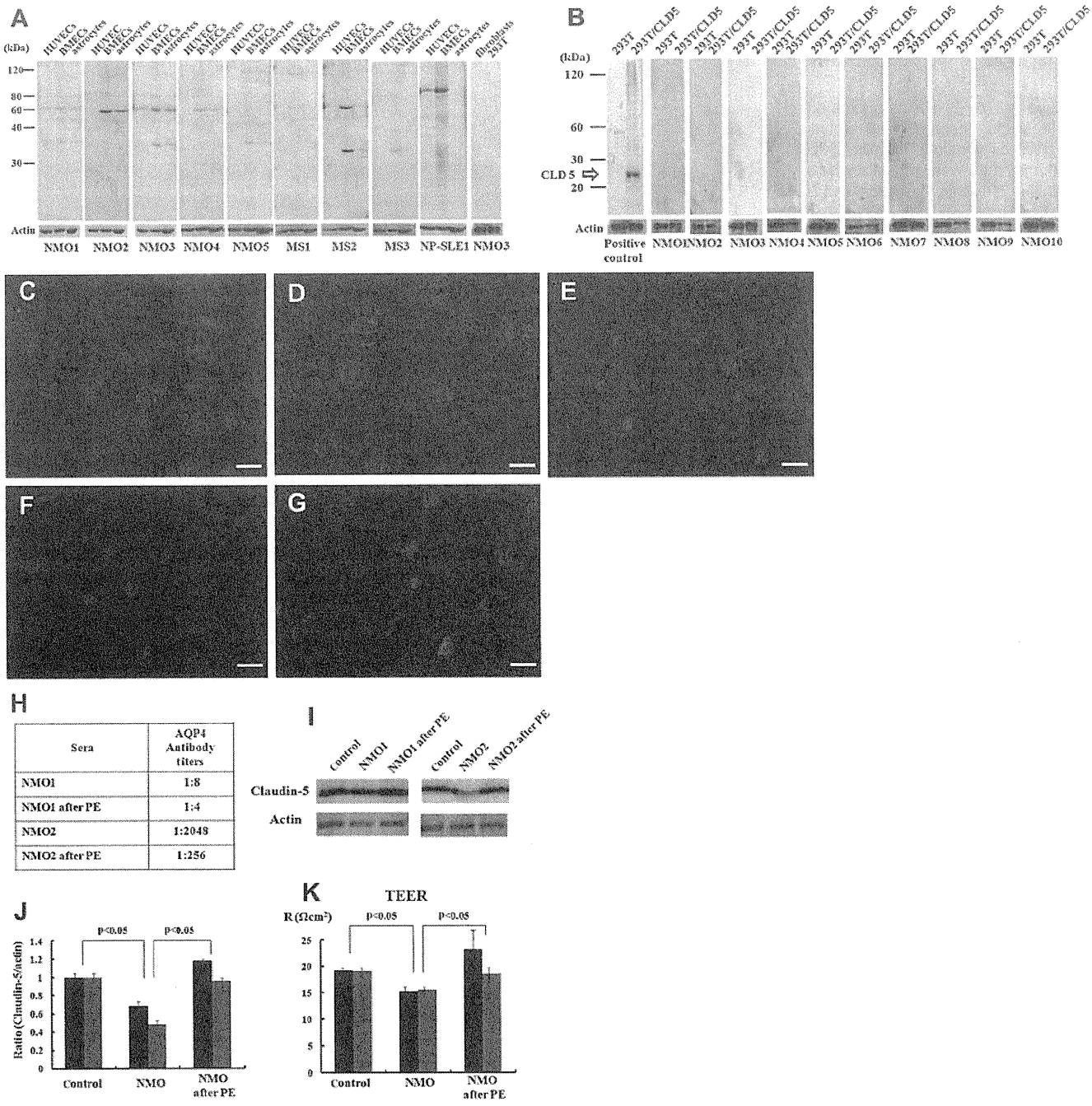

Figure 2 (A) Representative results obtained by immunoblotting of human brain microvascular endothelial cell (BMEC) lysates. The blots were exposed to sera from 14 neuromyelitis optica (NMO) and seven conventional MS (C-MS) patients, or to 11 neurological disease controls and 12 healthy controls after 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)

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 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

[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.

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 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

