Interferon Beta Promotes Nerve Growth Factor Secretion Early in the Course of Multiple Sclerosis

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Background: Interferon beta therapy has been shown to reduce the rate of clinical relapse and the frequency of magnetic resonance imaging–defined T2-weighted lesions in patients with multiple sclerosis (MS). When given early, interferon beta also reduces the rate of development of brain atrophy and improves axonal integrity. Nerve growth factor (NGF) can retard the severity and course of experimental allergic encephalomyelitis.

Objective: To determine whether interferon beta effects on patients with MS could be related to modulation of neurotrophin production within the central nervous system.

Design: We studied neurotrophin production by human glial and brain endothelial cells in response to coculture with MS patient–derived lymphocytes, and correlated levels of NGF secretion with clinical and magnetic resonance imaging–defined markers of disease.

Results: We demonstrate that production of NGF by human brain microvascular endothelial cells is triggered by interaction with T lymphocytes derived from MS patients. No such response was observed using human adult microglia or human fetal astrocytes. Nerve growth factor production by endothelial cells was potentiated by pretreating lymphocytes with interferon beta in vitro, and by using lymphocytes derived from MS patients treated with interferon beta in vivo. By using this assay, we show that levels of NGF induced by lymphocytes from MS patients inversely correlate with magnetic resonance imaging measures of brain atrophy and axonal injury.

Conclusion: These findings suggest that interferon beta–mediated production of NGF at the level of the blood-brain barrier, whether acting as an immunomodulator or directly on neural cells, is another potential mechanism contributing to the magnetic resonance imaging–defined effect of interferon beta on brain atrophy when given early in the course of MS.

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Multiple sclerosis (MS) is a chronic disease of the central nervous system (CNS) clinically characterized by an initial relapsing-remitting course that evolves into a secondary progressive phase in more than 50% of patients. Multiple sclerosis is considered to be initiated by autoreactive T lymphocytes that cross the blood-brain barrier and lead to the autoimmune destruction of myelin and axons.1,2 Over time, there is continued tissue loss, resulting in global brain atrophy.3,6 These latter processes have been referred to as the neurodegenerative component of MS.

Interferon beta is a cytokine administered as a therapy for relapsing MS. Interferon beta therapy has been shown to significantly reduce the frequency and severity of clinical relapses and to decrease the number of new and enhancing lesions seen on magnetic resonance imaging (MRI). Several large clinical trials have suggested that interferon beta administered late in the course of MS has marginal efficacy on disease progression.7,8 However, when given early, interferon beta can reduce the rate of development of brain atrophy,8 and can partially reverse the decrease in the density of N-acetylaspartate (NAA),10 a marker of axonal integrity. It is believed that attenuation of atrophy by interferon beta during the early stages of MS is considered a consequence of suppressed inflammation by a still unknown mechanism of action.

A family of molecules referred to as neurotrophins is postulated to mediate tissue protection and repair within the CNS. These molecules include nerve growth fac-
tor (NGF) and brain-derived neurotrophic factor (BDNF). Sources of neurotrophins within the CNS include microglia, astrocytes, and neurons.\textsuperscript{11-13} Regarding the endothelium, human dermal microvascular endothelial cells (ECs) have recently been shown to produce NGF.\textsuperscript{14} Evidence from experimental studies has underlined a protective role for NGF in immune-mediated CNS demyelinating diseases.\textsuperscript{15} Systemic administration of NGF is shown to reduce the severity of experimental allergic encephalomyelitis in nonhuman primates.\textsuperscript{16} Experimental allergic encephalomyelitis induced by injection of myelin-basic protein-specific T lymphocytes can be prevented by coinjection with NGF secreting antigen-specific T lymphocytes.\textsuperscript{37} The effects described are considered to reflect effects on immune cell trafficking to the CNS.

The present study aimed to evaluate whether interferon beta treatment may affect neurotrophin production by resident CNS glial or brain microvascular ECs. We used lymphocytes derived from MS patients to show that interferon beta enhances NGF production by brain ECs in T lymphocyte–EC cocultures. We further demonstrate that such NGF induction inversely correlates with disease duration, disability, and MRI-defined markers of brain atrophy.

