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CSF cytokine and chemokine profiles in acute disseminated encephalomyelitis

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Abstract

We simultaneously measured 16 cytokines/chemokines in cerebrospinal fluid (CSF) from 14 patients with acute disseminated encephalomyelitis (ADEM) and 20 controls using a fluorescent bead-based immunoassay. A variety of cytokines, such as IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IFN-γ, TNF-α, G-CSF and MIP-1β, were significantly elevated in ADEM. In particular, G-CSF showed a marked 38-fold increase compared to the control mean. Significant positive correlations with inflammatory parameters in CSF, such as cell counts and protein levels, were found for IFN-γ, IL-6 and IL-8. In contrast, IL-17 produced by activated $CD4^+$ memory T cells was not increased. The results suggested that various cytokines related to activation of macrophages/microglias and Th_1 and Th_2 cells are upregulated in CSF in ADEM.

Keywords: Acute disseminated encephalomyelitis; Cytokine; Chemokine; Cerebrospinal fluid

1. Introduction

Acute disseminated encephalomyelitis (ADEM) is an acute inflammatory demyelinating disease of the central nervous system (CNS) that preferentially affects children and young adults. It typically occurs after infections or vaccination (Dale et al., 2000) and is therefore believed to result from a transient autoimmune response against myelin, possibly via molecular mimicry or non-specific activation of autoreactive T cells (Garg, 2003). Cytokines and chemokines are key mediators of autoimmune diseases and may also play roles in the evolution of ADEM (Dale, 2003; Garg, 2003). However, just a few studies have measured limited numbers of cytokines in cerebrospinal fluid (CSF) from ADEM patients using enzyme-linked immunosorbent assays (ELISAs), and alterations were only found in interleukin (IL)-6 (Dale and Morovat, 2003; Ichiyama et al., 2002) and IL-10 (Ichiyama et al., 2002). A recently developed multiplexed fluorescent bead-based immunoassay is able to simultaneously measure multiple cytokines and chemokines using only small volumes of materials, and is therefore especially suitable for assaying cytokines in CSF. Using this method, we have successfully characterized disease-specific changes in CSF cytokines and chemokines in multiple sclerosis (MS) (Ishizu et al., 2005) and chronic inflammatory demyelinating polyneuropathy (CIDP) (Mei et al., 2005). In the present study we applied this method to simultaneously measure 16 cytokines/chemokines in ADEM CSF and clarify their possible roles in this condition.

2. Patients and methods

2.1. Patients

We performed cytokine and chemokine assays using CSF from 14 patients with ADEM (Table 1). The diagnosis of

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Table 1
Demographic features of the ADEM and OND patients

	ADEM	OND
Number of patients	14	20
Gender (male/female)	6/8	6/14
Age (years)	6.5±4.8	4.1±3.9
CSF		
Cell count (per μl)	96.1±120.9*	1.8±2.5
Total protein (mg/dl)	59.4±31.1*	22.8±11.7

Values are expressed as the mean±S.D. CSF data on cell counts and total protein amounts were not available in 2 ADEM patients.
ADEM=acute disseminated encephalomyelitis; OND=other non-inflammatory neurological diseases; CSF=cerebrospinal fluid.
* $p<0.001$ compared with OND.

ADEM was made based on MRI findings, consistent with a disseminated demyelinating process, as described previously in the Departments of Pediatrics in Yamaguchi and Kyushu University Hospitals (Ichiyama et al., 2002). Precipitating infections, such as upper respiratory tract infection, gastroenteritis and mumps, were observed in 7 of the 14 patients and a preceding fever in another 3. All 12 patients whose CSF data were available had either pleocytosis (≥5/μl) or total protein increase (≥40 mg/dl); mononuclear pleocytosis was seen in 11 of the 12 (92%) and protein increase in 8 (67%). All but one, who had residual mild hemiparesis, of the 14 patients showed complete recovery without any recurrence. The mean time from symptom onset to lumbar puncture (LP) was 8.5 days and patients who had received immunomodulatory therapies were excluded. In addition, 20 patients of similar ages with other non-inflammatory neurological diseases (OND) were used as controls (Table 1). The OND group consisted of 11 patients with epilepsy, 2 with cerebral palsy, and 1 each with congenital myopathy, narcolepsy, Leigh encephalopathy, Krabbe disease, holoprosencephaly, cortical dysplasia, and malingering. CSF was obtained from all patients by non-traumatic LP for diagnostic purposes and stored at −70 °C until analysis.

2.2. Multiplexed fluorescent bead-based immunoassay

CSF was analyzed simultaneously for 16 different cytokines and chemokines, namely IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 (p70), IL-13, IL-17, interferon (IFN)-γ, tumor necrosis factor (TNF)-α, granulocyte colony-stimulating factor (G-CSF), monocyte chemoattractant protein 1 (MCP-1) and macrophage inflammatory protein 1β (MIP-1β), using the Bio-Plex Cytokine Assay System (Bio-Rad Laboratories, Hercules, CA) as described previously (Ishizu et al., 2005; Mei et al., 2005). Briefly, 50 μl of each CSF and various concentrations of each cytokine standard were added to 50 μl of antibody-conjugated beads in a 96-well filter plate. After incubation for 30 min, the plate was washed and 25 μl of a biotinylated antibody solution was added to each well, followed by incubation for another 30 min. The plate was then washed and 50 μl of streptavidin-conjugated phycoerythrin was added to each well and incubated for 10 min. Following a final wash, the contents of each well were resuspended in 125 μl assay buffer and analyzed using a Bio-Plex Array Reader (Bio-Rad). Cytokine concentrations were calculated by comparison with a standard curve for each cytokine derived from various concentrations of the cytokine standards (0.2, 0.78, 3.13, 12.5, 50, 200, 800 and 3200 pg/ml) assayed in the same manner as the CSF samples. All 34 CSF samples were measured at one time; intra-assay variability, expressed as a coefficient of variation, is reported to be less than 10% (manufacturer's instructions). The detection limit of each cytokine was determined by the recovery of the corresponding cytokine standard, and the lowest values showing more than 50% recovery were set as the lower detection limits. The lower detection limit for each cytokine was as follows: 0.2 pg/ml for IL-2, IL-4, IL-5, IL-7, IL-8, IL-10, IL-12 (p70), IL-13, IL-17, IFN-γ and TNF-α; 0.78 pg/ml for IL-1β and IL-6; and 3.13 pg/ml for G-CSF, MCP-1 and MIP-1β. All samples were analyzed undiluted in duplicate. Intra-assay variability between the duplicate determinations was calculated and expressed with %CV as follows: IL-1β=3.8±2.7%, IL-2=4.6±3.7%, IL-4=2.9±2.7%, IL-5=4.5±3.5%, IL-6=6.9±8.7%, IL-7=3.6±2.5%, IL-8=3.1±4.6%, IL-10=4.9±3.0%, IL-12 (p70)=4.1±3.0%, IL-13=3.3±2.7%, IL-17=3.6±2.4%, IFN-γ=4.5±4.2%, TNF-α=5.1±4.1%, G-CSF=4.7±3.6%, MCP-1=5.3±4.7%, MIP-1β=3.6±5.7%.