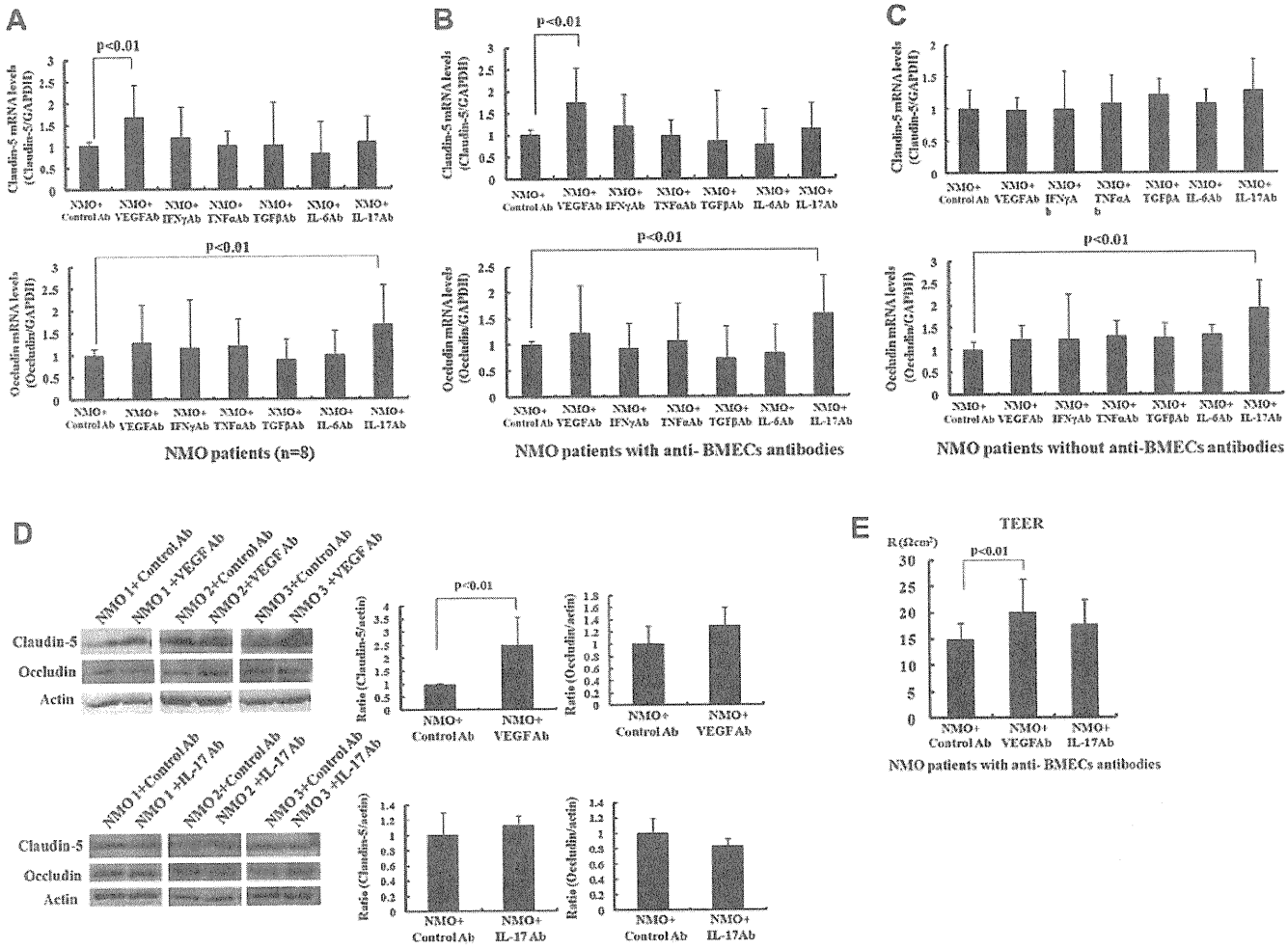

Figure 3 (A) Effects of anti-tumour necrosis factor α (TNFα), interleukin 6 (IL-6), interferon γ (IFNγ), interleukin 17 (IL-17), vascular endothelial growth factor (VEGF) and transforming growth factor β (TGFβ) neutralising antibodies on expression of tight junction molecules in human brain microvascular 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.

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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 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

