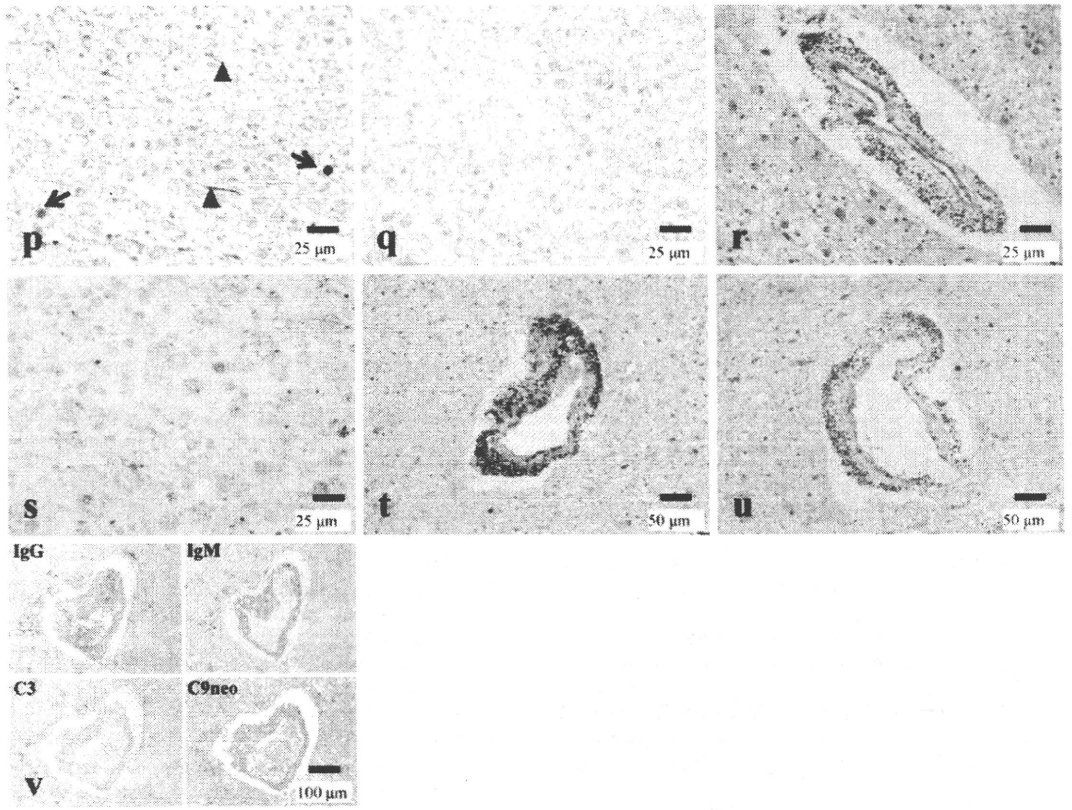

Fig. 3 continued

Table 2 Summary of the pathological findings in demyelinating lesions in cases with Baló's concentric sclerosis

Case	Lesion	Stage	AQP4 loss	T cell infiltration	Perivascular deposition of immunoglobulins and complement
Baló-1	Cr1	Active	Extensive	Perivascular	No
	Cr2	Active	Extensive	Perivascular	No
Baló-2	Cr1	Active	Extensive	Perivascular	No
Baló-3	Cr1	Active	Extensive	Perivascular	No
Baló-4	Cr1	Active	Extensive	Perivascular	No
	Cr2	Active	Extensive	Perivascular	No

AQP4 aquaporin-4, *Cr* cerebrum

AQP4 loss in the acute lesions without vasculocentric deposition of complement or immunoglobulin is considered to be characteristic of BCS.

There are several lines of experimental evidence that anti-AQP4 antibody-independent impairment of astrocytes causes AQP4 loss followed by demyelination. Sharma et al. [33] reported lipopolysaccharide-induced demyelinating lesions in rats showed loss of AQP4 and retraction of astrocytic vascular foot processes. Likewise, Wolburg-Buchholz et al. [40] demonstrated that ultrastructural distribution of AQP4 is altered in the astrocytic foot processes followed by destruction of the BBB in a murine experimental autoimmune encephalomyelitis model immunized with proteolipid protein peptide. Indeed, the astrocytes in the active lesions in our BCS cases showed extremely hypertrophic, abnormal reacting morphology suggestive of considerable functional impairment including loss of AQP4 expression. The mechanism underlying such anti-AQP4 antibody-independent AQP4 loss is currently unknown. However, phosphorylation-related internalization of AQP4 resulting from a variety of stimuli might initiate this process [5, 8, 28].

Future studies on astrocytopathy as well as the dynamic plasticity of astrocytes may shed light on the mechanisms underlying the alternating myelinated and demyelinated lesions observed in BCS.

Acknowledgments We thank Ms. Sachiko Nagae and Ms. Kimiko Sato, Department of Neuropathology, Kyushu University, for their excellent technical assistance and Dr. Takekazu Ohi, Department of Neurology, Kurashiki Central Hospital for providing materials. This work was supported in part by Grants from the Research Committees of Neuroimmunological Diseases, the Ministry of Health, Labor and Welfare, Japan (JK) and from the Ministry of Education, Culture, Sports, Science and Technology (JK), Japan.

Conflict of interest The authors declare that they have no conflict of interest.

References

1. Aoki K, Uchihara T, Tsuchiya K, Nakamura A, Ikeda K, Wakayama Y (2003) Enhanced expression of aquaporin 4 in human brain with infarction. Acta Neuropathol 106:121–124
2. Baló J (1928) Encephalitis periaxialis concentrica. Arch Neurol 19:242–263
3. Bennett JL, Lam C, Kalluri SR et al (2009) Intrathecal pathogenic anti-aquaporin-4 antibodies in early neuromyelitis optica. Ann Neurol 66:617–629
4. Bradl M, Misu T, Takahashi T et al (2009) Neuromyelitis optica: pathogenicity of patient immunoglobulin in vivo. Ann Neurol 66:630–643
5. Carmosino M, Procino G, Tamma G, Mannucci R, Svelto M, Valenti G (2007) Trafficking and phosphorylation dynamics of AQP4 in histamine-treated human gastric cells. Biol Cell 99:25–36
6. Chen CJ, Chu NS, Lu CS, Sung CY (1999) Serial magnetic resonance imaging in patients with Baló's concentric sclerosis: natural history of lesion development. Ann Neurol 46:651–656
7. Courville CB (1970) Concentric sclerosis. In: Vinken PJ, Bruyn GW (eds) Multiple sclerosis and other demyelinating diseases. North-Holland, Amsterdam, pp 437–451
8. Fenton RA, Moeller HB, Zelenina M, Snaebjornsson MT, Holen T, MacAulay N (2010) Differential water permeability and regulation of three aquaporin 4 isoforms. Cell Mol Life Sci 67:829–840
9. Frydenlund DS, Bhardwaj A, Otsuka T et al (2006) Temporary loss of perivascular aquaporin-4 in neocortex after transient middle cerebral artery occlusion in mice. Proc Natl Acad Sci USA 103:13532–13536
10. Fujita Y, Yamamoto N, Sobue K et al (2003) Effect of mild hypothermia on the expression of aquaporin family in cultured rat astrocytes under hypoxic condition. Neurosci Res 47:437–444
11. Graber JJ, Kister I, Geyer H, Khaund M, Herbert J (2010) Neuromyelitis optica and concentric rings of Baló in the brainstem. Arch Neurol 66:274–275
12. Jung JS, Bhat RV, Preston GM, Guggino WB, Baraban JM, Agre P (1994) Molecular characterization of an aquaporin cDNA from brain: candidate osmoreceptor and regulator of water balance. Proc Natl Acad Sci USA 91:13052–13056
13. Kinoshita M, Nakatsuji Y, Kimura T et al (2009) Neuromyelitis optica: passive transfer to rats by human immunoglobulin. Biochem Biophys Res Commun 386:623–627
14. Kinoshita M, Nakatsuji Y, Moriya M et al (2009) Astrocytic necrosis is induced by anti-aquaporin-4 antibody-positive serum. Neuroreport 20:508–512
15. Kuroiwa Y (1985) Neuromyelitis optica (Devic's disease, Devic's syndrome). In: Koetsier JC (ed) Handbook of clinical neurology. Demyelinating disease, vol 47. Elsevier, Amsterdam, pp 397–408
16. Lassmann H, Raine CS, Antel J, Prineas JW (1998) Immunopathology of multiple sclerosis: report on an international meeting held at the institute of neurology of the University of Vienna. J Neuroimmunol 86:213–217
17. Lee M, Lee SJ, Choi HJ et al (2008) Regulation of AQP4 protein expression in rat brain astrocytes: role of P2X7 receptor activation. Brain Res 1195:1–11
18. Lee TS, Eid T, Mane S et al (2004) Aquaporin-4 is increased in the sclerotic hippocampus in human temporal lobe epilepsy. Acta Neuropathol 108:493–502
19. Lennon VA, Kryzer TJ, Pittock SJ, Verkman AS, Hinson SR (2005) IgG marker of optic-spinal multiple sclerosis binds to the aquaporin-4 water channel. J Exp Med 202:473–477
20. Lennon VA, Wingerchuk DM, Kryzer TJ et al (2004) A serum autoantibody marker of neuromyelitis optica: distinction from multiple sclerosis. Lancet 364:2106–2112
21. Li L, Zhang H, Verkman AS (2009) Greatly attenuated experimental autoimmune encephalomyelitis in aquaporin-4 knockout mice. BMC Neurosci 10:94
22. Lucchinetti C, Brück W, Parisi J, Scheithauer B, Rodriguez M, Lassmann H (2000) Heterogeneity of multiple sclerosis lesions: implications for the pathogenesis of demyelination. Ann Neurol 47:707–717
23. Lucchinetti CF, Mandler RN, McGavern D et al (2002) A role for humoral mechanisms in the pathogenesis of Devic's neuromyelitis optica. Brain 125:1450–1461
24. Mahad D, Ziabreva I, Lassmann H, Turnbull D (2008) Mitochondrial defects in acute multiple sclerosis lesions. Brain 131:1722–1735
25. Manley GT, Fujimura M, Ma T, Noshita N, Filiz F, Bollen AW et al (2000) Aquaporin-4 deletion in mice reduces brain edema after acute water intoxication and ischemic stroke. Nat Med 6:159–163
26. Meng S, Qiao M, Lin L, Del Bigio MR, Tomanek B, Tuor UI (2004) Correspondence of AQP4 expression and hypoxic-ischaemic brain edema monitored by magnetic resonance imaging in the immature and juvenile rat. Eur J Neurosci 19:2261–2269
27. Misu T, Fujihara K, Kakita A et al (2007) Loss of aquaporin 4 in lesions of neuromyelitis optica: distinction from multiple sclerosis. Brain 130:1224–1234
28. Moeller HB, Fenton RA, Zeuthen T, Macaulay N (2009) Vasopressin-dependent short-term regulation of aquaporin 4 expressed in *Xenopus* oocytes. Neuroscience 164:1674–1684
29. Nag S, Manias JL, Stewart DJ (2009) Pathology and new players in the pathogenesis of brain edema. Acta Neuropathol 118:197–217
30. Roemer SF, Parisi JE, Lennon VA et al (2007) Pattern-specific loss of aquaporin-4 immunoreactivity distinguishes neuromyelitis optica from multiple sclerosis. Brain 130:1194–1205
31. Saadoun S, Waters P, Bell BA, Vincent A, Verkman AS, Papadopoulos MC (2010) Intra-cerebral injection of neuromyelitis optica immunoglobulin G and human complement produces neuromyelitis optica lesions in mice. Brain 133:349–361
32. Sabater L, Giralt A, Boronat A et al (2009) Cytotoxic effect of neuromyelitis optica antibody (NMO-IgG) to astrocytes: an in vitro study. J Neuroimmunol 215:31–35
33. Sharma R, Fischer MT, Bauer J et al (2010) Inflammation induced by innate immunity in the central nervous system leads to primary astrocyte dysfunction followed by demyelination. Acta Neuropathol 120:223–236
34. Stadelmann C, Ludwin S, Tabira T et al (2005) Tissue preconditioning may explain concentric lesions in Baló's type of multiple sclerosis. Brain 128:979–987
35. Verkman AS, Binder DK, Bloch O, Auguste K, Papadopoulos MC (2006) Three distinct roles of aquaporin-4 in brain function

revealed by knockout mice. Biochim Biophys Acta 1758:1085–1093
36. Vincent T, Saikali P, Cayrol R et al (2008) Functional consequences of neuromyelitis optica–IgG astrocyte interactions on blood–brain barrier permeability and granulocyte recruitment. J Immunol 181:5730–5737
37. Voskuhl RR, Peterson RS, Song B et al (2009) Reactive astrocytes form scar-like perivascular barriers to leukocytes during adaptive immune inflammation of the CNS. J Neurosci 29:11511–11522
38. Wang C, Zhang KN, Wu XM et al (2008) Baló's disease showing benign clinical course and co-existence with multiple sclerosis-like lesions in Chinese. Mult Scler 14:418–424
39. Wiendl H, Weissert T, Herrlinger U, Krapf H, Küker W (2005) Diffusion abnormality in Baló's concentric sclerosis: clues for the pathogenesis. Eur Neurol 53:42–44
40. Wolburg-Buchholz K, Mack AF, Steiner E, Pfeiffer F, Engelhardt B, Wolburg H (2009) Loss of astrocyte polarity marks blood–brain barrier impairment during experimental autoimmune encephalomyelitis. Acta Neuropathol 118:219–233

Research Paper

Multiple Sclerosis

Multiple Sclerosis
16(10) 1178–1188

DOI: 10.1177/1352458510375706
msj.sagepub.com
SAGE

Altered production of brain-derived neurotrophic factor by peripheral blood immune cells in multiple sclerosis

Satoshi Yoshimura, Hirofumi Ochi, Noriko Isobe, Takuya Matsushita, Kyoko Motomura, Takeshi Matsuoka, Motozumi Minohara and Jun-ichi Kira

Abstract

Background: Within multiple sclerosis lesions, brain-derived neurotrophic factor is detected in neurons and immunocytes.

