Interferon beta-1a induces expression of brain-derived neurotrophic factor in human T lymphocytes *in vitro* and not *in vivo*

Zarlascht Karmand¹, Hans-Peter Hartung¹ & Oliver Neuhaus^{*,1,2}
¹Heinrich Heine Universität Düsseldorf, Department of Neurology, 40225 Düsseldorf, Germany

²SRH Krankenhaus Sigmaringen, Department of Neurology, 72488 Sigmaringen, Germany

*Author for correspondence: Tel.: +49 75711002483; Fax: +49 75711002836; oliver.neuhaus@srh.de

Aim: To detect IFN β -1a-induced expression of brain-derived neurotrophic factor (BDNF) to undermine the hypothesis of IFN β -1a-associated neuroprotection in multiple sclerosis (MS). **Methods:** The influence of IFN β -1a on *in vitro* activated peripheral blood lymphocytes from healthy donors was tested. Proliferation analyses were made to detect T-cell growth. BDNF expression was measured by standard ELISA. To assess the influence of IFN β -1a on BDNF expression *in vivo*, BDNF serum levels of MS patients treated with IFN β -1a were compared with those of untreated patients. **Results:** IFN β -1a inhibited T-cell proliferation dose dependently. It induced BDNF expression at middle concentrations. MS patients treated with IFN β -1a exhibited significantly lower BDNF serum levels than untreated patients. **Conclusion:** IFN β -1a may promote neuroprotection by inducing BDNF expression, but its importance *in vivo* remains open.

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Multiple sclerosis (MS) is the most disabling neurological disease of the younger adulthood. Its pathogenesis is still not fully understood. Apart from a complex autoimmune cellular and humoral inflammatory process, demyelination and axonal damage enter the focus of scientific interest. Neurodegenerative axonal damage is putatively responsible for primary or secondary chronic progression of disablement. Whereas several immunomodulatory agents that have been approved for treatment of relapsing forms of MS achieve good clinical responses in this disease course ('no or minimal evidence of disease activity' has become a realistic treatment goal in relapsing MS), the treatment effects of the chronic progressive forms of MS are still disappointing. Thus, the search of agents with neuroprotective potential forms a major goal in MS science.

Apart from the search for new neuroprotectants or the assessment of the few agents already used in other neurodegenerative diseases (such as riluzole that has been approved for treatment of amyotrophic lateral sclerosis), the examination of current already approved MS drugs in terms of their neuroprotective potential is reasonable.

IFN β -1a (Rebif®) has been approved for treatment of relapsing forms of MS. Its efficacy in reducing the relapse rate had been proven in class I trials. Several immunomodulatory properties have been described. For example, it modulates the IFN γ -induced expression of MHC class II molecules on antigen-presenting cells [1,2]; it reverses the increased expression of co-stimulatory CD80 molecules on B lymphocytes [3]; it inhibits T-cell proliferation [4] and induces anti-apoptotic proteins [5]; furthermore, it inhibits secretion of pro-inflammatory cytokines such as IFN- γ and TNF- α [4] and stimulates anti-inflammatory cytokines such as IL-4 and IL-10 [6].

Neurotrophic factors (NF) form a protein family essential for the development of the nervous system. They regulate neuronal and glial functions such as axonal and dendritic growth and synaptic connectivity – functions essential for neuronal plasticity – and induce expression of neurotransmitters [7,8]. Interestingly, NF influence immune functions such as migration, activation, differentiation and antigen presentation [9,10].

Brain-derived neurotrophic factor (BDNF) is one of the key NF. It promotes neuronal survival and growth and differentiation of neurons [11]. Interestingly, it is expressed by immune cells such as T and B lymphocytes,

Table 1. Characteristics of multiple sclerosis patients.

Characteristic	Untreated patients (n = 6)	IFN β -1a-treated patients (n = 6)	p-value
Sex (female:male)	3:3	4:2	p = 0.60
Mean age (years)	38.5 $\pm$ 13.9	41.0 $\pm$ 8.9	p = 0.72
Mean duration of disease (years)	5.7 $\pm$ 3.8	6.0 $\pm$ 4.9	p = 0.92
Mean EDSS	1.9 $\pm$ 0.7	2.2 $\pm$ 2.1	p = 0.79
Mean treatment duration (years)	n.a.	2.5 $\pm$ 2.5	n.a.

