LOS antibodies were present irrespective of the type of antecedent infections.

Discussion

Disease pathology due to acetylcholine receptor antibodies has been clearly demonstrated for myasthenia gravis, although approximately 20% of myasthenia gravis patients with generalized disease lack the autoantibodies (so called seronegative myasthenia gravis), and some instead have antibodies reactive to a muscle specific kinase [23]. We have found a similar situation in FS: although most FS patients have anti-GQ1b autoantibodies, some FS patients have other autoantibodies, and clinical features are not useful for identifying GQ1b-seronegative FS patients. There are several individual case reports of GQ1b-seronegative FS, most of which describe a clinical picture atypical for FS, such as early infant onset (2 years old), complication of Burkitt's lymphoma, marked oculomotor nerve disturbance without abducens palsy predominance, and one-sided horizontal gaze palsy, irrespective of coexisting anti-ganglioside antibodies [24–27]. Unlike these reports, we did not find any obvious clinical differences between patients with and without anti-GQ1b antibodies, except for male dominance and antecedent gastrointestinal symptoms, and rarer onset of diplopia. This is the first study to extensively describe the clinical features of GQ1b-seronegative FS patients.

We found 24 GQ1b-seronegative FS patients among 207 FS cases and showed that some GQ1b-seronegative FS patients had IgG antibodies against single gangliosides such as GM1b (8.3%), GD1a (4.2%), GalNAc-GD1a (4.2%), or GT1a (8.3%). This is in agreement with our previous findings that some patients with GQ1b-seronegative FS have antibodies to GM1b (9/76 [12%]), GalNAc-GD1a (9/76 [12%]), or GD1b (9/76 [12%]) [4, 28]. A pioneering study found that anti-GT1a antibodies coexisted with anti-GQ1b antibodies in all FS patients, although both antibodies cross-reacted with each other, and a biochemical analysis detected GQ1b, but not GT1a, expression in human oculomotor nerves, but not GT1a. The authors speculated that anti-GQ1b antibodies are the primary effector in FS pathogenesis [2]. In contrast, our biochemical analysis detected GT1a as well as GQ1b in human oculomotor nerves [29]. The current study data, as well as our biochemical findings, raise the possibility that antibodies against gangliosides other than GQ1b play a pathogenic role in the development of FS, although negative result of anti-GQ1b antibodies might be partially due to insufficient sensitivity of the antibody assay used, as that of acetylcholine receptor antibodies in myasthenia gravis. Administration of anti-GQ1b monoclonal antibody can cause general weakness and respiratory failure in mice, but it remains unclear whether anti-GQ1b antibodies can induce FS-like deficits [30]. To investigate which anti-ganglioside antibodies play a pathogenic role of FS development, establishment of FS animal model by immunization with gangliosides or passive transfer of the antibodies is needed.

One recent study showed that three patients with GQ1b-seronegative FS had antibodies against ganglioside complexes [31]. Our larger study, including 24 GQ1b-seronegative FS patients, found that the frequency of GQ1b-seronegative FS patients with IgG reactive to heterologous ganglioside complexes was 17% (4 of 24 patients). Moreover, the GQ1b-seronegative FS patients with anti-ganglioside complex antibodies in our study did not show distinguishing clinical features, suggesting that clinical features are not helpful in identifying these patients among GQ1b-seronegative FS cases. In agreement with a previous report [31], our data showed that combinations of GT1a/GM1 and GQ1b/GM1, which contains a total of two sialic acids in the terminal residues, could be the alternative target antigens for serum IgG antibodies in patients with GQ1b-seronegative FS. GQ1b and GT1a are expressed diffusely in human cranial nerves [29], and therefore conspicuous ophthalmoplegia in FS appears not be explained only by the uneven expression of GQ1b and GT1a in cranial nerves. In order to clarify the molecular mechanisms underpinning FS pathogenesis, future studies should determine distribution of the alternative epitopes formed by ganglioside complexes containing two sialic acids in the terminal residues in the human nervous system to clarify the molecular mechanism in developing unique clinical picture of FS.

Structural analysis of the LOS of GC068 and GC219 confirmed previous studies that showed that FS-related *C. jejuni* isolates frequently displayed a GD1c-like LOS, and not the precise GQ1b-like LOS [8–11]. We also observed GalNAc-GM1b- and GalNAc-GD1c-like LOS, both of which had not been previously described in *C. jejuni*. GD1c, GQ1b, and GT1a have in common the structure (NeuAc α2–8 NeuAc α2–3 Gal β1–3 GalNAc) in the terminal residues (Fig. 1). All five patients with FS or FS/GBS overlap, from whom GD1c-like LOS-bearing *C. jejuni* was isolated (GC033 and GC068 in Table 2 and GC041, GC107, and GC125, unpublished data), had anti-GQ1b antibodies. It therefore can be assumed that GD1c-like LOS triggers the production of anti-GD1c antibodies that cross-react with GQ1b in some FS patients following *C. jejuni* enteritis. Mechanistically, IgG autoantibodies in FS patients are speculated to have higher affinity for GD1c than GQ1b, and our finding that some GQ1b-seronegative FS patients have antibodies against GD1c-like LOS supports this assertion. Anti-GD1c antibody testing should be performed using authentic ganglioside as the antigen to confirm our speculation.

Expression of GalNAc-GM1b-like LOS on FS-related *C. jejuni* strains suggests three possible mechanisms in the development of FS. First, GalNAc-GM1b-like LOS might be associated with production of antibodies against GM1b and GalNAc-GD1a. GalNAc-GM1b has a carbohydrate sequence (GalNAc β1-4 [NeuAc α2–3] Gal β1–3 GalNAc) in the terminal moiety in common with GalNAc-GD1a, and the inner moiety (NeuAc α2–3 Gal β1–3 GalNAc) is also common to GM1b (Fig. 1). In GBS and chronic neuropathies, anti-GalNAc-GM1b antibodies (IgG or IgM) can coexist with antibodies against GalNAc-GD1a or GM1b, and cross-react with each other [32–35]. Based on these findings, GalNAc-GM1b-like LOS may act as an immunogen for producing antibodies against GalNAc-GD1a and GM1b, which contribute to FS pathogenesis. This is supported by the detection of anti-GM1b and anti-GalNAc-GD1a autoantibodies in GQ1b-seronegative FS patients in this and another study [4], and the detection of anti-GalNAc-GD1a antibodies in a patient from whom *C. jejuni* bearing GalNAc-GM1b-like LOS (GC051, unpublished data) had been isolated. Second, anti-GalNAc-GM1b antibodies themselves might initiate the development of FS. GalNAc-GM1b is expressed in the brains of several animals, although it is unclear if this minor ganglioside is expressed in the human nervous system [32]. Some individuals may express GalNAc-GM1b as well as GQ1b in oculomotor nerves. Finally, GalNAc-GM1b-like LOS might be associated with the production of anti-GQ1b antibodies. Although non-reducing terminal of oligosaccharide of GalNAc-GM1b is different from that of GQ1b in looking at the second dimensional structure, the three-dimensional structure can be identical, as in the case of GM1b and GalNAc-GD1a [36]. This alternative explanation seems the most likely, as we found anti-GQ1b antibodies in all three patients with FS or FS/GBS overlap from whom *C. jejuni* bearing GalNAc-GM1b-like LOS [GC219 and GC068 in Table 2; GC051 (unpublished data)] was cultured. Thus, the presence of anti-GalNAc-GM1b antibodies should be examined using authentic ganglioside as an antigen.

In conclusion, this is the first study to examine the clinical and serological features of patients with GQ1b-seronegative FS. We showed that the clinical features of GQ1b-seronegative FS were similar to those of GQ1b-seropositive FS, except for extreme male predominance and a history of antecedent gastrointestinal illness. IgG antibodies against GM1b, GD1c, GalNAc-GM1b, and ganglioside complexes were found to be serological markers of GQ1b-seronegative FS, although a pathogenic role of these autoantibodies requires further study.

Acknowledgments We thank Dr Masaaki Odaka (Dokkyo Medical University) for proving detailed data from a previous study. We thank Denis Brochu (National Research Council Canada, Institute for Biological Sciences) for help with the analysis of the LOS outer core structures and Marie-France Karwaski (National Research Council Canada, Institute for Biological Sciences) for DNA sequencing of LOS biosynthesis genes.

Dr Koga received a grant from the Kimi Imai Memorial Foundation for Neuromuscular Diseases and a Grant-in-Aid for Scientific Research (C) (KAKENHI 20590446) from the Ministry of Education, Culture, Sports, Science and Technology of Japan. Drs Gilbert, Li, Takahashi, and Hirata report no disclosures. Dr Kanda received a Research Grant for Neuroimmunological Diseases from the Ministry of Health, Labour and Welfare of Japan. Dr Yuki received a grant from National Medical Research Council (IRG 10 Nov 086), Ministry of Health and Yong Loo School of Medicine start-up grant (Nobuhiro Yuki) in Singapore.

Conflicts of interest None.

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RESEARCH PAPER

Sera from neuromyelitis optica patients disrupt the blood–brain barrier

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Received 3 May 2011
Accepted 20 September 2011
Published Online First
19 November 2011

ABSTRACT

Objective In neuromyelitis optica (NMO), the destruction of the blood–brain barrier (BBB) has been considered to be the first step of the disease process. It is unclear whether sera from patients with NMO can open the BBB, and which component of patient sera is most important for this disruption.

Methods The effects of sera from antiaquaporin4 (AQP4) antibody positive NMO patients, multiple sclerosis patients and control subjects were evaluated for expression of tight junction proteins and for transendothelial electrical resistance (TEER) of human brain microvascular endothelial cells (BMECs). Whether antibodies against human BMECs as well as anti-AQP4 antibodies exist in NMO sera was also examined using western blot analysis.

Results Expression of tight junction proteins and TEER in BMECs was significantly decreased after exposure to NMO sera. However, this effect was reversed after application of an antivascular endothelial growth factor (VEGF) neutralising antibody. Antibodies against BMECs other than anti-AQP4 antibodies were found in the sera of NMO patients whereas no specific bands were detected in the sera of healthy and neurological controls. These antibodies apparently disrupt the BBB by increasing the autocrine secretion of VEGF by BMECs themselves. Absorption of the anti-AQP4 antibody by AQP4 transfected astrocytes reduced AQP4 antibody titres but was not associated with a reduction in BBB disruption.

Conclusions Sera from NMO patients reduce expression of tight junction proteins and disrupt the BBB. Autoantibodies against BMECs other than anti-AQP4 antibodies may disrupt the BBB through upregulation of VEGF in BMECs.

INTRODUCTION

Neuromyelitis optica (NMO) is defined as an inflammatory CNS disease predominantly affecting the spinal cord and the optic nerves.[1] This disorder was long regarded as a variant of multiple sclerosis (MS), with distinctive pathological features.[2] A breakthrough in our understanding of NMO was identification of an autoantibody response with high sensitivity and specificity for the disease, which was found to be directed against the astrocytic water channel aquaporin 4 (AQP4).[3] Several studies have suggested that the anti-AQP4 antibody is pathogenic and it also plays a key role in the development of NMO.[4–11]

Circulating anti-AQP4 antibodies need to pass through the blood–brain barrier (BBB) in order to reach the CNS parenchyma, which is the site affected by inflammation in this disease. Initiation of disease by transfer of these antibodies into normal animals has not been achieved to date[12] because the BBB restricts the entry of circulating anti-AQP4 antibodies into the CNS under normal conditions. Although destruction of the BBB causing leakage of anti-AQP4 antibodies and cytokines into the CNS space has been considered as a key step in the development of NMO, it remains unclear which components of patient sera is most important for disruption of the BBB. It is also unclear whether sera from an NMO patient containing circulating anti-AQP4 antibodies can open the BBB because no direct evidence has been presented indicating that the brain microvascular endothelial cells (BMECs), which comprise the BBB, express the AQP4 protein.[13 14] Various circulating inflammatory cytokines, including tumour necrosis factor α (TNFα) and vascular endothelial growth factor (VEGF), which have already been reported to induce disruption of the BBB, may be the candidate molecules leading to the breakdown of the BBB.[15 16] The existence of unknown pathogenic antibodies, apart from anti-AQP4 antibodies, may also cause BBB impairment.

The aim of the current study was to demonstrate the effects of sera from patients with NMO on impairment of BBB function and to clarify the roles of humoral factors, especially antibodies, against human BMECs, in the destruction of the BBB.