Objective: To clarify brain-derived neurotrophic factor production by peripheral blood immunocytes and its relationship with clinical parameters in multiple sclerosis.

Methods: Serum brain-derived neurotrophic factor levels were measured by conventional enzyme-linked immunosorbent assay while brain-derived neurotrophic factor production by immunocytes was determined by an *in situ* enzyme-linked immunosorbent assay in 74 multiple sclerosis patients, 32 healthy controls, and 86 patients with other neurological diseases. The tyrosine kinase receptor *TrkB* expression level in peripheral blood mononuclear cells was measured by real-time polymerase chain reaction.

Results: Multiple sclerosis patients showed significantly higher serum brain-derived neurotrophic factor levels than healthy controls and patients with other neurological diseases. Multiple sclerosis patients with high brain-derived neurotrophic factor levels were younger, and showed fewer relapse numbers than those with low brain-derived neurotrophic factor levels. Brain-derived neurotrophic factor production by T cells increased with age in healthy controls, but not in multiple sclerosis patients. Interferon beta induced a significant increase in serum brain-derived neurotrophic factor levels. Brain-derived neurotrophic factor production from T cells and *TrkB* expression levels in peripheral blood mononuclear cells were significantly enhanced in interferon beta-treated multiple sclerosis patients compared with untreated ones.

Conclusions: A high brain-derived neurotrophic factor level is related to early mild disease in young multiple sclerosis patients. Interferon beta potentiates brain-derived neurotrophic factor production and brain-derived neurotrophic factor receptor expression in peripheral blood mononuclear cells, which may act beneficially.

Keywords

Brain-derived neurotrophic factor, BDNF, multiple sclerosis, MS, interferon beta, IFNβ, T cells, age

Date received: 12th January 2010; revised: 28th April 2010; accepted: 21st May 2010

Introduction

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system (CNS). It is characterized by episodes of acute neurological dysfunction during the relapsing–remitting (RR) phase, leading to partial or full recovery.[1] However, with time, recovery from each episode becomes incomplete and persistent symptoms accumulate.[1] Thus, it is critical to understand the neuroprotection and repair mechanisms operating in this condition.

Among neuroprotective factors, brain-derived neurotrophic factor (BDNF), a member of the neurotrophin (NT) family which includes nerve growth factor (NGF), neurotrophin-3 (NT-3), and neurotrophin-4/5 (NT-4/5),[2] has recently received a lot of attention in MS research. BDNF has been shown to promote neuronal survival after experimental axotomy[3] and to enhance oligodendrocyte proliferation and myelination.[4]

Department of Neurology, Neurological Institute, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan.

Corresponding author:
Jun-ichi Kira, Department of Neurology, Neurological Institute, Graduate School of Medical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan
Email: kira@neuro.med.kyushu-u.ac.jp

Not only neurons but also immune cells, such as T cells, B cells, and monocytes, produce BDNF *in vitro* and in inflamed CNS lesions in MS patients.[5] Furthermore, tyrosine kinase receptor *TrkB*, a receptor for BDNF, was detected in reactive astrocytes within MS plaques and neurons in the immediate vicinity of such lesions.[6] Thus, BDNF and *TrkB* are supposed to be key players in neuroprotective immunity.[7]

There is accumulating evidence for a functional role of BDNF in the periphery, because BDNF is detected in both human serum and plasma.[8] More than 90% of blood BDNF is stored in platelets and can be released in serum on activation or clotting, which explains the 50–200-fold higher levels of BDNF in serum than in plasma.[8,9] Platelets and megakaryocytes have low levels of mRNA for BDNF;[8,10] however, platelets bind and internalize BDNF from other sources via the blood circulation, through high affinity receptors other than *TrkB*.[10] Alternative sources of blood BDNF identified to date include lymphocytes, monocytes,[5] eosinophils,[11] vascular endothelial cells,[12] and vascular smooth muscle.[13] Human platelets are known to circulate for about 10 days in peripheral blood,[14] while BDNF protein circulates in plasma for less than an hour.[15] Thus, serum or platelets are more stable components of blood to measure BDNF levels than plasma. Since BDNF is known to cross the blood–brain barrier in both directions, a substantial portion of circulating BDNF might originate from neurons and glia cells of the CNS.[16] Karege et al.[17] observed a positive correlation between serum and cortical levels of BDNF, indicating that cortical BDNF is a possible candidate source for circulating BDNF, and that peripheral measurement of BDNF could be used as a surrogate measure for BDNF levels in the CNS. Collectively, the presence of high levels of BDNF in platelets and serum suggests a role of circulating BDNF in neural repair at injured sites.

While no study reports platelet BDNF release in MS, there have been several studies describing circulating BDNF levels: serum BDNF levels are similar in relapsing–remitting MS (RRMS) patients in remission and controls;[18] they are lower in RRMS patients, either at relapse or in remission, than in healthy controls;[19] and they are higher in patients in relapse than in those in remission.[20] Concerning BDNF production by immune cells in MS, some studies have revealed that BDNF production by peripheral blood mononuclear cells (PBMCs) is higher during relapse and the recovery phase than during the stable phase in RRMS patients,[20,21] and that it is significantly associated with contrast-enhanced lesion volumes.[22] BDNF production by PBMCs was also reported to be higher in RRMS patients than in secondary progressive MS (SPMS) or primary–progressive MS (PPMS) patients.[20,23] In contrast, other studies have reported that BDNF production by PBMCs is lower in RRMS patients in remission than in controls.[24]

Regarding the effects of immunomodulatory therapies, PBMCs from glatiramer acetate (GA)-treated RRMS patients produced higher amounts of BDNF when compared with PBMCs from untreated RRMS patients and controls.[25] GA also significantly increased the serum levels of BDNF in MS patients,[19] especially in responders.[26] However, there is conflicting data on the effects of interferon beta (IFNβ) treatment on BDNF production; enhanced production of BDNF by PBMCs from IFNβ-treated patients[18] and by *in vitro* IFNβ-treated PBMCs[23] were reported in some studies, while no effect of IFNβ on BDNF expression was observed in others.[25,27] Thus, there is no consensus regarding serum BDNF levels and BDNF production by peripheral blood immune cells in MS patients, or their modification by IFNβ, and there have been no studies regarding BDNF levels and production in Asian MS patients in general. Therefore, in the present study, we aimed to measure serum BDNF levels, as well as BDNF production and *TrkB* expression by PBMCs, in Japanese MS patients with and without IFNβ treatment, and ascertain their relationships with clinical parameters. Our study is the first to measure BDNF production by separated T cells and monocytes from MS patients using an *in situ* enzyme-linked immunosorbent assay (ELISA), and the first to assess *TrkB* expression in PBMCs from untreated MS patients.

Methods

Subjects

In the present study, patients with MS, who were diagnosed as clinically definite MS according to the McDonald's criteria,[28] at the MS clinic in the Department of Neurology, Kyushu University Hospital between 1999 and 2008, were enrolled after informed consent was obtained. For conventional ELISA experiments to determine serum BDNF levels, 74 MS patients, 32 age-matched healthy controls (HCs), 29 patients with amyotrophic lateral sclerosis (ALS), 28 with spinocerebellar degeneration (SCD), and 29 with human T-cell lymphotropic virus associated myelopathy (HAM) were enrolled (Table 1). The disability status of the MS patients was scored by one of the authors (JK) throughout the study, according to the Kurtzke's Expanded Disability Status Scale (EDSS).[29] Among the MS patients, 66 patients had RRMS and eight had SPMS; no patients with PPMS were recruited. MS patients were clinically classified into two subtypes, opticospinal MS (OSMS) and conventional MS (CMS), as described previously.[30]

Table 1. Demographic features of subjects

	Number of patients (male/female)	Age at examination (years)	Disease duration (months)	EDSS	Number of relapses	Annualized relapse rates	RRMS/ SPMS	OSMS/ CMS	AQP4 (+)	NMO
Conventional ELISA for serum BDNF										
Untreated MS	74 (19/55)	39.9 ± 11.9	126.6 ± 115.4	3.38 ± 2.04	5.39 ± 4.88	0.94 ± 1.02	66/8	31/43	12	12
IFNβ-treated MS	12 (4/8)	40.8 ± 8.1	80.2 ± 51.1	2.79 ± 2.23	5.17 ± 6.07	1.97 ± 1.74	9/3	1/11	0	0
HCs	32 (15/17)	34.4 ± 10.2								
ALS	29 (13/16)	60.1 ± 12.4								
SCD	28 (14/14)	59.9 ± 14.0								
HAM	29 (10/19)	53.0 ± 13.2								
BDNF production from T cells and monocytes										
Untreated MS	17 (5/12)	50.8 ± 15.5	200.8 ± 122.9	3.26 ± 2.69	4.76 ± 3.73	0.31 ± 0.19	14/3	6/11	1	0
IFNβ-treated MS[a]	8 (3/5)	42.0 ± 8.3	91.9 ± 68.9	2.5 ± 2.2	3.13 ± 2.23	0.54 ± 0.38	6/2	0/8	0	0
HCs	18 (8/10)	44.9 ± 17.0								
TrkB expression in PBMCs										
Untreated MS	15 (1/14)	52.5 ± 15.8	220.7 ± 138	4.03 ± 2.45	6.87 ± 6.5	0.38 ± 0.27	13/2	4/11	0	0
IFNβ-treated MS[b]	11 (3/8)	37.2 ± 12.6	106 ± 68.7	2.64 ± 2.68	7.82 ± 8.22	0.79 ± 0.52	10/1	2/9	0	0
HCs	21 (10/21)	50.4 ± 15.2								

[a]Seven on IFNβ-1b and one on IFNβ-1a.
[b]Ten on IFNβ-1b and one on IFNβ-1a.
ALS, amyotrophic lateral sclerosis; AQP4 (+), anti-AQP4 antibody-positive; BDNF, brain-derived neurotrophic factor; CMS: conventional multiple sclerosis; EDSS, Expanded Disability Status Scale;[29] ELISA, enzyme-linked immunosorbent assay; HAM: human T-cell lymphotropic virus associated myelopathy; HCs, healthy controls; IFNβ, interferon beta; MS, multiple sclerosis; NMO: neuromyelitis optica; OSMS, opticospinal multiple sclerosis; PBMCs, peripheral blood mononuclear cells; RRMS, relapsing–remitting multiple sclerosis; SCD, spinocerebellar degeneration; SPMS, secondary progressive multiple sclerosis; *TrkB*, tyrosine kinase receptor.

