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# Downregulation of water channel aquaporin-4 in rats with experimental autoimmune encephalomyeritis induced by myelin basic protein

Tomoki Kaneyama [a,1], Sho Takizawa [b], Sayaka Tsugane [b], Satoshi Yanagisawa [b], Naoya Takeichi [b], Takashi Ehara [a], Motoki Ichikawa [b], Chang-Sung Koh [b,*]

[a] Department of Pathology, Graduate School of Medicine, Shinshu University, Matsumoto, Nagano 390-8621, Japan
[b] Department of Biomedical Laboratory Sciences, Graduate School of Medicine, Shinshu University, Matsumoto, Nagano 390-8621, Japan

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ABSTRACT

Characteristics of myelin basic protein (MBP)-induced experimental autoimmune encephalomyelitis (EAE) include acute edema and infiltration of mononuclear cells (MNCs) in the microvessels of central nervous system (CNS). Aquaporin-4 (AQP4) is a water channel protein expressed in astrocytes foot process throughout the CNS. We performed immunostaining, western blotting and semi-quantitative real-time RT-PCR of AQP4 and glial fibrillary acidic protein (GFAP) in CNS from rats immunized with MBP. Immunohistochemical analysis revealed that AQP4 is down-regulated in MNCs infiltrated microvessels of rats with EAE. Furthermore, western blotting and real-time RT-PCR analyses showed that AQP4 was significantly decreased at the stage of severe EAE compared with control rats. On the other hand, expression of GFAP-protein was significantly increased after stage of severe EAE. Our findings suggest that AQP4 may be involved in forming edema in the inflammatory lesions of EAE accompanying with up-regulation of reactive astrocyte.



## 1. Introduction

Experimental autoimmune encephalomyelitis (EAE) is an autoimmune disease of the central nervous system (CNS) that can be experimentally induced in susceptible rodents by sensitization to neural antigens such as myelin basic protein (MBP) [1]. Homeostasis of the CNS microenvironment is essential for its normal function and is maintained by the blood–brain barrier (BBB) and blood-cerebrospinal fluid barrier (BCSFB). During pathological conditions of the CNS such as in multiple sclerosis (MS), or in its animal model EAE, loss of barrier properties of the highly specialized cerebral endothelium leads to edema formation and exacerbation of clinical disease [2]. The pathology of EAE induced in rats by MBP is mainly characterized by edema along with increased BBB or BCSFB permeability and perivascular infiltrates of mononuclear cells (MNCs) in the CNS [1–4].

Recently, the presence of antibodies against aquaporin-4 (AQP4) has been reported in patients affected with neuromyelitis optica (NMO) [5,6]. NMO, also known as Devic's disease, is a demyelinating and inflammatory disorder [6–8]. The clinical features of NMO differ from MS in that NMO primarily affects the optic nerves and spinal cord and spares the brain, at least in the initial stages and histologically, there is loss of AQP4 expression and reactive astrocytes in NMO but not in MS [9,10].

Homeostatic control of water transport in cells and tissues is critical for survival and has been the subject of many studies, particularly following the discovery of aquaporins (AQPs) in the early 1990s [11]. AQPs, a family of water channel proteins, are now recognized as serving an important role in the distribution of fluid and in the reduction of osmotic and hydrostatic gradients with 13 different mammalian aquaporin proteins (AQP0-AQP12) [12]. AQPs are proteins that assemble in the cell membranes as tetramers [13,14]. Each monomer is ~30 kDa and has six membrane-spanning domains surrounding a water pore that can transport water in both directions.

AQPs are present on the plasma membrane at the boundary of various tissues and some members of the aquaporin family (AQP1, AQP4 and AQP9) have been identified in the CNS [15]. These CNS aquaporins appear to play important roles in the dynamic regulation of brain water homeostasis and in the regulation of cerebrospinal fluid (CSF) production. The most abundant water channel in the CNS is AQP4, which is found in three locations: the perimicrovessel astrocyte foot processes, glia limitans and ependyma [16]. Especially, AQP4 is highly expressed in a polarized fashion in the plasma cell membranes of astrocyte foot processes around microvascular endothelial cells [17].