MATERIALS AND METHODS

Sera and antibody

The acute phase sera from 14 consecutive NMO patients hospitalised at our institution were studied. All patients met the clinical criteria for NMO spectrum disorders.[17 18] None of the NMO patients had antinuclear antibodies or SS-A/SS-B antibodies. The human anti-AQP4 antibody was detected in all patients by a procedure previously described by Takahashi.[9] Blood samples were obtained within 7 days of onset and stored at −80°C until use. The sera from two patients who began plasma exchange (PE) treatment were also obtained. The acute phase sera from seven patients with conventional MS (C-MS), diagnosed by the McDonald criteria,[19] were also used in this study. The sera from 15 patients with autoimmune inflammatory neurological diseases, including three patients with neuropsychiatric systemic lupus erythematosus (NP-SLE), four patients with dermatomyositis, three patients with myasthenia gravis, three patients with multifocal motor neuropathy and two patients with microscopic polyangiitis were studied as inflammatory

disease controls. All NP-SLE, dermatomyositis and microscopic polyangiitis patients had antinuclear antibodies. In contrast, none of the myasthenia gravis and multifocal motor neuropathy patients had these antibodies. Sera from 12 patients with non-inflammatory neurological diseases, including four patients with amyotrophic lateral sclerosis, two patients with Parkinson's disease, four patients with cervical spondylosis and two patients with multiple system atrophy, were used as neurological disease controls. The sera from 12 healthy individuals also served as normal controls. All sera were incubated at 65°C for 30 min just prior to use. There were no statistically significant differences in the concentrations of IgG between the serum samples of the 14 NMO, 7 MS and 12 normal controls (means±SEM, NMO 1035±517 mg/dl; MS 1090±151 mg/dl; normal controls 1042±225 mg/dl) when the concentration of IgG in each of the samples was measured. The use of the patient's sera for this study was approved by the ethics committee of Yamaguchi University following the principles of the Declaration of Helsinki.

Cell culture and treatment

The immortalised human brain microvascular endothelial cells (BMECs) were generated previously.[20] Briefly, we previously established conditionally immortalised BBB derived endothelial cells, called TY08 cells, harbouring the temperature sensitive SV40 large T antigen (tsA58) protein.[20] The gene product of tsA58 is in an active conformation and binds to p53 at 33°C, thus facilitating the immortalisation of the cells, whereas the conformation of the gene product changes, leading to its degradation and the release of p53 when the cells are grown at 37°C. Therefore, these cells are conditionally immortal. The cells expressed all key tight junctional proteins, such as occludin, claudin-5, ZO-1 and ZO-2, and had low permeabiltity to inulin across monolayers. All of the analyses were determined 3 days after the temperature shift from 33°C to 37°C. Human umblilical vein endothelial cells (HUVECs), human fibroblasts and 293T cells were obtained from the Japan Health Sciences Foundation (Osaka, Japan) and human astrocytes were purchased from Lonza (Walkersville, Maryland, USA). BMECs were treated with culture medium containing 10% patient or healthy control sera in a humidified atmosphere of 5% CO_2/air. BMECs treated with culture medium with 10% fetal bovine serum (Sigma, St. Louis, Missouri, USA) were used as controls. The mRNAs were extracted 24 h later, and total proteins were obtained a day later.

Reagents

The culture medium for BMECs consisted of Dulbecco's modified Eagle's medium (DMEM; Sigma) containing 100 U/ml penicillin (Sigma), 100 μg/ml streptomycin (Sigma), 25 ng/ml amphotericin B (Invitrogen, Grand Island, New York, USA) and 10% fetal bovine serum (Sigma). Polyclonal anticlaudin-5 and antioccludin antibodies were purchased from Zymed (San Francisco, California, USA). The polyclonal antiactin antibody was obtained from Santa Cruz (Santa Cruz, California, USA). The polyclonal anti-transforming growth factor β (TGFβ), anti-VEGF, anti-interleukin (IL)-6, anti-IL-17, anti-interferon γ (IFNγ) and anti-TNFα antibodies were purchased from R&D systems (Minneapolis, Minnesota, USA). Lysates of human claudin-5 transfected 293T cells and control 293T cells were purchased from Santa Cruz. A total of 5 μg of protein lysates were loaded for the western blot analysis.

Quantitative real time PCR analysis

Total RNA was extracted from BMECs using an RNeasy Plus Mini Kit (Qiagen, Hilden, Germany). Single stranded cDNA was created from 40 ng of total RNA using the StrataScript First Strand Synthesis System (Stratagene, Cedar Greek, Texas, USA.). The sequence of each human primer pair and its reference are as follows: sense primer 5′-CTG TTT CCA TAG GCA GAG CG-3′ and antisense primer 5′-AAG CAG ATT CTT AGC CTT CC-3′ for claudin-5[21]; sense primer 5′-TGG GAG TGA ACC CAA CTG CT-3′ and antisense primer 5′-CTT CAG GAA CCG GCG TGG AT-3′ for occludin[22]; and sense primer 5′-GTC AAC GGA TTT GGT CTG TAT T-3′ and antisense primer 5′-AGT CTT CTG GGT GGC AGT GAT-3′ for glyceraldehyde-3-phosphate dehydrogenase.[23] Quantitative real time PCR analyses were performed using a Stratagene Mx3005P (Stratagene) with Full-Velocity SYBR Green QPCR master mix (Stratagene). Glyceraldehyde-3-phosphate dehydrogenase was used as an internal standard. The samples were subjected to PCR analysis using the following cycling parameters: 10 min at 95°C followed by 40 cycles for 15 s at 95°C, 1 min at 60°C and 1 min at 72°C. The standard reaction curve was analysed by the MxPro (Stratagene) software programme and the relative quantity according to standard reaction curve (R_v) was calculated by computer according to the formula $R_v=R_{Gene}/R_{GAPDH}$.

Western blot analysis

Protein samples (10–20 μg) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Biorad, Hercules, CA, USA) and transferred to nitrocellulose membranes (Amersham, Chalfont, UK). Expression of β-actin was used as an internal standard. The membranes were treated with blocking buffer (5% skimmed milk in 25 mM Tris-HCl, pH 7.6,125 nM NaCl, 0.5% Tween 20) for 1 h at room temperature and incubated with the relevant primary antibodies (dilution 1:100) for 2 h at room temperature. The membranes were then exposed to a peroxidase conjugated secondary antibody (1:2000), followed by a chemiluminescence reagent (Amersham), and exposure to X-Omat S films (Amersham) and quantification of the band intensity was obtained using the Fuji image analysis software package.

Immunocytochemistry

Cultured cells were fixed with 4% paraformaldehyde (Wako, Osaka, Japan) and permeabilised with 100% methanol. Cells were subsequently incubated overnight with 5% serum (as relevant primary antibodies (dilution 1:20)) and then were incubated with a FITC labelled secondary antibody at a dilution of 1:200 for staining. Fluorescence was observed by a fluorescence microscope (Olympus, Tokyo, Japan). The nuclei were stained with DAPI, and the fluorescence was detected with a fluorescence microscope (Olympus). Image stacks were analysed with the localisation module of the Olympus software program (Olympus).

Transendothelial electrical resistance studies

Transwell inserts (pore size 0.4 μm, effective growth area 0.3 cm^2, BD Bioscience, Sparks, Maryland, USA) were coated with rat tail collagen type I (BD Bioscience). Transendothelial electrical resistance (TEER) values of cell layers were measured with a Millicell electrical resistance apparatus (Endohm-6 and EVOM; World Precision Instruments, Sarasota, Florida, USA). BMECs were seeded (1×10^6 cells/insert) on the upper compartment and incubated with each medium (non-conditioned medium used as a control, conditioned medium contained 10% patient sera) for 24 h.

Studies with patient sera preincubated with neutralising antibodies against TNFα, IFNγ, VEGF, TGFβ, IL-6 or IL-17

BMECs were incubated with the sera from eight NMO patients containing 2.0 μg/ml of a neutralising antibody against TNFα,

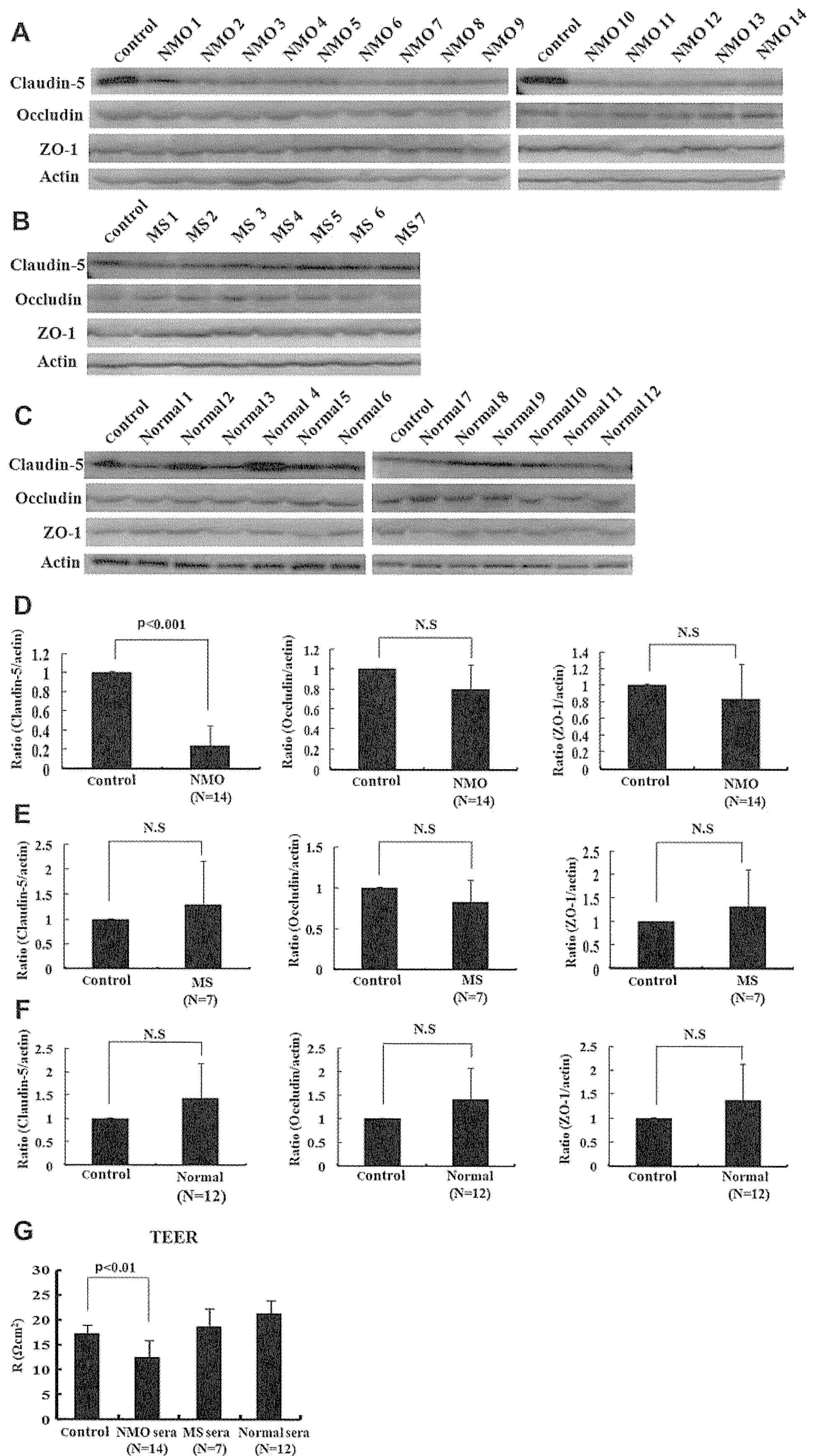
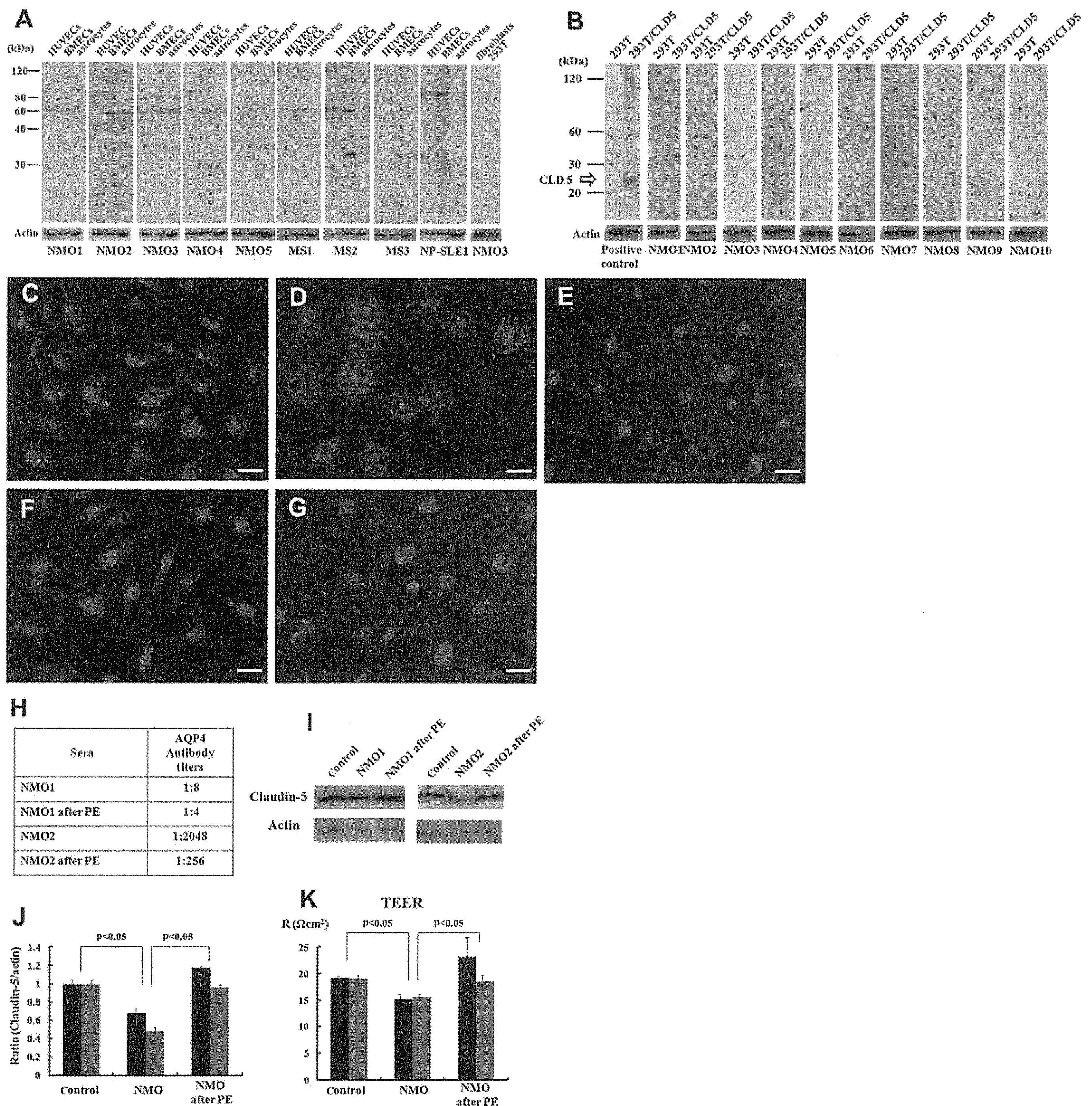