2.3. Statistical analysis

We used the following statistical tests for the appropriate applications: Fisher's exact probability test was used to compare the detection rates of cytokines and chemokines in each group; the non-parametric Mann–Whitney *U*-test was employed to compare the cytokine and chemokine levels in each group; and Spearman's rank correlation analysis was used to correlate various clinical parameters and CSF cytokine levels. Statistical significance was set at $p<0.05$.

3. Results

3.1. Comparisons of cytokine and chemokine detection rates in CSF

The detection rates of IL-1β, IL-2, IL-4, IL-5, IL-10, IFN-γ, TNF-α and G-CSF were significantly higher in ADEM than OND patients (100% vs. 5%, $p<0.0001$ for IL-1β; 71.4% vs. 15%, $p=0.001$ for IL-2; 100% vs. 65%, $p=0.02$ for IL-4; 100% vs. 50%, $p=0.001$ for IL-5; 100% vs. 45%, $p=0.0006$ for IL-10; 100% vs. 55%, $p=0.004$ for IFN-γ; 100% vs. 45%, $p=0.0006$ for TNF-α; 100% vs. 60%, $p=0.01$ for G-CSF). The detection rates of the other

cytokines did not differ significantly between ADEM and OND patients.

3.2. Comparisons of cytokine and chemokine levels in CSF

The following cytokines were significantly higher in ADEM than OND patients (Fig. 1): IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IFN-γ, TNF-α, G-CSF and MIP-1β. The other cytokines (IL-7, IL-12 (p70), IL-13, IL-17 and MCP-1) did not differ significantly between the two groups. Even when the cut-off line was uniformly set at 2 pg/ml, the following cytokines were significantly higher in ADEM than OND patients; IL-1β, IL-2, IL-5, IL-6, IL-8, IL-10, TNF-α, G-CSF and MIP-1β, while IL-4 and IFN-γ lost significance. Nine of the 20 OND patients showed relatively high IFN-γ levels (more than 5 pg/ml). The diagnoses of these patients were; epilepsy in 6, and cortical dysplasia, Krabbe disease and Leigh encephalopathy in 1 each. Thus, 6 of the 11 patients with epilepsy (54.5%) making up part of the non-inflammatory neurological controls had unexpectedly high IFN-γ levels.

3.3. Correlations between clinical parameters and CSF cytokine levels in ADEM

Among the elevated cytokines/chemokines in ADEM CSF, IL-6 and IFN-γ showed significant positive correlations with the CSF cell count, while IL-8 showed a significant positive correlation with the CSF protein concentration (Fig. 2). The age at onset, gender and days from symptom onset to LP did not show any significant correlations with CSF cytokine/chemokine levels.

4. Discussion

The present study is the first to reveal that a variety of cytokines related to the activation of macrophages and Th_1 and Th_2 cells were significantly elevated in ADEM CSF, while a cytokine produced by activated $CD4^+$ memory T cells (IL-17) was not. Even when the cut-off line was set at 2 pg/ml, most of these cytokines remained statistically significance; however, the validity of such low cytokine

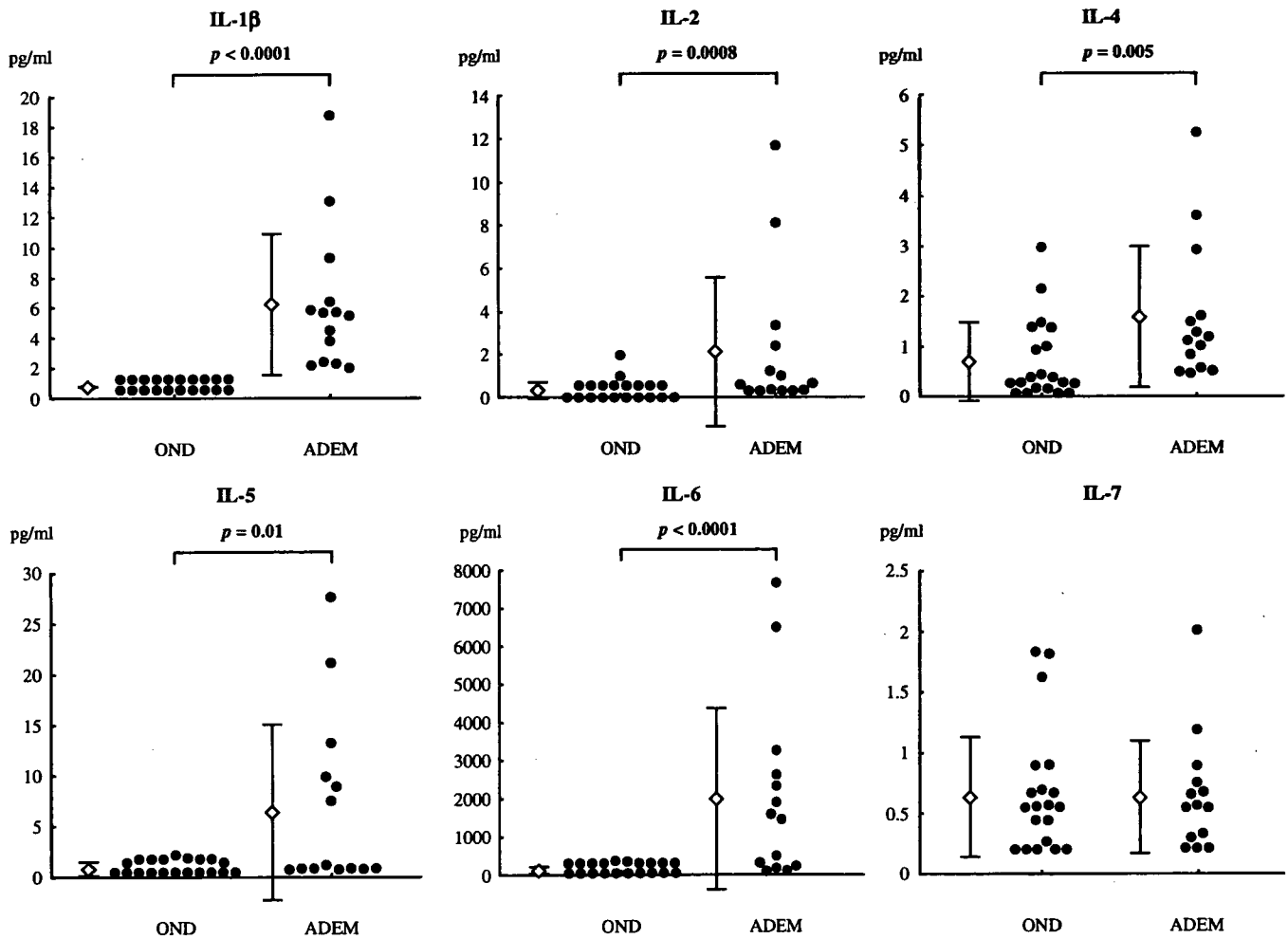

Fig. 1. Cytokine and chemokine levels in CSF from patients with ADEM and OND assessed by a multiplexed fluorescent bead-based immunoassay. Samples totaled 34: 14 ADEM and 20 OND. Open diamonds and bars indicate the mean ± S.D. for each group.

