Complement-dependent and -independent aquaporin 4-antibody-mediated cytotoxicity in human astrocytes: Pathogenetic implications in neuromyelitis optica

S. Nishiyama a,*, T. Misu b, M. Nuriya c, R. Takano d, T. Takahashi a, I. Nakashima a, M. Yasui c, Y. Itoyama a, M. Aoki a, K. Fujihara b

a Departments of Neurology Tohoku University Graduate School of Medicine, Sendai, Japan
b Multiple Sclerosis Therapeutics, Tohoku University Graduate School of Medicine, Sendai, Japan
c Department of Pharmacology, School of Medicine, Keio University, Tokyo, Japan

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ABSTRACT

Background: Neuromyelitis optica (NMO) is an inflammatory disease caused by the aquaporin (AQP)-4-antibody. Pathological studies on NMO have revealed extensive astrocytic damage, as evidenced by the loss of AQP4 and glial fibrillary acidic protein (GFAP), specifically in perivascular regions with immunoglobulin and complement depositions, although other pathological patterns, such as a loss of AQP4 without astrocyte destruction and clasmatodendrosis, have also been observed. Previous studies have shown that complement-dependent antibody-mediated astrocyte lysis is likely a major pathomechanism in NMO. However, there are also data to suggest antibody-mediated astrocyte dysfunction in the absence of complement. Thus, the importance of complement inhibitory proteins in complement-dependent AQP4-antibody-mediated astrocyte lysis in NMO is unclear. In most of the previous studies, the complement and target cells (astrocytes or AQP4-transfected cells) were derived from different species; however, the complement inhibitory proteins that are expressed on the cell surface cannot protect themselves against complement-dependent cytolysis unless the complements and complement inhibitory proteins are from the same species. To resolve these issues, we studied human astrocytes in primary culture treated with AQP4-antibody in the presence or absence of human complement and examined the effect of complement inhibitory proteins using small interfering RNA (siRNA).

Methods: Purified IgG (10 mg/mL) was obtained from 5 patients with AQP4-antibody-positive NMO, 3 patients with multiple sclerosis (MS), and 3 healthy controls. Confluent human astrocytes transfected with Venus-M1-AQP4-cDNA were incubated with IgG (5% volume). After washing, we cultured the cells with human complements with or without heat inactivation. We observed time-lapse morphological and immunohistochemical changes using a fluorescence microscope. We also evaluated cytotoxicity using a propidium iodide (PI) kit and the lactate dehydrogenase (LDH) assay.

Result: AQP4-antibody alone caused clustering and degradation followed by endocytosis of membraneous AQP4, thereby resulting in decreased cellular adherence and the shrinkage of astrocytic processes. However, these changes were partially reversed by the removal of IgG in culture. In contrast, following the application of AQP4-antibody and non-heated human complements, the cell bodies and nuclei started to swell. At 3 h, most of the astrocytes had lost mobility and adherence and were eventually destroyed or had swollen and were then destroyed. In addition, the remaining adherent cells were mostly PI-positive, indicating necrosis. Astrocyte lysis caused by rabbit complement occurred much faster than did cell lysis with human complement. However, the cell lysis was significantly enhanced by the transfection of astrocytes with siRNA against human CD55 and CD59, which are major complement inhibitory proteins on the astrocyte membrane. AQP4-antibody-negative IgG in MS or control did not induce such changes.

Conclusion: Taken together, these findings suggest that both complement-dependent and complement-independent AQP4-antibody-mediated astrocytopathies may operate in NMO, potentially contributing to diverse pathological patterns. Our results also suggest that the effect of complement inhibitory proteins should be considered when evaluating AQP4-antibody-mediated cytotoxicity in AQP4-expressing cells.