EDSS: Expanded disability status scale [21]; n.a.: Not applicable.

monocytes and microglia, which make it an interesting target in MS [12]. Upon antigen stimulation, the expression of BDNF by myelin-specific CD4⁺ T-cell lines is increased [12,13]. In addition, BDNF promotes proliferation of oligodendrocytes and myelination [14]. In MS lesions, BDNF expressing inflammatory cells were detected; these findings led to the concept of 'neuroprotective immunity', indicating that inflammatory processes in the central nervous system may promote not only harmful but also beneficial effects [12,15].

The putative neuroprotective effects of agents against MS have been assessed, especially their potential ability to induce BDNF expression. Glatiramer acetate (GA) normalizes BDNF serum levels in MS patients who – if untreated – exhibit reduced levels [16]. Ziemssen *et al.* showed that GA-reactive peripheral blood lymphocytes (PBL) produce BDNF upon GA stimulation [13].

The published data of IFN β -1a effects on the BDNF expression are heterogeneous. Some authors do not see any influence assessing BDNF production by PBL *in vitro* [17,18] whereas others described an IFN β -1a-dependent increased expression of BDNF [19]. Lalive *et al.* observed increased cellular BDNF levels in PBL in MS patients treated with IFN β as compared with untreated patients whereas serum BDNF levels did not differ [20].

In our study, we aimed to reconsider the effects of IFN β -1a on the expression of BDNF in human T lymphocytes *in vitro* and *in vivo* by modified methods. We assessed BDNF production by *in vitro* activated PBL obtained from healthy donors in the presence or absence of IFN β -1a; the *in vivo* effects were studied by measuring BDNF serum levels in untreated MS patients in comparison to BDNF serum levels in MS patients treated with IFN β -1a.

Methods

Patients & controls

A total of 12 patients with relapsing-remitting MS – six untreated patients and six patients treated with IFN β -1a – donated serum with informed consent. The study protocol was formally approved by the Ethics Committee of the Medical Faculty of the Heinrich Heine Universität Düsseldorf. There were no significant differences between the two groups (Table 1). Serum was frozen at -21°C. For the *in vitro* tests, PBL were prepared from blood obtained from healthy donors.

Lymphocyte culture

PBL were isolated by standard density gradient using Lymphoprep[®] (Axis Shield, Oslo, Norway). Cells were cultured in RPMI 1640 medium with 2 mM L-glutamin and 5 % heat-inactivated fetal calf serum (all reagents from Gibco, Paisley, UK), and 100 U/ml penicillin, 100 µg/ml streptomycin (both from Gibco) and 200 µg/ml ciprofloxacin (Bayer, Leverkusen, Germany) were added in order to prevent bacterial contamination. All cultures were held at 37°C in a 5% CO₂ atmosphere (Heraeus Instruments, Hanau, Germany).

Freshly isolated PBL (1.5 $\times$ 10⁴/100 µl/well) were activated with concanavalin A (ConA, 5 µg/ml; Sigma, Steinheim, Germany) or kept inactivated. ConA had been chosen as mitogen as it provided the highest proliferative potential as compared with phytohemagglutinine or phorbol-12-myristate-13-acetate (both from Calbiochem, Darmstadt, Germany; data not shown). IFN β -1a (Merck, Darmstadt, Germany) was added in different concentrations (0–100,000 IU/ml). After 4 days, cell pellets and supernatants were collected separately and frozen at -21°C.

T-cell proliferation assay

T-cell proliferation was assessed by standard [methyl-³H] thymidine intake assay (0.5 µCi per well). A total of 16–18 h prior to harvesting, [methyl-³H] thymidine (Amersham Biosciences, Buckinghamshire, UK) was added to the

Figure 1. Concentration-dependent inhibition of peripheral blood proliferation *in vitro* by IFN β -1a. PBL remained non-activated (column 1) or were activated by Concanavalin A (ConA) 5 μ g/ml (columns 2–10). IFN β -1a was added in different concentrations. Proliferation was measured by scintillation detection and is given as mean cpm + standard deviation. Results are from one representative of six experiments.