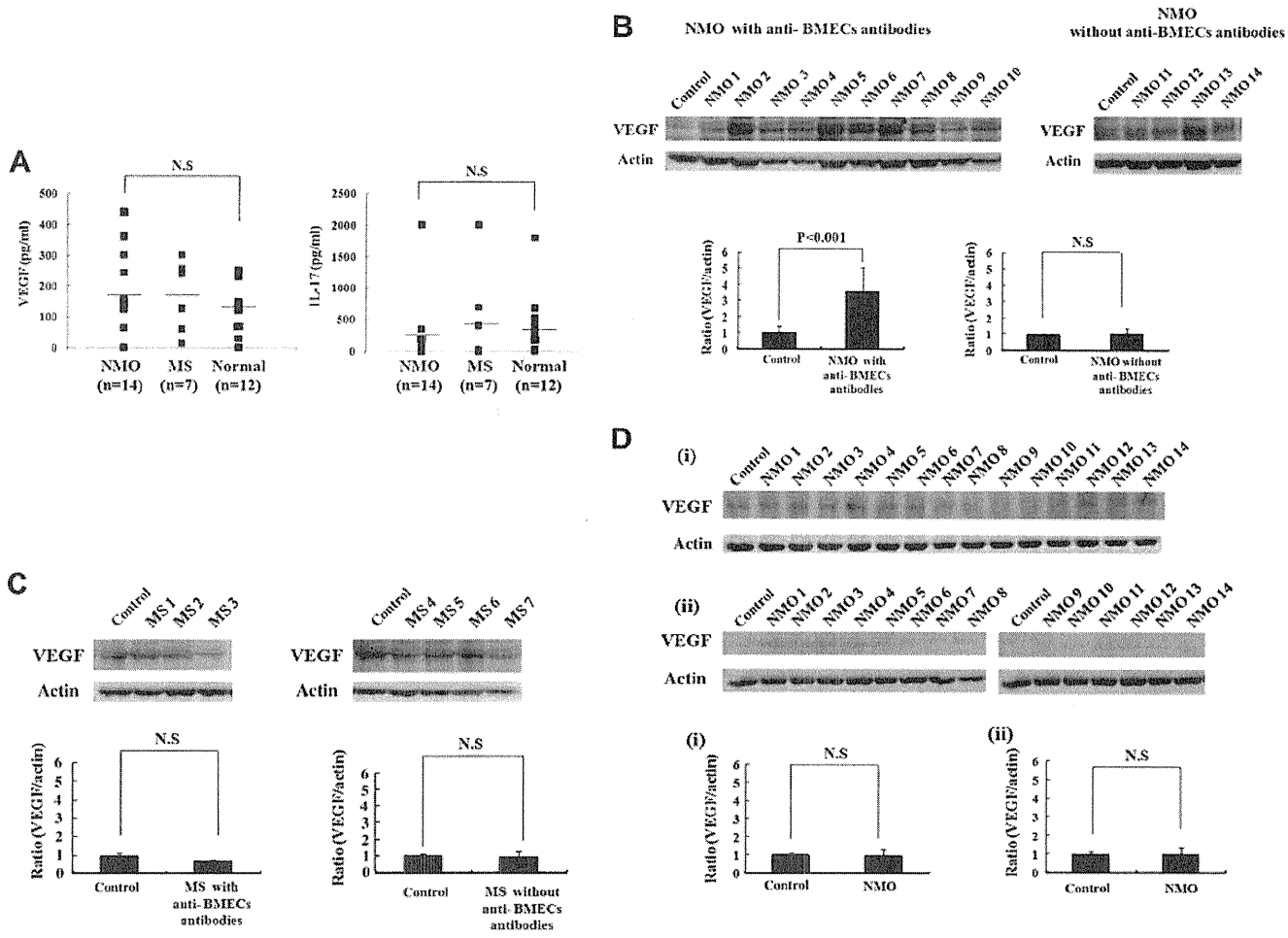
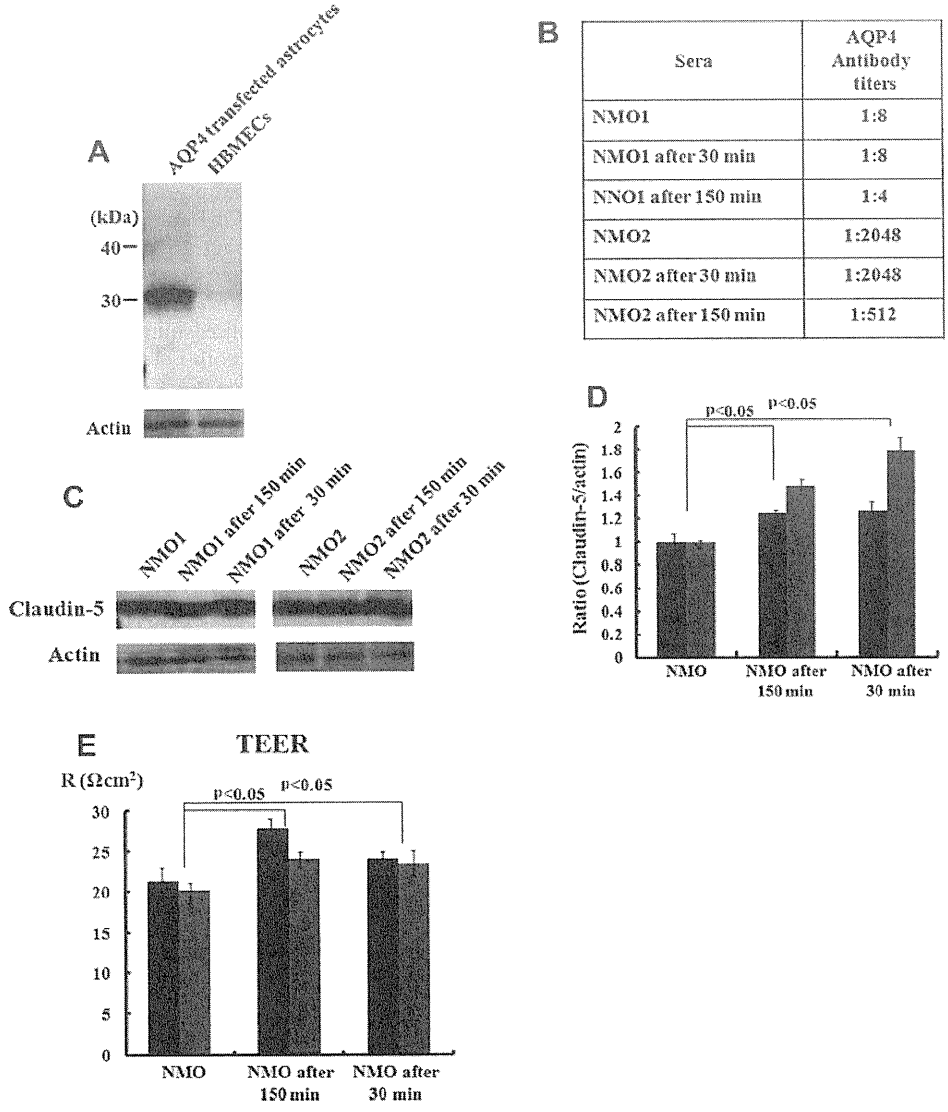


Figure 4 (A) Vascular endothelial growth factor (VEGF) concentration was analysed in the sera of patients with neuromyelitis optica (NMO), conventional MS (C-MS) or from healthy control subjects. The bars indicate the mean of each group. No significant differences were observed between the three groups. (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.



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.

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.

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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.