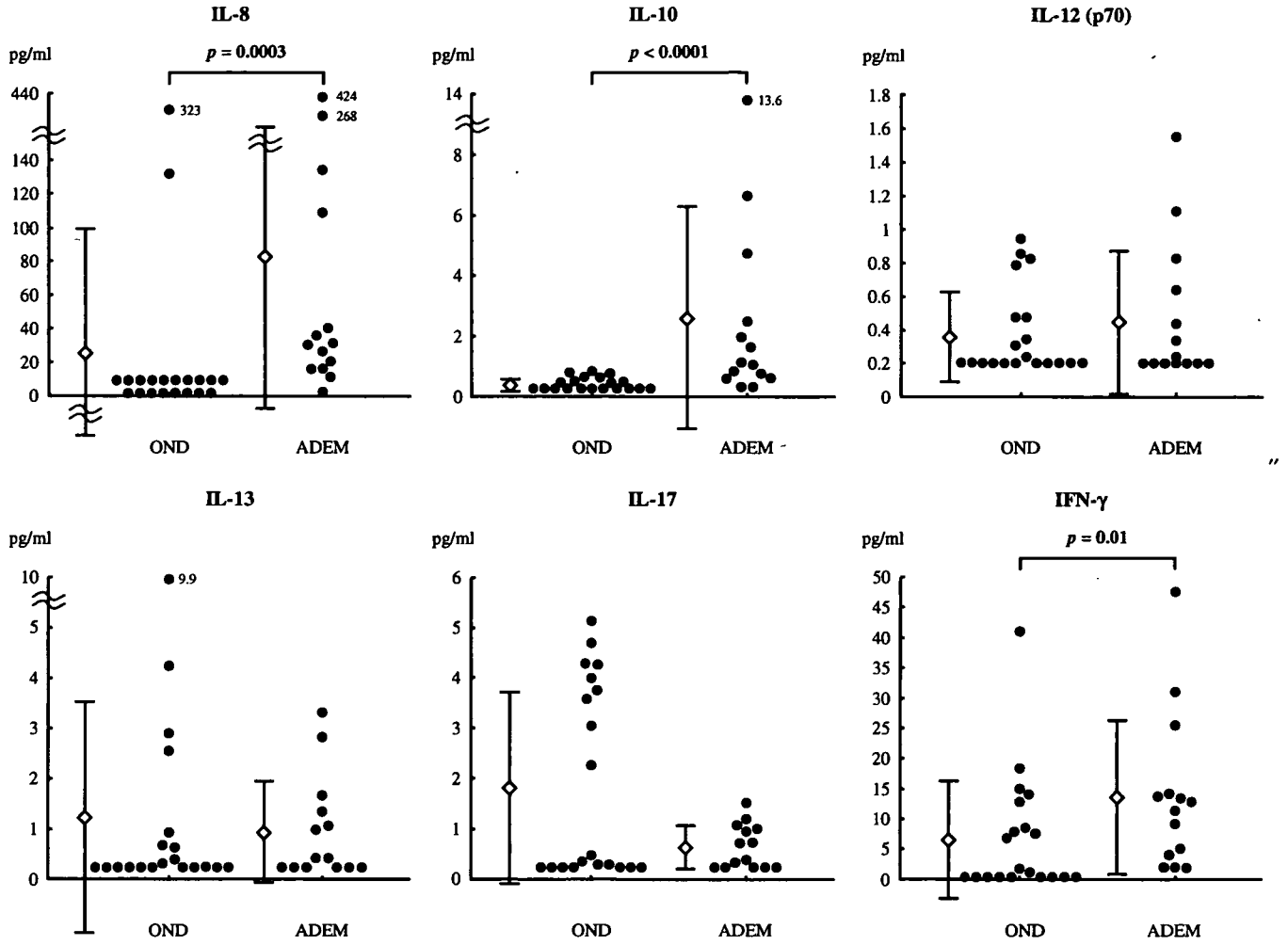

Fig. 1 (*continued*).

levels, less than 2 pg/ml, needs further study on a larger scale.

Involvement of type 1 cytokines in ADEM has been extremely difficult to demonstrate by ordinary ELISAs for CSF, with just one previous study reporting an increase in just TNF-α in a fraction of ADEM patients (17%), primarily due to the short half-lives of these Th_1 cytokines (Ichiyama et al., 2002). However, we have successfully demonstrated upregulation of Th_1 cytokines and found positive correlations between CSF inflammatory parameters and Th_1 (IFN-γ) and downstream inflammatory (IL-6 and IL-8) cytokines, further underscoring the contribution of Th_1 cells to inflammation of the CNS in ADEM. Th_1 cells have been shown to be deeply involved in organ-specific autoimmune diseases and are well known to induce experimental autoimmune encephalomyelitis (EAE), an animal model of acute inflammatory demyelinating diseases in humans. Taking all these observations into account, although the mechanism remains to be elucidated, Th_1 cells also appear to be involved in the inflammation of the CNS in ADEM.

In this study, we also noted a marked increase in G-CSF (38-fold) that has not been observed in other autoimmune inflammatory demyelinating diseases such as MS (Ishizu et al., 2005) and CIDP (Mei et al., 2005). G-CSF is mainly produced by monocytes/macrophages, endothelial cells, fibroblasts and mesothelial cells. G-CSF is not only a potent inducer of granulocytes, but also an inducer of type 2 cytokines, such as IL-4, IL-5 and IL-6, and a strong suppressor of EAE (Zavala et al., 2002), a Th_1-mediated autoimmune disease against CNS myelin. These facts suggest that G-CSF behaves like a Th_2 cytokine. Therefore, together with other Th_2 cytokines, G-CSF may be upregulated due to the host's effort to overcome Th_1-mediated inflammation. Alternatively, since anti-myelin reactive T cells isolated from peripheral blood of ADEM patients show a Th_2-biased profile (Jorens et al., 2000), and CNS myelin-reactive Th_2 cells by themselves can induce EAE (Lafaille et al., 1997), upregulated Th_2 cytokines may contribute to the exacerbation of ADEM, by working with the markedly increased amount of IL-6 and IL-10, through enhancement of