Figure 1 (A–C) Effects of sera on tight junction proteins in human brain microvascular endothelial cells (BMECs) determined by western blot analysis. Changes in claudin-5, occludin and ZO-1 expression in BMECs were determined after exposure to sera from patients with neuromyelitis optica (NMO) or conventional MS (C-MS), or from healthy controls. (D–F) Each bar graph reflects the combined densitometry data from each independent experiment. (D) Expression of claudin-5 protein in BMECs was significantly decreased after exposure to sera from NMO patients (mean±SEM, n=14, $p<0.001$). (E, F) Expression levels of claudin-5 and occludin were not affected by exposure to sera from patients with C-MS (mean±SEM, n=7) or from healthy controls (mean±SEM, n=12). (G) The transendothelial electrical resistance (TEER) value of BMECs was significantly decreased after exposure to NMO sera but was not influenced by exposure to sera from patient with C-MS or from healthy controls. NMO, conditioned medium with 10% serum from an NMO patient diluted with non-conditioned Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS); MS, conditioned medium with a 10% concentration of serum from an MS patient diluted with non-conditioned DMEM containing 10% FBS; Normal, conditioned medium with 10% serum from a healthy control diluted with non-conditioned medium of DMEM containing 10% FBS.



Sera	AQP4 Antibody titers
NMO1	1:8
NMO1 after PE	1:4
NMO2	1:2048
NMO2 after PE	1:256

Figure 2 (A) Representative results obtained by immunoblotting of human brain microvascular endothelial cell (BMEC) lysates. The blots were exposed to sera from 14 neuromyelitis optica (NMO) and seven conventional MS (C-MS) patients, or to 11 neurological disease controls and 12 healthy controls after a total of 20 μg of protein lysates from human umblilical vein endothelial cells (HUVECs), BMECs and astrocytes were loaded. The NMO sera predominantly reacted with one or more antigens of approximately 35, 60, 80 and 110 kDa in both BMEC and astrocyte lysates. The anti-BMEC antibodies were present in sera (1:100 dilutions) from 10 of 14 NMO patients (71.4%), three of seven C-MS patients (42%) and one of three neuropsychiatric systemic lupus erythematosus (NP-SLE) patients (33%) but no protein bands against human fibroblasts or 293T cells (as negative controls) were detected in any of the NMO serum samples. No bands were demonstrated in the samples from 14 patients with autoimmune inflammatory neurological diseases, and 12 patients with non-inflammatory neurological diseases or 12 healthy controls, but the sera from one of three NP-SLE patients also reacted with the 80 kDa antigens of both HUVECs and BMECs. Expression of actin was used as an internal standard. (B) The anti-claudin-5 antibodies were present in the sera of NMO patients, as determined by western blot analysis. The whole cell lysates prepared from 293T cells with or without transfection of the human claudin-5 gene were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis. No protein bands corresponding to anti-claudin-5 antibodies were demonstrated in any of the NMO sera. Claudin-5 was detected using anti-claudin-5 antibodies as a positive control. β-Actin was detected with an anti-β-actin antibody as an internal standard. 293T, 293T cell lysates without transfection; 293T/CLD5, 293T cells lysates with transfection of claudin-5. (C–G) Immunocytochemical analysis of BMECs (C–E), HUVECs (F) or human fibroblasts (G) using 5% serum from five NMO patients (C, D) or five normal controls (E). The anti-BMECs antibodies in the NMO sera were localised in the cytoplasm of BMECs, showing a granular staining pattern (C, D) although no immunopositive samples were detected in the sera from normal controls (E). No immunopositive staining against human fibroblasts (as negative controls) were detected in any of the NMO serum samples (G).

IFNγ, VEGF, TGFβ, IL-6 or IL-17, or normal rabbit IgG. Total RNA was extracted and the TEER value was measured 24 h later. Total proteins were obtained the next day.

Sera from NMO patients were pretreated with 2.0 μg/ml of a neutralising antibody against TNFα, IFNγ, VEGF, TGFβ, IL-6 or IL-17, or normal rabbit IgG (control antibody) for 6 h at 4°C. BMECs were cultured with the sera from eight NMO patients containing each neutralising antibody at 37°C. Total RNA was extracted and the TEER value was measured 24 h later. Total proteins were obtained the next day.

Absorption of the anti-AQP4 antibody by AQP4 transfected cells

Human astrocytes were transfected with a retrovirus incorporating the shorter isoform of human AQP4 (M-23) in order to overexpress the AQP4 protein. Expression of the AQP4 protein in astrocytes was verified by western blot analysis. Sera from two NMO patients were added to the transfected cells. After a 30 min incubation period at 37°C in 5% CO_2 with gentle shaking, the patients' sera were removed and used for the subsequent analyses. This process was repeated at least five times (total exposure time 150 min).

Data analysis

Unless otherwise indicated, all data represent means±SEM. An unpaired two-tailed Student t test was used to determine the significance of differences between the means of two groups. A p value of <0.05 was considered to be statistically significant.

RESULTS

Sera from patients with NMO reduced the expression of tight junction molecules in BMECs

To analyse whether the sera from NMO patients affects the BBB, we first examined the effect of sera from patients with NMO or C-MS on BMECs. The amount of claudin-5 in BMECs was significantly decreased after exposure to sera from patients with NMO whereas it was not affected by the sera from patients with C-MS or from healthy controls, as determined by a western blot analysis (figure 1A,D). Expression levels of occludin and the ZO-1 protein were not significantly influenced by the application of sera from patients with NMO, C-MS or from healthy controls (figure 1B,C,E,F). The TEER value of BMECs was significantly decreased after exposure to sera from patients with NMO although it was not changed by incubation with sera from patients with C-MS or from healthy controls (figure 1G).

Anti-BMEC antibodies were present in the sera from NMO patients, and plasmapheresis reduced the ability of sera from NMO patients to disrupt the BBB

Next we analysed whether autoantibodies against human BMECs were present in the sera of NMO patients by a western blot analysis. Antibodies that bound to both BEMCs and astrocytes were seen in the sera from 10 of 14 NMO patients (71.4%) and three of seven C-MS patients (42%) (figure 2A), but no protein bands against human fibroblasts or 293T cells (as negative controls) were detected in the sera from any of the NMO patients (figure 2A). No protein bands were demonstrated in the sera from any of the 14 patients with autoimmune inflammatory neurological diseases, from the 11 non-inflammatory neurological controls or from the 12 healthy control serum samples (data not shown) although anti-BMEC antibodies were present in the sera of one NP-SLE patient. The sera from NMO patients predominantly reacted with one or more antigens of approximately 35, 60, 80 and 110 kDa in both BMECs and astrocytes (figure 2A). The 60 kDa bands in BMECs and astrocytes were commonly detected in all NMO patients but sera from some NMO cases also showed antibodies against the 60 kDa antigens in HUVECs. The bands corresponding to the 35 and 110 kDa antigens of BMECs were specific in BMECs and astrocytes, and were not detected in HUVECs. The 80 kDa bands in BMECs, HUVECs and astrocytes were commonly detected in NMO patients although the sera from NP-SLE patients also reacted with 80 kDa antigens from both HUVECs and BMECs (figure 2A). Serum samples from patients with C-MS reacted with approximately 32, 38, 60 and 110 kDa antigens of BMECs (figure 2A). Notably, antibodies against the antigens corresponding to 32 and 38 kDa were specific for MS patients and were not seen in NMO patients. We next examined whether anticlaudin-5 antibodies were present in the sera of NMO patients by western blot analysis. No protein bands corresponding to anticlaudin-5 antibodies were demonstrated in any of the NMO sera by immunoblotting of the whole cell lysates prepared from 293T cells with or without transfection of the human claudin-5 gene (figure 2B). Immunocytochemical analysis also showed that the anti-BMECs antibodies in NMO sera were localised in the cytoplasm of BMECs, thus showing a granular pattern (figure 2C–G). Furthermore, PE treatment reduced the titres of the anti-AQP4 antibodies (figure 2H) and led to an increase in the expression of claudin-5, and an increase in TEER values in BMECs, suggesting that the removal of anti-BMEC antibodies or anti-AQP4 antibodies decreased the ability of sera from NMO patients to disrupt the BBB (figure 2I–K). The effects on claudin-5 and TEER values in NMO patient No 1 seemed almost the same as those in NMO patient No 2 who had high titres of anti-AQP4 antibodies although AQP4 antibody titre in patient No 1 was very low (1:8) and the reduction to 1:4 after PE was not significant, suggesting that the effect of NMO sera to BBB disruption was not due to anti-AQP4 antibodies but other factors in the serum constituents (figure 2H–K).