There were 31 patients with OSMS (42%) and 43 patients with CMS (58%). The ages at examination were not significantly different between the two subtypes of patients (mean ± SD in years; 42.6 ± 14.3 in OSMS and 37.8 ± 9.3 in CMS). Anti-aquaporin-4 (AQP4) antibody was tested in all MS patients as previously described,[31,32] and only 12 were positive for the antibody (10 OSMS patients and two CMS patients); 62 were negative. Nine patients with anti-AQP4 antibody and three patients without anti-AQP4 antibody also fulfilled the criteria for neuromyelitis optica (NMO).[33] Thirty-one samples were obtained from 28 MS patients in the relapse phase, and 85 samples were obtained from 57 MS patients in the remission phase. All of the recruited patients were untreated for at least 6 months before study entry. Relapse was defined by the appearance of new neurological symptoms lasting at least 48 h in a patient who had been neurologically stable or improving for the previous 30 days, accompanied by objective changes on neurological examination. Thus, in the present study, the relapse phase was regarded as within 1 month after the onset of acute exacerbation, while the remission phase was regarded as either the stable stage or more than 1 month after exacerbation. We classified MS patients under treatment as clinical responders and suboptimal responders to IFNβ therapy, based on EDSS progression and the number of relapses; the occurrence of more than one relapse or an increase in EDSS score of at least 1 point during the one year of IFNβ treatment, according to Pozzilli et al.[34]

For the assay of BDNF production from T cells and monocytes using an *in situ* ELISA, 17 untreated MS patients, eight IFNβ-treated MS patients, and 18 HCs were enrolled (Table 1). For examination of *TrkB* expression in PBMCs, 15 untreated MS patients, 11 IFNβ-treated MS patients, and 21 HCs were enrolled (Table 1). The IFNβ-treated patients had received the drug for at least 6 months before the commencement of the study and none underwent any additional immunomodulatory therapy while they were on IFNβ.

Conventional ELISA for serum BDNF levels

Serum samples of MS patients and controls were stored at −80°C until use. Serum BDNF levels were determined using sandwich ELISA kits for BDNF (Promega, Madison, Wisconsin, USA) according to the procedure supplied by the manufacturer. In this procedure, flat-bottomed 96-well plates were coated with anti-human BDNF monoclonal antibody (mAB) to bind soluble BDNF, and the plates were incubated overnight at 4°C. After washing with wash buffer (Tris-HCl, pH 7.6), and blocking for nonspecific binding with Block & Sample

Buffer, the plates were incubated at room temperature for 1 h without shaking and washed once. Samples were diluted 80-fold with a calibrator prior to the assay. One hundred microliters of samples and BDNF standards, in duplicate, were added to the appropriate wells and the plates were incubated for 2 h at room temperature. A second BDNF-specific polyclonal antibody was added and the plates were incubated for 2 h at room temperature so that the captured BDNF could bind the polyclonal antibody. After washing, the amount of specifically bound polyclonal antibody was then detected using species-specific anti-IgY antibody conjugated to horseradish peroxidase (HRP) as a tertiary reactant. Unbound conjugate was removed by washing, which was followed by incubation with a chromogenic substrate and stopping the reaction with 1 N hydrochloric acid. The absorbencies were measured at 450 nm using an automatic ELISA microplate reader (IMMUNO-MINI NJ-2300, Tokyo, Japan). The sensitivity of the assay (expressed as the minimum amount of BDNF that could be detected) was 15.6 pg/ml, and the intra- and inter-assay coefficients of variation were 6.0% and 8.5%, respectively.

In situ ELISA for BDNF produced by peripheral blood immunocytes

PBMCs were isolated from venous blood containing 0.2% ethylenediamine tetraacetic acid (EDTA), diluted in a 1:1 ratio with saline, and subjected to density gradient centrifugation for 20 min at 2000 × g at 20°C, using lymphoprep tubes (Nycomed Pharma AS, Oslo, Norway). Dead cells were removed using the MACS Dead Cell Removal Kit (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). Viable PBMCs were determined by trypan blue exclusion. T cells and monocytes were purified from PBMCs by negative immunoselection (Miltenyi Biotec). The purity of the isolated T cells and monocytes was >90% as determined by flow cytometry using anti-CD3 IgG and anti-CD14 IgG and fluorescein isothiocyanate (FITC)-conjugated antibodies. Isolated T cells and monocytes were washed twice and resuspended in X-VIVO 15 (Takara, Tokyo, Japan) at a concentration of 5×10^6 cells per milliliter. Then, 200 µl of purified peripheral blood T cells and monocytes (1×10^5 cells) was added to the UV-sterilized 96-well ELISA plates precoated with anti-BDNF mAB, which were incubated at 37°C in a 5% CO_2-humidified atmosphere for 48 h. Forty-six hours after the start of the assay, BDNF samples used to generate the standard curve were incubated in the same plate as the cells. At the end of the cell culture period, plates were extensively washed to remove all cells and cell debris, and the anti-BDNF polyclonal antibody was applied, followed by subsequent steps according to Promega's ELISA protocol. All experiments were performed in duplicate.

Real-time PCR for TrkB expression

TrkB mRNA expression in PBMCs from MS patients and HCs was examined by real-time PCR. First, approximately 5×10^6 to 1×10^7 PBMCs were isolated from venous blood containing 0.2% EDTA by density gradient centrifugation for 10 min at 2000 × g at 20°C on Ficoll-Paque. mRNA was extracted from total cellular RNA using a commercially available mRNA isolation kit (RNeasy Mini Kit, Qiagen GmbH, Hilden, Germany). Reverse transcription was performed using 750 ng of mRNA from each sample in a 20 µl reaction for 10 min at 65°C followed by 30 min at 55°C according to the manufacturer's instructions (Transcriptor First Strand cDNA Synthesis Kit; Roche Diagnostics GmbH, Mannheim, Germany). As a control, 750 ng of mRNA from each sample was treated according to the same protocol with the addition of distilled water instead of the reverse transcriptase. Real-time PCR was performed on the LightCycler® Instrument (Bio-Rad MiniOpticon™, Bio-Rad Laboratories, Hercules, California, USA) using the DNA-binding dye SYBR Green I (Takara SYBR Premix Ex Taq™ II, Takara, Kyoto, Japan). Specific primers for PCR were designed against glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) (sense: 5′-GAGTCAACGGATTTGGTC GT-3′; antisense: 5′-TTGATTTTGGAGGGATCT CG-3′; expected product length: 238 bp), and *TrkB* (sense: 5′-CGAGATTGGAGCCTAACAGT-3′; antisense: 5′-CACCAGGATCAGTTCAGACA-3′; expected product length: 272 bp). After an initial denaturation step at 95°C for 5 s, the PCR reaction was performed with an annealing temperature of 55°C for 10 s, followed by an extension phase at 72°C for 15 s. At the end of each extension phase, fluorescence was observed at 72°C. The PCR reaction was completed after 45 cycles. Melting point analysis was performed by heating the amplicon from 50 to 95°C and revealed the characteristic melting point for each product. After cooling to 40°C, the product was extracted from the capillary. Ten microliters of each reaction was separated on a 1.5% agarose gel and visualized by staining with ethidium bromide.

Statistical analysis

Statistical analyses of numerical variables were initially performed using the Kruskal–Wallis *H*-test. When a significant difference was found, the Mann–Whitney *U*-test was used to determine the significance of differences between subgroups. *K*-means cluster analyses were used to identify subgroups among the MS patients according to serum BDNF levels. For comparisons among the subgroups, we performed logistic regression analyses. All analyses were performed using JMP

6.0.3 (SAS Institute, Cary, North Carolina, USA). Changes in serum BDNF levels were compared using the Wilcoxon signed rank test.

Results

Serum BDNF levels determined by conventional ELISA

MS patients showed significantly higher serum BDNF levels than HCs, and patients with ALS, SCD, and HAM ($p < 0.0001$ in all, Figure 1A). Serum BDNF levels tended to be higher in the relapse phase than in the remission phase ($p = 0.094$, Figure 1B) while they were not significantly different between males and females (Supplementary Figure 1A). Serum BDNF levels in OSMS patients were significantly higher than those in CMS patients ($p = 0.015$, Figure 1C), while they were not significantly different between anti-AQP4 antibody-positive and antibody-negative patients, or between those who fulfilled the NMO criteria and those who did not (Supplementary

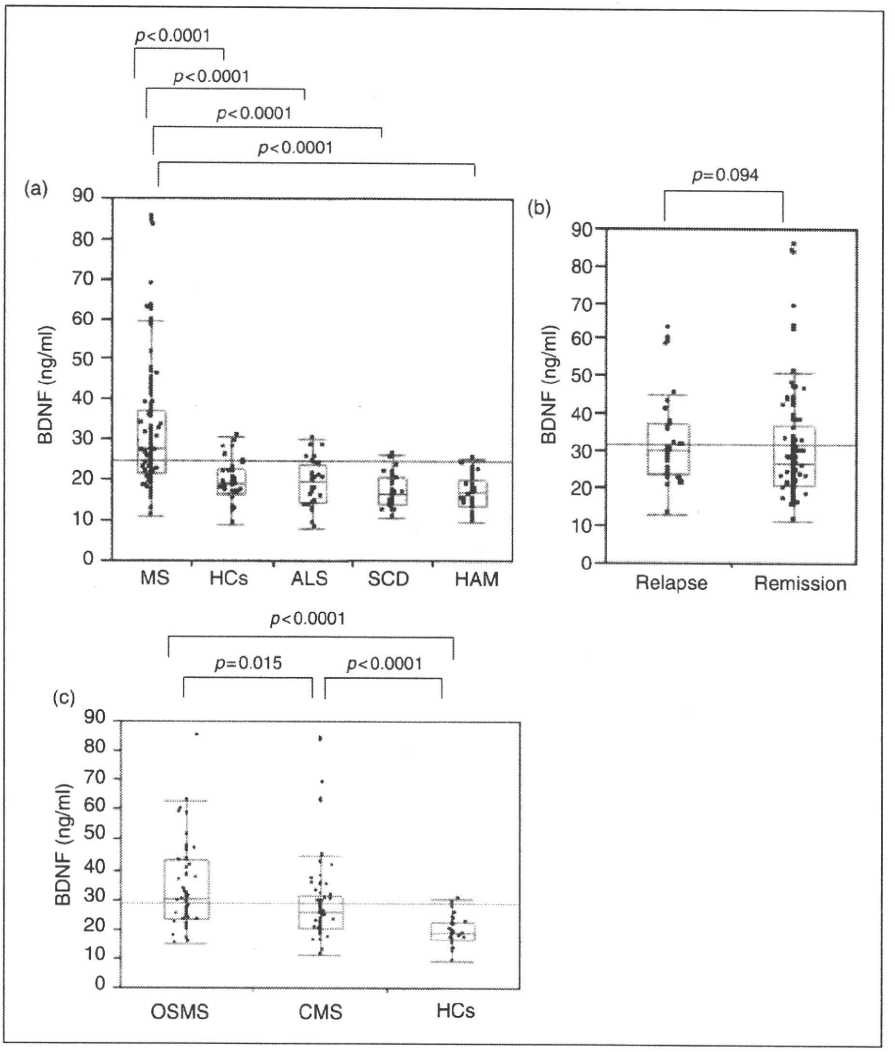

Figure 1. Serum BDNF levels determined by conventional ELISA. MS patients show significantly higher serum BDNF levels compared with HCs, and patients with ALS, SCD, and HAM (A). Serum BDNF levels tends to be higher in the relapse phase than in the remission phase (B). Serum BDNF levels are significantly higher in OSMS patients than in CMS patients, although both OSMS and CMS patients show significantly increased levels of BDNF compared with HCs (C).
ALS, amyotrophic lateral sclerosis; BDNF, brain-derived neurotrophic factor; CMS, conventional multiple sclerosis; ELISA, enzyme-linked immunosorbent assay; HAM, human T-cell lymphotropic virus associated myelopathy; HCs, healthy controls; MS, multiple sclerosis; NS, not significant; OSMS, opticospinal multiple sclerosis; SCD, spinocerebellar degeneration.

Figure 1B, C). Exclusion of anti-AQP4 antibody-positive patients and those who met the NMO criteria gave essentially the same results. Serum BDNF levels were not significantly different between RRMS (mean ± SD = 32 ± 14.9) and SPMS patients (mean ± SD = 24.9 ± 4.9), in part due to the small sample size of SPMS patients, while none of the SPMS patients had BDNF levels higher than 1 SD above the mean level in RRMS patients (Supplementary Figure 1D).