## METHODS

### STUDY POPULATION

Informed consent was obtained from all patients/donors, and ethical approval was given by the local ethics committee. For the first set of studies, peripheral venous blood was obtained from 23 healthy volunteers, 8 untreated patients with relapsing-remitting MS, and 8 interferon beta–treated patients with relapsing-remitting MS followed up at the Montreal Neurological Hospital MS Clinic, Montreal. Healthy volunteers were younger than the untreated and interferon beta–treated MS groups studied (mean ± SEM age, 27.0 ± 1.4, 36.0 ± 2.6, and 37.0 ± 2.2 years, respectively). Male-female ratios were comparable between all 3 groups (1:2.3). For the untreated MS and interferon beta–treated groups, mean ± SEM Expanded Disability Status Scale (EDSS) scores (2.5 ± 0.4 and 3.0 ± 0.7, respectively) and disease duration (9.8 ± 1.7 and 11.0 ± 2.5 years, respectively) did not significantly differ.

For the subsequent NGF-MRI correlation studies, 13 untreated MS patients with a relapsing course were selected from the Montreal Neurological Institute MS–magnetic resonance spectroscopy database. These patients had a similar EDSS score range (mean ± SEM, 2.7 ± 0.4) as the first cohort of patients, but were selected to cover a wide range of brain atrophy measurements.

### ISOLATION AND CULTURE OF HUMAN BRAIN ECs AND GLIAL CELLS

Primary adult human brain ECs (HBECs) were isolated from CNS tissue specimens derived from young adults undergoing temporal lobe resection surgery for the treatment of non–tumor-related intractable epilepsy, as previously described.\textsuperscript{18-20} Immunoreactivity for α-myosin and glial fibrillary acidic protein could not be detected on the HBEC cultures, suggesting the absence of contaminating astrocytes and smooth muscle cells. Human microglia were isolated from the same initial surgical specimens as previously described.\textsuperscript{21} The microglial cultures were greater than 95% pure based on CD68 staining and the absence of glial fibrillary acidic protein–positive cells. Astrocytes were derived from fetal human CNS tissues, as previously described;\textsuperscript{22} cultures were shown to be greater than 95% glial fibrillary acidic protein–positive by immunocytochemistry.

### ISOLATION OF T LYMPHOCYTES FROM HUMAN PERIPHERAL BLOOD MONONUCLEAR CELLS

CD3-positive T lymphocytes were isolated from healthy adult volunteer donors or from untreated or interferon beta–treated (either Avonex or Betaseron) MS patients. CD3-positive T lymphocytes were isolated from peripheral blood mononuclear cells through positive selection using magnetic cell sorting (MACS; Miltenyi, Toronto, Ontario), according to the manufacturer's instructions. In our study and as previously reported by others,\textsuperscript{23-25} CD3-positive T-lymphocyte isolation using anti–CD3 antibodies does not result in lymphocyte proliferation (mean ± SEM, 66 ± 27 cpm [n = 15]) or cell activation, as assessed by the expression of CD25, CD69, very late activation antigen 4, and leukocyte function–associated antigen 1 markers, suggesting that the antibody used does not cross-link CD3 molecules on the surface of lymphocytes. T-lymphocyte viability and purity by flow cytometry was 95% or greater. The relative composition of CD3 cells in terms of lymphocyte subset was as follows: CD4, 64%; CD8, 21%; CD45RA, 50%; and CD45RO, 45%. The composition was similar between control subjects and MS patients.\textsuperscript{26}

### HBEC–T-LYMPHOCYTE COCULTURE ASSAYS FOR NGF PRODUCTION

Human brain ECs were plated at 25,000 cells per well, and interferon beta (Medicorp, Montreal) was added to the cultures for 24 hours. CD3-positive cells (10\textsuperscript{4}) obtained from healthy volunteers, untreated patients, or interferon beta–treated MS patients were plated in complete RPMI media (Invitrogen, Toronto, Montreal) in the presence or absence of interferon beta for 24 hours. The following day, 10\textsuperscript{6} CD3 cells were added to HBECs for an additional 24 hours of coculture. Supernatants were harvested, and levels of NGF and BDNF were assessed using enzyme-linked immunosorbent assay kits according to the manufacturer’s directions (Promega, Madison, Wis). It has previously been shown that HBECs do not support T-lymphocyte activation and proliferation in a mixed leukocyte-type reaction, despite HLA disparity.\textsuperscript{19}

In this study, a dose–response curve to interferon beta was performed in HBEC–T-lymphocyte cocultures (10, 100, 500, 1000, and 10,000 U/mL) (data not shown). A modest but significant effect of interferon beta on NGF production was seen at 100 U/mL. Because of limitations imposed by lymphocyte numbers, we selected a dose of interferon beta at 1000 U/mL for all experiments.