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1. Introduction

Neuromyelitis optica (NMO) is an inflammatory disease that is characterized by severe optic neuritis and longitudinally extended transverse myelitis. Aquaporin-4 (AQP4)-antibody is a diagnostic biomarker of NMO [1]. AQP4 is a transmembrane water channel that is richly expressed on the endfeet of astrocytes in the central nervous system [2]. AQP4 has the two main isoforms: full-length M1 and a shorter form, M23. Both isoforms are present in astrocytes, and endogenous AQP4 is a tetramer that consists of various combinations of M1 and M23 subunits [3]. The expression ratios of these AQP4 isoforms are different, but both isoforms are expressed in the cerebrum, brainstem, spinal cord, and optic nerve [4]. Pathological studies have revealed an extensive loss of AQP4 and glial fibrillary acidic protein (GFAP), an astrocytic cytoplasmic protein, in perivascular regions with complement in NMO [5]. Other pathological patterns, including the loss of AQP4 without astrocyte destruction and clasmatodendrosis, have also been observed. There is a marked increase in GFAP in the cerebrospinal fluids during relapse of NMO [6]. A loss of AQP4 and GFAP has also been observed in experimental autoimmune encephalomyelitis with AQP4-antibody administration [7,8]. Taken together, these findings suggest astrocyte lysis by complement-dependent AQP4-antibody-mediated cytotoxicity to astrocytes. In addition, pure AQP4 loss without astrocyte damage has been reported in neuropathological studies of NMO [9–11]. Astrocyte lysis may also be mediated by either complement-dependent cytotoxicity (CDC) [12–17] or antibody-dependent cell-mediated cytotoxicity [18–20], and AQP4 loss without astrocyte lysis might be caused by AQP4-antibody alone (Supplement table 1). For CDC, ‘homologous restriction’ cannot be ignored. Homologous restriction indicates that the membrane complement regulators in human cells inhibit the human complement and thereby prevent cell damage, but such a cell protective mechanism does not operate when the complements are from other species [21]. Thus, in vivo or in vitro studies using cells and complements derived from heterogeneous species may result in an over-estimation of CDC, although there have been no comprehensive studies of cytotoxicity in AQP4-expressing human astrocytes by human AQP4-antibody and human complement.

2. Materials and methods

2.1. Purified IgG

Sera were obtained from 5 patients with AQP4-antibody-positive NMO, 3 patients with multiple sclerosis (MS), and 3 healthy controls. MS patients and controls were seronegative for the AQP4-antibody. IgG was purified using ProteinA Sepharose Fast Flow (GE healthcare, Tokyo, Japan), and the concentration was adjusted to 10 mg/mL.

2.2. Cells

Primary human astrocytes (CC-2565, Lonza Japan, Tokyo, Japan) were incubated with AGM BulletKit (Lonza Japan, Tokyo, Japan). All astrocytes were confirmed to be positively stained for GFAP by immunofluorescence. Prior to each assay, astrocytes were incubated at 37 °C and 5% CO₂ for 4 days. Confluent primary human astrocytes were incubated with IgG (5% volume) for 30 min. After the removal of unbound IgG, we further cultured the cells with human complement (S1764, Sigma-Aldrich Japan, Tokyo, Japan) (CH50=42) or rabbit complement (20% volume) with or without heat inactivation (56 °C, 30 min). In each experiment, we observed the morphological changes of the cells in each well at 2–24 h of culture. For the positive controls for AQP4 expression, we used HEK293 cells transfected with M1-AQP4 vector, which have been previously reported [22].

2.3. Time-lapse microscopic analysis

For the time-lapse analysis, primary astrocytes were seeded onto 8-well Lab-Tek II chamber slides (Nalge Nunc International, Rochester, USA). We observed time-lapse morphological changes of the cells using fluorescence microscopes (BZ-9000, Keyence, Osaka, Japan, and FV1000-D and FV1000MPE, Olympus, Tokyo, Japan). A microscopic cell culture system with an incubator (INU-ONICS-BP, Tokai-hit, Fujinomiya, Shizuoka, Japan) was also employed. Time-lapse images of living astrocytes were captured at 15- or 30-second intervals. FM 4–64 dyes (10 μM) were used to label the plasma membrane. Moreover, to stop the endocytosis of cells, we used 5 μM of phenylarsine oxide (PAO). To visualize the AQP4 dynamics in astrocytes, we transfected Venus-M1-AQP4 cDNA (provided by Dr. Abe, Keio University). Venus-M1-AQP4 is a fusion protein of the fluorescent protein, Venus C-terminal and N-terminal of M1-AQP4. The Kozak sequence in Venus-M1-AQP4 is optimally transcribed from Venus protein, and it is less possible to express through leaky scanning or re-initiation.