Correlation of serum BDNF levels with clinical parameters

Serum BDNF level showed a significant positive correlation with age at examination in HCs ($p = 0.042$,

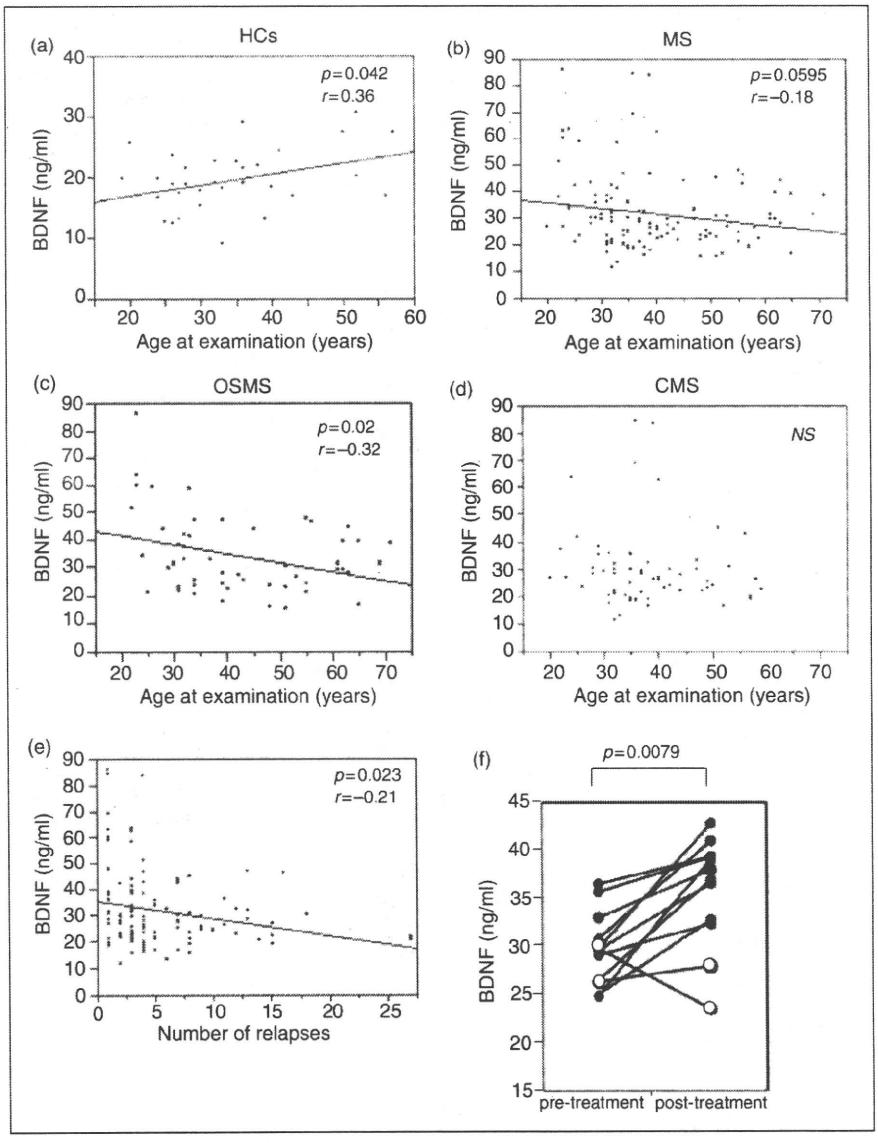

Figure 2. Relationship between serum BDNF levels and clinical parameters. Serum BDNF levels show a significant positive correlation with age at examination in HCs (A), whereas it tends to decrease with age in MS patients (B). Serum BDNF levels are significantly negatively correlated with age at examination in OSMS patients (C), but not in CMS patients (D). Serum BDNF level is also negatively correlated with the number of relapses (E). Serum BDNF levels are significantly higher after IFNβ treatment than before treatment (F). Closed circles represent responders, while open circles indicate suboptimal responders to IFNβ.
BDNF, brain-derived neurotrophic factor; CMS, conventional multiple sclerosis; HCs, healthy controls; MS, multiple sclerosis; *NS*, not significant; OSMS, opticospinal multiple sclerosis.

$r = 0.36$), while in MS patients it tended to show a weak negative correlation ($p = 0.059$, $r = -0.18$) (Figure 2A, B). Serum BDNF levels were significantly negatively correlated with age at examination in patients with OSMS ($p = 0.02$, $r = -0.32$), but not in those with CMS (Figure 2C, D). Serum BDNF level was negatively correlated with number of relapses ($p = 0.023$, $r = -0.21$) (Figure 2E) but not with disease duration, EDSS scores, or annualized relapse rate (Supplementary Figure 2A–C). There was no significant correlation between serum BDNF levels and EDSS after correcting for age and disease duration by multiple logistic regression analyses (data not shown). In addition, there was no significant correlation of serum BDNF levels at relapse (31 samples) with EDSS scores either at the peak of relapse or at three months after the relapse (Supplementary Figure 2D, E).

K-means cluster analyses revealed three subgroups on the basis of serum BDNF levels: high (58.1–85.2 ng/ml, $n = 10$), medium (31.4–50.8 ng/ml, $n = 30$), and low (11.2–31.0 ng/ml, $n = 76$). Logistic regression analyses of the subgroups identified by K-means clustering disclosed that as compared with the BDNF-low and -medium groups, the BDNF-high group showed significantly younger age at examination ($p = 0.0108$ and $p = 0.0237$, respectively) and fewer relapses ($p = 0.0123$ and $p = 0.0184$, respectively) (Supplementary Figure 3A, B). Disease duration had a tendency to be shorter in the BDNF-high group than in the BDNF-low and -medium groups ($p = 0.0644$ and

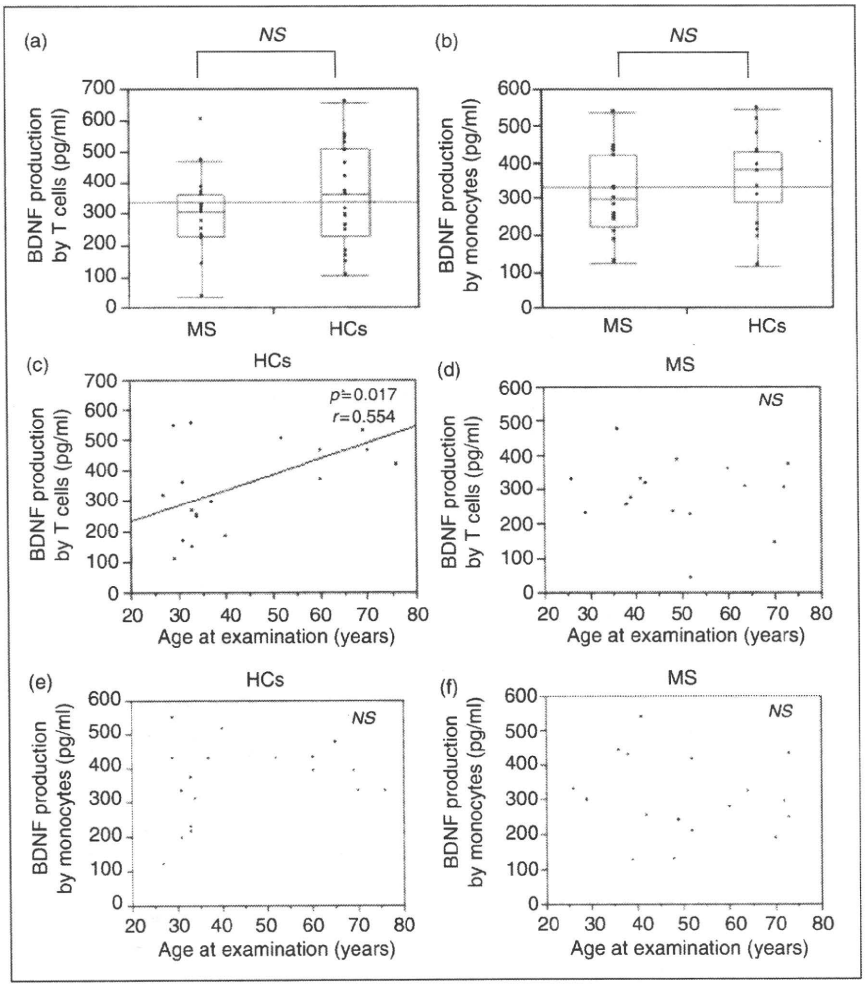

Figure 3. ELISA *in situ* analysis of BDNF production by immunocytes. BDNF production by T cells (A) and monocytes (B) is not significantly different between untreated MS patients and HCs. In HCs, BDNF production by T cells shows a significant positive correlation with age at examination (C). In MS patients, such a correlation between BDNF production and age is not seen (D). BDNF production by monocytes has no correlation with age in either HCs (E) or MS patients (F).
BDNF, brain-derived neurotrophic factor; ELISA, enzyme-linked immunosorbent assay; HCs, healthy controls; MS, multiple sclerosis; *NS*, not significant.

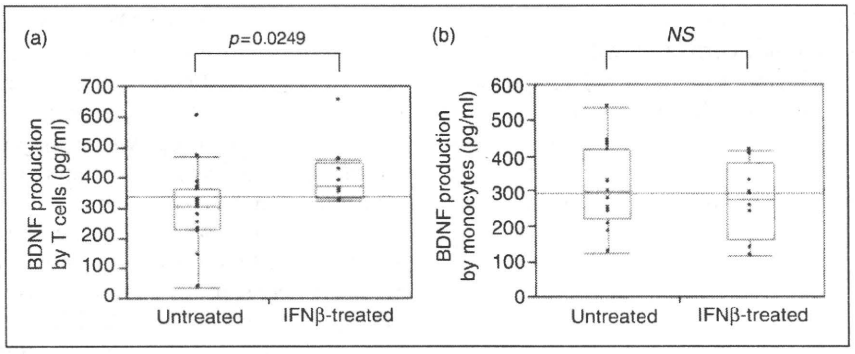

Figure 4. Comparison of BDNF production by immunocytes between IFNβ-treated MS patients and untreated ones. BDNF production by T cells shows a significant increase in IFNβ-treated MS patients compared with untreated ones (A). On the other hand, BDNF production by monocytes is not significantly different between IFNβ-treated and untreated patients (B).
BDNF, brain-derived neurotrophic factor; IFNβ, interferon beta; MS, multiple sclerosis; *NS*: not significant.

$p = 0.0994$, respectively), while neither EDSS scores nor annualized relapse rate differed significantly among the three groups (Supplementary Figure 3C–E). Introduction of IFNβ induced a significant increase in serum BDNF levels in MS patients ($p = 0.0079$) (Figure 2F).

Among the 12 IFNβ-treated patients, 10 were regarded as responders (nine were CMS patients and one was an OSMS patient who neither had anti-AQP4 antibody nor met the diagnostic criteria for NMO)[33] and two were regarded as suboptimal responders (both with CMS). The two suboptimal responders (both with CMS) had the lowest and the second lowest serum BDNF levels among the IFNβ-treated MS patients after IFNβ administration. The only patient who showed a decrease in serum BDNF levels after therapy was one of the two suboptimal responders, who had the lowest serum BDNF level after IFNβ administration.

BDNF production from peripheral blood immunocytes by in situ ELISA

Levels of BDNF production by T cells and monocytes were not significantly different between untreated MS patients and HCs (Figure 3A, B). In HCs, BDNF production by T cells demonstrated a significant positive correlation with age at examination ($p = 0.017$, $r = 0.554$), whereas in MS patients, such a correlation was not seen (Figure 3C, D). By contrast, BDNF production by monocytes had no correlation with age in either MS patients or HCs (Figure 3E, F). BDNF production by either T cells or monocytes was not correlated with disease duration, number of relapses, annualized relapse rate, or EDSS scores (data not shown). Levels of BDNF production by T cells and monocytes were not significantly different between RRMS and SPMS patients (data not shown). BDNF production by T cells was significantly increased in IFNβ-treated MS patients compared with untreated ones ($p = 0.0249$), while BDNF production by monocytes did not differ significantly with IFNβ treatment (Figure 4A, B). Among IFNβ-treated MS patients, six responders and two suboptimal responders showed similar levels of BDNF production by either T cells or monocytes (data not shown).

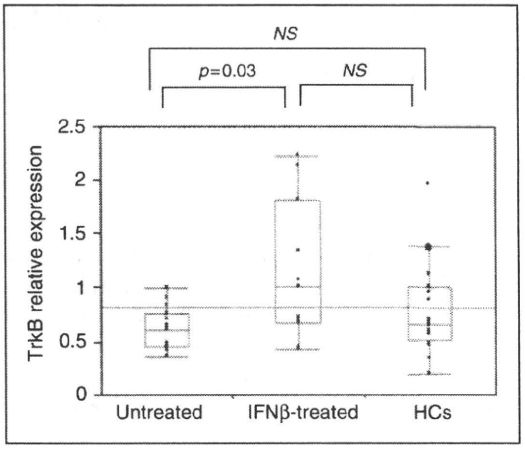

Figure 5. Real-time PCR analysis of *TrkB* expression levels. *TrkB* expression levels in PBMCs are significantly higher in IFNβ-treated MS patients than untreated ones, but are not significantly different either between untreated MS patients and HCs or between IFNβ-treated MS patients and HCs.
BDNF, brain-derived neurotrophic factor; HCs, healthy controls; IFNβ, interferon beta; MS, multiple sclerosis; *NS*, not significant.

Real-time PCR analysis of TrkB expression levels

TrkB expression levels in PBMCs were significantly higher in IFNβ-treated MS patients than in untreated ones ($p = 0.03$), but were not significantly different either between untreated MS patients and HCs or between

IFNβ-treated MS patients and HCs (Figure 5). *TrkB* expression levels were not correlated with age, disease duration, number of relapses, annualized relapse rate, or EDSS scores (data not shown). *TrkB* expression levels were not significantly different either between RRMS and SPMS patients or between the eight responders and the three suboptimal responders (data not shown).

