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Impact of glatiramer acetate on paraclinical markers of neuroprotection in multiple sclerosis: A prospective observational clinical trial

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ABSTRACT

Data from *in vitro* and animal studies support a neuroprotective role of glatiramer acetate (GA) in multiple sclerosis (MS). We investigated prospectively whether treatment with GA leads to clinical and paraclinical changes associated with neuroprotection in patients with relapsing-remitting (RR) MS. Primary aim of this clinical study was to determine serum BDNF levels in RR-MS patients who were started on GA as compared to patients who remained therapy-naive throughout 24 months. Secondary outcomes included relapses and EDSS, cognition, quality of life, fatigue and depression, BDNF expression levels on peripheral immune cells (FACS, RT-PCR), serum anti-myelin basic peptide (MBP) antibody status, evoked potential and cerebral MRI studies. While GA treatment did not alter serum levels or expression levels on peripheral immune cells of BDNF over time it resulted in a transient increase of serum IgG antibody response to MBP, mainly due to subtype IgG1 ($p < 0.05$), after 3 months. However, no significant differences were found between GA treated and therapy-naive patients with regard to serum BDNF and intracellular BDNF expression levels, nerve conduction (including median and tibial nerve somatosensory, pattern-shift visual and upper and lower limb motor evoked potentials) or MRI (including volume of hyperintense lesions, volume of hypointense lesions after CE, mean diffusivity and fractional anisotropy) outcome parameters. In conclusion, our findings do not support a major impact of GA treatment on paraclinical markers of neuroprotection in human RR-MS.

1. Introduction

In multiple sclerosis (MS), approved disease modifying treatments (DMTs) have a beneficial impact on the number of clinical relapses and on MRI-correlates of disease activity. Both outcomes are mainly representing the influence of DMTs on the inflammatory component of the disease. The potential impact on the neurodegenerative processes is less evident. However, neuronal loss occurs early in the disease and is the driving force behind accumulation of irreversible clinical disability (De Stefano et al., 1998). Sustained worsening in the expanded disability status scale (EDSS) and specific MRI-parameters are established outcome measures of disease progression, yet both markers are insensitive to subtle changes in a two-year period of standard clinical trials (Kurtzke, 1983). Therefore, paraclinical markers for monitoring neuronal survival were of essential value.

Several lines of evidence suggest that brain-derived neurotrophic factor (BDNF) is associated with neuroprotection, especially in MS. In experimental autoimmune encephalomyelitis (EAE) BDNF strongly supports neuronal and axonal survival and injection of BDNF overexpressing T cells ameliorates EAE symptoms (Kerschensteiner et al., 1999; Linker et al., 2010). BDNF immunoreactivity is not only present in activated T and B cells and monocytes in lesions of EAE, but also in lesions of MS patients, where the number of BDNF immunopositive cells is correlated with the extent of demyelination, giving indirect

Abbreviations: BDNF, brain-derived neurotrophic factor; CE, contrast enhancement; CMCT, central motor conduction time; CSCT, central sensory conduction time; CSF, cerebrospinal fluid; DMTs, disease modifying treatments; EAE, experimental autoimmune encephalomyelitis; EDSS, expanded disability status scale; HRQoL, health related quality of life; LL-MEP, lower limb motor evoked potential; MBP, myelin basic protein; M-SEP, median nerve somatosensory evoked potential; MOG, myelin oligodendrocyte glycoprotein; MS, multiple sclerosis; RR, relapsing-remitting; T-SEP, tibial nerve somatosensory evoked potential; UL-MEP, upper limb motor evoked potential; VEP, pattern-shift visual evoked potential.

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evidence for neurotrophic interactions between infiltrating immune cells and resident CNS cells (Kerschensteiner et al., 1999; Stadelmann et al., 2002). Therapeutic intervention with glatiramer acetate (GA), a first-line DMT in relapsing-remitting MS (RR-MS) is able to alter BDNF response *in vitro* and animal studies. While GA leads to enhanced BDNF expression and to a reduction in neuronal and axonal damage in classic EAE (Aharoni et al., 2005a,b), inducible deletion of BDNF limits clinical efficacy of GA in conditional EAE knock-out (Lee et al., 2012). Stimulation of human and murine T cell lines with GA enhances BDNF production (Ziemssen et al., 2002; Aharoni et al., 2003). However, there is currently no evidence whether these effects are also associated with reduced neuronal damage in humans.

Investigations on serum BDNF concentrations in patients with MS have provided conflicting results. Levels in stable RR-MS patients have been reported as decreased, increased or unchanged when compared to healthy controls (Gold et al., 2003; Azoulay et al., 2005; Lalive et al., 2008). During and after acute relapses, serum BDNF levels have been found increased or unchanged (Azoulay et al., 2005; Frota et al., 2009). A cross-sectional study reported similar serum BDNF levels in RR-MS patients undergoing GA treatment and in healthy controls, whereas untreated patients exhibited significantly lower levels (Azoulay et al., 2005). Whether these increased BDNF levels are associated with a clinical meaningful benefit remains unclear.

The present study intended to longitudinally investigate serum BDNF and its expression levels on peripheral immune cells in RR-MS patients after treatment initiation with GA as compared to treatment naive patients. Clinical, radiological and electrophysiological outcome parameters were implemented to account for clinically meaningful levels of neuroprotection.