* $p < 0.05$. Non-activated PBL (column 1) versus ConA-activated PBL (column 2).

** $p < 0.05$. ConA-activated PBL without IFN β -1a (column 2) versus IFN β -1a-treated ConA-activated PBL (columns 3–10).

cpm: Counts per min; PBL: Peripheral blood lymphocyte.

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cell cultures. After harvesting onto a nitrocellulose filter, the intake was measured using a beta counter (Packard BioScience, CT, USA). Results are given as counts per min (cpm).

Quantification of BDNF levels

BDNF levels were measured in culture supernatants (nondiluted) or serum (diluted in phosphate buffered saline at 1:10 or 1:100, respectively) by standard ELISA (Thermo Fisher Scientific, Vantaa, Finland). A commercially available BDNF ELISA kit was used (R&D Systems, Abingdon, UK).

Statistical analysis. Student's t-test was performed for statistical analysis. A p -value of <0.05 was accepted to be significant.

Results

IFN β -1a inhibits PBL proliferation dose dependently

PBL were kept inactivated or were activated by ConA. IFN β -1a was added in different concentrations. Proliferation was inhibited dose dependently (Figure 1).

IFN β -1a induces BDNF expression in PBL at middle doses

BDNF levels were measured in supernatants of cell cultures (Figure 2). Interestingly, only at middle doses of IFN β -1a between 1000 and 5000 IU/ml, the BDNF levels increased, whereas at lower and higher doses the levels remained low.

BDNF levels are lower in IFN β -1a-treated MS patients than in untreated MS patients

BDNF levels were measured by ELISA in 12 patients with relapsing-remitting MS (Table 1). Six of these had been treated with IFN β -1a (mean treatment duration 2.5 years) and six had been untreated at the time of blood sampling. Four of the treated patients had received 22 μ g IFN β -1a sc. three-times per week and the other two had received 44 μ g IFN β -1a sc. three-times per week.

Figure 2. IFN β -1a-induced BDNF expression by activated PBL *in vitro*. PBL were activated by Concanavalin A 5 μ g/ml. IFN β -1a was added in different concentrations. BDNF levels were measured by ELISA. Note the highest BDNF levels at middle doses (columns 3 and 4). Results are from one representative of three experiments. *Significant reduction ($p < 0.05$) and **significant increase ($p < 0.05$) of BDNF levels upon IFN β -1a treatment (column 1 vs columns 2–6).

BDNF: Brain-derived neurotrophic factor; PBL: Peripheral blood lymphocyte.
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Figure 3. BDNF serum levels in untreated and IFN β -1a-treated multiple sclerosis patients. BDNF serum levels of six untreated multiple sclerosis patients and six IFN β -1a-treated MS patients were measured by ELISA. Open circles, individual data; black bars and error bars, mean values $\pm$ standard deviation.

BDNF: Brain-derived neurotrophic factor.

Adapted with permission from the medical thesis of Z Karmand [22].

Serum levels of BDNF were significantly lower in IFN β -1a-treated patients as compared with untreated patients ($p < 0.001$) (Figure 3). The mean serum level of BDNF in IFN β -1a-treated patients was 16.6 ng/ml as compared with 21.6 ng/ml in untreated patients.

Discussion

One of the major therapeutic principles in MS is immunomodulation by IFN β . IFN β -1a has been proven to reduce relapse rate, progression of disability and MRI inflammation [23]. For example, in one more recent controlled trial with IFN β -1a as control, it reduced the relapse rate by 39%; 59% of IFN β -1a-treated patients remained relapse-free over 2 years [24].

The exact mechanism of action of IFN β -1a in MS is still not understood. Suggestions are the induction of anti-inflammatory cytokines such as IL-4 and IL-10 [6], the inhibition of secretion of pro-inflammatory cytokines such as IFN- γ and TNF- α [4] and the antiproliferative effects on T-cells as well as the inhibition of their migration into the CNS by stabilization of the blood–brain barrier [25,26].

In this study we could confirm the antiproliferative effect of IFN β -1a on human PBL *in vitro* in a dose-dependent manner [4].