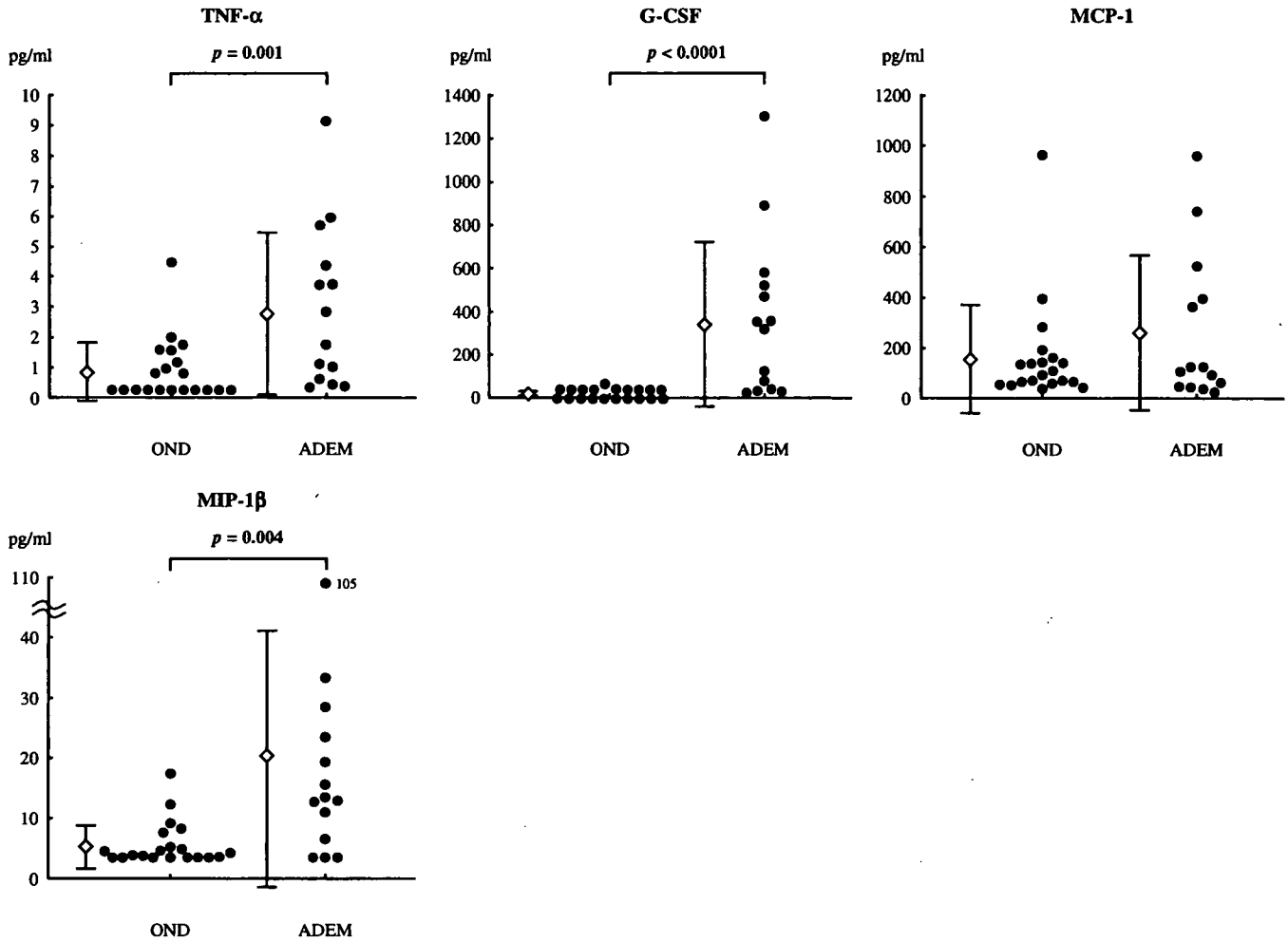

Fig. 1 (*continued*).

humoral immunity involving autoantibodies, complements and immune complexes (Dale et al., 2001; Jorens et al., 2000).

In addition, cytokines reflecting activation of macrophages/microglias, such as IL-1β, IL-6 and MIP-1β, as well as those acting on other effectors, such as IL-5 on eosinophils and IL-8 and G-CSF on granulocytes, were markedly elevated in CSF, indicating contribution of these cells to ADEM pathology. This may explain the infiltration of various types of inflammatory cells into ADEM lesions (Dale, 2003; Dale et al., 2000). However, it is intriguing to note that the marked upregulation of IL-17 that is observed in persistent autoimmune diseases against myelin such as MS (Ishizu et al., 2005) and CIDP (Mei et al., 2005), was not evident in ADEM. Activated $CD4^+$ memory T cells induced by IL-23 to mediate autoimmune inflammation mainly produce IL-17 (Kolls and Linden, 2004). It has been shown that IL-23, but not IL-12, is required for CNS autoimmunity (Cua et al., 2003; Langrish et al., 2005). IL-23 promotes the development of IL-17-producing $CD4^+$ T cells, whereas IL-12 drives IFN-γ-producing $CD4^+$ T cells (Langrish et al., 2005, 2004). The former are considered essential for organ-specific autoimmune inflammation, while the latter are critical for host defense against pathogens (Langrish et al., 2005, 2004; Shtrichman and Samuel, 2001). According to the results of our study, the latter process appears to be activated in ADEM, but not the former, which makes sense since ADEM usually follows infection by pathogens, as also seen in our series. We recently found that IL-17 was significantly higher in MS CSF at relapse than in non-inflammatory controls, while IFN-γ was not (Ishizu et al., 2005). Contrarily, the present study revealed that IFN-γ was increased in ADEM, while IL-17 was not. Therefore, these two conditions have distinct CSF cytokine profiles, suggesting that distinct immune mechanisms are operating. IL-17 may be involved in a persistent autoimmune attack against myelin, but not in a transient autoimmune attack such as ADEM, or at least in the first attack against myelin since the possibility that our ADEM patients may later relapse cannot be completely ruled out.

To summarize, this study has revealed a distinct CSF cytokine/chemokine profile for ADEM that may be useful for differentiating this disease from persistent autoimmune