VEGF in NMO sera disrupted the BBB

Various circulating inflammatory cytokines may be candidate agents disrupting the BBB. To clarify the contribution of

Figure 2 [Continued]

Anti-BMECs antibodies in NMO sera were also present in the cytoplasm of HUVECs in a granular pattern (F). Scale bars, 50 mm. (H) Titres of anti-AQP4 antibody from the sera of two different patients with NMO (NMO 1 and NMO 2) were decreased after plasma exchange (PE). (I) PE led to an increase in expression of claudin-5 in BMECs. (J) The bar graph reflects the combined densitometry data from three independent experiments. Each column reflects the combined densitometry data from three independent experiments for the two different NMO patients (mean±SEM, n=6, $p<0.05$; black bars, NMO 1; grey bars, NMO 2). (K) The transendothelial electrical resistance (TEER) value of BMECs significantly increased after PE. Each column reflects the combined densitometry data from three independent experiments for the two different NMO patients (mean±SEM, n=6, $p<0.05$; black bars, NMO 1; grey bars, NMO 2). Control, non-conditioned Dulbecco's modified Eagle's medium (DMEM) containing 20% fetal bovine serum (FBS); NMO, conditioned medium with 10% NMO patient serum diluted with non-conditioned DMEM containing 10% FBS; NMO after PE, conditioned medium with 10% NMO serum after PE treatment.

inflammatory cytokines to BBB breakdown, TNFα, IL-6, IFNγ, IL-17, VEGF and TGFβ activities were neutralised using the corresponding neutralising antibodies. Expression of claudin-5 or occludin mRNA in BMECs increased after exposure to NMO sera pretreated with the anti-VEGF or IL-17 neutralising antibodies, as determined by relative quantification with a real time RT-PCR analysis (figure 3A). We classified the sera of the eight NMO patients into two different groups: five with anti-BMEC antibodies (group 1) and three without anti-BMEC antibodies (group 2) (figure 3B,C). Expression of claudin-5 or occludin mRNA in BMECs was significantly increased by preincubation with an anti-VEGF antibody or an anti-IL-17 antibody in group 1 NMO sera (figure 3B). In contrast, pretreatment with an anti-IL-17 antibody significantly increased expression levels of occludin mRNA in group 2 NMO sera although pretreatment with the anti-VEGF antibody did not influence expression in that group (figure 3C). Next, changes in claudin-5 and occludin protein levels in BMECs after exposure to group 1 NMO sera pretreated with anti-VEGF or IL-17 antibodies were determined by western blot analysis (figure 3D). After confirming the effects seen at the

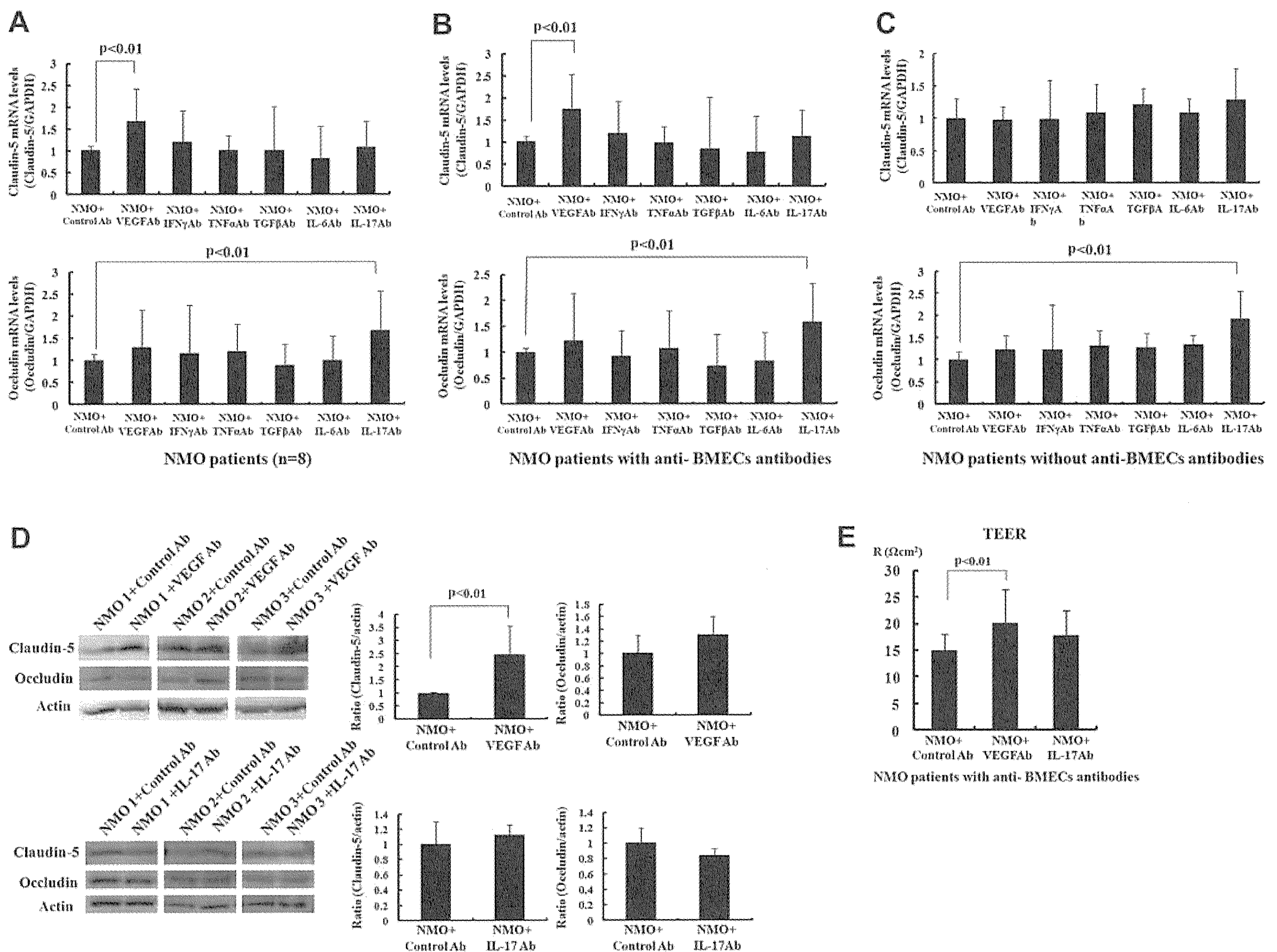


Figure 3 (A) Effects of anti-tumour necrosis factor α (TNFα), interleukin 6 (IL-6), interferon γ (IFNγ), interleukin 17 (IL-17), vascular endothelial growth factor (VEGF) and transforming growth factor β (TGFβ) neutralising antibodies on expression of tight junction molecules in human brain microvascular endothelial cells (BMECs) after exposure to sera from a patient with neuromyelitis optica (NMO), as determined by relative quantification with a real time RT-PCR analysis. Preincubation of anti-VEGF antibodies showed increased expression of claudin-5 mRNA in BMECs. Preincubation with the anti-IL-17 antibody induced the expression of occludin mRNA in BMECs. (B, C) Effects of anti-TNFα, IL-6, IFNγ, IL-17, VEGF or TGFβ neutralising antibodies on tight junction molecules in BMECs after exposure to sera from five NMO patients with anti-BMEC antibodies or to that of three patients without anti-BMEC antibodies. (B) Expression levels of claudin-5 mRNA in BMECs were increased by preincubation of the anti-VEGF antibody in NMO sera with anti-BMEC antibodies while expression of occludin mRNA in BMECs was increased after pretreatment with the anti-IL-17 antibodies (mean±SEM, n=5). (C) Expression of claudin-5 was not changed although expression of occludin mRNA was increased by preincubation of anti-IL-17 antibodies with the sera from NMO patients without the anti-BMEC antibodies (mean±SEM, n=3). (D) Effects of anti-VEGF or anti-IL-17 neutralising antibodies on expression of the claudin-5 and occludin proteins in BMECs after exposure to sera from an NMO patient with anti-BMEC antibodies, as determined by western blot analysis. Claudin-5 expression in BMECs was increased after preincubation with an anti-VEGF antibody while expression of occludin was not influenced after pre-exposure to the anti-IL-17 antibody (mean±SEM, n=3). (E) Transendothelial electrical resistance (TEER) value of BMECs significantly increased after incubation with sera from NMO patients with anti-BMEC antibodies pretreated with an anti-VEGF antibody but did not change after preincubation with an anti-IL-17 antibody (mean±SEM, n=3). NMO, conditioned medium with 10% NMO patient serum diluted with Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS); NMO+VEGFAb, conditioned medium with 10% NMO sera pretreated with an anti-VEGF neutralising antibody; NMO+IL-17Ab, conditioned medium with 10% NMO sera pretreated with an anti-IL-17 neutralising antibody.

mRNA level, expression of claudin-5 in BMECs significantly increased after preincubation with anti-VEGF antibodies whereas it did not change after preincubation with anti-IL-17 antibodies (figure 3D). The TEER value of the BMECs was also significantly increased after exposure to the group 1 NMO sera pretreated with an anti-VEGF antibody but was not affected after anti-IL-17 antibody pretreatment (figure 3E).

Anti-BMEC antibodies in NMO sera disrupted the BBB through upregulation of autocrine VEGF in BMECs

The concentration of VEGF was not significantly different between the sera from NMO patients and from healthy controls, as determined using ELISA (figure 4A). We thus hypothesised that anti-BMEC antibodies may disrupt the BBB by increasing the autocrine secretion of VEGF in BMECs. Expression of VEGF in BMECs was significantly increased after exposure to sera from group 1 NMO patients with anti-BMEC antibodies although it did not change after exposure to sera from group 2 NMO patients without anti-BMEC antibodies, or after sera from C-MS patients or healthy controls (figure 4B,C). Expression of VEGF secreted by astrocytes and HUVECs did not change after exposure to the sera of NMO patients (figure 4D).

Reduction of the anti-AQP4 antibody titre did not influence the ability of sera from NMO patients to disrupt the BBB

The 30 kDa single band corresponding to the shorter isoform of the AQP4 protein (AQP4-M23) was detected in BMECs by western blot analysis (figure 5A). We next examined whether anti-AQP4 antibodies in NMO sera were indeed responsible for disruption of the BBB. For this purpose, we preabsorbed anti-AQP4 antibodies from the sera of two different NMO patients using human astrocytes expressing human AQP4. These cells were generated via transduction with a retrovirus incorporated shorter isoform of the human AQP4 gene (M-23) into immortalised human astrocytes. The method used in our study can absorb not only anti-AQP4 antibodies but also other antibodies