Discussion

In the present study, we found that, compared with controls, BDNF levels in the sera of RRMS patients were significantly higher, especially in younger patients with fewer relapses and mild disease. Interestingly, a significant positive correlation of BDNF levels with age was found in HCs, whereas a trend toward a negative correlation was observed in MS patients. Such a reverse trend was apparent in OSMS patients while it was not in CMS patients. However, even the lower levels in older MS patients were compatible with those in HCs at the same ages. Thus, it is suggested that serum BDNF levels are increased in younger MS patients with an early disease course, but decreased in older patients in the late stage of the disease to the levels seen in HCs of similar ages. Thus, the reverse trend of BDNF levels in relation to age appears to be explained by the existence of young MS patients with high BDNF levels, but without anti-AQP4 antibody.

Our results are in line with previous studies showing that the PBMCs of RRMS patients produce more BDNF than those of SPMS patients.[20,23] Furthermore, BDNF levels in our series showed a tendency to be higher at relapse than in remission, which is in accordance with previous results demonstrating that BDNF production by PBMCs is higher in the active phase than in the stable phase in RRMS patients.[20,21] Therefore, at least in young patients, acute inflammation appears to induce enhanced BDNF production in peripheral blood during early relapses in MS patients, which is consistent with the concept of neuroprotective immunity.[7] Because BDNF production appears to decline during later relapses, such neuroprotective immunity may contribute to good recovery and tissue repair only in the early course of the disease. However, our results are somewhat discrepant from those studies reporting similar or lower serum BDNF levels in MS patients compared with controls.[18,19] Differences in the proportion of younger patients or ethnic backgrounds may account for these discrepancies. In the present study, considerable numbers of our MS patients presented with an OSMS phenotype, and most of these were seronegative for anti-AQP4 antibody (68% were seronegative), as reported previously.[31,32] Moreover, OSMS patients had significantly greater serum BDNF levels than CMS patients. It is the OSMS patients who show a significant negative correlation of BDNF level with age. Thus, young OSMS patients without anti-AQP4 antibody in the early course of the disease appear to have high BDNF levels, which may relate to the rare occurrence of a progressive course reflecting neuroaxonal degeneration in this subgroup.[35,36]

We could not find any increase in BDNF production by peripheral blood T cells or monocytes by an *in situ* ELISA, whereas previous studies have shown increased BDNF production by PBMCs.[21,27] Differences in assay methods, stimuli employed, and the number of patients studied may in part explain these discrepancies. Hamamcioglu and Reder[23] reported a decrease in BDNF production by unstimulated PBMCs in SPMS patients compared with HCs. Petereit et al.[27] described an increase in BDNF production by phorbol myristate acetate (PMA)- and ionomycin-stimulated PBMCs in RRMS patients in remission compared with HCs. Caggiula et al.[21] disclosed that BDNF production by unstimulated PBMCs in RRMS patients was higher in relapse than in remission. Lalive et al.[18] revealed that BDNF levels in PBMC lysates were higher in lysates of PBMCs from IFNβ-treated RRMS patients than in those from untreated ones. All of the above-mentioned studies were done with unseparated PBMCs using an ELISA of the culture supernatants or cell lysates, whereas we separated PBMCs into T cells and monocytes and employed an *in situ* ELISA. The only other study using separated T cells and monocytes, performed by Azoulay et al,[24] examined only two MS patients, and found similar BDNF levels to those in controls, which is in accord with our results.

In our hands, BDNF production by only T cells significantly increased with age in HCs, which may relate to an age-associated increase in serum BDNF levels in HCs. However, such a trend was completely absent in MS patients. Therefore, BDNF production by T cells in aged MS patients may be dampened, and this may partly account for the age-associated decrease in serum BDNF levels in MS patients. It is also possible that BDNF production may be up-regulated in other cell populations, such as B cells, platelets, and eosinophils, which are also known to produce it.[11,37,38] Further studies on these populations are required to clarify the source of elevated BDNF levels in sera from young MS patients. Alternatively, secreted BDNF may not be utilized efficiently in the periphery. However, because *TrkB* expression levels in PBMCs from untreated MS patients did not differ significantly from those in PBMCs obtained from HCs, a comparison that had not previously been made, this possibility seems less likely.

Several reports have indicated that serum BDNF levels are lower in drug-free subjects suffering from major depression than in healthy controls.[39–42] In our

series, no MS patient had major depression at the time of examination. However, follow-up studies may be necessary to clarify whether low BDNF patients experience future development of depression.

Finally, for the first time, we have shown that IFNβ treatment in MS patients potentiates BDNF production in T cells but not in monocytes, which may account for the increase in serum BDNF levels after introduction of IFNβ observed in the present study. These findings are in line with the results of previous studies showing that BDNF production by PBMCs from IFNβ-treated patients is enhanced,[18] and that GA, another disease-modifying drug for MS, also potentiates BDNF production in T cells.[43] We also found that *TrkB* expression levels in PBMCs were significantly elevated in IFNβ-treated MS patients compared with untreated ones. Among PBMCs, *TrkB* expression has been reported in human T[44,45] and B cells.[46] Although there is a need for further study to exactly identify the cell populations showing an enhancement of *TrkB* expression by IFNβ, IFNβ-induced enhanced BDNF production in T cells and increased levels of serum BDNF thus appear to at least partly contribute to its beneficial effects in MS. Therefore, enhancement of neuroprotective immunity, as seen in early MS patients, may be an important target for future disease-modifying therapy in MS.

Funding

This work was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology, Japan; the Neuroimmunological Disease Research Committee; and the Ministry of Health, Labour and Welfare, Japan.

Conflict of interest statement

None declared.

Acknowledgments

We thank Dr Junji Kishimoto for the assistance with the statistical analyses.

References

1. Compston A and Coles A. Multiple sclerosis. *Lancet* 2008; 372: 1502–1517.
2. Lewin GR and Barde YA. Physiology of the neurotrophins. *Annu Rev Neurosci* 1996; 19: 289–317.
3. Gravel C, Gotz R, Lorrain A and Sendtner M. Adenoviral gene transfer of ciliary neurotrophic factor and brain-derived neurotrophic factor leads to long-term survival of axotomized motor neurons. *Nat Med* 1997; 3: 765–770.
4. McTigue DM, Horner PJ, Stokes BT and Gage FH. Neurotrophin-3 and brain-derived neurotrophic factor induce oligodendrocyte proliferation and myelination of regenerating axons in the contused adult rat spinal cord. *J Neurosci* 1998; 18: 5354–5365.
5. Kerschensteiner M, Gallmeier E, Behrens L, et al. Activated human T cells, B cells, and monocytes produce brain-derived neurotrophic factor in vitro and in inflammatory brain lesions: a neuroprotective role of inflammation? *J Exp Med* 1999; 189: 865–870.
6. Stadelmann C, Kerschensteiner M, Misgeld T, Bruck W, Hohlfeld R and Lassmann H. BDNF and gp145trkB in multiple sclerosis brain lesions: neuroprotective interactions between immune and neuronal cells? *Brain* 2002; 125: 75–85.
7. Kerschensteiner M, Stadelmann C, Dechant G, Wekerle H and Hohlfeld R. Neurotrophic cross-talk between the nervous and immune systems: implications for neurological diseases. *Ann Neurol* 2003; 53: 292–304.
8. Rosenfeld RD, Zeni L, Haniu M, et al. Purification and identification of brain-derived neurotrophic factor from human serum. *Protein Expr Purif* 1995; 6: 465–471.
9. Radka SF, Holst PA, Fritsche M and Altar CA. Presence of brain-derived neurotrophic factor in brain and human and rat but not mouse serum detected by a sensitive and specific immunoassay. *Brain Res* 1996; 709: 122–301.
10. Fujimura H, Altar CA, Chen R, et al. Brain-derived neurotrophic factor is stored in human platelets and released by agonist stimulation. *Thromb Haemost* 2002; 87: 728–734.
11. Nassenstein C, Braun A, Erpenbeck VJ, et al. The neurotrophins nerve growth factor, brain-derived neurotrophic factor, neurotrophin-3, and neurotrophin-4 are survival and activation factors for eosinophils in patients with allergic bronchial asthma. *J Exp Med* 2003; 198: 455–467.
12. Nakahashi T, Fujimura H, Altar CA, et al. Vascular endothelial cells synthesize and secrete brain-derived neurotrophic factor. *FEBS Lett* 2000; 470: 113–117.
13. Donovan MJ, Miranda RC, Kraemer R, et al. Neurotrophin and neurotrophin receptors in vascular smooth muscle cells. Regulation of expression in response to injury. *Am J Pathol* 1995; 147: 309–324.
14. Leeksma CH and Cohen JA. Determination of the life of human blood platelets using labelled diisopropylfluorophosphanate. *Nature* 1955; 175: 552–553.
15. Poduslo JF and Curran GL. Permeability at the blood-brain and blood-nerve barriers of the neurotrophic factors: NGF, CNTF, NT-3, BDNF. *Brain Res Mol Brain Res* 1996; 36: 280–286.
16. Pan W, Banks WA, Fasold MB, Bluth J and Kastin AJ. Transport of brain-derived neurotrophic factor across the blood-brain barrier. *Neuropharmacology* 1998; 37: 1553–1561.
17. Karege F, Schwald M and Cisse M. Postnatal developmental profile of brain-derived neurotrophic factor in rat brain and platelets. *Neurosci Lett* 2002; 328: 261–264.
18. Lalive PH, Kantengwa S, Benkhoucha M, Juillard C and Chofflon M. Interferon-beta induces brain-derived neurotrophic factor in peripheral blood mononuclear cells of multiple sclerosis patients. *J Neuroimmunol* 2008; 197: 147–151.
19. Azoulay D, Vachapova V, Shihman B, Miler A and Karni A. Lower brain-derived neurotrophic factor in