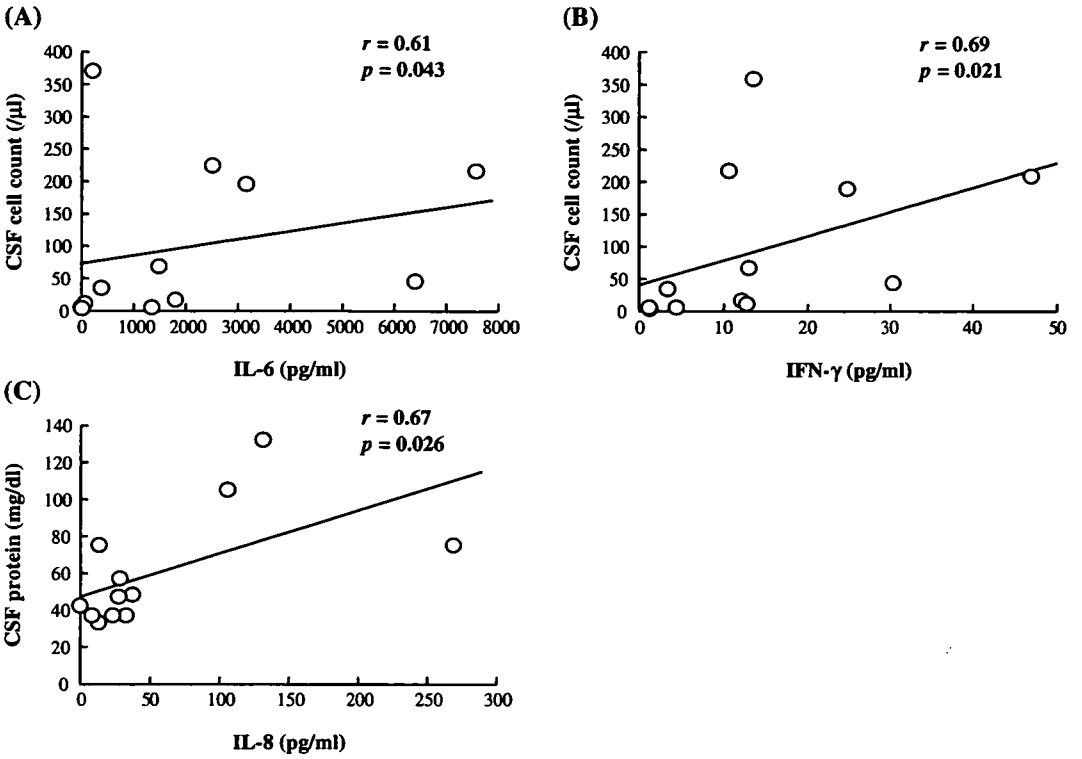

Fig. 2. Correlations between CSF parameters and cytokine/chemokine levels in patients with ADEM evaluated by Spearman's rank correlation analysis. (A) CSF cell count and IL-6 levels. (B) CSF cell count and IFN-γ levels. (C) CSF protein concentration and IL-8 levels.

demyelinating diseases, such as MS. Although we found no significant correlation between any of the cytokines in CSF and the days from symptom onset to LP, future larger-scale studies using CSF taken at multiple time points may clarify the relationship between cytokines and disease course.

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Interleukin-1β induces the expression of aquaporin-4 through a nuclear factor-κB pathway in rat astrocytes

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Abstract

Interleukin (IL)-1β is known to play a role in the formation of brain edema after various types of injury. Aquaporin (AQP)4 is also reported to be involved in the progression of brain edema. We tested the hypothesis that AQP4 is induced in response to IL-1β. We found that expression of AQP4 mRNA and protein was significantly up-regulated by IL-1β in cultured rat astrocytes, and that intracerebroventricular administration of IL-1β increased the expression of AQP4 protein in rat brain. The effects of IL-1β on induction of AQP4 were concentration and time dependent. The effects of IL-1β on AQP4 were mediated through IL-1β receptors because they were abolished by co-incubation with IL-1 receptor antagonist. It appeared that IL-1β increased the level of AQP4 mRNA without involvement of *de novo* protein synthesis because cycloheximide, a protein synthesis inhibitor, did not inhibit the effects of IL-1β. Inhibition of the nuclear factor-κB (NF-κB) pathway blocked the induction of AQP4 by IL-1β in a concentration-dependent manner. These findings show that IL-1β induces expression of AQP4 through a NF-κB pathway without involvement of *de novo* protein synthesis in rat astrocytes.

Keywords: aquaporin-4, interleukin-1β, nuclear factor-κB.
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The aquaporins (AQPs) are water channel proteins that increase water permeability via the plasma membrane and thus provide a route for rapid fluid movement (King and Agre 1996; Verkman and Mitra 2000). They are expressed in many tissues, including kidney, lung, salivary glands, eyes and brain, where rapid, regulated transport of water is necessary (Deen *et al.* 1994; Hasegawa *et al.* 1994).

In the brain, AQP4 is abundantly expressed, and is highly concentrated in astrocyte membranes that are in direct contact with capillaries and the pia mater. AQP4 is considered a major pathway for massive water shift across the plasma membrane under various conditions and is involved in brain edema formation (King *et al.* 1996; Nielsen *et al.* 1997). Taniguchi *et al.* reported that AQP4 expression in brain is up-regulated after ischemic injury (Taniguchi *et al.* 2000). In one study two peaks of AQP4 expression were demonstrated at 1 and 48 h after ischemic injury (de Castro Ribeiro *et al.* 2006), whereas another study showed that AQP4 expression is decreased immediately after ischemic injury (Meng *et al.* 2004). Manley *et al.* have shown that brain tissue water content is significantly reduced in AQP4-deficient mice, and that these mice exhibit better survival than wild-type counterparts in models of brain

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Abbreviations used: AQP, aquaporin; con, contralateral; DMSO, dimethyl sulfoxide; ERK, extracellular signal-regulated kinase; GFAP, glial fibrillary acidic protein; IκB, inhibitor of nuclear factor-κB; IL, interleukin; IL-1R1, IL-1 receptor type 1; IL-1ra, recombinant IL-1 receptor antagonist; ipsi, ipsilateral; JNK, Jun-N-terminal kinase; l-DMEM, low-glucose Dulbecco's modified Eagle's medium; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; NF-κB, nuclear factor- κB; PBS(–), Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline; PKC, protein kinase C; PSI, proteasome inhibitor I; SDS, sodium dodecyl sulfate; TBS, Tris-buffered saline; TBS-T, TBS containing 0.1% Tween 20; TK, thymidine kinase; TNF-α, tumor necrosis factor-α.

edema caused by acute water intoxication and focal ischemic stroke produced by middle cerebral artery occlusion (Manley *et al.* 2000). Moreover, they found that elimination of water was also impaired in AQP4-deficient mice, and demonstrated that water flow through AQP4 is bi-directional (Papadopoulos *et al.* 2004). There are contradictory findings concerning the role of AQP4 in brain edema formation. Little is known about the mechanisms by which AQP4 expression is regulated in the brain, although they are of great interest.

Interleukin (IL)-1β is one of the pivotal pro-inflamamatory cytokines in the brain (Touzani *et al.* 1999; Wang and Shuaib 2002). It plays roles in pathological conditions such as ischemic and traumatic brain injury, as well as in other brain disorders (Nguyen *et al.* 1998; Lemke *et al.* 1999). However, opinions vary concerning the function of IL-1β in astrocytes in brain injury. On the one hand, IL-1β might exhibit pathological effects by interfering with the function of residual neuronal circuits, and thus prevent axonal remyelination and inhibit axonal regeneration (Wang *et al.* 2002). On the other hand, astrocytes reactivated by IL-1β may also participate in wound healing. Reactive astrocytes regulate molecular and ionic contents of the extracellular space at sites of injury and induce the secretion of neurotrophic factors (Gadient *et al.* 1990; Yu *et al.* 1991).

On the assumption that IL-1β regulates AQP4 expression, this study was designed to assess the effect of IL-1β on AQP4 expression, and to identify the signal transduction pathways mediating this effect in cultured rat astrocytes. We found that expression of AQP4 mRNA and protein is induced in response to IL-1β in cultured rat astrocytes and in brain of rats administered IL-1β intracerebroventricularly, and that this induction might be regulated by the nuclear factor (NF)-κB pathway. To our knowledge, this study provides the first example of induction of an AQP in response to a pro-inflammatory cytokine in the brain.

Experimental procedures

Cell culture

Cultures of cortical astrocytes were prepared from rat postnatal cortex (P2) according to the methods described by Kato *et al.* (1979). Trypsinized and dissociated cortical cells were cultured in 75-cm^2 culture flasks (Sigma-Aldrich, St Louis, MO, USA) containing low-glucose (1000 mg/L) Dulbecco's modified Eagle's medium (l-DMEM; Invitrogen, San Diego, CA, USA) supplemented with 10% fetal bovine serum (Sigma). After incubation for 5–7 days, the cells were trypsinized and subcultured in culture dishes with a diameter of 60 mm (Falcon; PGC Scientifics, Frederick, MD, USA). All cultures were maintained in 5% CO_2, 95% air at 37°C. Cell populations consisted of over 95% astrocytes as determined by immunocytochemical examination with anti-glial fibrillary acidic protein (GFAP).