2. Methods

2.1. Ethics statement

The ethical committee of the Medical University of Innsbruck, Austria (study nr. UN2045, 217/4.12) approved the study and all participants gave written informed consent.

2.2. Patients, study design and procedures

Study participants were recruited prospectively at the Clinical Department of Neurology, Medical University of Innsbruck, Austria. Key eligibility criteria included diagnosis of RR-MS with recent disease activity as evidenced by at least one clinically documented relapse within 12 months before enrolment (Polman et al., in review). A key exclusion criteria was any previous immunosuppressive or immunomodulatory treatment.

At study entry, patients were either started on treatment with GA (20 mg Copaxone© subcutaneously once daily) or remained therapy naive, in case of individual refusal of any immunomodulatory therapy due to diverse reasons, *e.g.* needle phobia (control group). The decision to start treatment or not was independently made by the treating physician before patients entered the study. Patients were followed over a period of 24 months. Study visits were scheduled every 3 months for standardized neurological assessments including EDSS.

2.3. Neuropsychological assessment

Cognition was assessed at baseline, months 12 and 24 using a modified brief repeatable battery of neuropsychological tests (Rao, 1990) comprising the following subtests: 1) verbal learning and memory test, 2) faces recognition test to examine figural learning and memory, 3) test of everyday attention and digit span to examine sustained and divided attention; and 4) Word List Generation to assess semantic verbal fluency and flexibility. Abnormal sub-testing was defined as a score below the sixteenth percentile of the performance of healthy controls as provided by the authors. Self-rating scales were used to assess health related quality of life (HRQoL) (Gold et al., 2001), anxiety and depression (hospital anxiety and depression scale; cut-off value used for abnormal testing >7) and fatigue (cut-off values indicating physical fatigue or cognitive fatigue >16 or >17 retrospectively, indicating any fatigue >32) (Flachenecker et al., 2006).

2.4. Laboratory parameters

2.4.1. Serum BDNF and anti-myelin basic peptide antibody measurement

Blood samples were taken at baseline and every 3 months thereafter and investigated for serum BDNF using a commercially available ELISA kit (DuoSet ELISA Development System, Minneapolis, MN, USA) and serum anti-myelin basic peptide antibodies (anti-myelin basic protein, MBP; IgM and IgG including subclasses 1 to 4) using ELISA as described previously (Tomassini et al., 2007).

2.4.2. FACS of intracellular BDNF production

FACS staining of EDTA samples from patients was performed within 60 min after collection at baseline and every 3 months thereafter. 100 μl of whole blood were stained with 5 μl of anti-human CD3FITC and 5 μl of anti-human CD45PerCP (both BD Biosciences, San Diego, CA, USA) and incubated for 15 min, followed by incubation with 2 ml of 1× BD FACS lysing solution (BD Biosciences, San Diego, CA, USA) for 15 min. Cells were gained through centrifugation (RT, 5 min, 500 *g*), fixed in FACS Cytofix/Cytoperm solution (BD Biosciences, San Diego, CA, USA) and incubated on ice for 20 min. After washing and blocking with 10% normal goat serum for 30 min cells were incubated with 5 μl of anti-human BDNF mouse IgG1 (R&D Systems) and 5 μl of Mouse IgG1 (BD Biosciences, San Diego, CA, USA) for isotype control for 45 min. Stainings were performed in duplicate, one for isotype control and one for staining of intracellular produced BDNF and analyzed on a FACScan using Cell-Quest software (Becton Dickinson, Heidelberg, Germany). Lymphocytes and monocytes were gated in CD45/side scatter and quadrants were set on isotype controls. A total of 10,000 events for lymphocytes were acquired. Fluorescence compensation was adjusted with FACSComp software using CaliBRITE Beads (CaliBRITE™ 3, Becton Dickinson, Heidelberg, Germany). Compensation settings were adapted for acquisition of stained cells.

2.4.3. RT-PCR for BDNF

Blood samples were collected at baseline and every 3 months thereafter. RNA from whole blood was extracted using Tempus™ Blood RNA tubes and purified RNA reverse transcribed to cDNA using High-Capacity cDNA Reverse transcription kit (Applied Biosystems, CA, USA). mRNA BDNF expression was quantified on a 7300 Real Time PCR system (Applied Biosystems, CA, USA) with a two-step PCR protocol (95 °C for 10 min, followed by 40 cycles for 15 s and 60 °C for 1 min) using BDNF-specific primers (forward 5′-GGCCCTTACCATGGATAGCAAAA-3′; reverse 5′-TCCCCTTTTAATGGTCAATGTACATACAC-3′), fluorescent probes (NFQ-MGB™ FAM-5′-TTGGCTGGCGATTCAT-3′) and 18S rRNA primers (all Applied Biosystems, CA, USA). Measurements were performed in duplicates and with each run, a no template control and/or a control from the reverse transcription (without RNA or without enzyme) was added. Random RNA samples were added to exclude genomic contamination. Relative BDNF expression levels were calculated according to the comparative method of $2^{-\Delta\Delta Ct}$ using fetal brain as calibrato (Livak and Schmittgen, 2001).

2.5. Nerve conduction studies

Median and tibial nerve somatosensory evoked potentials (M-SEP and T-SEP), pattern-shift visual evoked potentials (VEP) and upper and lower limb motor evoked potentials (UL-MEP and LL-MEP) were recorded at baseline and at 12 and 24 months using a Nicolet Viking IV (Nicolet Biomedical, Madison, WI). MEPs were elicited by transcranial