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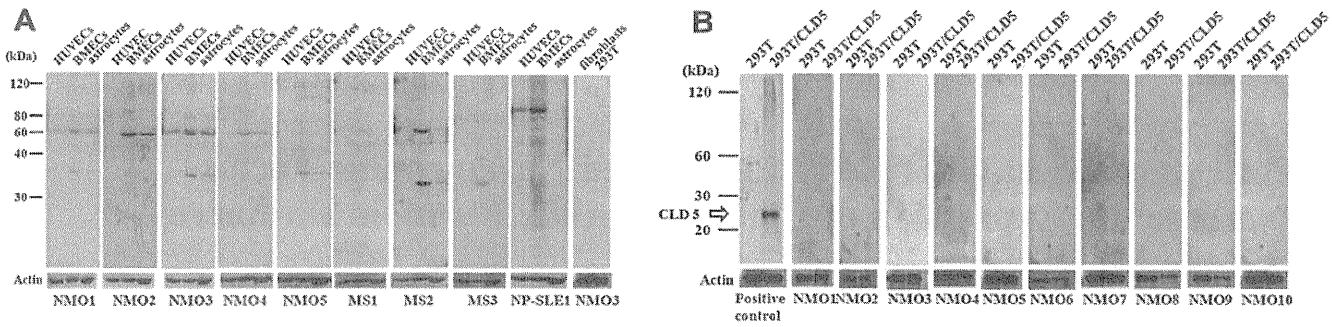
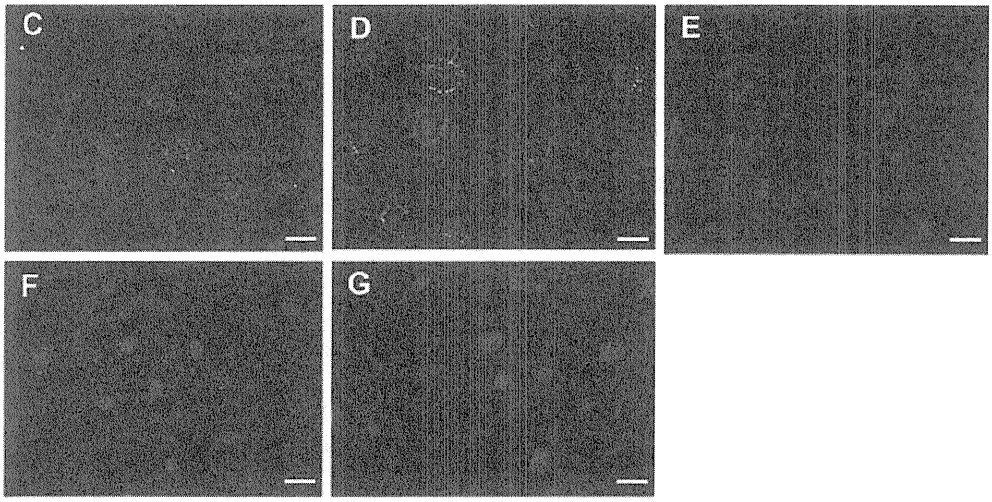
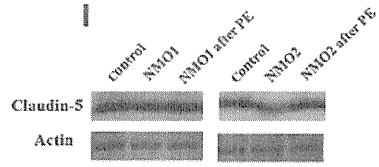
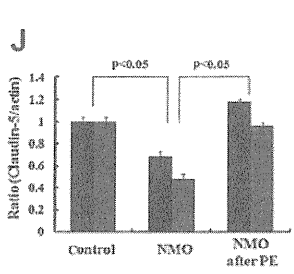


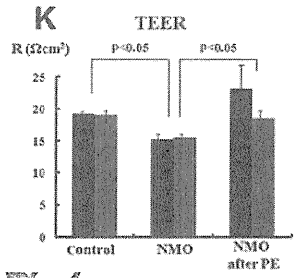


H

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







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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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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

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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

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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 (STRATAGENE, 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.

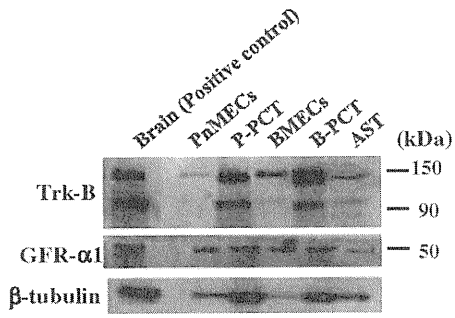

Fig. 1 A Western blot analysis of GFR-α1 and Trk-B in BMECs, PnMECs, the brain and peripheral nerve pericytes, and astrocytes. Human brain tissues specimens were used as positive controls