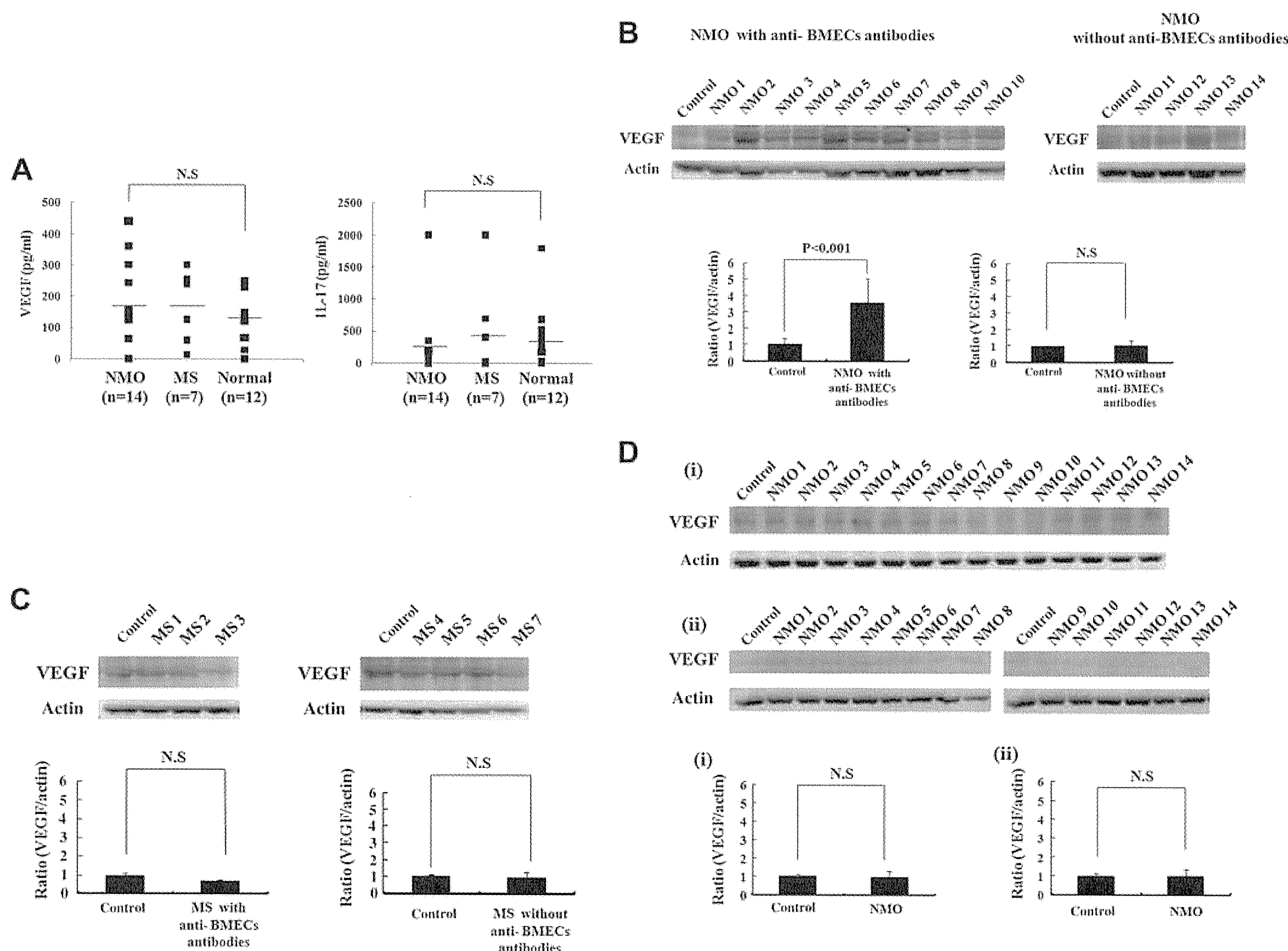
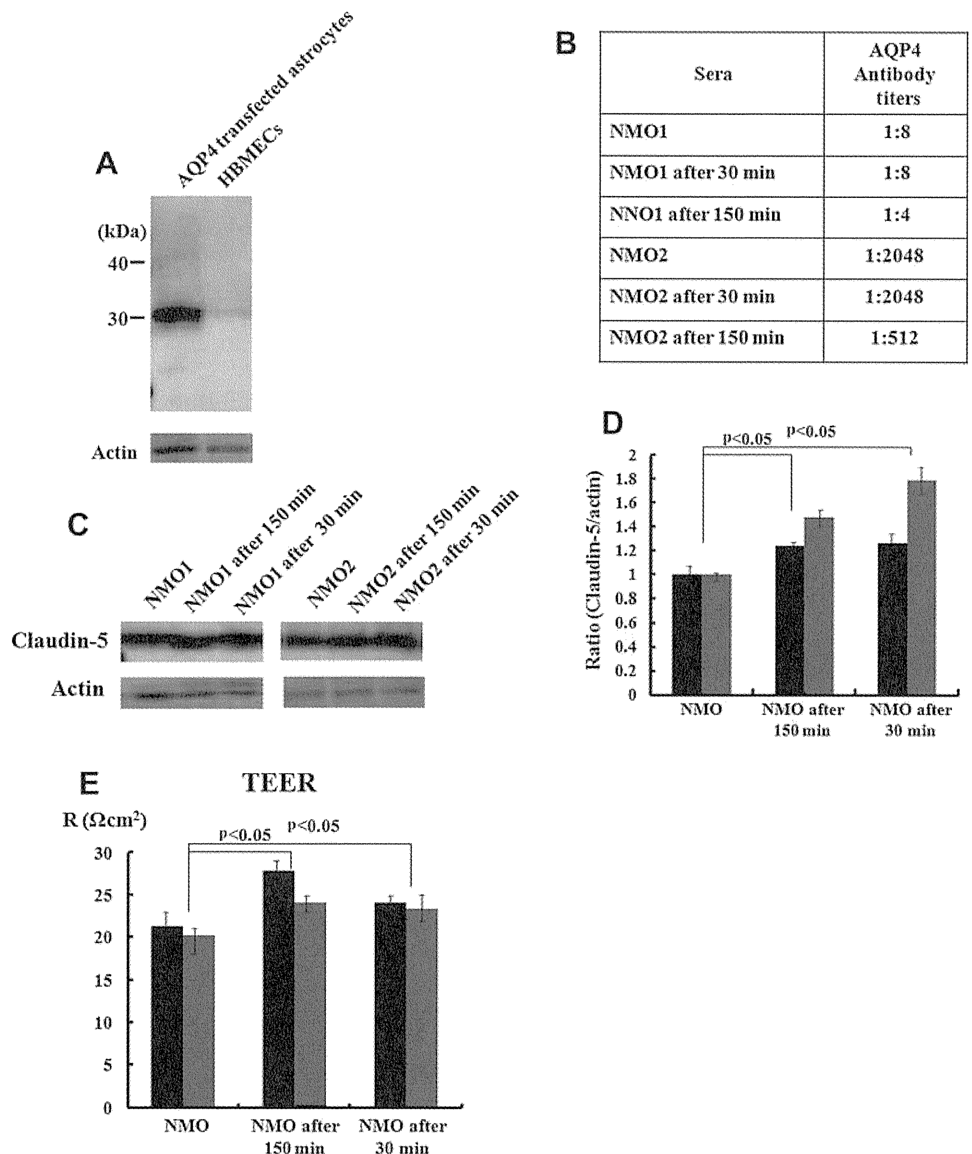


Figure 4 (A) Vascular endothelial growth factor (VEGF) concentration was analysed in the sera of patients with neuromyelitis optica (NMO), conventional MS (C-MS) or from healthy control subjects. The bars indicate the mean of each group. No significant differences were observed between the three groups. (B) Effect of VEGF expression in BMECs after exposure to sera from 10 NMO patients with anti-BMEC antibodies and four patients without anti-BMEC antibodies. Expression of VEGF in BMECs was significantly increased after exposure to sera from NMO patients with anti-BMEC antibodies (mean±SEM, n=10) although it did not change after exposure to sera from NMO patients without anti-BMECs antibodies (mean±SEM, n=4). (C) Expression of VEGF in BMECs did not change after exposure to sera from C-MS patients both with (mean±SEM, n=3) and without (mean±SEM, n=4) anti-BMEC antibodies. (D) VEGF secreted by astrocytes (i) and human umblilical vein endothelial cells (HUVECs) (ii) was not altered by exposure to sera from NMO patients with anti-BMEC antibodies (mean±SEM, n=14). NMO, conditioned medium with 10% NMO patient serum diluted with Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS); MS, conditioned medium with 10% serum from an MS patient diluted with DMEM containing 10% FBS.



B

Sera	AQP4 Antibody titers
NMO1	1:8
NMO1 after 30 min	1:8
NNO1 after 150 min	1:4
NMO2	1:2048
NMO2 after 30 min	1:2048
NMO2 after 150 min	1:512

Figure 5 (A) The 30 kDa single band corresponding to the shorter isoform of AQP4 (AQP4-M23) was detected in brain microvascular endothelial cells (BMECs) by western blot analysis. The AQP4-M23 transfected astrocytes were used as a positive control. (B) The anti-AQP4 antibody was absorbed from the sera of two different neuromyelitis optica (NMO) patients (NMO1 and NMO2) using astrocytes expressing human AQP4. In both cases the titres of anti-AQP4 antibody were decreased by at least 50% after a 150 min incubation period with cells although the titre was not affected after a 30 min incubation period. (C) Effects of reduction of the anti-AQP4 antibody on expression of claudin-5 protein in BMECs. The sera after both the 150 min and 30 min incubations with astrocytes led to an increase in expression of claudin-5 in BMECs. (D) Each column reflects the combined densitometry data from three independent experiments for the two different patients with NMO (mean±SEM, n=6, $p<0.05$; black bars, NMO1; grey bars, NMO2). (E) The transendothelial electrical resistance (TEER) value of BMECs was significantly increased after exposure to sera from NMO patients after both the 150 min and 30 min incubations with AQP4 transfected astrocytes. Each column reflects the combined densitometry data from three independent experiments for the two different patients with NMO (mean±SEM, n=6, $p<0.05$; black bars, NMO1; grey bars, NMO2). NMO, conditioned medium with 10% serum from an NMO patient diluted with Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS); NMO after 150 min, conditioned medium with 10% NMO sera after a 150 min incubation with astrocytes; NMO after 30 min, conditioned medium with 10% NMO sera after 30 min incubation with astrocytes.

that react with the cell surfaces antigens expressed by astrocytes. In both patients, the titres of the anti-AQP4 antibodies from NMO sera were decreased to one half or less than that of unadsorbed antibodies after a 150 min incubation period with the astrocytes although there was no significant change after a 30 min incubation period (figure 5B). Both the sera with and without reduction of the anti-AQP4 antibodies led to an increase in the expression of claudin-5 and in the TEER values of BMECs (figure 5C–E), suggesting that a reduction in anti-AQP4 antibody titre did not influence the ability of NMO sera to disrupt the BBB.

DISCUSSION

In this study, we used our established conditionally immortalised BBB derived endothelial cells to analyse the effects of sera from patients with NMO on impairment of BBB function. Although it would have been better to elucidate barrier function using microvascular endothelial cells derived from the spinal cord and optic nerve, no optimal endothelial cell lines originating from the spinal cord or optic nerve have been developed to date in any laboratory due to the difficulty in isolating a sufficient amount of microvascular endothelial cells from a minuscule amount of spinal cord and optic nerve tissue. We believe it was reasonable to use BMECs because several studies have shown a high incidence of brain lesions in approximately 60% of patients with NMO.[24 25] It is unclear why NMO predominantly affects the spinal cord and optic nerves despite the fact that destruction of the BBB occurs in NMO, but one possibility may be that the barrier properties of the microvascular endothelial cells derived from the spinal cord and optic nerve are more leaky than those of the BBB and, as a result, the destruction of barrier property causes more leakage of the anti-AQP4 antibodies and cytokines into the spinal cord and optic nerve spaces.

Several lines of evidence suggest that the bulk of the anti-AQP4 antibody is synthesised in the peripheral lymphoid compartment in affected individuals.[9] The anti-AQP4 antibody contained in the sera of NMO patients did not induce NMO-like lesions when injected into normal rats but did cause disease in experimental animals with T cell mediated brain inflammation.[12] This indicates that a leaky BBB that allows the intrusion of circulating anti-AQP4 antibodies thus plays a crucial role in the development of NMO. However, the molecular mechanism of BBB breakdown in NMO has not been adequately explained. Our present study is

the first to demonstrate that sera from patients with NMO can open the BBB. Expression of tight junction proteins and TEER value in BMECs was significantly decreased after exposure to sera from patients with NMO. Together, these results indicate that humoral factors in NMO sera disrupt the BBB; we therefore first tried to identify the most important substance involved in opening the BBB in NMO patients.

Antiendothelial cell (EC) antibodies binding to HUVECs have been detected in patients with several autoimmune diseases, such as SLE and MS.[26–29] Several studies demonstrated that anti-EC antibodies containing SLE sera activated ECs and facilitated the recruitment and trafficking of leucocytes into the inflamed vessels by increasing the expression of adhesion molecules and proinflammatory cytokines, including E-selectin and intercellular adhesion molecule 1, IL-1, TNFα and VEGF in an autocrine or paracrine manner.[30–37] No anti-EC antibodies have been detected in the sera from NMO patient to date but some reports have demonstrated that these may be a marker of disease activity in MS.[28] Therefore, based on this information and the fact that anti-AQP4 antibodies were insufficient to induce NMO lesions in the absence of inflammation, we hypothesised that anti-BMEC antibodies other than the anti-AQP4 antibodies might be involved in causing BBB disruption in NMO patients. Our study demonstrated that anti-BMEC antibodies were present in the sera of 10 of 14 NMO patients (71.4%) whereas no specific bands were detected in the sera from healthy or neurological disease controls. In contrast, anti-BMEC antibodies were present in the sera from one of three NP-SLE patients but several studies demonstrated that anti-EC antibodies binding to HUVECs have been detected in patients with NP-SLE. Immunocytochemical analysis showed that the anti-BMEC antibodies in NMO sera were localised in the cytoplasm of BMECs showing a granular pattern, similar to anti-EC antibodies in NP-SLE patients, thus suggesting that the anti-BMEC antibodies present in NMO sera as well as NP-SLE sera might contribute to the pathogenesis of BBB breakdown.

