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NMO sera down-regulate AQP4 in human astrocyte and induce cytotoxicity independent of complement

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ABSTRACT

Autoantibodies against astrocyte water channel aquaporin-4 (AQP4) are highly specific for neuromyelitis optica (NMO). However, the molecular mechanism of NMO still remains unclear.
The purpose of this study was to identify the possible humoral mechanisms responsible for the occurrence of astrocytic damage. Human primary astrocytes (AST) were immortalized by retroviral vectors harboring temperature-sensitive SV40 T antigen gene and AQP4 cDNA (M23), designated as hAST-AQP4. The effects of NMO sera on the content and localization of AQP4, including cytotoxicity and astrocytic morphology, were evaluated. In addition, this study examined whether the amount and localization of AQP4 protein in astrocytes were influenced by direct contact with the immortalized human brain microvascular endothelial cell line, TY09. NMO sera alone induced cytotoxicity and addition of complement had a more harmful effect on hAST-AQP4. NMO sera also decreased AQP4 mRNA and protein. NMO sera alone up-regulated TNFα and IL-6 in astrocytes and co-incubation with anti-TNFα and anti-IL-6 neutralizing antibodies blocked both the cytotoxicity and reduction of AQP4 in astrocytes. In the experiment using the in vitro BBB models, AQP4 protein mainly localized at the astrocytic membrane after co-culture with TY09, in contact with TY09. The future elucidation of factors that up-regulate AQP4 in astrocytes presumably released by blood brain barrier forming endothelial cells and that block the production of inflammatory cytokines may therefore lead to the development of a novel therapeutic strategy.

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1. Introduction

Neuromyelitis optica (NMO) is an inflammatory disorder of the central nervous system that affects mainly the optic nerves and the spinal cord [1]. A highly disease-specific autoantibody named NMO-IgG wasdiscovered in NMO [2] and its target antigen was identified as aquaporin-4 (AQP4) [3]. AQP4 exists as two different heterotetramers, M-1 and M-23 AQP4, which result from usage of different start codons [4–6]. Although AQP4 antibodies have been reported to alter the subcellular localization of AQP4 proteins or down-regulate AQP4 protein [7–10], Ratelade et al. demonstrated that NMO-IgG brings little internalization to in vivo mouse brain and mouse primary astrocytes [11]. These studies used HEK 293 cells transfected with M1-AQP4 [7,8] or rodent astrocytes [8–11]. On the other hand, in vitro studies using human astrocytes, particularly cells dominantly expressing the M23 isoform, are rare. In addition, although there is only one report in which a model of co-culture of human astrocytes and endothelial cells was used in NMO research [12], no studies performing quantification of AQP4 using Western blotting (WB) with co-culture of conditionally-immortalized human brain microvascular endothelial cells (HBMECs) [13] and human astrocytic cell lines have been reported.

This study established a human astrocytic cell line expressing dominantly M23 protein. This line was used to determine whether NMO sera influence the amount of AQP4 and inflammatory cytokines. In addition, the effects of NMO sera on the morphology and viability of astrocytes and the subcellular localization of AQP4 were investigated. The influence of co-culture with HBMECs on the expression level and localization pattern of AQP4 protein in astrocytes was also evaluated using both immunofluorescence and WB. The results suggest a novel underlying mechanism which can provide a better understanding of the pathophysiology of NMO.

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2. Materials and methods

2.1. Antibodies and sera

Polyclonal rabbit anti-AQP4 (H 80), polyclonal rabbit anti-GFAP, polyclonal goat anti-excitatory amino acid transporter 2 (EAAT2), and polyclonal mouse anti-β-actin were obtained from Santa Cruz Biotechnologies (Santa Cruz, CA, USA). The polyclonal anti-interleukin (IL)-6 and anti-TNFα were purchased from R&D systems (Minneapolis, Minnesota, USA). FITC-conjugated secondary antibodies were obtained from Invitrogen (CA, USA). HRP-conjugated secondary antibodies were purchased from Merck Millipore (Frankfurt, Germany). The sera were collected from five patients who tested positive for the anti-AQP4 antibody (AQP4-Ab), and five healthy volunteers (control subjects). Five AQP4-Ab-positive patients fulfilled the clinical criteria for NMO [14]. We detected anti-AQP4 antibodies according to the method described in our previous report [15]. Briefly, human embryonic kidney (HEK-293) cells were stably transfected with the vector with or without AQP4 cDNA, and the specimens were tested using an indirect immunofluorescent assay with these two cell lines (with or without AQP4) as the substrates. We used Alexa Fluor 488 goat anti-human IgG (Invitrogen, Eugene, Oregon, USA) instead of fluorescein-conjugated antibodies, which have been used in previous studies [15], as the second antibody in the indirect immunofluorescent assay. This modification resulted in slightly higher antibody titers in this study compared with those observed in previous reports [15]. The serum samples were titrated in doubling dilutions to ascertain the maximum dilution that positively stained the AQP4-transfected HEK-293 cells. Blood samples obtained from NMO patients were collected within seven days from onset and stored at −80 °C until use. All sera were incubated at 65 °C for 30 min just prior to use.