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

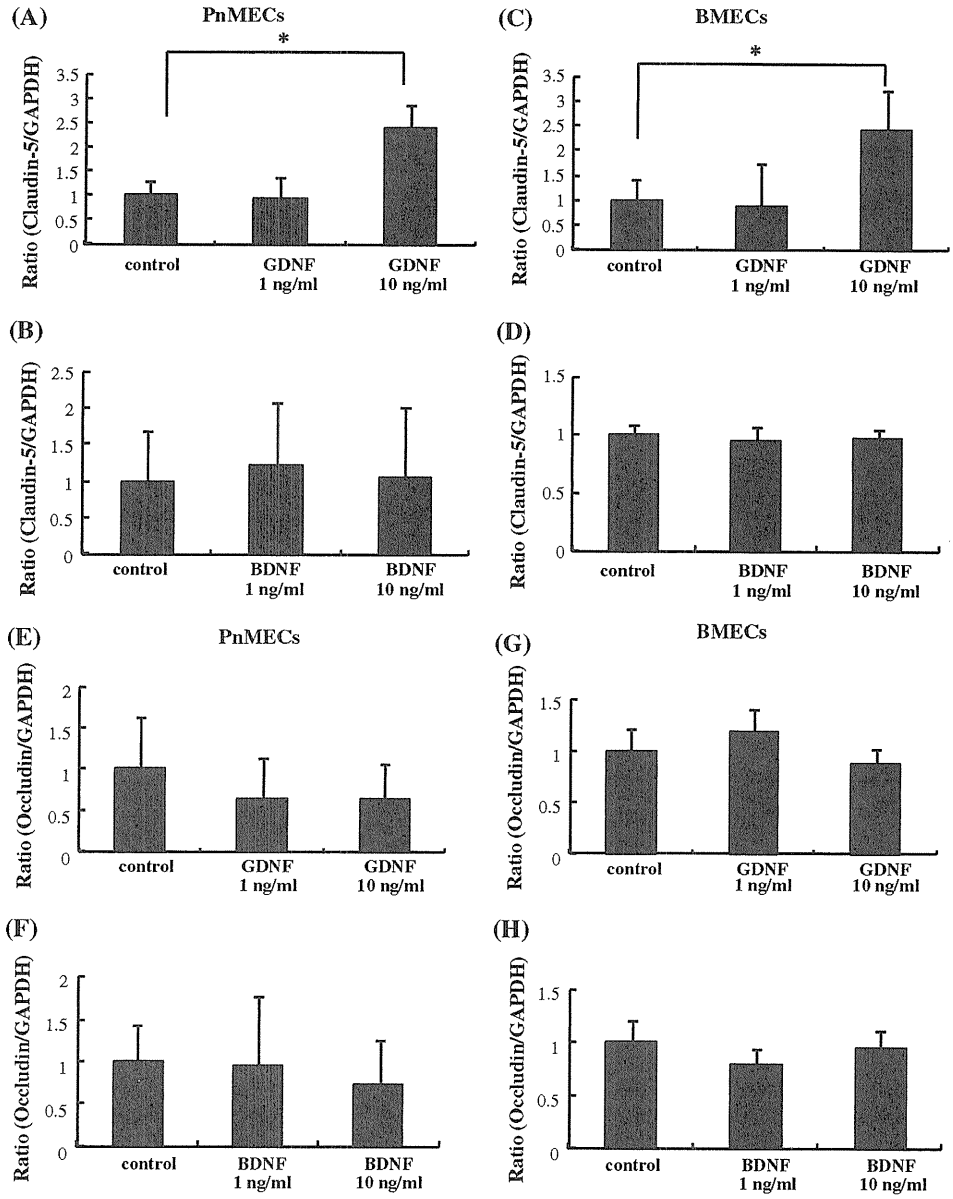

Fig. 2 The *claudin-5* and *occludin* mRNA level after a 24 h application of GDNF or BDNF in PnMECs (**a**, **b**, **e**, **f**) and BMECs (**c**, **d**, **g**, **h**). The *claudin-5* and *occludin* mRNA levels in BMECs and PnMECs were quantified by real-time RT–PCR and expressed as the ratio of target 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

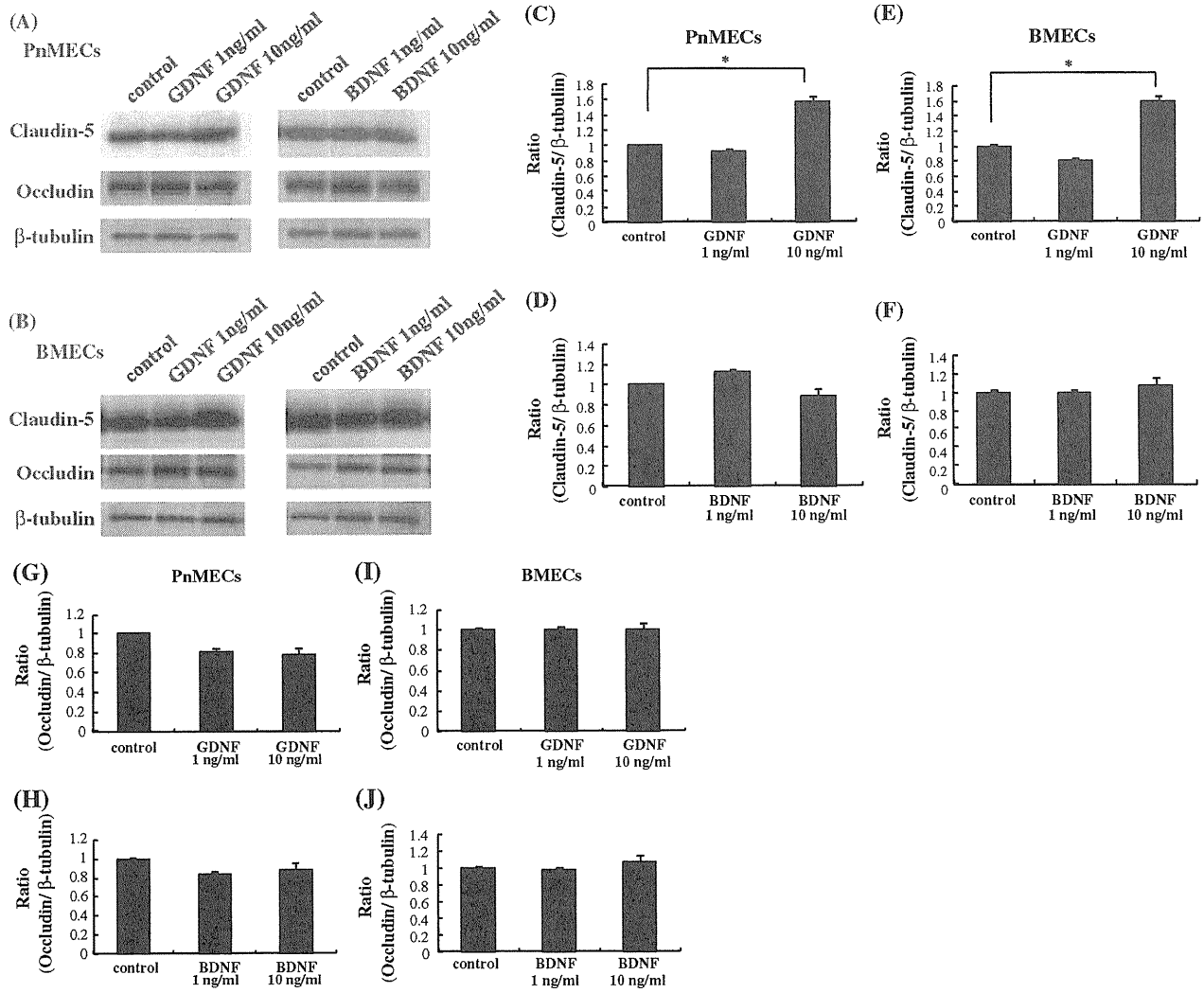

Fig. 3 **a** The effect of GDNF or BDNF on the claudin-5 or occludin protein expression in PnMECs after a 2-day treatment. **b** The effect of GDNF or BDNF on the claudin-5 or occludin protein expression in BMECs. The expression of β-tubulin was used as an internal standard. The bar graph reflects the 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