### POLYMERASE CHAIN REACTION ANALYSIS OF NGF MESSENGER RNA LEVELS

Human brain EC RNA after CD3 coculture was obtained following the removal of CD3 cells through positive selection using magnetic cell sorting. RNA, 2 μg, was transcribed with random primers and mouse Maloney virus reverse transcriptase (all from Invitrogen). The polymerase chain reaction was performed as follows: 94°C for 60 seconds, 60°C for 60 seconds, and 72°C for 35 cycles, followed by refrigeration at 4°C. A polymerase chain reaction for β-actin was run in parallel as an internal loading control. The oligonucleotide primer sequences used were as follows: β-actin forward, 5′-GAGGCGATCCCTCGTAGAT-3′, and β-actin reverse, 5′-CAGAAG-
separated on an agarose gel. (expected size, 185 bp). The amplified DNA fragments were and NGF reverse, 5'-GAGTGTGGTTCCGCCTGTAT-3' (expected size, 185 bp). The amplified DNA fragments were separated on an agarose gel.

**PROTON MRI**

Magnetic resonance imaging examinations of the brain were performed in a single session for each examination using a scanner operating at 1.5 T (Philips Gyroscan ACS II). A transverse dual-echo, turbo, spin-echo sequence (repetition time, 2075 milliseconds; echo times, 32 and 90 milliseconds; 256 × 256 matrix; 1 signal average; and 250-mm field of view) yielded proton density–weighted and T2-weighted images with 5 mm slice thickness. Two-dimensional spectroscopic images were obtained parallel to the line connecting the anterior and posterior commissures. The magnetic resonance images were used to select an intracranial volume of interest for spectroscopy, measuring approximately 90 mm anteroposteriorly by 20 mm craniocaudally by 90 mm left to right. This volume of interest was centered on the corpus callosum to include mostly white matter and some mesial cortex of both hemispheres. Two-dimensional spectroscopic images were obtained using a point-resolved spectroscopy sequence (repetition time, 2000 milliseconds; echo time, 272 milliseconds; 250-mm field of view; 32 × 32 phase-encoding steps; and 1 signal average per step), as previously described.27

N-Acetylaspartate values were expressed relative to intravoxel creatine (Cr) values to compensate for machine-dependent variations of signal intensity over the volume of interest. The NAA/Cr ratios for all the voxels in the spectroscopic volume of interest (excluding edge voxels containing spectra that were artifactually distorted) were then averaged to obtain a summary NAA/Cr ratio for each subject.

**MEASUREMENTS OF THE BRAIN–INTRACRANIAL CAPACITY RATIO**

The brain–intracranial capacity ratio (BICCR) is calculated from the volume of automatically segmented cerebrospinal fluid, gray matter, white matter, and lesions. The BICCR is the ratio obtained by dividing the sum of the volumes of gray matter, white matter, and lesion by the sum of the volumes of gray matter, white matter, lesion, and cerebrospinal fluid.

**STATISTICAL ANALYSIS**

Data were analyzed by a 1-way analysis of variance followed by either a t test or a Bonferroni post hoc test correcting for multiple comparisons, using computer software (Prism; GraphPad, San Diego, Calif). P < .05 was considered statistically significant. Data are given as mean ± SEM. For the correlative NGF-MRI experiments, NGF production by cell coculture was plotted against MRI variables and EDSS score, and goodness of fit to a theoretical exponential association was tested using computer software (Prism).