The presence of circulating cytokines, including TNFα, IL-6, IFNγ, IL-17A, VEGF and TGFβ, appears to be linked to the pathogenesis of BBB breakdown in NMO patients. Recent data suggest that these cytokines can disrupt the BBB[15 16 38–40]; in particular, VEGF was able to induce BBB impairment.[16] Our present study demonstrated that BBB function was restored after adding a neutralising anti-VEGF antibody to NMO sera, indicating that VEGF was the key molecule responsible for disruption of the BBB in NMO patients. Although concentration of VEGF in sera from NMO patients was not increased compared with sera from healthy control, secretion of VEGF in BMECs was increased after exposure to NMO sera in an autocrine manner. This suggests that anti-BMECs antibodies in sera from NMO patients activated BMECs and stimulated the secretion of VEGF by BMECs themselves, thus causing disruption of the BBB by reducing the production of claudin-5 by BMECs. We speculate that serum levels of VEGF were not increased because VEGF released by BMECs was not sufficiently high to increase serum concentrations but was still enough to influence BMECs by increasing local concentration.

Our study also provides confirmation that the anti-AQP4 antibody is one of the key mediators of BBB impairment in NMO patients because this study was the first to demonstrate that the AQP4 protein was expressed in BMECs using western blot analysis. However, while this antibody may have a role, it appears to be less important that the effects of VEGF or other anti-BMEC antibodies because reduction of the amount of anti-AQP4 antibody after exposure to transfected astrocytes did not influence the ability of sera from NMO patients to induce BBB disruption. Furthermore, we observed that the TEER value and expression of claudin-5 in BMECs were both increased after PE treatment. Removal of humoral factors, including various proinflammatory cytokines as well as presumed antibodies, is now the best explanation for the therapeutic effect following PE in NMO patients.[9 10] Removal of these serum constituents, including anti-BMEC antibodies, also restored BBB integrity, providing an additional rationale for PE during the acute stage of NMO. Therapy directed specifically towards BBB repair in the acute stage might also be a promising therapeutic strategy for NMO.

In conclusion, the present study demonstrated that anti-BMEC antibodies in the sera from NMO patients disrupted the BBB through upregulation of VEGF secreted by BMECs. These data provide new pathological explanations concerning the triggers for BBB breakdown and trafficking of anti-AQP4 antibodies into the CNS in the acute stage of NMO. Further studies of the pathological processes underlying NMO lesion formation should help in the development of therapies for this severe and disabling disease.

Funding This work was supported by research grants (Nos 22790821 and 21390268) from the Japan Society for the Promotion of Science, Tokyo, Japan and also by a research grant (K2002528) from Health and Labour Sciences Research Grants for research on intractable diseases (Neuroimmunological Disease Research Committee) from the Ministry of Health, Labour and Welfare of Japan.

Competing interests None.

Ethics approval The study was approved by the ethics committee of Yamaguchi University.

Contributors FS and TK conceived and designed the study. All authors reviewed, amended and agreed on the final version of the manuscript.

Provenance and peer review Not commissioned; externally peer reviewed.

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Neurochem Res (2012) 37:401–409
DOI 10.1007/s11064-011-0626-8

ORIGINAL PAPER

Pericyte-derived Glial Cell Line-derived Neurotrophic Factor Increase the Expression of Claudin-5 in the Blood–brain Barrier and the Blood-nerve Barrier

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Received: 23 May 2011 / Revised: 15 September 2011 / Accepted: 30 September 2011 / Published online: 16 October 2011
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Abstract The destruction of blood–brain barrier (BBB) and blood-nerve barrier (BNB) has been considered to be a key step in the disease process of a number of neurological disorders including cerebral ischemia, Alzheimer's disease, multiple sclerosis, and diabetic neuropathy. Although glial cell line-derived neurotrophic factor (GDNF) and brain-derived neurotrophic factor (BDNF) facilitate neuronal or axonal regeneration in the brain or peripheral nerves, their action in the BBB and BNB remains unclear. The purpose of the present study was to elucidate whether these neurotrophic factors secreted from the brain or peripheral nerve pericytes increase the barrier function of the BBB or BNB, using our newly established human brain microvascular endothelial cell (BMEC) line or peripheral nerve microvascular endothelial cell (PnMEC) line. GDNF increased the expression of claudin-5 and the transendothelial electrical resistance (TEER) of BMECs and PnMECs, whereas BDNF did not have this effect. Furthermore, we herein demonstrate that the GDNF secreted from the brain and peripheral nerve pericytes was one of the key molecules responsible for the up-regulation of claudin-5 expression and the TEER value in the BBB and BNB. These results indicate that the regulation of GDNF secreted from pericytes may therefore be a novel therapeutic strategy to modify the BBB or BNB functions and promote brain or peripheral nerve regeneration.

Keywords GDNF · Blood–brain barrier · Blood-nerve barrier · Pericytes · Endothelial cells

Special issue: In honor of Dr. Abel Lajtha.

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Introduction

The blood–brain barrier (BBB) and the blood-nerve barrier (BNB) are formed from a continuous monolayer of highly specialized endothelial cells, constituting the functional barriers sheltering the nervous system from circulating blood [1, 2]. The BBB is comprised of brain microvascular endothelial cells (BMECs), astrocytes and pericytes of microvascular origin, whereas the BNB comprises peripheral nerve microvascular endothelial cells (PnMECs) and pericytes of endoneurial microvascular origin [1, 3]. Astrocytes have been demonstrated to strengthen the barrier function of the BBB via their secretion of soluble factors, as demonstrated in in vitro BBB models [4, 5], although pericytes have also been shown to have this effect in in vitro BBB and BNB models [6, 7]).

The glia cell line-derived neutrophic factor (GDNF) is a member of the transforming factor-β superfamily, and its neurotrophic action is mediated by a unique multi-component receptor system consisting of GDNF-family of receptors (GFRα1-4) [8]. Several studies have demonstrated the exogenous administration of GDNF to support long-term neuronal survival, while it also protects and repairs dopaminergic neurons in Parkinson's disease [9, 10], and the motor neurons in amyotrophic lateral sclerosis (ALS) [11, 12]. On the other hand, brain-derived neurotrophic factor (BDNF) is a neurotrophin that promotes the

survival and growth of developing neurons in vitro, and its effects are mediated by the tropomyosin receptor kinase family of receptors (Trk-B) [13].

Astrocytes, the most abundant glial cell type in the brain, have various physiological roles; for example, the maintenance of BBB function and the production of neurotrophic factors, including nerve growth factor (NGF), BDNF, and GDNF [14–16]. Some reports have indicated that the GDNF secreted from astrocytes modulates the barrier function of tight junctions in the BBB and blood-retinal barrier (BRB) [17, 18]. Although we have previously demonstrated that brain and peripheral nerve pericytes secrete several neurotrophic factors including NGF, GDNF and BDNF [7], it remains unclear whether these growth factors modulate the BBB and BNB function. In the present study, we examined the effects of GDNF and BDNF secreted from pericytes on an endothelial cell line which originated from the human BBB and BNB.

Materials and Methods

Reagents

The culture medium for pericytes and PnMECs consisted of Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, MO, USA) containing 100 U/ml penicillin (Sigma), 100 μg/ml streptomycin (Sigma), 25 ng/ml amphotericin B (Invitrogen, Grand Island, NY, U.S.A), 10% fetal bovine serum (FBS) (Sigma) and 2.5 mM D-glucose (Sigma). Polyclonal anti-claudin-5 and anti-occludin antibodies were purchased from Zymed (San Fransisco, CA, USA). The polyclonal anti-BDNF antibodies were purchased from Calbiochem (Darmstadt, Germany). Polyclonal anti-GDNF antibody was purchased from R&D Systems (Minneapolis, USA). The polyclonal anti-β-tubulin antibody, anti-GFRα antibodies, and anti-Trk-B antibodies were obtained from Santa Cruz (Santa Cruz, CA, USA). Human astrocytes were purchased from Lonza (Walkersville, MD, USA).

Cell Culture and Treatment

The immortalized human BMECs and PnMECs, and brain and peripheral nerve pericyte cell lines were generated as described previously [7, 19, 20]. The study protocol for human tissue was approved by the ethics committee of the Medical Faculty of the University of Yamaguchi Graduate School and was conducted in accordance with the Declaration of Helsinki, as amended in Somerset West in 1996. Written informed consent was obtained from the families of the participants before they entered the study. The cell lines were isolated from human sciatic nerve and brain tissue, and retained the morphological characteristics of primary brain and peripheral nerve endothelial cells, or brain and peripheral nerve pericytes, and expressed either endothelial or pericyte markers [7, 19, 20]. The cultures were maintained at 37°C in 5% CO_2, and the DMEM medium was replaced every 3 days.

Quantitative Real-time PCR Analysis

Total RNA was extracted from BMECs or PnMECs using an RNeasy® Plus Mini Kit (Qiagen, Hilden, Germany). Single-stranded cDNA was created from 40 ng of total RNA using the StrataScript First Strand Synthesis System (STRAT AGENE, Cedar Greek, TX, USA). The sequence of each human primer pair and its reference used in the present studies were as follows; sense primer 5′-CTGTTTCCAT AGGCAGAGCG-3′ and anti-sense primer 5′-AAGCAGA TTCTTAGCCTTCC-3′ for claudin-5 [21]; sense primer 5′-TGGGAGTGAACCCAACTGCT-3′ and anti-sense primer 5′-CTTCAGGAACCGGCGTGGAT-3′ for occludin [22]; sense primer 5′-GTCAACGGAT TTGGTCTGTATT-3′ and anti-sense primer 5′- AGTCT TCTGGGTGGCAGTG AT-3′ for G3PDH [23]. The quantitative real-time PCR analyses were performed using a Stratagene Mx3005P instrument (STRATAGENE®, Cedar Greek, TX, USA) with FullVelocity® RSYBR® Green QPCR master mix (STRATAGENE®). Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) was used as an internal standard. The samples were subjected to a PCR analysis using the following cycling parameters: 95°C for 10 min, 95°C for 15 s and 60°C for 1 min, for 40 cycles. The standard reaction curves were analyzed using the MxProTM (STRATAGENE®) software program, and the relative quantity according to the standard reaction curve (Rv) was calculated according to the formula Rv = RGene/RGAPDH on a computer.

Western Blot Analysis

The protein samples (10–20 μg) were separated by SDS–PAGE (Biorad) and transferred to nitrocellulose membranes (Amersham, Chalfont, UK). The membranes were treated with blocking buffer (5% skim milk in 25 mM Tris–HCl pH 7.6, with 125 nM NaCl and 0.5% Tween 20) for 1 h at room temperature and incubated with the relevant primary antibodies (dilution 1:100) for 2 h at room temperature. The membranes were then exposed to a peroxidase-conjugated secondary antibody (1:2000) at room temperature for 1 h. The membranes were visualized by enhanced chemiluminescence detection (ECL-plus, Amersham, UK), and recorded on a CCD camera (The Bio-Rad ChemiDoc XRS System. BIO-RAD, Hercules, CA). Quantification of the band intensity was performed using the Quantity One software program (BIO-RAD, Hercules, CA).

Transendothelial Electrical Resistance (TEER) Studies

Transwell inserts (pore size 0.4 μm, effective growth area 0.3 cm^2, BD Bioscience, Sparks, MD, USA) were coated with rat-tail collagen type-I (BD Bioscience). The TEER values of cell layers were measured with a Millicell electrical resistance apparatus (Endohm-6 and EVOM, World Precision Instruments, Sarasota, FL, USA). The BMECs were seeded (1×10^6 cells/insert) on the upper compartment and incubated with each medium (non-conditioned medium used as a control, conditioned medium contained 10% patient sera) for 24 h.

Analysis of the Effects of GDNF or BDNF on the Expression of Tight Junction Molecules in BMECs or PnMECs

BMECs and PnMECs were either left untreated, or were treated with human GDNF (1, 10 ng/ml) or BDNF (1, 10 ng/ml) for 24 h. The total RNA was then extracted, or the TEER value was measured. Total proteins were obtained the next day.

GDNF Inhibitory Study

The conditioned media of brain pericytes (BPCT-CM), of peripheral nerve pericytes (PPCT-CM), or of astrocytes (AST-CM) were pre-treated with 2.0 μg/ml of a neutralizing antibody against GDNF or with normal rabbit IgG (control) for 12 h at 4°C. BMECs and PnMECs were cultured with the GDNF-neutralized- BPCT-CM, PPCT-CM or AST-CM at 37°C. The total proteins were extracted, and the TEER value was measured 2 days later.

Data Analysis

Unless otherwise indicated, all data represent the means ± SEM. An unpaired, two-tailed Student's *t*-test was used to determine the significance of differences between the means of two groups. A *P* value of <0.01 was considered to be statistically significant.