serum of relapsing remitting MS: reversal by glatiramer acetate. *J Neuroimmunol* 2005; 167: 215–218.
20. Sarchielli P, Greco L, Stipa A, Floridi A and Gallai V. Brain-derived neurotrophic factor in patients with multiple sclerosis. *J Neuroimmunol* 2002; 132: 180–188.
21. Caggiula M, Batocchi AP, Frisullo G, et al. Neurotrophic factors and clinical recovery in relapsing–remitting multiple sclerosis. *Scand J Immunol* 2005; 62: 176–182.
22. Weinstock-Guttman B, Zivadinov R, Tamano-Blanco M, et al. Immune cell BDNF secretion is associated with white matter volume in multiple sclerosis. *J Neuroimmunol* 2007; 188: 167–174.
23. Hamamcioglu K and Reder AT. Interferon-beta regulates cytokines and BDNF: greater effect in relapsing than in progressive multiple sclerosis. *Mult Scler* 2007; 13: 459–470.
24. Azoulay D, Urshansky N and Karni A. Low and dysregulated BDNF secretion from immune cells of MS patients is related to reduced neuroprotection. *J Neuroimmunol* 2008; 195: 186–193.
25. Sarchielli P, Zaffaroni M, Floridi A, et al. Production of brain-derived neurotrophic factor by mononuclear cells of patients with multiple sclerosis treated with glatiramer acetate, interferon-beta 1a, and high doses of immunoglobulins. *Mult Scler* 2007; 13: 313–331.
26. Blanco Y, Moral EA, Costa M, et al. Effect of glatiramer acetate (Copaxone) on the immunophenotypic and cytokine profile and BDNF production in multiple sclerosis: a longitudinal study. *Neurosci Lett* 2006; 406: 270–275.
27. Petereit HF, Lindemann H and Schoppe S. Effect of immunomodulatory drugs on in vitro production of brain-derived neurotrophic factor. *Mult Scler* 2003; 9: 16–20.
28. Polman CH, Reingold SC, Edan G, et al. Diagnostic criteria for multiple sclerosis: 2005 revisions to the "McDonald Criteria". *Ann Neurol* 2005; 58: 840–846.
29. Kurtzke JF. Rating neurologic impairment in multiple sclerosis: an expanded disability status scale (EDSS). *Neurology* 1983; 33: 1444–1452.
30. Kira J, Kanai T, Nishimura Y, et al. Western versus Asian types of multiple sclerosis: immunogenetically and clinically distinct disorders. *Ann Neurol* 1996; 40: 569–574.
31. Matsuoka T, Matsushita T, Kawano Y, et al. Heterogeneity of aquaporin-4 autoimmunity and spinal cord lesions in multiple sclerosis in Japanese. *Brain* 2007; 130: 1206–1223.
32. Matsushita T, Isobe N, Matsuoka T, et al. Aquaporin-4 autoimmune syndrome and anti-aquaporin-4 antibody-negative opticospinal multiple sclerosis in Japanese. *Mult Scler* 2009; 15: 834–847.
33. Wingerchuk DM, Lennon VA, Pittock SJ, Lucchinetti CF and Weinshenker BG. Revised diagnostic criteria for neuromyelitis optica. *Neurology* 2006; 66: 1485–1489.
34. Pozzilli C, Prosperini L, Sbardella E, De Giglio L, Onesti E and Tomassini V. Post-marketing survey on clinical response to interferon beta in relapsing multiple sclerosis: the Roman experience. *Neurol Sci* 2005; 26: S174–S178.
35. Kira J. Multiple sclerosis in the Japanese population. *Lancet Neurol* 2003; 2: 117–127.
36. Ishizu T, Kira J, Osoegawa M, et al. Heterogeneity and continuum of multiple sclerosis phenotypes in Japanese according to the results of the fourth nationwide survey. *J Neurol Sci* 2009; 280: 22–28.
37. Edling AE, Nanavati T, Johnson JM and Tuohy VK. Human and murine lymphocyte neurotrophin expression is confined to B cells. *J Neurosci Res* 2004; 77: 709–717.
38. Yamamoto H and Gurney ME. Human platelets contain brain-derived neurotrophic factor. *J Neurosci* 1990; 10: 3469–3478.
39. Karege F, Perret G, Bondolfi G, Schwald M, Bertschy G and Aubry JM. Decreased serum brain-derived neurotrophic factor levels in major depressed patients. *Psychiatry Res* 2002; 109: 143–148.
40. Shimizu E, Hashimoto K, Okamura N, et al. Alterations of serum levels of brain-derived neurotrophic factor (BDNF) in depressed patients with or without antidepressants. *Biol Psychiatry* 2003; 54: 70–75.
41. Karege F, Bondolfi G, Gervasoni N, Schwald M, Aubry JM and Bertschy G. Low brain-derived neurotrophic factor (BDNF) levels in serum of depressed patients probably results from lowered platelet BDNF release unrelated to platelet reactivity. *Biol Psychiatry* 2005; 57: 1068–1072.
42. Piccinni A, Marazziti D, Catena M, et al. Plasma and serum brain-derived neurotrophic factor (BDNF) in depressed patients during 1 year of antidepressant treatments. *J Affect Disord* 2008; 105: 279–283.
43. Linker R, Lee D-H, Siglienti I and Gold R. Is there a role for neurotrophins in the pathology of multiple sclerosis? *J Neurol* 2007; 254(Suppl): I33–I40.
44. Besser M and Wank R. Cutting edge: clonally restricted production of the neurotrophins brain-derived neurotrophic factor and neurotrophin-3 mRNA by human immune cells and Th1/Th2-polarized expression of their receptors. *J Immunol* 1999; 162: 6303–6306.
45. De Santi L, Cantalupo L, Tassi M, Raspadori D, Cioni C and Annunziata P. Higher expression of BDNF receptor gp145trkB is associated with lower apoptosis intensity in T cell lines in multiple sclerosis. *J Neurol Sci* 2009; 277: 65–70.
46. Schenone A, Gill JS, Zacharias DA and Windebank AJ. Expression of high- and low-affinity neurotrophin receptors on human transformed B lymphocytes. *J Neuroimmunol* 1996; 64: 141–149.

Journal of the Neurological Sciences 295 (2010) 41–45

Contents lists available at ScienceDirect

Journal of the Neurological Sciences

journal homepage: www.elsevier.com/locate/jns

CSF angiotensin II and angiotensin-converting enzyme levels in anti-aquaporin-4 autoimmunity

T. Matsushita [a], N. Isobe [a], M. Kawajiri [b], M. Mogi [c], K. Tsukuda [c], M. Horiuchi [c], Y. Ohyagi [c], J. Kira [a,*]

[a] Department of Neurology, Neurological Institute, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan
[b] Department of Neurology, Saiseikai Fukuoka General Hospital, Fukuoka, Japan
[c] Department of Molecular Cardiovascular Biology and Pharmacology, Ehime University, Graduate School of Medicine, Ehime, Japan

ARTICLE INFO

Article history:
Received 4 March 2010
Received in revised form 11 May 2010
Accepted 17 May 2010
Available online 11 June 2010

Keywords:
Angiotensin II
Multiple sclerosis
Neuromyelitis optica
Anti-aquaporin-4 antibody
Cerebrospinal fluid

ABSTRACT

Background: Anti-aquaporin-4 (AQP4) antibody targets perivascular astrocyte foot processes, which contain abundant angiotensinogen, a precursor of angiotensin II, angiotensin-converting enzyme (ACE) and ACE2.
Objective: To disclose any abnormality in the intrathecal angiotensin II metabolic pathway in Japanese patients with neuromyelitis optica (NMO) or NMO spectrum disorders (NMOs) and positive for anti-AQP4 antibody.
Methods: We measured CSF angiotensin II, ACE and ACE2 levels in 15 anti-AQP4 antibody-positive patients with NMO or NMOs, 21 anti-AQP4 antibody-negative multiple sclerosis (MS) patients, 32 patients with other neurological diseases (OND) and 24 non-neurologic controls, using established ELISAs.
Results: CSF angiotensin II levels were lower in patients with NMO/NMOs (2.01 ± 1.82 pg/ml) and those with MS (3.15 ± 1.67 pg/ml) than in the OND (5.41 ± 2.34 pg/ml) and control groups (6.71 ± 2.65 pg/ml) ($P^{corr} < 0.005$). The difference in CSF angiotensin II levels between NMO/NMOs and MS patients was nearly significant ($P^{uncorr} = 0.052$). In NMO/NMOs and MS patients, angiotensin II levels were negatively correlated with CSF/serum albumin ratio ($P < 0.05$). ACE levels in CSF were lower in patients with NMO/NMOs (34.3 ± 5.61 ng/ml) than in MS patients (42.5 ± 8.19 ng/ml, $P^{corr} = 0.035$) and controls (44.7 ± 4.02 ng/ml, $P^{corr} < 0.0003$) while ACE2 levels were lower in NMO/NMOs (1.13 ± 0.49 ng/ml) and MS (1.75 ± 0.86 ng/ml) patients than in controls (2.76 ± 0.23 ng/ml, $P^{corr} < 0.001$ for both).
Conclusion: CSF angiotensin II, ACE, and ACE2 levels are decreased in NMO/NMOs patients with anti-AQP4 antibody, reflecting severe destruction of perivascular astrocytes.

1. Introduction

Angiotensin II is a potent vasoactive substance of the renin–angiotensin system (RAS), which plays an important role in regulating blood volume and systemic vascular resistance. Our previous work has demonstrated that angiotensin II attenuates brain damage and enhances neural differentiation via its receptor expressed on neuronal cells [1,2]. Moreover, we recently discovered reduced levels of angiotensin II in cerebrospinal fluid (CSF) from patients with multiple sclerosis (MS) [3], suggesting that the RAS may also be involved in the abnormal neural damage and repair processes in MS.

Angiotensin II is produced from its precursor, angiotensinogen; renin produces angiotensin I from angiotensinogen, and then angiotensin-converting enzyme (ACE) converts angiotensin I to angiotensin II [4]. Recently, a homologue of ACE, ACE2, was discovered; this enzyme cleaves angiotensin II to produce angiotensin-(1–7) (see Fig. 1 for the angiotensin metabolic pathway) [4]. Perivascular astrocytes, a key constituent of the blood–brain barrier (BBB), abundantly express angiotensinogen, ACE and ACE2, and secrete angiotensin II in the CNS [5,6]. Secreted angiotensin II is supposed to up-regulate tight junction proteins in endothelial cells, which play a critical role in keeping the BBB tight [6]. In the chronic stage of MS, perivascular astrocytes are progressively lost [6], rendering the BBB leaky. The reduction of angiotensin II levels in the CSF that we observed may in part reflect such a process [3,7].

Neuromyelitis optica (NMO) is characterized by severe and selective involvement of the optic nerves and spinal cord. A specific IgG against NMO, designated NMO-IgG [8], targets aquaporin-4 (AQP4) on the perivascular astrocyte foot process [9]. We recently reported occurrences of extensive vasogenic edema in patients with anti-AQP4 antibody [10], possibly due to disruption of the AQP4 molecule regulating water flux in the CNS [11]. In Japanese patients with opticospinal MS (OSMS), some of whom also fulfill the proposed criteria for NMO, anti-AQP4 antibody is frequently detected [12,13]. These observations prompted us to study the levels of angiotensin II and its catalytic enzymes in the CSF of anti-AQP4 antibody-positive NMO patients, to uncover the relationship between the presence of anti-AQP4 antibody and BBB dysfunction.

* Corresponding author. Department of Neurology, Neurological Institute, Graduate School of Medical Sciences, Kyushu University, 311 Maidashi, Higashi-ku, Fukuoka 8128582, Japan. Tel.: +81 926425340; fax: +81 926425352.
E-mail address: kira@neuro.med.kyushuu.ac.jp (J. Kira).

0022-510X/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.jns.2010.05.014

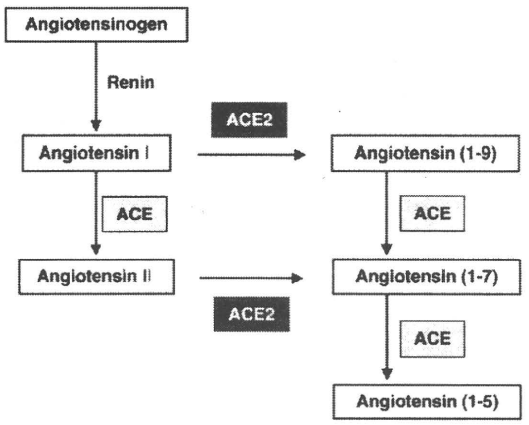

Fig. 1. Schematic illustration of the metabolic pathway of the renin–angiotensin system. ACE = angiotensin-converting enzyme.

2. Materials and methods

2.1. Patients

Patients were examined in the Department of Neurology at Kyushu University Hospital and the Departments of Anesthesiology and Resuscitology at Ehime University Hospital, Japan between 2000 and 2008. Informed consent was obtained from each individual and the study protocol was approved by the ethics committees of both hospitals. We obtained CSF samples from 15 patients with anti-AQP4 antibody, the presence of which was determined by an immunofluorescence method using green fluorescence protein-AQP4 fusion protein-transfected human embryonic kidney cells, as described previously [12,13]. Among them, 12 patients fulfilled the revised NMO criteria [14] while three with myelitis or optic neuritis or both, who did not fully meet the criteria, were considered to have NMO spectrum disorders (NMOs). In addition, 21 MS patients who fulfilled the McDonald's MS criteria [15] and did not have anti-AQP4 antibody, 32 patients with other neurological diseases (OND) and 24 controls were also recruited (Table 1). The MS patients comprised 18 women and three men, while the 15 NMO/NMOs patients were all women. All MS and NMO/NMOs patients showed a relapsing–remitting course, and all CSF specimens were taken at relapse (within 30 days of the initiation of relapse). Among the 21 MS patients, four received interferon β-1b (8 million units every other day) while two received low-to-medium doses of oral prednisolone (5.0 and 10 mg/day) at the time of lumbar puncture. Among the 15 patients with NMO/NMOs, four were treated with interferon β-1b and six were treated with chronic administration of oral prednisolone (2.5–40 mg/day) at the time of lumbar puncture. Among patients with MS and NMO/NMOs, the average ages at examination were 36.4 ± 12.5 (mean ± SD) and 47.9 ± 12.7 years, respectively, which were significantly different ($P = 0.023$). The average disease duration in these groups was 8.0 ± 9.7 and 8.1 ± 5.3 years, respectively (Table 1). Kurtzke's Expanded Disability Status Scale (EDSS) scores [16] ($P = 0.0086$) and the appearance rate of longitudinally extensive spinal cord lesions (LESCLs) extending over three or more vertebral segments during the entire course ($P = 0.0059$) were higher in NMO/NMOs patients than in MS patients. CSF total protein amount and CSF/serum albumin ratio were not different between patients with NMO/NMOs and those with MS ($P > 0.1$), while the IgG index and the positivity rate for oligoclonal IgG bands were higher in MS patients than in NMO/NMOs patients ($P = 0.015$ and $P = 0.0006$, respectively). The OND group comprised 15 patients with chronic inflammatory demyelinating polyneuropathy (CIDP), nine with spinocerebellar degeneration and eight with Guillain–Barré syndrome. The control group of 24 patients (10 men and 14 women; age at examination: 45.3 ± 14.6 years) did not have any neurological or other diseases that might affect the angiotensin II levels in the CSF (18 patients underwent lumbar puncture during spinal anesthesia for operations for either urological or gynecological disorders; the other six cases underwent lumbar puncture because of suspected neurological diseases, but turned out to be neurologically normal). None of the patients or controls had hypertension, hypotension, or a metabolic disorder, and none took antihypertensive drugs. CSF samples were obtained by non-traumatic lumbar puncture and centrifuged within 30 min at 800 rpm at 4 °C for 5 min. The liquid phase of the CSF, which excluded sedimented cells, was stored at −80 °C until use.