Drug treatment

When astrocytes became confluent in culture dishes, cells received equal volumes of l-DMEM or various concentrations of recombinant rat IL-1β (R & D Systems, Minneapolis, MN, USA) for the indicated periods of time. In the recombinant IL-1 receptor antagonist (IL-1ra) blockade and mitogen-activated protein kinase (MAPK) or NF-κB inhibition studies, antagonist or inhibitors were added at the specified concentrations 1 h before the addition of 5 ng/mL IL-1β.

RT–PCR

Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA) at the indicated times. Complementary DNAs were generated from 1 μg total RNA by Superscript II RNase H^- (Invitrogen) reverse transcriptase primed with oligo $(dT)_{18}$ (Invitrogen). They were amplified with primers designed according to published sequences. The PCR protocols and primers for AQP4 and β-actin have been reported previously (Yamamoto *et al.* 2001). The oligonucleotide primers were as follows: AQP4, 5′-TTGGACCAATCATAGGCGC-3′ (forward) and 5′-GGTCAATGTCGATCACATGC-3′ (reverse); β-actin, 5′-GACCTGACTGACTACCTCAT-3′ (forward) and 5′-TCGTCATACTCCTGCTTGCT-3′ (reverse). Multitarget PCRs were performed, co-amplifying β-actin as an internal standard. The reactions for AQP4 contained 2 μL reverse transcriptase reaction product as template DNA and were carried out for 20 cycles, using a 95°C, 30-s denaturing step, a 57°C, 30-s annealing step, and a 72°C, 1-min extension step.

Semiquantification of mRNA

The PCR products were visualized by ethidium bromide staining following separation on 2% agarose gels, and quantified with a 2100 bioanalyzer with the DNA 7500 assay kit (Agilent Technologies, Palo Alto, CA, USA). PCR products of AQP4 and β-actin were compared by integration of peak areas.

Western blot analysis

Following the specified incubation, cultured cells were harvested with Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline [PBS (–)] and centrifuged at 800 *g* for 10 min at 4°C. Cell pellets were suspended in 100 μL Tris-buffered saline (TBS) containing 200 mM phenylmethylsulfonyl fluoride, 10 μM pepstatin A, 10 μM leupeptin, 2 mM EDTA and 0.5% Igepal CA-630 (Sigma), and sonicated at 8-W output for 10 s on ice. Samples were left at room temperature (20°C) with 5 μL sodium dodecyl sulfate (SDS) sample buffer for 15 min. Protein content was determined with a bicinchoninic protein assay reagent kit (Pierce Biotechnology, Inc., Rockford, IL, USA). Equal amounts of protein, i.e. 3 μg protein for AQP4 and GFAP, and 20 μg protein for inhibitor of NF-κB (IκB-α), were separated by SDS–polyacrylamide gel electrophoresis (12.5% gel) (ATTO, Tokyo, Japan) and then transferred to a Clear Blot Membrane-P (ATTO). Blotted membranes were blocked for 1 h with 5% skimmed milk in TBS-T (20 mM Tris, pH 7.6, 137 mM NaCl, 0.1% Tween 20) and incubated overnight with a 1 : 750 dilution of polyclonal AQP4 antibody (Chemicon International, Inc., Temecula, CA, USA) or a 1 : 1000 dilution of IκB-α antibody (Cell Signaling Technology, Beverly, MA, USA) in TBS-T containing 5% skimmed milk. After four 15-min washes with TBS-T, blots for AQP4 and IκB-α were incubated for 1 h with a 1 : 1000 dilution of horseradish

peroxidase-conjugated secondary antibody in TBS-T buffer containing 5% skimmed milk. Protein was visualized using the enhance chemiluminescence detection system (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Relative band intensities were determined by densitometry using Kodak Digital Science 1D version 2.0 (Eastman Kodak Company, Rochester, NY, USA).

Isolation of the 5′ flanking regions of human *AQP4* gene

The 5′ flanking region of the human *AQP4* gene was isolated as described previously (Umenishi and Verkman 1998). The *AQP4* gene is regulated by alternative promoters upstream of exons 0 and 1. The 5′ flanking regions of the exon 0 promoter (bp − 1930 to + 28) (nucleotide + 1 corresponds to the initiation codon of exon 0) and the exon 1 promoter (bp −1600 to + 240) (nucleotide + 1 corresponds to the initiation codon of exon 1) were subcloned into the vector pCR2.1 (Invitrogen), and 5′ deletion mutants were constructed. Deletion mutants corresponding to bp − 1930 to + 28 in the exon 0 promoter (eight constructs) and from bp −1600 to + 240 in the exon 1 promoter were fused to the promoterless vector PGV-B (Toyo-ink, Tokyo, Japan) containing the luciferase reporter gene. The mutants were sequenced completely, and BLAST searches were performed on the National Center for Biotechnology Information (NCBI) sites. The sequence of the human *AQP4* gene was identical to GenBank Accession number AC025431.

Cell transfection and luciferase reporter gene assay

Reporter assays were performed on *AQP4* gene fragments fused upstream of a firefly luciferase reporter using the plasmid PGV-B (Toyo-ink) and dual-luciferase reporter assay systems (Promega, Madison, WI, USA) in which the pRL-thymidine kinase (TK) plasmid containing the *Renilla* luciferase gene under control of the TK promoter was co-transfected as an internal control. For transient transfection, astrocytes in l-DMEM without serum were plated at 3×10^5 per well in a 12-well plate for 18 h and transfected with 1.6 μg each of test plasmid along with 0.016 μg pRL-TK (Promega) using 4 μL Lipofectamine 2000 (Invitrogen). After 8 h, the astrocytes received equal volumes of l-DMEM or 5 ng/mL IL-1β in l-DMEM. After 24 h, the astrocytes were washed and harvested. The activities of control *Renilla* luciferase and firefly luciferase were measured in triplicate. After normalization with respect to *Renilla* luciferase activity, promoter activity was calculated as a percentage of the activity of the *AQP4* exon 0 promoter (bp − 1930 to + 29) or exon 1 (bp −1600 to + 240) under control conditions, which was assayed in each experiment and assigned a value of 100%.

Intracerebroventricular administration of IL-1β in rats

All animal studies were undertaken after approval of the protocols by the Animal Care and Use Committee of Nagoya City University Graduate School of Medical Sciences. All efforts were made to minimize the number of animals used and their suffering. Pathogen-free male Wistar rats weighing 273 ± 21 g at the time of surgery were used. Animals were housed under standard conditions in plastic transparent cages with unrestricted access to food and water in a room maintained at 24–25°C and illuminated for 12 h (from 06.00 to 18.00 hours). After acclimatization, animals were anesthetized with pentobarbital (Schering-Plough Animal Health, Kenilworth, NJ, USA) (3.2 mg/100 g body weight). They were placed in a stereotaxic apparatus (Kopf Instruments, Tujunga, CA, USA), and a hole was drilled in the skull with a no. 6 (1 mm) dental burr, and a 23-G stainless steel cannula was implanted into the right lateral ventricle at 2 mm lateral to midline, 0.6 mm posterior to bregma and 4.3 mm below the skull.