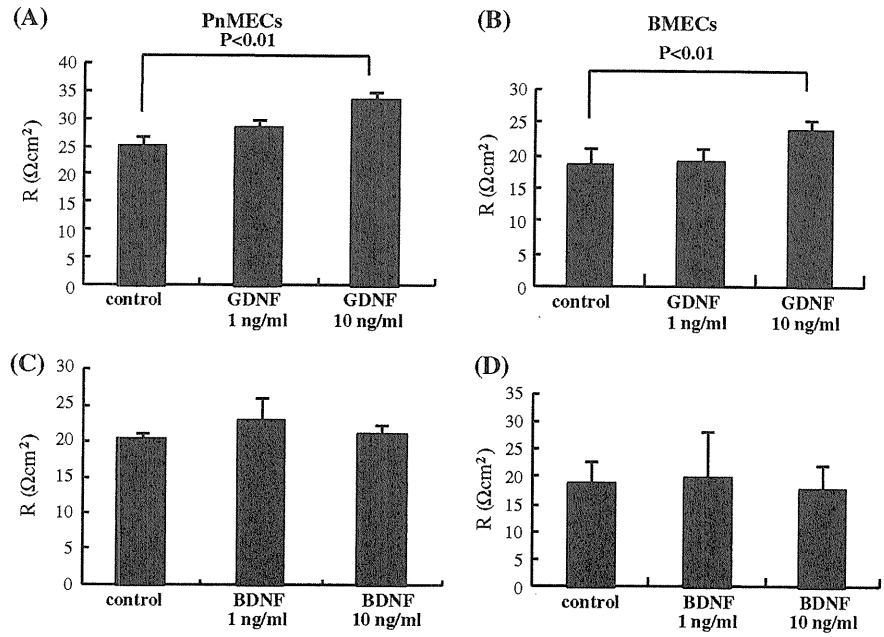

Fig. 4 The effect of GDNF or BDNF on the TEER values across PnMECs (**a**, **c**) or BMECs (**b**, **d**) monolayer (mean ± SD, $n = 5$, $^*P < 0.01$)

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

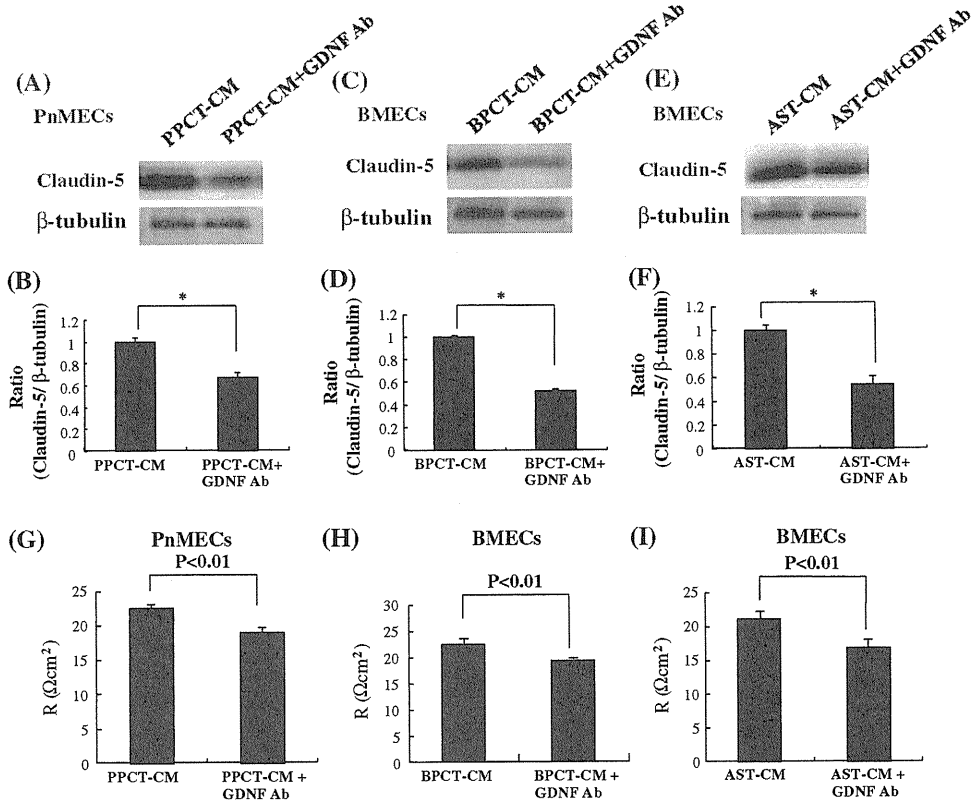

Fig. 5 Effects of GDNF neutralizing antibody on the induction of claudin-5 and TEER changes induced by brain- or peripheral nerve pericyte-conditioned media. **a** The PnMECs were cultured with PPCT-CM or PPCT-CM pre-treated with anti-GDNF antibody for 2 days. Claudin-5 protein was inhibited after pretreatment 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.

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Research Committee) from the Ministry of Health, Labor and Welfare of Japan.

Conflict of interest The authors declare that there is no duality of interest associated with this manuscript.