2.4. Immunohistochemical staining

Cells were grown on coverslips with water-shedding coating (Matsunami, Kishiwada, Japan). Purified IgG (0.5 mg/mL) from patients was applied to the culture media, and the cells were incubated for 30 min at room temperature. Next, the cells were fixed in 4% paraformaldehyde for 30 min, permeabilized with 0.3% Triton X-100 in PBS for 10 min, blocked with 10% goat serum for 20 min, and then incubated with primary antibodies overnight. We used rabbit polyclonal anti-AQP4-IgG (Merck Millipore, Darmstadt, Germany) directed against the intracellular domain near the C-terminal and mouse monoclonal anti-Csb-9-IgG (DakoCytomation, Glostrup, Denmark) as primary antibodies. Goat anti-human Alexa fluor 488, goat anti-rabbit Alexa fluor 488, goat anti-rabbit Alexa fluor 568, and goat anti-mouse Alexa fluor 568 (Invitrogen, Carlsbad, USA), were used for secondary antibodies.

2.5. Western blotting for CD55 and CD59

Astrocytes grown on dishes were washed three times with PBS, and the membrane fraction was isolated using ProteoExtract (Merck Millipore, Darmstadt, Germany). Total protein was determined using the BCA assay. Each sample (30 μg per well) was separated using SDS-PAGE and subsequently transferred onto polyvinylidene difluoride membrane (Merck Millipore, Darmstadt, Germany). The membranes were incubated with mouse CD55-antibody (Acris Antibodies, Herford, Germany) or rabbit CD59-antibody (Proteintech Group, Rosemont, USA) as primary antibodies for 8 h and then with horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibody (DakoCytomation, Glostrup, Denmark). The bands were detected using an enhanced chemiluminescent detection system (ECLPlus; GE healthcare, Tokyo, Japan) with an image analyzer (LAS-3000; Fuji Photo Film Japan, Kanagawa, Japan).

2.6. siRNA transfection

CD55- and CD59-specific small interfering RNAs (siRNAs) were purchased from Invitrogen, Carlsbad, USA (Invitrogen stealth RNAi). The transfection was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, USA) according to the manufacturer’s instructions.
2.7. Propidium iodide assay

Cellular cytotoxicity with or without apoptosis was analyzed using a propidium iodide (PI) kit (TACS Annexin V-FITC, R&D Systems, Minneapolis, USA). Astrocytes on an 8-well slide were incubated with purified IgG (0.5 mg/mL) or normal medium for 30 min. The media were changed to non-heated complement or heat-inactivated complement. Next, these cells were incubated for 6 h. After incubation, PI staining was performed according to the manufacturer’s instructions.

2.8. LDH release assay

LDH release into the supernatant was measured using the Cytotoxicity Detection Kit Plus (Roche Diagnostics, Tokyo, Japan). Prior to the assay, human astrocytes in primary culture were incubated in a 6-well plate for four days, and the media were completely exchanged prior to the assay. The supernatants in each well were harvested before and after the application of purified IgG (0.5 mg/mL). Next, we removed unbound IgG, and each well was cultured in the presence of non-heated or heat-inactivated complement for 3 h. After incubation, we harvested the supernatants of each well. The conditions were performed in duplicate for this assay.

2.9. Statistical analysis

To compare the PI positivity, LDH release and cellular nucleic size, one-factor analysis of variance (ANOVA) was performed using GraphPad Prism 5.04. When a significant difference was observed, a post-hoc test was performed using the Bonferroni test. Statistical significance was assumed when P < 0.05. Image J was used to determine the size of the astrocyte or for densitometry analyses.