There are increasing evidences that decrease of AQP4 immunoreactivity is well correlated with spinal cord edema after spinal

* Corresponding author. Fax: +81 263 37 3430.
*E-mail address:* kshosei@shinshu-u.ac.jp (C.-S. Koh).
[1] Research Fellow of the Japan Society for the Promotion of Science.

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cord injuries in rodents [18,19]. Whereas, it has been reported that AQP4 is up-regulated in mice with EAE induced by $MOG_{35-55}$ [20]. Furthermore, in mice lacking AQP4, the development of EAE is greatly attenuated [21]. In the rat EAE induced with GPMBP, which is characterized with spinal cord edema, however, the expression of AQP4 has not been investigated. Moreover, the role of AQP4 is not clarified in edema of rat EAE.

Here, we have described for the first time that expression of AQP4-protein and AQP4-mRNA were down-regulated in inflammatory lesions of the CNS of rats with EAE induced with MBP. These findings may be beneficial for understanding of edema formation in the CNS and contribute to control EAE.

## 2. Materials and methods

### 2.1. Rat

Female Lewis rats, 9 weeks old, were purchased from the Charles River Laboratories Japan, Inc. (Ibaraki, Japan), housed and cared for in an approved facility, in accordance with the Shinshu University Guide for Laboratory. The animals were kept in plastic cages containing pine chips, and given food and water ad libitum. The protocol for animal experiments was approved by the Animal Care Committee of Shinshu University.

### 2.2. Sensitization and assessment of EAE

Guinea pig myelin basic protein (GPMBP) was isolated from guinea pig spinal cords (Pel-Freeze, Rogers, AR, USA) by the method of Swanborg et al. (1974) [22]. MBP was suspended in phosphate buffered saline (PBS, pH 7.2) and emulsified in an equal volume of complete Freund's adjuvant (CFA) using a Sorvall omnimixer (model 17105, Dupont Instruments, Newtown, CT, USA). Each Lewis rat was sensitized in the hind footpads with 10 μg of GPMBP emulsified in CFA containing 200 μg of *Mycobacterium tuberculosis* H37Rv Jamaican strain (MTB Difco, Detroit MI, USA). Control rats were sensitized with CFA only. Immunized rats were examined twice a day for clinical neurologic signs, which were recorded using the following grading system: 0, no disease; 1, distal limp tail; 2, limp tail; 3, ataxic gait; 4, early paresis; and 5, paralysis. AQP4 was not detected in our GPMBP preparation by using western blot.

### 2.3. Antibodies

The following antibodies were used to detect specific water channel and BBB associate proteins: Polyclonal rabbit anti-aquaporin-4 antibody (Abcam, Tokyo, Japan), Polyclonal rabbit anti-glial fibrillary acidic protein (GFAP) (DAKO, Glostrup, Denmark), Polyclonal rabbit anti-fibronectin (DAKO), Polyclonal rabbit anti-VE-cadherin (Alexis Biochemicals, USA), Polyclonal rabbit anti-ZO-1 (Zymed, invitrogen, Karlsruhe, Germany). If not otherwise mentioned, all antibodies were used in a dilution of 1:100. Antibodies were labeled by using Zenon Alexa Fluor 594 Rabbit IgG Labeling Kit (invitrogen).