Apart from the various immunomodulatory effects of IFN β -1a in MS, an additional neuroprotective mode of action is a matter of intensive discussion. Neurotrophic factors, especially BDNF, have been assessed. BDNF promotes neuronal survival and growth of dendrites [27,28] and induces axonal regeneration [29]. In MS lesions, immunoreactivity against BDNF has been shown in T-cells, macrophages, reactive astrocytes and neurons [15].

The influence of IFN β -1a on BDNF serum levels has been reported inconspicuously. In one study, the BDNF levels were higher in IFN β -1a-treated patients as compared with untreated controls [17]. In contrast, two other groups did not find IFN β -1a-induced BDNF expression *in vitro* and *in vivo* [18,30]. Reduced BDNF serum levels in IFN β -1a-treated patients have not yet been described.

Here we investigated the influence of IFN β -1a on the expression of BDNF *in vitro* and *in vivo*. IFN β -1a induced the expression of BDNF in cultured PBL *in vitro* predominantly at middle concentrations between 1000 and 5000 IU/ml, in other words, not at lower or at higher concentrations. We postulate that for a neuroprotective effect of IFN β -1a via BDNF, middle concentrations would be necessary as lower doses are putatively too weak to induce BDNF expression whereas in higher doses the anti-proliferative effects counterpart the induction of BDNF expression.

To further interpret these data, it is useful to have a look at the pharmacokinetics of IFN β -1a after subcutaneous injection: 22 μ g of IFN β -1a represent 6×10^6 IU and 44 μ g represent 1.2×10^7 IU. Subcutaneous injection provides a depot in the fatty tissue. Pharmacokinetic studies of IFN β -1a (44 μ g three-times a week) have shown serum steady-state concentrations between 188 pg/ml (51 IU; trough serum concentration) and 266 pg/ml (73 IU; maximum serum concentration) [31]. We interpret that our observation of BDNF induction by IFN β -1a at doses between 1000 and 5000 IU/ml *in vitro* is improbable to reflect conditions *in vivo*.

Our results of the BDNF serum levels were surprising. In contrast to the observation of Azoulay *et al.*, who described higher BDNF levels in IFN β -1a-treated patients [16], we found lower BDNF serum levels in IFN β -1a-treated MS patients as compared with untreated patients. This discrepancy could be explained by putative heterogeneity of blood donors (BDNF serum levels may be influenced by several conditions such as the time interval between IFN β -1a injection and blood sampling), by different assessment kits or by chance. As our result was highly significant ($p < 0.001$), the last option seems unlikely. However, limitation of our cohort is its relatively small sample size.

Serum levels of BDNF in MS patients can be measured quite easily by ELISA. An open question remains if these serum levels correlate to BDNF levels in functionally more relevant compartments such as cerebrospinal fluid or lymphatic tissue [32]. Moreover, the link between BDNF serum levels and real neuroprotective effects has not yet been proven.

Taken together, our findings partially did confirm and partially did not confirm published data. The putative neuroprotective effect of IFN β -1a was seen at middle concentrations *in vitro* but not *in vivo*. Here, *in vivo*, in contrast, an inhibitory effect of IFN β -1a on the serum expression of BDNF was found.

Other neurotrophins or other neuroprotective pathways should be analyzed in order to shed more light on the mechanisms of action of IFN β -1a.

The effects of other immunomodulatory agents on BDNF expression are currently under investigation. GA increased BDNF levels *in vivo* and *in vitro* [13,16], fingolimod promoted BDNF expression in an experimental autoimmune encephalitis model [33] and natalizumab was shown to increase BDNF serum levels *in vivo* [34].

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Ethical conduct of research

The study protocol was formally approved by the Ethics Committee of the Medical Faculty of the Heinrich Heine Universität Düsseldorf. The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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Summary points

- IFN β -1a provides immunomodulatory properties.
- Published data on neuroprotective properties of IFN β -1a are heterogeneous.
- *In vitro*, IFN β -1a inhibits T-cell proliferation dose dependently.
- *In vitro*, IFN β -1a induces expression of brain-derived neurotrophic factor (BDNF) at doses between 1000 and 5000 IU/ml.
- *In vivo*, IFN β -1a reduces serum expression of BDNF.
- This study does not confirm a neuroprotective effect of IFN β -1a via the BDNF pathway.

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