Results

The Effects of GDNF or BDNF on the Expression of Tight Junctional Molecules by BMECs and PnMECs

In order to determine the sensitivity of BMECs and PnMECs to GDNF or BDNF, we examined the expression of the GDNF receptor, GFR-α1, and BDNF receptor, Trk-B, in these cell lines using a Western blot analysis. The single band at 51 kDa for GFR-α1 was detected in BMECs, PnMECs, brain and peripheral nerve pericytes, and astrocytes (Fig. 1). The bands at 145 kDa for Trk-B, corresponding to the full-length tyrosine kinase receptor, were also observed in BMECs, PnMECs, brain and peripheral nerve pericytes, and astrocytes. In contrast, the bands at 95 kDa for the truncated isoform of Trk-B were detected in brain and peripheral nerve pericytes, and astrocytes, although it was not observed in BMECs and PnMECs.

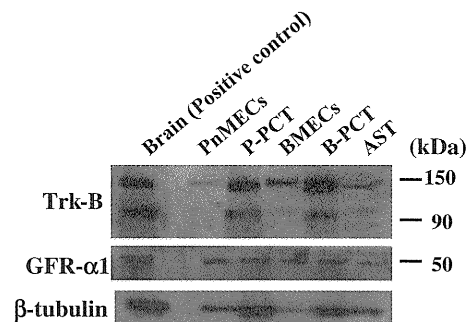

Fig. 1 A Western blot analysis of GFR-α1 and Trk-B in BMECs, PnMECs, the brain and peripheral nerve pericytes, and astrocytes. Human brain tissues specimens were used as positive controls

To determine the effects of GDNF or BDNF on the barrier function of the BBB and BNB, we also examined the changes in *claudin-5* or *occludin* expression by BMECs and PnMECs after exposure to GDNF or BDNF by means of relative quantification with a real-time RT–PCR analysis (Fig. 2a–h). The expression of *claudin-5* mRNA in BMECs and PnMECs significantly increased after incubation with GDNF (10 ng/ml) (Fig. 2a, c), whereas it was not affected by incubation with BDNF (Fig. 2b, d). In contrast, the expression of the *occludin* mRNA level in BMECs or PnMECs did not change following treatment with GDNF or BDNF (Fig. 2e–h).

Furthermore, the claudin-5 and occludin protein expression levels in BMECs and PnMECs after incubation with GDNF (1, 10 ng/ml) or BDNF (1, 10 ng/ml) were quantified using a Western blot analysis (Fig. 3a–j). The claudin-5 protein expression was increased after treatment with GDNF (10 ng/ml; Fig. 3c, e), whereas it was not changed by incubation with BDNF (Fig. 3d, e). On the other hand, the expression of the occludin protein expression did not significantly change after treatment with GDNF or BDNF (Fig. 3g–h).

The Changes in the TEER in BMECs and PnMECs after Incubation with GDNF or BDNF

The TEER across the monolayer of BMECs and PnMECs in response to treatment with GDNF or BDNF was measured to determine whether GDNF or BDNF enhances the barrier properties of BMECs and PnMECs (Fig. 4a–d). The TEER value of BMECs and PnMECs increased after

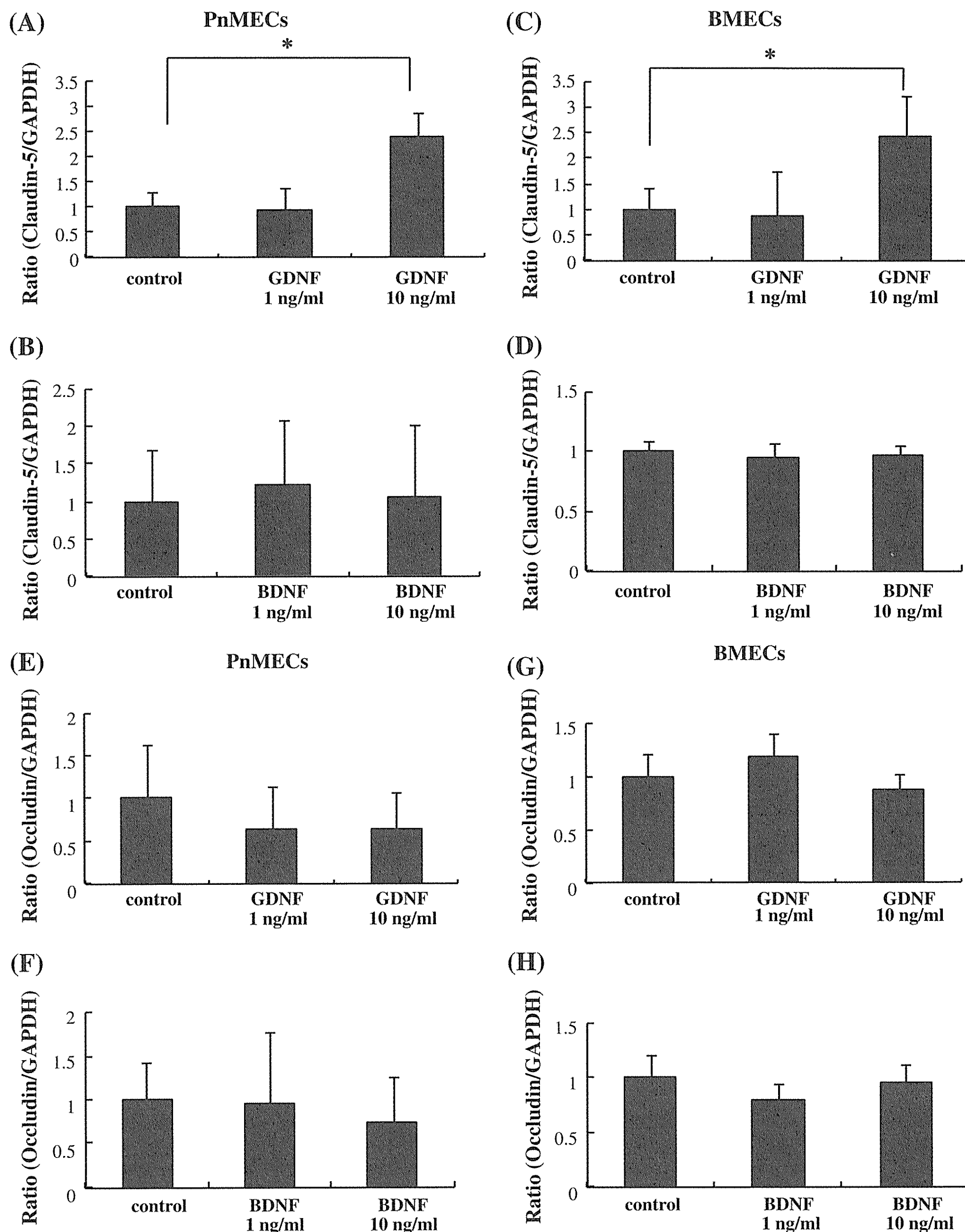

Fig. 2 The *claudin-5* and *occludin* mRNA level after a 24 h application of GDNF or BDNF in PnMECs (**a**, **b**, **e**, **f**) and BMECs (**c**, **d**, **g**, **h**). The *claudin-5* and *occludin* mRNA levels in BMECs and PnMECs were quantified by real-time RT–PCR and expressed as the ratio of target gene/GAPDH. Data are presented as the mean (±SEM) of six independent PCR runs

incubation with GDNF (10 ng/ml) (Fig. 4a, b), but there was no change after treatment with BDNF (Fig. 4c, d).

The Effects of the GDNF Neutralizing Antibody on the Induction of claudin-5 and TEER Changes Induced by Brain or Peripheral Nerve Pericyte-conditioned Media

To clarify the contribution of GDNF to the induction of claudin-5 in BMECs and PnMECs by the BPCT-CM, PPCT-CM or AST-CM, the GDNF activities were neutralized using an anti-GDNF antibody (Figs. 5a–f). The expression of the claudin-5 protein in BMECs was decreased after incubation with BPCT-CM (Fig. 5d) or AST-CM (Fig. 5f) that were pretreated with the anti-GDNF antibody. In addition, the claudin-5 protein expression level was reduced after incubation with PPCT-CM pretreated with the anti-GDNF antibody (Fig. 5b). Furthermore, the TEER value of BMECs was significantly reduced following treatment with BPCT-CM (Fig. 5h) or AST-CM (Fig. 5i) that were pretreated with the anti-GDNF neutralizing antibody. The TEER value of PnMECs was also significantly decreased in PPCT-CM

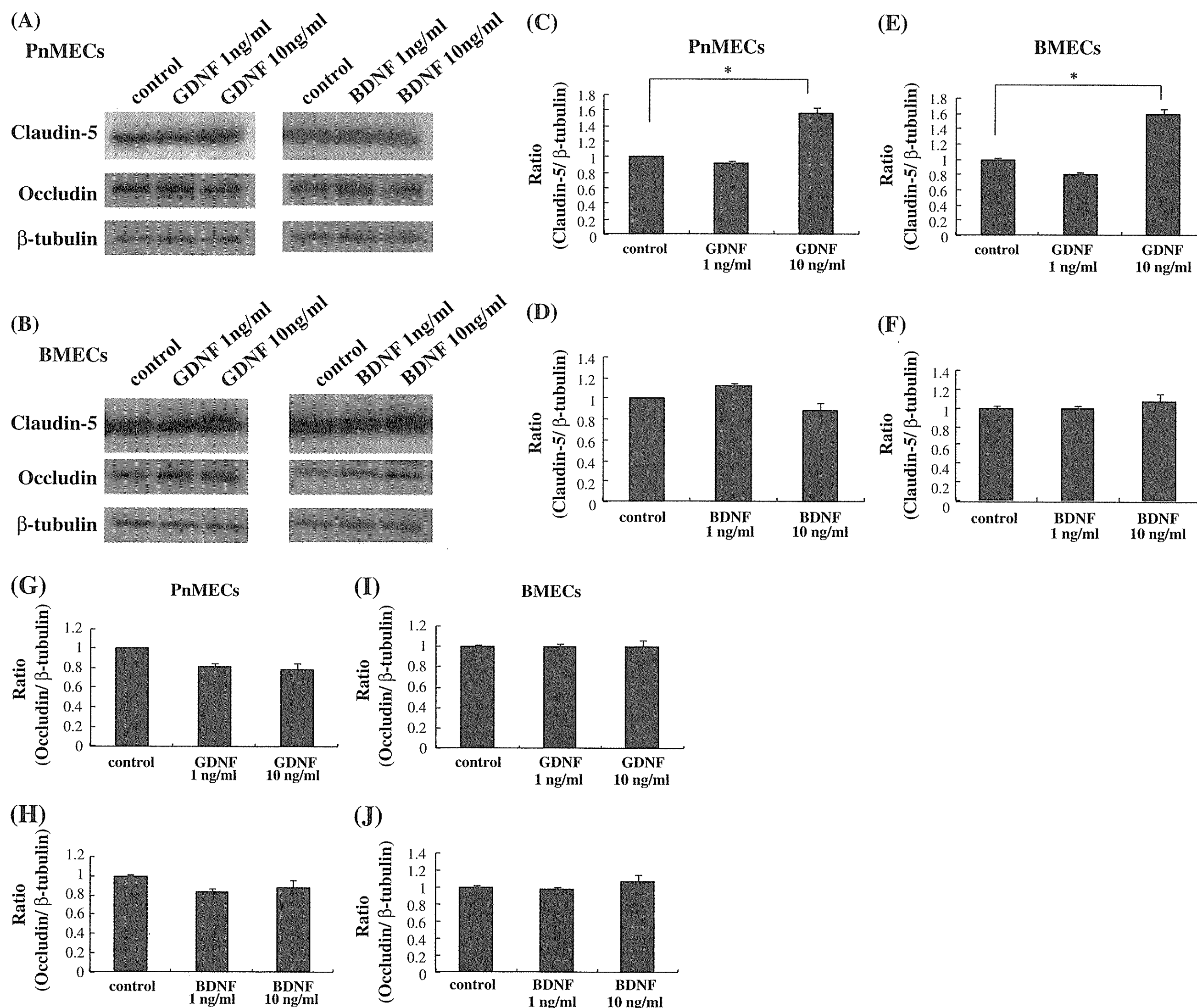

Fig. 3 **a** The effect of GDNF or BDNF on the claudin-5 or occludin protein expression in PnMECs after a 2-day treatment. **b** The effect of GDNF or BDNF on the claudin-5 or occludin protein expression in BMECs. The expression of β-tubulin was used as an internal standard. The bar graph reflects the combined densitometry data from three independent experiments (mean ± SEM, $n = 3$, $^{*}P < 0.01$)

pretreated with the anti-GDNF neutralizing antibody (Fig. 5g).