Table 1
Demographic features of patients.

	NMO/NMOs	MS	OND
No. of patients	15	21	32
Men/women	0/15	3/18	25/7
Age at examination, years, mean ± SD	47.9 ± 12.7*	36.4 ± 12.5*	52.5 ± 16.4
Disease duration, years, mean ± SD	8.1 ± 5.3	8.0 ± 9.7	NA
Number of relapses, mean ± SD	9.7 ± 8.1	5.4 ± 3.9	NA
EDSS score, mean ± SD	5.7 ± 1.7*	3.9 ± 2.1*	NA
LESCLs during the entire course, *n* (%)	12 (80.0)*	6 (28.6)*	NA
CSF			
Cell count per μl, mean ± SD	9.1 ± 13.7	5.0 ± 6.6	1.5 ± 1.9
Total protein amount, mg/dl, mean ± SD	39.3 ± 19.1	33.5 ± 17.1	66.9 ± 38.7
CSF/serum albumin ratio, $\times 10^{-3}$, mean ± SD	75.1 ± 38.9	53.6 ± 23.2	NA
IgG index, mean ± SD	0.56 ± 0.082*	0.74 ± 0.17*	NA
Oligoclonal IgG bands	0 (0.0)*	12 (57.1)*	NA

EDSS = Kurtzke's Expanded Disability Status Scale; LESCLs = longitudinally extensive spinal cord lesions; MS = multiple sclerosis; NMO = neuromyelitis optica; NMOs = neuromyelitis optica spectrum disorders; OND = other neurological diseases; NA = not applicable.

* $P < 0.05$ in comparison between MS and NMO/NMOs patients. Percentages in parentheses.

2.2. Measurement of angiotensin II, ACE and ACE2 concentrations in the CSF

Angiotensin II, ACE and ACE2 concentrations in CSF were measured by established ELISA systems for angiotensin II (SPI-bio, Montigny-le-Bretonneux, France), ACE (R&D Systems, Minneapolis, MN), and ACE2 (AdipoGen Inc., Seoul, Korea), as described previously [3,7]. According to the manufacturer's data, the precision, recovery, and linearity of these assays are as follows: for the human angiotensin II ELISA, the intra-assay coefficients of variation (CVs) are 2.0–10.0%, inter-assay CVs are 5.0–15.0%, the recovery range is 80–115% and the linearity is around 90% of expected; for the human ACE ELISA, the intra-assay CVs are 3.4–4.0%, inter-assay CVs are 4.9–7.7%, the recovery range is 91–106% and linearity is around 105% of expected; for the human ACE2 ELISA, the intra-assay CVs are 5.3–9.9%, inter-assay CVs are 5.4–10.8%, the recovery range is 81–113%, and linearity is around 100% of expected.

2.3. Statistical analysis

Statistical analyses of age at examination, disease duration, number of relapses, EDSS score, CSF cell count and total protein amount, CSF/serum albumin ratio, and IgG index between two subgroups were performed using the Mann–Whitney *U*-test. Comparisons of sex and numbers of LESCLs between two subgroups were performed using Fisher's exact probability test. Statistical analyses of angiotensin II, ACE and ACE2 levels were initially performed using the Kruskal–Wallis *H*-test. When differences were significant, the Mann–Whitney *U*-test was used to determine the significance of differences between groups. Uncorrected *P* values were corrected by multiplying them by the number of comparisons (Bonferroni–Dunn's correction)

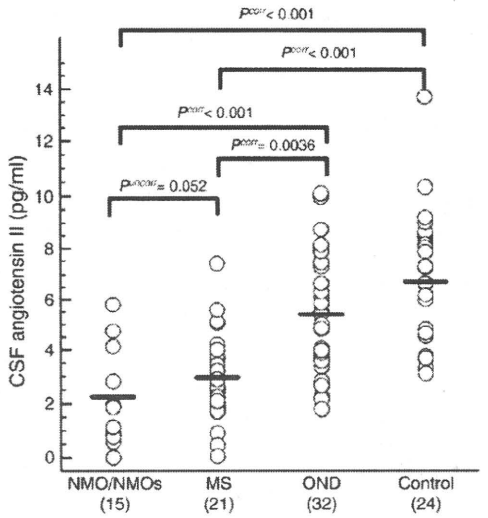

Fig. 2. Angiotensin II levels in the CSF from patients with NMO/NMOs, MS, OND and controls. Bars indicate the mean concentration in each group. MS = multiple sclerosis; NMO = neuromyelitis optica; NMOs = neuromyelitis optica spectrum disorders; OND = other neurological diseases.

to calculate corrected *P* values (P^{corr}). Spearman's rank correlation test was used for statistical analyses of the correlations between angiotensin II levels in CSF and various clinical parameters. In all assays, significance was set at $P<0.05$.

3. Results

3.1. Angiotensin II levels in the CSF

Angiotensin II levels in the CSF were 2.01 ± 1.82 pg/ml in the NMO/NMOs group, 3.15 ± 1.67 pg/ml in the MS group, 5.41 ± 2.34 pg/ml in the OND group and 6.71 ± 2.65 pg/ml in the control group (Fig. 2). The levels were significantly lower in the NMO/NMOs group and the MS group than in the OND group ($P^{corr}=0.0036$ and $P^{corr}<0.001$) and the control group ($P^{corr}<0.001$ and $P^{corr}<0.001$). The difference in CSF angiotensin II levels between NMO/NMOs and MS patients was nearly significant ($P^{uncorr}=0.052$). The level in the OND group was not different from that in the control group ($P^{corr}>0.1$). Exclusion of patients on prednisolone therapy gave practically the same results (data not shown). Among patients with MS or NMO/NMOs, there was no significant difference in angiotensin II levels between treated (prednisolone or interferon β-1b) and untreated patients ($P>0.1$).

3.2. Correlations between angiotensin II levels in the CSF and clinical parameters

Among patients with MS and NMO/NMOs, there were significant correlations of CSF angiotensin II levels with the CSF total protein amount ($R=-0.44$, $P=0.0078$) and CSF/serum albumin ratio ($R=-0.45$, $P=0.030$) (Fig. 3A, B). No significant correlations with clinical parameters, such as age at examination, disease duration, number of relapses, EDSS score, CSF cell count and IgG index, were noted in patients with NMO/NMOs.

3.3. ACE and ACE2 levels in the CSF

ACE levels in the CSF were 34.3 ± 5.61 ng/ml in NMO/NMOs patients, 42.5 ± 8.19 ng/ml in MS patients, and 44.7 ± 4.02 ng/ml in controls (Fig. 4A). The levels were significantly lower in NMO/NMOs patients than in MS patients ($P^{corr}=0.035$) and controls ($P^{corr}<0.0003$), while they were not different between MS patients and controls ($P>0.1$). ACE2 levels

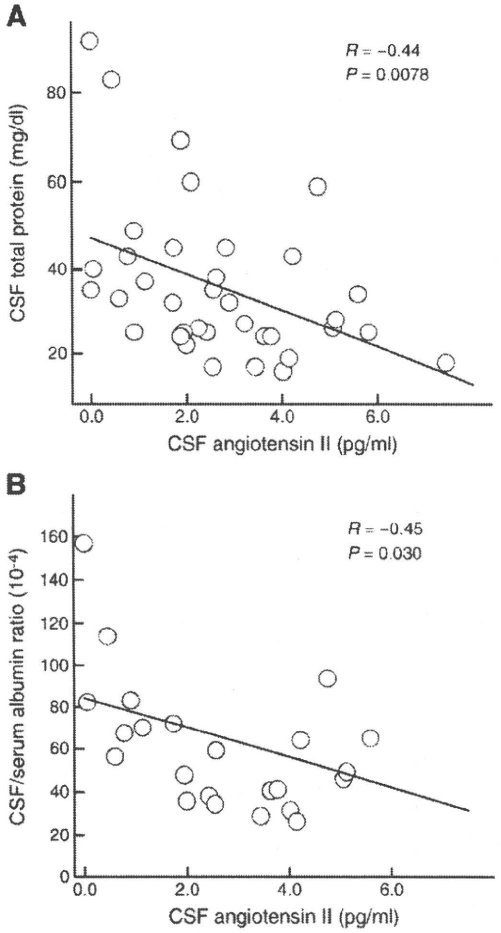

Fig. 3. (A) Correlation between angiotensin II levels and total protein amounts in the CSF in NMO/NMOs and MS patients. The number of patients represented is 36. (B) Correlations between CSF angiotensin II levels and the CSF/serum albumin ratio in NMO/NMOs and MS patients. The number of patients represented is 23.

in the CSF were 1.13 ± 0.49 ng/ml in NMO/NMOs patients, 1.75 ± 0.86 ng/ml in MS patients, and 2.76 ± 0.23 ng/ml in controls (Fig. 4B). The levels were significantly lower in patients with NMO/NMOs and MS than in controls ($P^{corr}<0.0003$ and $P^{corr}=0.0006$, respectively), while they did not differ between NMO/NMOs and MS patients ($P>0.1$). Among patients with MS or NMO/NMOs, neither ACE nor ACE2 levels were different between treated (prednisolone or interferon β-1b) and untreated patients ($P>0.1$ and $P=0.097$, respectively).

4. Discussion

In this study, we uncovered severe reductions in angiotensin II, ACE and ACE2 levels in the CSF of NMO/NMOs patients. As an increase in CSF angiotensin II levels has been reported in patients with essential hypertension [17], we carefully excluded those with hypertension or on antihypertensive drugs. Moreover, we obtained essentially the same results even when removing those on corticosteroids. Therefore, we consider that the decrease in angiotensin II levels in the CSF of NMO/NMOs patients is not an artifact.

Angiotensin II levels became lower as the CSF/serum albumin ratio became higher, indicating that angiotensin II levels successively decrease as BBB breakdown becomes more severe. There was a more pronounced decrease in angiotensin II levels in NMO/NMOs patients, who were all positive for anti-AQP4 antibody in this study, than in MS patients, suggesting more severe BBB disruption in the former group.

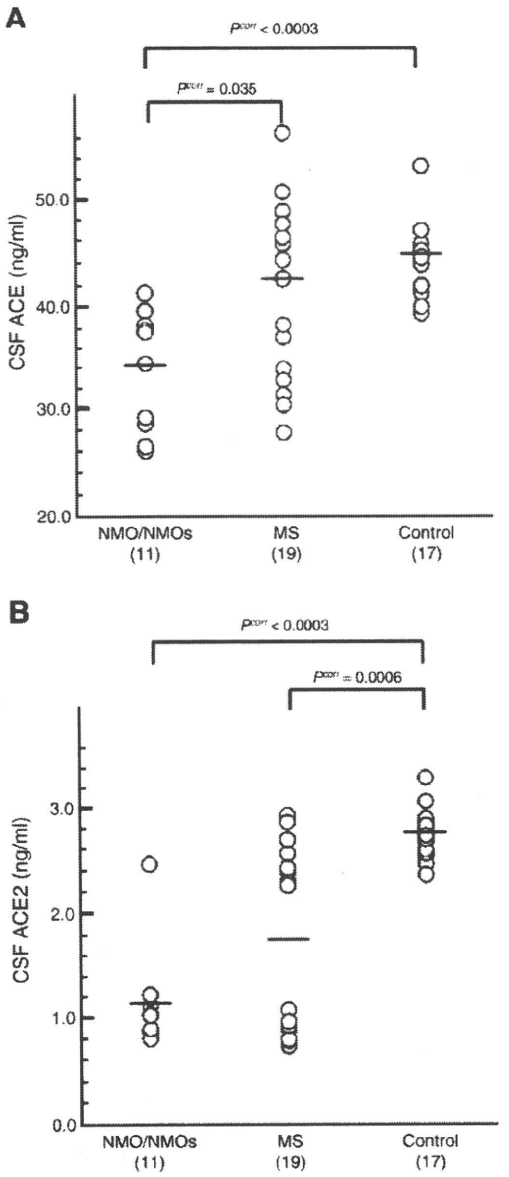

Fig. 4. ACE (A) and ACE2 (B) levels in the CSF from patients with NMO/NMOs and MS, and controls. Bars indicate the mean concentration in each group. ACE = angiotensin-converting enzyme; NMO = neuromyelitis optica; NMOs = neuromyelitis optica spectrum disorders.