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ARTICLE

Advanced glycation end-products induce basement membrane hypertrophy in endoneurial microvessels and disrupt the blood–nerve barrier by stimulating the release of TGF-β and vascular endothelial growth factor (VEGF) by pericytes

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Abstract
Aims/hypothesis The breakdown of the blood–nerve barrier (BNB) is considered to be a key step in diabetic neuropathy. Although basement membrane hypertrophy and breakdown of the BNB are characteristic features of diabetic neuropathy, the underlying pathogenesis remains unclear. The purpose of the present study was to identify the possible mechanisms responsible for inducing the hypertrophy of basement membrane and the disruption of the BNB after exposure to AGEs.
Methods The newly established human peripheral nerve microvascular endothelial cell (PnMEC) and pericyte cell lines were used to elucidate which cell types constituting the BNB regulate the basement membrane and to investigate the effect of AGEs on the basement membrane of the BNB using western blot analysis.
Results Fibronectin, collagen type IV and tissue inhibitor of metalloproteinase (TIMP-1) protein were produced mainly by peripheral nerve pericytes, indicating that the basement membrane of the BNB is regulated mainly by these cells. AGEs reduced the production of claudin-5 in PnMECs by increasing autocrine signalling through vascular endothelial growth factor (VEGF) secreted by the PnMECs themselves. Furthermore, AGEs increased the amount of fibronectin, collagen type IV and TIMP-1 in pericytes through a similar upregulation of autocrine VEGF and transforming growth factor (TGF)-β released by pericytes.
Conclusions/interpretation These results indicate that pericytes may be the main regulators of the basement membrane at the BNB. AGEs induce basement membrane hypertrophy and disrupt the BNB by increasing autocrine VEGF and TGF-β signalling by pericytes under diabetic conditions.

Keywords Advanced glycation end-products · Basement membrane hypertrophy · Blood–nerve barrier · Diabetic neuropathy

Abbreviations

Act-	Active-
AGEs	Advanced glycation end-products
BBB	Blood–brain barrier
bFGF	Basic fibroblast growth factor
BNB	Blood–nerve barrier
BRB	Blood–retinal barrier
ECM	Extracellular matrix
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
MMP	Matrix metalloproteinase
NF-κB	Nuclear factor κB
PnMECs	Peripheral nerve microvascular endothelial cells
RAGE	Receptor of advanced glycation end-product
TEER	Transendothelial electrical resistance
TGF	Transforming growth factor
TIMP	Tissue inhibitor of metalloproteinase
VEGF	Vascular endothelial growth factor

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Introduction

Diabetic neuropathy is one of the most frequent complications of diabetes. Microangiopathy of the endoneurium is a common finding in the nerves of diabetic patients, and is thought to be a contributing factor to the development of diabetic neuropathy. The blood–nerve barrier (BNB) is localised in the microvessels of the endoneurium or perineurium, and consists of peripheral nerve microvascular endothelial cells (PnMECs), pericytes of endoneurial microvascular origin, and the basement membrane [1–3]. The basement membrane of the BNB plays important roles in maintaining the structure and function of capillary vessels [4]. Recently, breakdown of the BNB has been suggested to be an initial key step in diabetic neuropathy [4–6]. Although hypertrophy of the basement membrane in the endoneurium or perineurium, pericyte loss and endothelial cell hyperplasia at the BNB are characteristic features of diabetic neuropathy [6], the pathology and pathogenesis remain unclear. It is known, however, that the basement membrane is constructed from extracellular matrix (ECM) components such as collagen type IV, laminin and fibronectin, and is regulated by matrix metalloproteinases (MMPs) at the blood–brain barrier (BBB) [7, 8]. MMP-9 and MMP-2 digest collagen type IV, fibronectin and laminin, and are inhibited by tissue inhibitor of metalloproteinase (TIMP) [9]. Several studies have suggested that brain pericytes or astrocytes synthesise fibronectin, MMP-2 and MMP-9, and regulate the state of the basement membrane in the BBB [10, 11]. However, the mechanisms underlying in the formation and maintenance of the basement membrane in the BNB have not yet been elucidated.

Hyperglycaemia is responsible for the presence of high levels of non-enzymatically produced AGEs in the diabetic patients [12]. Their accumulation on proteins in the microvasculature appears to be a key factor in the development of retinopathy and nephropathy [12], but their role remains unclear with regard to their importance in diabetic neuropathy. AGEs have been reported to directly stimulate the production of basement membrane by activating transforming growth factor (TGF)-β1 secretion during the development of diabetic nephropathy [13], and to induce blood–retinal barrier (BRB) dysfunction through vascular endothelial growth factor (VEGF) production in diabetic retinopathy [14]. However, to date, the molecular mechanism by which AGEs induce hypertrophy of the basement membrane and breakdown of the BNB during diabetic neuropathy is unclear. The purpose of this study was to elucidate which type of cells regulate the basement membrane of the BNB using our newly developed PnMEC and pericyte cell lines, and to identify the possible mechanisms responsible for increasing the basement membrane thickness of the BNB after exposure to AGEs.

Methods

Reagents The culture medium for pericytes and PnMECs consisted of 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, USA) and 10% fetal bovine serum (FBS) (Sigma). Polyclonal anti-TIMP-1, anti-fibronectin, anti-MMP-2, and anti-MMP-9 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Polyclonal anti-claudin-5 and anti-occludin antibodies were purchased from Zymed (San Francisco, CA, USA). Polyclonal anti-collagen type IV was obtained from ARP American Research Products (Belmont, MA, USA). AGEs–BSA was purchased from Calbiochem (Darmstadt, Germany). Polyclonal anti-basic fibroblast growth factor (bFGF), anti-TGF-β and anti-VEGF antibodies were purchased from R&D Systems (Minneapolis, MN, USA). Human astrocytes were purchased from Lonza (Walkersville, MD, USA).