**RESULTS**

Levels of NGF secretion by CD3 cells or HBECS, either untreated or treated with interferon beta, were at the lower limits of detectability, when cultured separately. **Figure 1 A** demonstrates that coculture of CD3 cells and HBECS results in a significant increase in the production of NGF (n = 12, P < .05). Addition of interferon beta to HBECS results in significantly increased NGF release (188 ± 14 pg/mL [n = 7] vs 139 ± 14 pg/mL [n = 12]; P < .05 for the presence vs absence of interferon beta).

To confirm the source of NGF in our T-lymphocyte–HBECS coculture conditions, we analyzed NGF messenger RNA production in HBECS and T lymphocytes by reverse transcription–polymerase chain reaction analysis.
Figure 2. Effect of in vivo and in vitro interferon beta on nerve growth factor (NGF) production. Human brain endothelial cells (HBECs) were cultured in the presence of CD3-positive T lymphocytes derived from healthy volunteers (A), untreated patients with multiple sclerosis (MS) (B), or MS patients treated with interferon beta (C). T lymphocytes and HBECs were cocultured either in the absence of interferon beta (−) or following 24-hour pretreatment of each cell type with interferon beta (+). Interferon beta significantly increased NGF production in coculture assays of HBECs and T lymphocytes obtained from healthy donors or untreated MS patients (the asterisk indicates P < .05). In the absence of in vitro interferon beta, NGF production by HBECs cultured with T lymphocytes derived from interferon beta–treated MS patients was also significantly increased compared with T lymphocytes from untreated patients, suggesting that the in vitro effect attributed to interferon beta also occurs in vivo.

MRI METRICS AND NGF PRODUCTION

For these experiments, CD3 cells were collected from untreated MS patients and CD3/EC cocultures were supplemented with in vitro interferon beta, 1000 U/mL. Figure 3A-C show that CD3 cells obtained from patients with high T2-weighted lesion volumes, low NAA/Cr ratios, and low BICCt, respectively, had lost the ability to induce NGF production by HBECs. In contrast, CD3 cells derived from patients with low T2-weighted lesion volumes, high NAA/Cr ratios, and high BICCt retained the capacity to stimulate HBECs to produce NGF. Nerve growth factor levels were predictive of the extent of axonal loss (NAA/Cr ratio) (by linear regression, \( r^2 = .80, P < .001 \)). Regression analysis using curve fit models also revealed that the level of NGF produced by HBECs in contact with CD3 cells was predictive of the volume of T2-weighted lesions \((y = 32.7 + 0.197x + 34.8; r^2 = 0.63, P < .001)\), the extent of brain atrophy (BICCt) \((y = -20.4/x + 84.700x + 116.000x^2 + 53.600x^3; r^2 = 0.51, P < .01)\), and the EDSS score \((y = 574.1e^{-0.710x} + 31.3; r^2 = 0.61, P < .01)\). There was no correlation between NGF levels when plotted against age of the patient for a given BICCt.

NGF PRODUCTION BY GLIAL CELLS AND EFFECT OF OTHER CYTOKINES

In contrast to HBECs, levels of NGF during CD3 lymphocyte coculture with microglia (26 ± 13 pg/mL) or as-
were performed in the presence of in vitro interferon beta (n=13). The NGF response was graphed against T2-weighted lesion volumes (A), the N-acetylaspartate (NAA)–creatine (Cr) ratio (B), the brain–intracranial capacity ratio (BICCR) (C), and the Expanded Disability Status Scale (EDSS) score (D). Regression curve analysis is provided in the “MRI Metrics and NGF Production” subsection of the “Results” section.

Figures 3A–D: Correlation of nerve growth factor (NGF) production by human brain endothelial cells (HBECs) with disability and measures of axonal integrity and brain atrophy. The HBECs were cultured in the presence of CD3 T lymphocytes derived from untreated patients with multiple sclerosis (MS). All coculture experiments were performed in the presence of interferon gamma, tumor necrosis factor alpha, or interleukin 1 failed to modulate NGF production in our HBEC–T-lymphocyte or glial–T-lymphocyte coculture assays (data not shown, n=6 for each cell type).