### 2.4. Morphological and immunohistochemical studies

In each experiment, six rats were randomly selected from each group beforehand for histologic examination and sacrificed on day 8 (stage of before onset), day 12 (stage of severe EAE) and day 18 (stage of complete remission) post immunization. Rats were perfused under anesthesia by the intraventricular route with 4% paraformaldehyde in PBS (pH 7.2). Spinal cords were removed and fixed in 4% paraformaldehyde. Spinal cord sections from paraffin-embedded tissues were prepared at 3 μm thickness. Sections were stained with hematoxylin-eosin or Kluver-Barrera's staining. Immunohistochemical staining was performed by using indirect immunoperoxidase techniques. Tissue sections were pretreated with 0.1% trypsin in PBS (pH 7.2) at 37 °C for 30 min, and were incubated overnight at 4 °C with the polyclonal rabbit anti-aquaporin-4 antibody (1:100, Abcam), polyclonal rabbit anti-GFAP (1:500, DAKO). After three washes in PBS for 5 min, sections were incubated with peroxidase-labeled Histofine simple stain Rat MAX-PO (MULTI) (Nichirei, Tokyo, Japan) for 60 min at room temperature. Following washes in PBS, peroxidase reaction was visualized by incubation in a 3′3-diaminobenzidine-tetrachloride (DAB)/$H_2O_2$ solution. The sections were counterstained with hematoxylin. For negative controls, primary antibodies were omitted.

### 2.5. Spinal cord edema measurement

Water content of the spinal cord was measured by a wet-dry method to provide a quantitative measure of edema by using the method as previously reported [23]. Briefly, spinal cords were immediately removed and weighed, then dried at 85 °C for 48 h and reweighed. Percent water content was calculated as (wet weight − dry weight)/wet weight × 100.

### 2.6. Gene expression measurements

Total RNA was extracted from spinal cord and brain using ISOGEN (Nippon gene, Tokyo, Japan) following the manufacturer's instructions. Prior to the reverse transcription reaction, potentially contaminating residual genomic DNA was eliminated with DNase I (Roche, Basel, Switzerland). Messenger RNA (mRNA) was reverse transcribed using SuperScript™ III (Invitrogen, Carlsbad, DA, USA). mRNA was quantified by real-time RT-PCR using a TaqMan Fast Universal Master Mix (Applied Biosystems, Foster City, CA, USA) in an Applied Biosystems StepOnePlus™ real-time PCR System (Applied Biosystems). The primers for AQP4 (Rn00563196_m1), GFAP (Rn00566603_m1) and β-actin (Rn00667869_m1) were obtained from Applied Biosystems. Amplification was conducted in a total volume of 20 μl for 40 cycles of 1 s at 95 °C and 20 s at 60 °C. AQP4 and GFAP mRNA levels were normalized to β-actin expression.

### 2.7. Western blot

Spinal cord and brain tissues were homogenized in a lysis buffer (20 mM Tris–HCl, 2.5 mM EDTA, 10% glycerol, 1% sodium deoxycholic acid, 0.1% SDS, 1% Triton X-100, 1 mM PMSF, 50 mM NaF, 25 mM glycerophosphate, 0.1 mM sodium orthovanadate, and protease inhibitor cocktail [Nacalai Tesque]). The supernatants were collected after centrifugation at 15,000 rpm at 4 °C for 30 min. Protein concentration was determined using a BCA protein assay kit (Thermo Scientific, Rockford, IL, USA), and whole-tissue lysate were mixed with an equal amount of 2 × loading buffer (100 mM Tris–HCl, 4% SDS, 20% glycerol, 0.1% mercaptoethanol, and 0.1% bromophenol blue). Samples were heated at 100 °C for approximately 5 min before loading and were separated on 10% SDS–polyacrylamide gels. The proteins were electrotransferred to a PVDF membrane (Bio-Rad, California, USA). Nonspecific binding to the membrane was blocked for 45 min at room temperature with 5% nonfat milk in TBS buffer. The membranes were then incubated for overnight at 4 °C with polyclonal rabbit anti aquaporin-4 antibody (1:1000, Abcame) or polyclonal rabbit anti-GFAP antibody (1:5000, DAKO) or mouse monoclonal anti-β-actin antibody (1:20000, A5316, Sigma, USA) in blocking buffer containing 5% nonfat milk. After extensive washing in TBS buffer, the membranes were then incubated with appropriate horseradish peroxidase-