidelberg, Germany)] to enhance differentiation of OPC to mature oligodendrocytes.

Astrocytes were prepared after most microglia and oligodendrocytes were removed by shaking the cultures for a second time at 150 rpm for 16–20 h. One day later, antimitotic arabinosylcytosine (Ara-C, 100 μM, Sigma–Aldrich) was added to the culture medium to avoid the growth of newly arising oligodendrocytes and microglia. Medium containing Ara-C was then removed after 72 h, cells were washed with PBS and allowed to recover for a few hours. Primary astrocytes were harvested on day 14 by 0.25% trypsin/0.05% EDTA (PAA, Coelbe, Germany) and seeded at a density of 1×10^4 cells on poly-L-lysine coated glass coverslips.

To measure proliferation of OPC, mixed glial cells were plated on poly-L-lysine coated glass coverslips at a density of 5×10^4 cells. Medium was changed the next day and on day 4 and 7 with fresh medium supplemented with 10% of control sera from healthy donors or MS patients. Controls were incubated with DMEM (10% FBS/1% penicillin/streptomycin) and B104 medium, respectively. A concentration of 10 μM of 5-bromo-2′-deoxyuridine (BrdU, Roche) was added after 8 days to the mixed glial cell cultures. After 24 h incubation, OPC were stained by applying the A2B5 antibody (1:2, hybridoma supernatant, clone 105, European Collection of Cell Cultures) to living cells for 30 min at 37 °C. Cells were fixed with 4% paraformaldehyde (PFA) and permeabilized with methanol for 20 min at −20 °C. The DNA was denatured with 2 N HCl for 60 min at 37 °C and the acid neutralized by washing the cells twice with 0.1 M borate buffer, pH 8.5. After 1 h incubation with anti-BrdU antibody (1:100) at RT the cells were labelled with the immunofluorescent secondary antibodies Cy3 rabbit anti-mouse IgM (Dianova, Hamburg, Germany) for A2B5 and Cy2 rabbit anti-mouse IgG (Dianova) for BrdU. Cells of 7–11 different preparations were used for this assay. Proliferation was quantified in a blinded manner by counting the total number of A2B5 positive and A2B5/BrdU double positive OPC in 5 visual fields per coverslip using a magnification of ×40 (Leica DMLB, Germany). The proliferation index shows the proportion of proliferating OPC from the total population of A2B5 positive OPC. Data were normalized to the respective daily control.

Differentiation of OPC was determined by double staining for the OPC marker A2B5 and the oligodendrocyte marker GalC. 1×10^4 astrocytes and 5×10^2 microglia were plated on poly-L-lysin coated glass coverslips and were allowed to attach overnight. On the next day 2×10^4 oligodendrocytes were seeded on the astrocyte–microglia layer and were cultured in DMEM/10% FBS/1% antibiotics. Medium was replaced on the next day and on day 3 after plating with N2B3 medium supplemented with 10% of control or MS serum. For controls, cells were incubated with DMEM and N2B3 supplemented with 10% FBS. On day 6 cells were double stained for A2B5 and GalC (1:2, hybridoma supernatant, clone IC-07, European Collection of Cell Cultures). Both antibodies were applied for 30 min at 37 °C. Cells were fixed with 4% PFA, washed with PBS, and incubated with secondary antibodies Cy3 rabbit anti-mouse IgM for A2B5 and Cy2 rabbit anti-mouse IgG for GalC. Cells of 3–5 different preparations were stained and counted. The number of A2B5 and GalC positive cells was counted in 5 visual fields per coverslip using a magnification of ×40. All enumerations were performed in a blinded manner. The differentiation index was calculated by the ratio of differentiated oligodendrocytes to OPC. Results were normalized to the ratio of untreated control cultures.

Serum neopterin was measured in the samples from untreated MS patients and patients treated with IFN-β 6 and ≥12 months, respectively, using a sensitive ELISA (RE59321, IBL International GmbH, Hamburg, Germany). The measurements were performed according to the manufacturers' instructions.

Data are expressed as mean ± standard deviation (SD). For each assay, normality of data distribution was tested with the Kolmogorov–Smirnov test. Normality of paired samples in the pro-