3. Results

3.1. NMO-IgG and complement bind to the extracellular-domain of AQP4-transfected HEK293 cells and human astrocytes in primary culture

AQP4-transfected HEK293 cells were co-labeled with NMO’s purified serum IgG and mouse AQP4-antibody, indicating that the NMO’s purified IgG did bind to the extracellular domain of AQP4 on the membrane of HEK293 cells (Supplement Fig. 1A). Human primary astrocytes were also co-labeled with the NMO AQP4-antibody and mouse AQP4-antibody (Supplement Fig. 1B). After incubation with purified IgG following non-heated complement, the cells were co-labeled for AQP4 and C5b-9 (Supplement Fig. 1C), which suggested complement activation by the patients’ AQP4-antibody.

3.2. Reversible shrinkage of astrocytes by the patients’ AQP4-antibody alone

Normal human astrocytes retain their adherent ability and mobility, even if the medium is exchanged to PBS or medium (Fig. 1(A)). Purified AQP4-antibody-positive IgG alone caused shrinkage of the astrocytic processes and a loss of adherent ability (Fig. 1(B)). The morphology of the cell bodies changed from star-shaped to spherical without a change in the nucleus. Moreover, the astrocytic processes connecting neighboring astrocytes also shrunk and appeared similar to threads. The connections became sparse with passing time. However, these changes were reversible to some extent by the removal of IgG (Fig. 1(C)). The cytoplasmic area was significantly smaller than that of control astrocytes (P < 0.001, Fig. 1(D)). In addition, the cytoplasmic area recovered shortly after the patient’s AQP4-antibody was removed from media (Fig. 1(E)). These findings were recorded (supplement video 1).
Supplementary material related to this article can be found online at http://dx.doi.org/10.1016/j.bbrep.2016.05.012.

3.3. Clustering and endocytosis of membraneous AQP4 on astrocytes by AQP4-antibody alone

We used Venus-human AQP4-transfected astrocytes to observe the real-time changes in AQP4 expression. In the normal condition, we observed dominant AQP4 expression on the astrocytic cell membrane (Fig. 2(A), D). In contrast, AQP4-transfected astrocytes cultured in media containing the NMO’s purified IgG showed Venus-AQP4 clusters on the membrane after from 30 min (Fig. 2(B) and supplement video 2). Next, endocytosis of Venus-AQP4 occurred sequentially (Fig. 2(C)). During the cluster formation of Venus-AQP4, the astrocytic processes gradually shrank and became detached from the dish (Fig. 2(B), C, supplement video 1). Furthermore, when the medium was changed to fresh medium that did not contain AQP4-antibody, the normal morphology of the astrocytes were gradually restored within 4 h. In addition, the recovery of AQP4 expression on the membrane of astrocytes was visible after 24 h (Fig. 2(E)), when some internalized AQP4 proteins in the cytoplasm could still be observed.

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Purified IgG obtained from MS patients and healthy controls did not cause these astrocytic changes. PAO-treated astrocytes with Venus-AQP4 vector showed AQP4 clusters on the membrane but not AQP4 endocytosis (Fig. 2(F)).

3.4. Differential cytotoxic effects between human complement and rabbit complement

Following the application of non-heated human complements, the cellular AQP4-immunoreactivity was lost, and the cell bodies and nuclei started to gradually swell. The LDH release in supernatants of astrocytes treated with AQP4-antibody and non-heated complements were significantly higher compared to other groups (Fig. 3(A)). Moreover, astrocytes treated with the patient’s AQP4-antibody and non-heated rabbit complements had a significantly larger nuclear area than did those treated with patient’s...
AQP4-antibody and non-heated human complements \((P < 0.01, \text{Fig. 3(B)})\). Taken together, these data suggest that “homologous restriction” occurred in this in vitro model; that is, human astrocytic complement inhibitory protein had a limited protective effect against rabbit complement.

Pl positivities were increased in both rabbit and human complement over time (Fig. 3(C)). The percentage of PI-positive cells after exposure to the patient’s AQP4-antibody and non-heated rabbit complement was significantly increased \((P < 0.01)\) compared with the value of AQP4-antibody and non-heated human complement. These data indicated that the cytotoxic effect of human complement against astrocyte treated with AQP4-antibody was much slower than previously reported [12].