BDNF PRODUCTION BY HBECs

Only low concentrations of BDNF were detected in supernatants derived from T lymphocytes cultured alone (44±14 pg/mL [n=7]). The addition of interferon beta did not enhance BDNF production by T lymphocytes (20±5 pg/mL). Levels of BDNF remained at the limit of detection in supernatants derived from HBECs (10±3 pg/mL) or in HBEC–T-lymphocyte cocultures in the absence (9±8 pg/mL) or presence (9±10 pg/mL) of interferon beta.

COMMENT

An ongoing debate since the initial descriptions of MS as a clinical-pathologic entity has related to the relative contribution of immune-mediated and neurodegenerative mechanisms to the overall disease pathogenesis. Neurotrophins represent a family of pleiotropic molecules that could impact both the inflammatory process and neurodegeneration. In the present study, we demonstrate that CNS microvascular ECs are a potential source of neurotrophins and that their secretion is regulated by interferon beta and T-lymphocyte contact. Lymphocytes from interferon beta–treated MS patients induced significantly higher levels of NGF in HBECs than did those derived from untreated MS patients. Levels of NGF were inversely correlated with MRI measures of brain axonal integrity. Although causality cannot be inferred from a correlation, this finding is consistent with the idea that NGF induction by infiltrating lymphocytes may aid in the mitigation of bystander axonal damage, and that eventual failure of NGF induction is associated with axonal dysfunction and degeneration. This NGF-related effect could reflect either effects on immune cell migration in the CNS, as suggested from experimental allergic encephalomyelitis–related studies, or a neuroprotective/reparative function. It is unlikely that the ability of T lymphocytes to induce HBECs to produce NGF is the only cause of the clinical and radiographic findings reported. It is, however, plausible that this effect could lead to a simple assay indicating the severity of the MS disease process at an early stage. The ability of interferon beta to stimulate NGF secretion may explain in part the observation that NAA/Cr can show partial recovery in some patients after treatment with interferon beta.

The inverse relation between NGF induction and brain atrophy was nonlinear. Patients with BICCRs in the normal range had lymphocytes that exhibited a range of NGF induction ability, whereas those with evident atrophy seemed to have lost this ability, suggesting that failure of NGF production is one prelude to cell loss and atrophy in MS patients. The inverse relation between NGF induction and EDSS score mirrors the atrophy results in that patients with an EDSS score of less than 2 (indicating the presence of mild signs and symptoms) exhibited a range of NGF induction, while patients with an EDSS score of 2 or more seemed to have lost this ability. We did not find any correlation between NGF secretion and age of the patients. Given that axonal damage and tissue loss are the likely substrates for irreversible clinical impairment, these results suggest that preservation of neural tissue after immune-mediated damage would be an important therapeutic avenue to explore.

Observations derived from pharmacokinetic studies suggest that it is unlikely that interferon beta penetrates through the blood–brain barrier in humans to mediate a direct effect on resident neural cells. The enhanced NGF production by brain ECs in response to interferon beta could, thus, play a functionally important role in MS, affecting both the immune and nervous systems. Within the CNS, NGF could exert local functional effects on neuronal and glial cell populations. Although NGF expression has not been previously reported in ECs, to our knowledge, secretion of the neurotrophin BDNF has been shown in other sources of human ECs. Our data indicate that brain ECs were the only CNS resident cells from which we could trigger an enhanced NGF secretion with interferon beta–treated lymphocytes, by a contact-dependent mechanism. We could not induce such a response from either adult brain–derived microglia or
fetal brain–derived astrocytes. To our knowledge, the precise signals that modulate NGF secretion are not yet defined. To identify the precise molecules expressed on the interferon beta–treated lymphocytes that signal the NGF response by HBECs remains a challenge because several hundred genes are up- or down-regulated in lymphocytes in response to interferon beta.31

In our study, NGF induction by lymphocytes derived from interferon beta–treated MS patients inversely correlated with clinical measure of disability and with MRI metrics of tissue injury. We, thus, postulate that cells of the blood-brain barrier not only provide the first line of defense against immune cell infiltration but also can act to promote neural or oligodendrocyte protection by being a source of NGF in response to protecting inflammatory cells. The apparent loss of this response in concert with disease progression suggests that the loss of NGF secretion may be a mechanism involved in the secondary degenerative process affecting MS patients later during the disease.

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