A 100-ng portion of recombinant rat IL-1β protein dissolved in sterile normal saline was administered at a constant rate over 5 min through a 30-G inner cannula. Control animals received an identical volume of carrier solution. The dosage was determined from previous animal studies (Proescholdt *et al.* 2002). A 10-μL Hamilton syringe (Hamilton Bonaduz AG, Bonaduz, Swizerland) and a stereotaxic injector (Muromachi Kikai Co., Tokyo, Japan) were used. Animals were removed from the stereotaxic instrument and allowed to recover from anesthesia, which required between 4 min and about 1 h.

The animals were killed by decapitation 1, 3, 6, 12, 24 or 48 h after initiation of administration. Following quick removal, the brains were divided into right (ipsilateral) and left (contralateral) cerebral hemispheres. Western blot analyses were performed as described above after homogenizing each cerebral hemisphere. Samples for immunohistochemical staining and measurement of water content were obtained from the cerebral sections (5 mm thick) and from a portion of the frontal lobe of ipsilateral or contralateral cerebral hemisphere respectively.

Immunohistochemical staining

Coronal sections of the brain were embedded in paraffin by the AMeX method (Sato *et al.* 1986). Briefly, tissues were fixed in acetone at 4°C for 1 day, cleared in methyl benzoate and xylene, and then embedded in paraffin. Sections 3 μm thick were prepared and deparaffinized with xylene. Immunoreactivity was visualized using the streptavidin–biotin method (Histofine SAB-PO kit; Nichirei, Tokyo, Japan). After washing the sections with PBS(−), they were treated with 0.3% (v/v) hydrogen peroxide in methanol for 30 min to inactivate endogenous peroxidase. They were then immersed in a 1 : 200 dilution of non-immune goat serum for 10 min to block non-specific binding and, after blotting to remove excess serum, they were incubated at room temperature for 1 h with AQP4 antibody diluted 1 : 400 in PBS(−) containing 1% bovine serum albumin. Control sections were treated with non-immune rabbit immunoglobulins (MBL, Nagoya, Japan). The sections were rinsed three times with PBS(−), incubated for 30 min each with secondary antibody, rinsed three times, and then incubated with streptavidin–biotin–peroxidase complex for 15 min. After washing the sections with PBS(−), the peroxidase reaction was developed by incubating sections in 0.02% (w/v) 3,3′-diaminobenzidine tetrahydrochloride (Dojindo, Kumamoto, Japan) solution containing 0.003% (v/v) hydrogen peroxide and 10 mM sodium azide. The sections were counterstained with hematoxylin.

Determination of water content

The amount of water contained in brain was determined by volumetric Karl Fisher titration with a Mettler Toledo DL31 titrator (Mettler-Toledo GmbH, Tokyo, Japan). This method involves heating the brain section, evaporating water in the coulometric titration cell, and collection of all moisture into methanol–formamide. All moisture inside and outside of the cells is then evaporated. The water is titrated at 50°C with Karl Fisher reagent (Sigma) based on imidazole.

Approximately 10 mg brain tissue was obtained 1, 3, 6, 12, 24 and 48 h after the administration of IL-1β. The samples were quickly weighed, and then inserted into a glass tube and heated at 150°C for 15 min. The amount of water contained in the samples was determined by Karl Fisher titration.

Data analysis

Values are the mean ± SEM of at least three or four independent experiments. Statistical examination was performed by one-way factorial ANOVA combined with Scheffe's test for all comparison pairs or Student's *t*-test with equal variance. Differences with *p*-values < 0.05 were considered significant.

Results

Induction of AQP4 in cultured rat astrocytes by IL-1β

Cultured rat astrocytes were used to examine the effects of IL-1β on AQP4 expression. In a preliminary experiment, 1 μg total astrocyte RNA was amplified by RT–PCR, and the amounts of PCR products for AQP4 and β-actin were measured every two cycles with a 2100 bioanalyzer. The rate of amplification of β-actin mRNA paralleled that of AQP4 between 18 and 24 cycles (Yamamoto *et al.* 2001). PCR was therefore terminated at 20 cycles. Under these conditions, the amplification curves for AQP4 and β-actin were linear when plotted against the amount of added RT reaction product. PCR products were confirmed by direct sequencing.

To evaluate whether the effects of IL-1β on AQP4 expression were concentration dependent, astrocytes were incubated with various concentrations of IL-1β for designated periods of time. Astrocytes were incubated in medium supplemented with 0.1, 0.3, 1, 3 or 10 ng/mL IL-1β for 6 h before isolation of total RNA for RT–PCR, or for 12 h before harvesting for western blot analysis. The levels of AQP4 mRNA and protein were significantly increased with treatment with 3 and 10 ng/mL IL-1β (Fig. 1). Light microscopy demonstrated that astrocytes treated with IL-1β remained adherent and morphologically similar to untreated cells.

In order to determine whether the effects of IL-1β on AQP4 expression were time dependent, astrocytes were treated with 5 ng/mL IL-1β for various periods of time before isolation of total RNA for RT–PCR or harvesting for western blot analysis. The AQP4 mRNA level was significantly increased after 3, 6 and 12 h of IL-1β treatment. The maximal increase in AQP4 mRNA level was observed after 6 h (Fig. 2a). After 24 h, the AQP4 mRNA level had almost returned to baseline level. AQP4 protein levels were significantly increased after 12, 24 and 48 h of treatment with IL-1β (Fig. 2b).

To determine whether the induction of AQP4 in response to IL-1β was mediated through IL-1 receptors, astrocytes were preincubated with various concentrations of IL-1ra (R & D Systems) for 1 h before treatment with 5 ng/mL IL-1β. As Fig. 3 shows, IL-1ra effectively blocked the induction of expression of AQP4 mRNA and protein by IL-1β. The highest concentration of IL-1ra, a 20-fold excess over IL-1β, almost completely blocked the induction of AQP4 by IL-1β.