3.5. Effect of siRNA of human complement inhibitory proteins CD55 and CD59

We suspected that the relative protection of human astrocytes against the cytotoxic effect of human complement was due to the complement inhibitory protein on the astrocytic membrane. There are four known complement inhibitory proteins: CD35, CD46, CD55, and CD59. In this model, CD55 and CD59 were expressed on the astrocytic membrane, as been confirmed using immunostaining and western blotting (Fig. 4(A)). The PI assays showed a significant increase in the percentage of PI-positive cells after CD55-siRNA-transfection compared with non-siRNA-transfected astrocyte. Transfection of CD59 siRNAs also showed a smaller effect in PI positivity. PI positivity in CD55- and CD59-siRNA-transfected astrocytes treated with human complement was not different from astrocytes treated with rabbit complement (Fig. 4(B)). Furthermore, LDH release in the supernatant of these siRNA-transfected astrocytes showed similar results to the PI positivity data \((P=0.1105, \text{Supplement Fig. 1E})\).

4. Discussion

In the present study, we found that transmembrane AQP4 became clustered on the cell surface of human astrocytes following the application of the patient’s AQP4-antibody in the absence of human complements and that it was subsequently endocytosed. This process was partially reversible after removing AQP4-antibody from the culture media, as previously reported for M1-AQP4-transfected kidney cells (HEK293) by Hinson et al. [12]. However, we further showed that these AQP4-antibody-mediated changes in AQP4 most likely resulted in altered motility and adherence of astrocytes, which may contribute to the pathological process, including abnormal neuron-glial interactions in NMO. Selective AQP4 loss without astrocyte destruction was reported by Misu and colleagues as Type 4 lesion in six different lesion types in NMO [10]. Thus, it is interesting to compare the localization of Type 4 lesions and IgG deposition in autopsied NMO cases. Anchoring proteins, such as syntrophin or dystrophin, are closely related to AQP4 expression, and the altered expression of AQP4 observed in the present study might affect both these anchoring proteins as well as other membrane proteins that function to maintain...
membrane homeostasis. The swelling of AQP4-transfected astrocytes treated with AQP4-antibody could result from altered water permeability in the astrocytic membrane; however, competing reports on whether AQP4-antibody affects the passage of water molecules through AQP4 water channel have emerged [18,23,24]. Complement-dependent AQP4-antibody-mediated astrocytic lysis has been previously reported, and such lesions correspond to Type 1 lesion in Misu’s classification of NMO lesion. However, because a majority of previous studies used target cells and complements that were from different species, as shown in Supplement table 1, the effect of “homologous restriction” by complement inhibitory proteins was not evaluated. In our study, we found that the patient’s AQP4-antibody-mediated cytotoxicity to human AQP4-transfected astrocytes by human complements was a much slower process than that by rabbit complements. In experiments with CD55-/CD59-siRNA-transfected astrocytes, CD55 was more closely related to “homologous restriction” than CD59 was. Recently, Saadoun and Papadopulos reported that these complement inhibitory proteins were expressed in other organs but not in the brain, which may explain why NMO primarily damages the central nervous system but spares peripheral organs [25]. It is intriguing to attribute the selective central nervous system involvement in NMO to no “homologous restriction”, but another study reported the expression of CD55 and CD59 in the brain [26]. Matiello et al. showed that AQP4-mRNA and proteins (both M1- and M23-AQP4) were much more highly expressed in the optic nerves and spinal cord than in other brain regions, which may contribute to the predilection of the optic nerve and spinal cord to NMO pathology [4]. Thus, we should analyze the localization of complement inhibitory proteins in the optic nerves, spinal cord and other regions of the central nervous system to determine whether “homologous restriction” is unevenly distributed.

There are some limitations in this study: 1. because we used short-term primary human astrocyte cultures, longitudinal changes in astrocytes treated with the patient’s AQP4-antibody and complements need to be evaluated in in vivo models. 2. Only M1-AQP4-transfected astrocytes were used in the present study. M23-AQP4 forms an orthogonal array of particles, and AQP4-antibody tends to bind more tightly to M23-AQP4 [25]. Thus, the morphological and functional changes and cytolyis observed in M23-AQP4-transfected astrocytes may not be identical to those observed in M1-AQP4-transfected astrocytes in the present study.

Disclosure

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Appendix A. Transparency Document

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2016.05.012.

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