We also found that both ACE and ACE2 levels were significantly decreased in NMO/NMOs patients. Because perivascular astrocytes are the main reservoirs of angiotensin II precursors and its metabolizing enzymes [5,6], the decrease in the levels of angiotensin II-metabolizing enzymes in NMO/NMOs patients' CSF suggests more severe destruction of astrocytes among anti-AQP4 antibody-positive patients than among MS patients without the antibody. Indeed, anti-AQP4 antibody, which mostly belongs to the IgG1 subclass [13], is supposed to disrupt astrocyte foot processes by fixing complements [18].

We previously reported an increase in the level of ACE and a decrease in the level of ACE2 in MS patients without anti-AQP4 antibody [7], and suggested a compensatory mechanism to be operative in response to a decrease in the level of angiotensin II, namely, up-regulation of the synthesizing enzyme (ACE) and down-regulation of the catabolizing enzyme (ACE2). Although we could not reproduce the increase in ACE level in this series of MS patients, at least the ACE level was not decreased as compared with that in controls. It is possible that perivascular astrocyte destruction in MS is variable among cases, and sufficiently mild for the compensatory mechanism to work in some cases. Such a compensatory mechanism is no way operative in NMO/NMOs.

Wosik et al. [6] recently demonstrated that angiotensinogen knock-out mice had disorganized occludin strands at the level of the BBB, and that the numbers of perivascular astrocytes immunopositive for angiotensinogen were decreased in MS lesions; this decrease was correlated with reduced expression of the tight junction protein occludin. Angiotensin II is thus considered to up-regulate expression of this tight junction protein, which strengthens the BBB. Therefore, the reduction in the level of angiotensin II may in turn contribute to BBB dysfunction through the loss of its up-regulating effect on tight junction proteins in NMO/NMOs patients with anti-AQP4 antibody, who had a tendency for more frequent relapses than MS patients without the antibody [12]. Moreover, a recent study on experimental autoimmune encephalomyelitis using transgenic mice with targeted ablation of reactive astrocytes indicates that lack of perivascular astrocyte scar formation induces severe inflammation, further supporting a protective barrier function of perivascular astrocytes [19]. Grave disruption of perivascular astrocytes in NMO as suggested by the present study may well explain the frequent relapses and severe inflammation in this condition.

Angiotensin II binds two major receptors, angiotensin II type-1 (AT1) and -2 (AT2) receptors. Most of its actions on cardiovascular regulation and fluid balance are mediated by the AT1 receptor. This receptor is also expressed on T cells and macrophages, and angiotensin II induces a Th1 shift via stimulation of AT1 receptors [20]. As in NMO, Th2 cells are assumed to play a contributory role [21,22]: reduction of angiotensin II levels may enhance Th2 responses in NMO/NMOs patients, and thus, aggravate the illness. Angiotensin II also stimulates AT2 receptors expressed on the vascular wall and neuronal cells, and not only protects brain tissues from ischemia, but also enhances neural differentiation [1,2]. Therefore, the decrease in the levels of angiotensin II in NMO/NMOs patients' CSF may also indicate attenuated neuroprotection via the AT2 receptor. It is thus assumed that the marked reduction in the level of CSF angiotensin II in NMO/NMOs patients may result in detrimental effects on the disease process through BBB dysfunction, a Th2 shift, and attenuated neuroprotection.

Acknowledgements

This work was supported in part by grants from the Research Committees of Neuroimmunological Diseases, the Ministry of Health, Labor and Welfare, Japan and from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

References

[1] Mogi M, Li J-M, Iwanami J, Min L-J, Tsukuda K, Iwai M, et al. Angiotensin II type-2 receptor stimulation prevents neural damage by transcriptional activation of methyl methanesulfonate sensitive 2. Hypertension 2006;48:141–8.
[2] Li J-M, Mogi M, Tsukuda K, Tomochika H, Iwanami J, Min L-J, et al. Angiotensin II-induced neural differentiation via angiotensin II type 2 (AT2) receptor-MMS2 cascade involving interaction between AT2 receptor-interacting protein and Src homology 2 domain-containing protein-tyrosine phosphatase 1. Mol Endocrinol 2007;21:499–511.
[3] Kawajiri M, Mogi M, Osoegawa M, Matsuoka T, Tsukuda K, Kohara K, et al. Reduction of angiotensin II in the cerebrospinal fluid of patients with multiple sclerosis. Mult Scler 2008;14:557–60.
[4] Ferrario CM. Angiotensin-converting enzyme 2 and angiotensin-(1–7): an evolving story in cardiovascular regulation. Hypertension 2006;47:515–21.
[5] Gallagher PE, Chappell MC, Ferrario CM, Tallant EA. Distinct roles for ANG II and ANG-(1–7) in the regulation of angiotensin-converting enzyme 2 in rat astrocytes. Am J Physiol Cell Physiol 2006;290:C420–6.
[6] Wosik K, Cayrol R, Dodelet-Devillers A, Berthelet F, Bernard M, Moumdjian R, et al. Angiotensin II controls occludin function and is required for blood brain barrier maintenance: relevance to multiple sclerosis. J Neurosci 2007;27:9032–42.

[7] Kawajiri M, Mogi M, Higaki N, Matsuoka T, Ohyagi Y, Tsukuda K, et al. Angiotensin-converting enzyme (ACE) and ACE2 levels in the cerebrospinal fluid of patients with multiple sclerosis. Mult Scler 2009;15:262–5.
[8] Lennon VA, Wingerchuk DM, Kryzer TJ, Pittock SJ, Lucchinetti CF, Fujihara K, et al. A serum autoantibody marker of neuromyelitis optica: distinction from multiple sclerosis. Lancet 2004;364:2106–12.
[9] Lennon VA, Kryzer TJ, Pittock SJ, Verkman AS, Hinson SR. IgG marker of optic-spinal multiple sclerosis binds to the aquaporin-4 water channel. J Exp Med 2005;202:473–7.
[10] Matsushita T, Isobe N, Matsuoka T, Ishizu T, Kawano Y, Yoshiura T, et al. Extensive vasogenic edema of anti-aquaporin-4 antibody-related brain lesions. Mult Scler 2009;15:1113–7.
[11] Tait MJ, Saadoun S, Bell BA, Papadopoulos MC. Water movements in the brain: role of aquaporins. Trends Neurosci 2008;31:37–43.
[12] Matsuoka T, Matsushita T, Kawano Y, Osoegawa M, Ochi H, Ishizu T, et al. Heterogeneity of aquaporin-4 autoimmunity and spinal cord lesions in multiple sclerosis in Japanese. Brain 2007;130:1206–23.
[13] Matsushita T, Isobe N, Matsuoka T, Shi N, Kawano Y, Wu X, et al. Aquaporin-4 autoimmune syndrome and anti-aquaporin-4 antibody-negative opticospinal multiple sclerosis in Japanese. Mult Scler 2009;15:834–47.
[14] Wingerchuk DM, Lennon VA, Pittock SJ, Lucchinetti CF, Weinshenker BG. Revised diagnostic criteria for neuromyelitis optica. Neurology 2006;66:1485–9.
[15] Polman CH, Reingold SC, Edan G, Filippi M, Hartung H-P, Kappos L, et al. Diagnostic criteria for multiple sclerosis: 2005 revisions to the "McDonald Criteria". Ann Neurol 2005;58:840–6.
[16] Kurtzke JF. Rating neurologic impairment in multiple sclerosis: an expanded disability status scale (EDSS). Neurology 1983;33:1444–52.
[17] Finkielman S, Fischer-Ferraro C, Diaz A, Goldstein DJ, Nahmod VE. A pressor substance in the cerebrospinal fluid of normotensive and hypertensive patients. Proc Natl Acad Sci USA 1972;69:3341–4.
[18] Jacob A, Matiello M, Wingerchuk DM, Lucchinetti CF, Pittock SJ, Weinshenker BG. Neuromyelitis optica: changing concepts. J Neuroimmunol 2007;187:126–38.
[19] Voskuhl RR, Peterson RS, Song B, Ao Y, Morales LBJ, Tiwari-Woodruff S, et al. Reactive astrocytes form scar-like perivascular barriers to leukocytes during adaptive immune inflammation of the CNS. J Neurosci 2009;29:11511–22.
[20] Shao J, Nangaku M, Miyata T, Inagi R, Yamada K, Kurokawa K, et al. Imbalance of T-cell subsets in angiotensin II-infused hypertensive rats with kidney injury. Hypertension 2003;42:31–8.
[21] Lucchinetti CF, Mandler RN, McGavern D, Bruck W, Gleich G, Ransohoff RM, et al. A role for humoral mechanisms in the pathogenesis of Devic's neuromyelitis optica. Brain 2002;125:1450–61.
[22] Ishizu T, Osoegawa M, Mei F-J, Kikuchi H, Tanaka M, Takakura Y, et al. Intrathecal activation of the IL-17/IL-8 axis in opticospinal multiple sclerosis. Brain 2005;128:988–1002.

ELSEVIER

Pathophysiology 18 (2011) 69–79

ISP
PATHOPHYSIOLOGY

www.elsevier.com/locate/pathophys

Neuromyelitis optica and opticospinal multiple sclerosis: Mechanisms and pathogenesis

Jun-ichi Kira *

Department of Neurology, Neurological Institute, Graduate School of Medical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan

Received 12 March 2010; received in revised form 24 March 2010; accepted 8 April 2010

Abstract

Multiple sclerosis (MS) is a demyelinating disease of the central nervous system (CNS) while neuromyelitis optica (NMO) is an inflammatory disease of the CNS that selectively affects the optic nerves and spinal cord. In Asians, MS is rare; however, when it appears, the selective and severe involvement of the optic nerves and spinal cord is characteristic. This form, termed opticospinal MS (OSMS), has similar features to the relapsing form of NMO in Western populations. Recently, a specific IgG against NMO, designated NMO-IgG, was discovered, and the relevant antigen was found to be aquaporin-4 (AQP4), one of the major water channel proteins in the CNS. Because NMO-IgG has been reported to be present in 30–60% of OSMS patients, OSMS in Asians has been suggested to be the same entity as NMO.

The sensitivity of NMO-IgG/anti-AQP4 antibody for NMO varies from 30% to 80%, while the specificity is 90–100%. Pathological studies on NMO have revealed perivascular immune complex (IgM, IgG and C9neo) deposition and extensive loss of AQP4 in active lesions, where myelin basic protein (MBP) staining was relatively preserved. IgG from NMO-IgG-seropositive NMO patients induces astrocyte death in culture in the presence of complements, and reproduces astrocyte loss *in vivo* when MBP-specific T cells are co-transferred to cause experimental autoimmune encephalomyelitis. It is thus postulated that the complement-activating anti-AQP4 antibody plays a pivotal role in the development of NMO lesions through astrocyte necrosis, and that demyelination is a secondary event.

However, in autopsied cases of NMO, we and others found that some demonstrated selective AQP4 loss while others showed preservation of AQP4, even in the acute lesions. We also found that, in some MS lesions, AQP4 was lost extensively far beyond the areas of myelin loss. In the CSF, proinflammatory cytokines such as IL-17, IL-8, IFNγ, and G-CSF are markedly elevated in OSMS patients, irrespective of the presence or absence of anti-AQP4 antibody. In OSMS and NMO patients, T cells reactive to myelin proteins show intra- and inter-molecular epitope spreading, suggesting that T cells are already stimulated with myelin antigens *in vivo*. These findings suggest that mechanism of NMO and OSMS in Asians is heterogeneous, anti-AQP4 antibody-related and -unrelated, and that not only anti-AQP4 antibody but also myelin-autoreactive Th17 or Th1 cells may also play a role in triggering CNS inflammation. Possible mechanisms for NMO and OSMS are discussed in this review.

Keywords: Multiple sclerosis; Neuromyelitis optica; Aquaporin-4; Opticospinal multiple sclerosis; Th17; Astrocyte necrosis; Asians and Westerners

1. Introduction

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system (CNS) thought to be caused by autoimmune attacks targeting CNS myelin. The pathological hallmark in MS is sharply demarcated demyelinating plaques with axons relatively preserved. By contrast, neuromyelitis optica (NMO) is an inflammatory disease of the CNS selectively affecting the optic nerves and spinal cord. In this condition, longitudinally extensive spinal cord lesions (LESCLs) extending over three vertebral segments are said to be characteristic on magnetic resonance imaging (MRI). Pathologically, both axons and myelin are involved, resulting in necrotic cavitation. In Asians, MS is rare; however, when it appears, the selective but severe involvement of the optic nerves and spinal cord is characteristic [1]. This form, termed opticospinal MS (OSMS), has similar features to the relapsing form of NMO in Western populations [2]. The nosological position of NMO has long been a matter of

* Tel.: +81 92 642 5340; fax: +81 92 642 5352.
E-mail address: kira@neuro.med.kyushu-u.ac.jp.

0928-4680/$ – see front matter © 2010 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.pathophys.2010.04.008