Cell culture The immortalised human PnMECs, and brain and peripheral nerve pericyte cell lines were generated as described previously by our group [15, 16]. The study protocol for human tissue was approved by the ethics committee of the Medical Faculty, 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 entering the study. The cell lines, which were isolated from human sciatic nerve and brain tissue, retain the morphological characteristics of primary peripheral nerve endothelial cells or brain and peripheral nerve pericytes and produce either endothelial or pericyte markers. The cultures were maintained at 37°C in 5% (vol./vol.) CO_2 and the DMEM medium was replaced every 3 days.

Quantitative real-time PCR analysis Total RNA was extracted using an RNeasy Plus Mini Kit (Qiagen, Hilden, Germany). Single-stranded cDNA was created from 40 ng total RNA using the StrataScript First Strand Synthesis System (Stratagene, Cedar Creek, TX, USA). The sequence specificity of each human primer pair is shown in the electronic supplementary material (ESM) Table 1. Quantitative real-time PCR analysis was performed using the Mx3005P (Stratagene) with FullVelocity SYBR Green QPCR master mix (Stratagene). Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as an internal standard. The samples were subjected to 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. Negative controls (cDNA-free solutions) were included in

Table 1 Effect of the two AGE concentrations (100 and 200 μg/ml) on production of basement-membrane-related molecules in peripheral nerve pericytes

Protein	Ratio of target protein production (protein:actin)	
	AGE 100 μg/ml	AGE 200 μg/ml
Collagen type IV	2.18±0.39**	2.93±0.61**
Fibronectin	4.83±0.52**	5.33±0.96**
TIMP-1	2.38±0.17**	2.86±0.14**
Act-MMP-9	0.68±0.06	0.36±0.18**
Act-MMP-2	1.15±0.07	0.94±0.05
RAGE	2.03±0.38**	2.40±0.42**
TGF-β	1.87±0.13**	2.68±0.18**
VEGF	2.26±0.29**	2.21±0.21**
bFGF	1.18±0.08	0.96±0.05

Data shown are means±SEM (n=3)

Each value reflects the combined densitometry data from three independent experiments and is shown as a fold increase above control

**$p<0.01$ compared with control

each reaction. The standard reaction curve (R_v) was analysed using MxPro software (Stratagene), with the relative quantity was calculated according to the formula $R_v = R_{gene}R_{gene}/R_{GAPDH}$ by the software.

Western blot analysis The protein samples (10–20 μg) were separated by SDS-PAGE (Bio-Rad), and transferred to nitrocellulose membrane (Amersham, Chalfont, UK). The membranes were treated with blocking buffer (5% skimmed milk in 25 mmol/l Tris-HCl pH 7.6,125 nmol/l NaCl, 0.5% Tween 20) for 1 h at room temperature and incubated with relevant primary antibodies (dilution 1:100) for 2 h at room temperature as the primary antibodies. The membrane was exposed to peroxidase-conjugated secondary antibody (1:2,000) followed by chemiluminescence reagent (Amersham), exposure to X-Omat S films (Amersham) and quantification of bands intensity using the Fuji image analysis software package.

Analysis of the effect of TGF-β1 or VEGF on the expression of basement-membrane-related genes Peripheral nerve pericytes were either left untreated or treated with human TGF-β1 (10 ng/ml) or VEGF (10 ng/ml). The total protein was collected 2 days later.

Treatment of human PnMECs and peripheral nerve pericytes with AGEs–BSA PnMECs and peripheral nerve pericytes were cultured in DMEM with unmodified BSA (100 μg/ml) and AGE-modified BSA (100 μg/ml, 200 μg/ml). AGEs–BSA contained <0.2 ng/ml of endotoxin. The cells were cultured for 2 days prior to collection of total protein.

VEGF or TGF-β inhibitory study Peripheral nerve pericytes were cultured with AGEs–BSA containing 2.0 μg/ml antibody against VEGF or TGF-β, or normal rabbit IgG. PnMECs were also cultured with AGEs containing 2.0 μg/ml antibody against VEGF or normal rabbit IgG. The total RNA was extracted 24 h later, and total protein was obtained a further 2 days later.

Transendothelial electrical resistance (TEER) study Transwell inserts (pore size 0.4 μm, effective growth area 0.3 cm^2; BD Biosciences, Franklin Lakes, NJ, USA) were coated by rat-tail collagen type-I (BD Biosciences). TEER values of cell layers were measured with a Millicell electrical resistance apparatus (Endohm-6 and EVOM, World Precision Instruments, Sarasota, FL, USA). The PnMECs were seeded (1×10^6 cells per insert) on the upper compartment and cultured for 24 h. In order to estimate the effect of AGEs against PnMECs, the upper compartment was incubated with each medium (control, AGE, AGE+ VEGF antibody) for 24 h.

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

Results

The expression of basement-membrane-related molecules secreted from the peripheral nerve pericytes We quantified the mRNA expression of fibronectin, collagen type IV (*COL4A4*), *TIMP1*, *MMP2* and *MMP9* by means of relative quantification with real-time RT-PCR using PnMECs and peripheral nerve pericytes (ESM Fig. 1a–e). The expression of *COL4A4* (ESM Fig. 1a), fibronectin (ESM Fig. 1b) and *TIMP1* (ESM Fig. 1c) mRNA in peripheral nerve pericytes was significantly higher than those in PnMECs ($p<0.01$). There were no significant differences in expression of *MMP2* (ESM Fig. 1d) and *MMP9* (ESM Fig. 1e) between these two cells. In addition, we determined fibronectin, collagen type IV, TIMP-1, MMP-2 and MMP-9 protein production using PnMECs and peripheral nerve cells by a western blot analysis (Fig. 1a). The fibronectin, collagen type IV, TIMP-1, MMP-2, and MMP-9 bands, corresponding to 190, 25, 38, and 92 kDa single bands, respectively, were detected in these cell lines (Fig. 1a). Although the collagen type IV and