This study was approved by the ethics committee of Yamaguchi University Hospital, and was conducted in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki), as amended in the British Medical Journal in 1964. The experiments were undertaken with the understanding and 'WRITTEN CONSENT' of each subject.

2.2. Reagents and cells

Human primary astrocytes were purchased from Lonza (Walkersville, USA). DH-BNBs are conditionally immortalized human endothelial cell lines derived from the blood–nerve barrier as previously described [16]. TY09 cell, a conditionally-immortalized human microvascular endothelial cell line proven to sustain barrier-specific properties, has been previously described [13]. The retrovirus vector pDON-AI 2 was purchased from TAKARA Bio Inc. (Otsu, Japan). Human AQP4 cDNA (M23) was constructed as previously described [17]. The culture medium (CM) for astrocytes was 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, USA) and 10% fetal bovine serum (FBS; Sigma). EC growth medium (ECM) for TY09 cells contained EBM-2 medium (Cambrex, Walkersville, MD) supplemented with 20% FBS, EGM-2 MV (Cambrex), 100 U/ml penicillin (Sigma), 100 ml/ml streptomycin (Sigma), and 25 ng/ml amphotericin B (Invitrogen).

2.3. Establishment of cell lines

To obtain astrocytic cell lines that not only preserve the physiological astrocytic functions but also have the ability to grow over 10 or more passages, which stand for the various analyses required to elucidate NMO pathology, human primary astrocytes were transfected with retroviral vectors (pDON-AI2) harboring the temperature-sensitive SV40 T antigen (tsA58) gene. The cells were named "hAST". Conditionally-immortalized astrocytic cell lines harboring the tsA58 gene have been reported to retain in vivo astrocytic function [18,19]. hAST was sequentially transfected by retroviral vectors (pDON-AI2) harboring human AQP4 cDNA (M23), and the obtained cells were termed "hAST-AQP4".

2.4. Exposure of sera and complement to the human astrocyte cell line

4×10^4/well cells were seeded on type-I collagen-coated 35-mm dishes for immunocytochemical and RT-PCR analyses, 1×10^5/well cells were seeded on type-I collagen-coated 60-mm dish for WB and grown at 33 °C for 48 h. The culture media was replaced with fresh medium containing 10% heat-inactivated NMO or healthy control (HC) sera and were incubated at 4 °C, the degrees Celsius which has been used in the same sort of experiment [7]. After 1 h incubation at 4 °C, we added Low-Tox-M rabbit complement (final concentration 2%) and cells were incubated at 37 °C for 23 h. The cells without complement were incubated at 37 °C for 24 h.

2.5. Reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total RNA was prepared using an RNeasy Plus Mini kit (Qiagen, Hilden, Germany). RT and PCR amplification were carried out with TAKARA PCR Thermal Cycler Dice (TakaRa, Otsu, Japan). Single-stranded cDNA was synthesized from 250 ng of total RNA using the StrataScript First Strand Synthesis System (STRATAGENE, Cedar Greek, TX, USA) with an oligo-dT primer and sequential PCR was performed with TaKaRa Ex Taq (TaKaRa). The sequences of human primer pairs for AQP4 and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) were previously reported [20,21].

2.6. Quantitative real-time PCR analysis

A quantitative real-time PCR analysis was performed using a Stratagene's Mx3005P (Strategene®) with Full Velocity® SYBR® Green QPCR master mix (Strategene®) according to the manufacturer's protocol. The sequences of human primers for AQP4, G3PDH, IL-6, TNFα, and IL-1β were previously described [22–26]. 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. The standard reaction curve was analyzed by the MxPro™ (Strategene®) software package and the relative quantity according to the formula $R_V = R_{GENE} / R_{GAPDH}$ by computer.

2.7. Western blot (WB) analysis

Twenty micrograms of proteins was mixed with an equal volume of Laemmli sample buffer (Bio-Rad Laboratories, CA, USA) containing 4 M urea and separated on a 12% SDS-polyacrylamide gel containing urea at a final concentration of 6 M, followed by blotting onto polyvinylidene difluoride membranes. The membrane was blocked at room temperature for 10 min with 5% powdered skimmed milk in PBS containing 0.5% Tween 20 (PBS-T-milk). The membrane was incubated with a primary antibody in PBS-T-milk (1:50) at room temperature for 1 h, followed by incubation with a secondary antibody in PBS-T-milk (1:2000) at room temperature for 1 h. The membranes were visualized by enhanced chemiluminescence detection (ECL-advance, Amersham, UK). A densitometric analysis using Quantity One (Bio-Rad Laboratories) was performed for quantification purposes.

2.8. Immunocytochemistry

The cells were fixed in 4% paraformaldehyde (Wako, Osaka, Japan) for 30 min and permeabilized with 0.5% Triton X-100 (Sigma) for 30 min. The cells were blocked with 3% BSA in PBS for 20 min, incubated with anti-AQP4-antibodies (1:50 dilution) at 4 °C overnight and

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