Discussion

In this study, we examined whether GDNF and BDNF could alter the expression amount of tight junction proteins, including claudin-5 and occludin, in the BBB and BNB. Claudin-5 is now recognized as the most important component involved in maintaining BBB function [24] and was reported to be the most abundantly expressed subtype among the claudins in mouse brain capillary endothelial cells at the mRNA level [25]. Several reports have previously demonstrated that the expression of claudin-5 in BMECs was increased by humoral factors such as adrenomedullin [26] and bFGF [27], and was reduced by VEGF [28]. We also previously demonstrated the expression of claudin-5 in PnMECs to increase by bFGF, while it decreased by VEGF or TGF-β [7]. The current results demonstrated that the capillary endothelial cells forming the BBB and BNB express the GDNF receptor, GFR-α1, and that GDNF could increase the expression of claudin-5 in BMECs and PnMECs at both the mRNA and protein levels, whereas the BDNF did not have such an effect. Furthermore, the TEER values of BMECs and PnMECs were increased after incubation with GDNF, suggesting that GDNF could enhance the barrier functions of the BBB and BNB. GDNF has been generally accepted as a neurotrophic factor that enhances the survival of dopaminergic

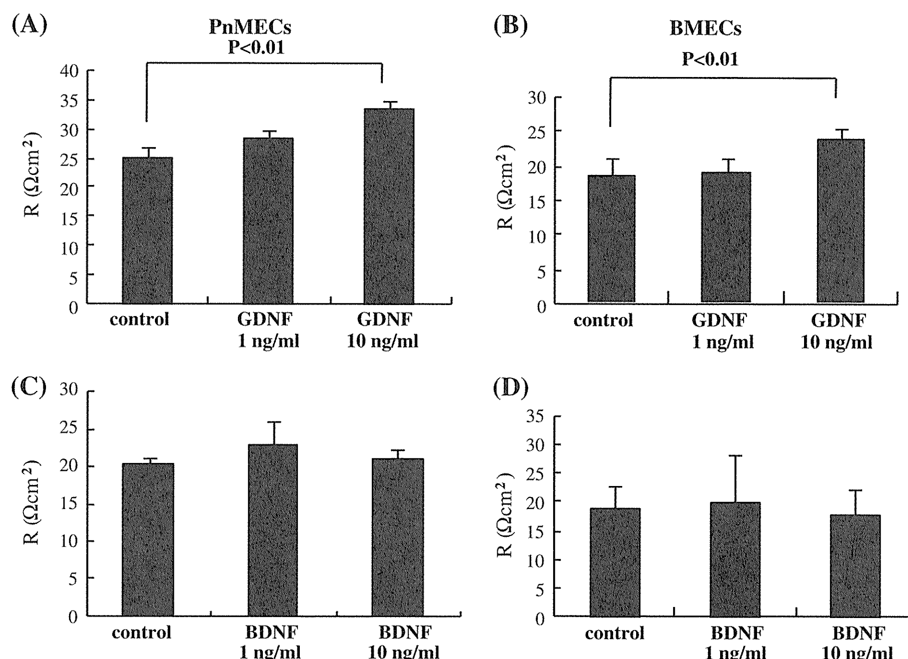

Fig. 4 The effect of GDNF or BDNF on the TEER values across PnMECs (**a**, **c**) or BMECs (**b**, **d**) monolayer (mean ± SD, $n = 5$, $*P < 0.01$)

and motor neurons [8]. However, a few reports have recently demonstrated that the capillary endothelial cells forming the BBB and BRB expressed the GDNF receptor, GFR-α1, and that GDNF could modulate the barrier function of the BBB and BRB [17, 18, 29]. The present findings demonstrated that GDNF is a critical factor that enhances the barrier properties of the BBB/BNB.

Recently, the breakdown of the BBB has been considered to be a critical event in the development and progression of several disorders that affect the central nerve system (CNS), including cerebral infarction, Alzheimer's disease, and multiple sclerosis [30–32]. In addition, the breakdown of the BNB has also been reported to be a key initial step in many diseases of peripheral nerve system (PNS), such as Guillain-Barré syndrome, chronic inflammatory demyelinating polyradiculoneuropathy (CIDP), and diabetic neuropathy [33–36]. This finding suggests that the modification of the integrity of tight junctions in the BBB and BNB may thus provide novel therapeutic avenues for many CNS and PNS diseases. The present study suggested that the intravenous administration of GDNF might thus have a therapeutic potential for repairing and modifying the barrier properties of the BBB and BNB in several CNS and PNS disorders.

Several studies have indicated that astrocytes strengthen the barrier function of BMECs via the secretion of soluble factors in the in vitro BBB model [4, 5], and that GDNF secreted from astrocytes has been reported to modulate the barrier function of BBB and blood-retinal barrier (BRB) [17, 18, 29]. We have previously demonstrated that brain and peripheral nerve pericytes produce GDNF, and that soluble factors including bFGF secreted from brain and peripheral nerve pericytes strengthen the barrier function of the BBB and BNB by increasing the expression of claudin-5 [7]. We therefore hypothesized that GDNF secreted from brain and peripheral nerve pericytes may contribute to the enhancement of the BBB and BNB function by up-regulating the expression of claudin-5. Our present study demonstrated that the expression of claudin-5 and the TEER value decreased after adding a neutralizing anti-GDNF antibody to the conditioned medium of pericytes, thus indicating that the GDNF secreted from the brain and peripheral nerve pericytes was one of the key molecules responsible for the up-regulation of claudin-5 expression and the TEER value in the BBB and BNB. Several studies have indicated that the exogenous administration of GDNF helps to promote neuronal survival and axonal regeneration in animal models of neurodegeneration disorders, stroke, and peripheral nerve injury. However, neurotrophic factors such as GDNF and BDNF cannot be used for neuroprotection following intravenous administration because the BBB and BNB interrupt the entrance of neurotrophic factor into the CNS and PNS under normal conditions. Several studies have demonstrated various methods for delivering GDNF into the CNS and PNS in animal models, including direct tissue infusion, as well as adenoviral and lentiviral infection [37], however, these methods also have several shortcomings and concerns. On the other hand, the intraputamenal infusion of supraphysiological levels of GDNF have been shown to possibly have a toxic effect in an animal model of Parkinson's disease [38], thus suggesting the importance of physiologically relevant doses of

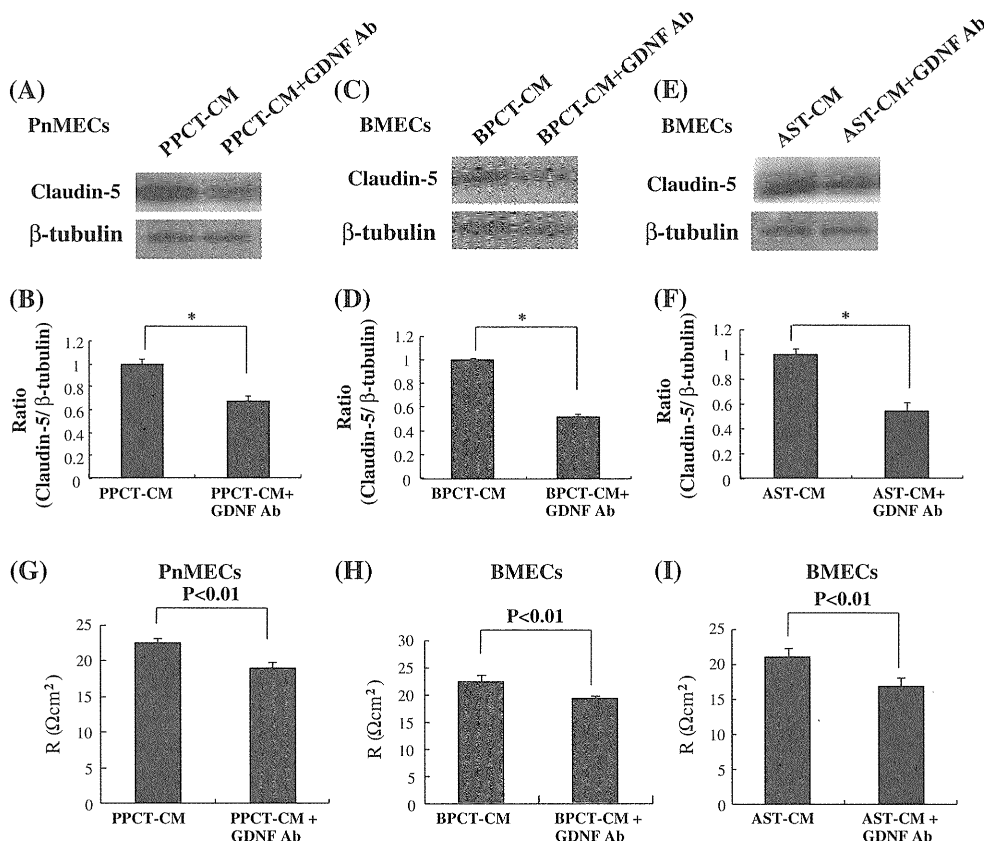

Fig. 5 Effects of GDNF neutralizing antibody on the induction of claudin-5 and TEER changes induced by brain- or peripheral nerve pericyte-conditioned media. **a** The PnMECs were cultured with PPCT-CM or PPCT-CM pre-treated with anti-GDNF antibody for 2 days. Claudin-5 protein was inhibited after pretreatment with anti-GDNF neutralizing antibody. **b** The bar graph reflects the combined densitometry data from three independent experiments (mean ± SEM, $n = 3$, $*P < 0.01$). **c**, **e** The BMECs were cultured with BPCT-CM or AST-CM pre-treated with anti-GDNF antibody for 2 days. The claudin-5 protein level decreased after pretreatment with anti-GDNF neutralizing antibody. **d**, **f** The bar graph reflects the combined densitometry data from three independent experiments (mean ± SEM, $n = 3$, *: $P < 0.01$). **g**, **h**, **i** The effect of PPCT-CM, BPCT-CM, or AST-CM on the TEER values across PnMEC or BMECs monolayer. (mean ± SD, $n = 5$, $*P < 0.01$). BPCT-CM, conditioned medium of brain pericytes; PPCT-CM, conditioned medium of peripheral nerve pericytes; AST-CM, conditioned medium of astrocytes. PPCT-CM + GDNF Ab; conditioned medium of peripheral nerve pericytes pre-treated with GDNF neutralizing antibody. BPCT-CM + GDNF Ab; conditioned medium of brain pericytes pre-treated with GDNF neutralizing antibody. AST-CM + GDNF Ab; conditioned medium of astrocytes pre-treated with GDNF neutralizing antibody

GDNF to achieve both safety and efficacy. New studies for the development of better and safer viral vectors or engineered cell lines suitable for the CNS grafting and delivery have been launched [39–41]. Indeed, promising results have been obtained with cell grafts including astrocytes, which have been successfully engineered to deliver GDNF within therapeutic window [41–44]. Pericytes as well as astrocytes are also very promising candidates for new methods for carrying out GDNF delivery, and they may be easily engineered to produce and secrete the most beneficial dose of this neurotrophin in the BBB and BNB. Further investigation to establish the optimal methods to increase the production of endogenous GDNF from brain and peripheral nerve pericytes into the CNS and PNS space within the therapeutic window may therefore be useful for enhancing the barrier property in the BBB and BNB as well as neuroprotection, and it may have a therapeutic potential for intractable disorders of both the CNS and PNS.

In conclusion, GDNF secreted from the brain or peripheral nerve pericytes strengthened the barrier function of the BBB or BNB by increasing the expression of claudin-5. Further research is therefore necessary to elucidate the molecular mechanisms by which pericytes regulate the BBB and the BNB function under both physiological and pathological conditions in order to establish new therapies for various neurological disorders of the CNS and PNS.

Acknowledgments This work was supported by research grants (Nos. 22790821 and Nos. 21390268) from the Japan Society for the Promotion of Science, Tokyo, Japan and also by research grant (K2002528) from Health and Labor Sciences Research Grants for research on intractable diseases (Neuroimmunological Disease