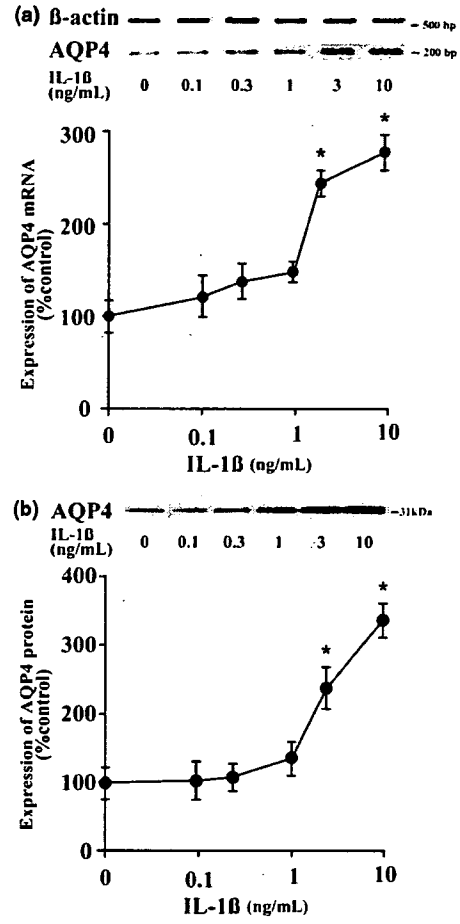

Fig. 1 Dose–response relationship of expression of AQP4 mRNA and protein. (a) Rat astrocytes were incubated in control medium or medium containing designated concentrations of IL-1β for 6 h. Cells were processed for RT–PCR and semiquantification of AQP4 mRNA. Relative expression (normalized with respect to β-actin) ($n = 8$ for each group) is shown as a mean ± SEM percentage of the control value. *$p < 0.05$ versus control. (b) Rat astrocytes were treated as in (a) and processed for western blot with affinity-purified AQP4 antibody. Western blots ($n = 8$ for each group) were analyzed by densitometry, and results expressed as a mean ± SEM percentage of the control value. *$p < 0.05$ versus control (one-way factorial ANOVA combined with Scheffe's test).

To examine whether the induction of AQP4 in response to IL-1β required *de novo* protein synthesis, astrocytes were pretreated with a protein synthesis inhibitor, cycloheximide (CN Biosciences, Inc., La Jolla, CA, USA) 20 μg/mL, 1 h before IL-1β treatment. The effects of IL-1β on AQP4 mRNA expression were the same in cycloheximide-treated and non-treated astrocytes (Fig. 4), suggesting that IL-1β increased the level of AQP4 mRNA without involvement of *de novo* protein synthesis.

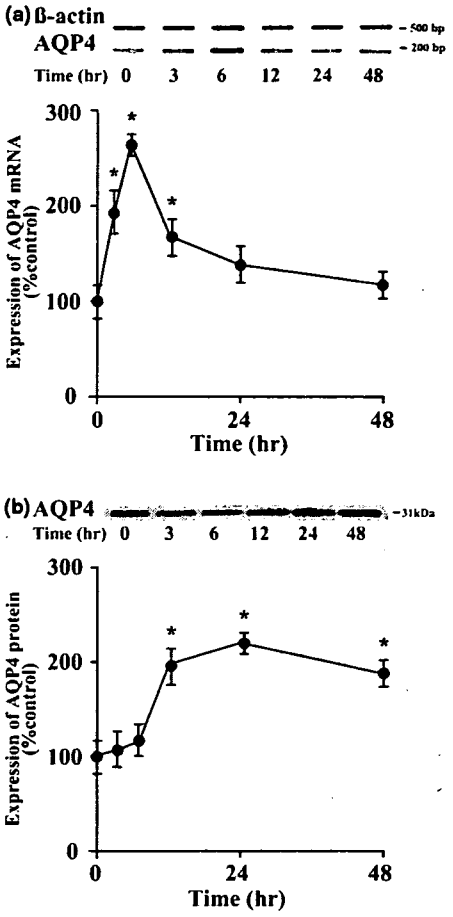

Fig. 2 Time course of AQP4 mRNA and protein expression with IL-1β treatment. (a) Rat astrocytes were incubated in medium containing 5 ng/mL IL-1β. At the designated times, cells were processed for RT–PCR and semiquantification of AQP4 mRNA. Relative expression (normalized with respect to β-actin) ($n = 8$ for each group) is shown as a mean ± SEM percentage of the value at time 0. *$p < 0.05$ versus time 0. (b) Rat astrocytes were treated as in (a) and processed for western blot with affinity-purified AQP4 antibody. Western blots ($n = 8$ for each group) were analyzed by densitometry, and results expressed as a mean ± SEM percentage of the value at time 0. *$p < 0.05$ versus time 0 (one-way factorial ANOVA combined with Scheffe's test).

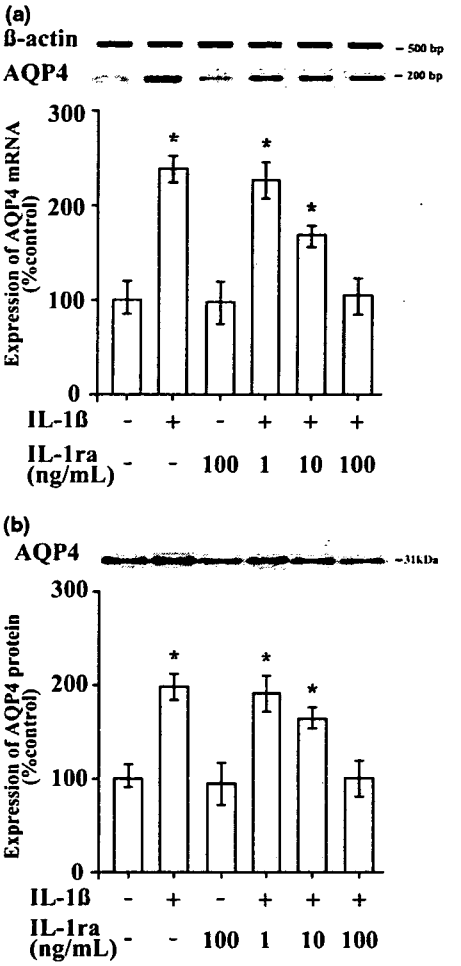

Fig. 3 Effect of IL-1ra on induction of AQP4 mRNA and protein by IL-1β. (a) Rat astrocytes were incubated in control medium or medium containing 5 ng/mL IL-1β, with various concentrations of IL-1ra for 12 h. IL-1ra competes with IL-1β for receptor binding, but does not interact with IL-1β. Cells were processed for RT–PCR and semi-quantification of AQP4 mRNA. Relative expression (normalized with respect to β-actin) ($n = 4$ for each group) is shown as a mean ± SEM percentage of the control value. *$p < 0.05$ versus control. (b) Rat astrocytes were treated as in (a) and processed for western blot with affinity-purified AQP4 antibody. Western blots ($n = 4$ for each group) were analyzed by densitometry, and results expressed as a mean ± SEM percentage of the control value. *$p < 0.05$ versus control (Student's *t*-test with equal variance).

Role of the NF-κB pathway in induction of AQP4 by IL-1β

To examine the involvement of three MAPK cascades (extracellular signal-regulated kinase [ERK] and Jun-N-terminal kinase [JNK]) and the NF-κB pathway in the induction of AQP4 in response to IL-1β, the effects of the p38 MAPK inhibitor SB203580 (Calbiochem, La Jolla, CA, USA), the MAPK/ERK kinase 1/2 (MEK1/2) inhibitor PD98059 (Calbiochem), the JNK inhibitor SP600125 (Calbiochem) and the NF-κB inhibitor SN-50 (Calbiochem) were examined. MEK1/2 are the upstream kinases that activate ERK.

Addition of SN-50 prevented the effects of IL-1β on AQP4 mRNA and protein expression, but MAPK inhibitors did not (Fig. 5). To determine the involvement of NF-κB activation in the induction of AQP4 in response to IL-1β, several inhibitors of NF-κB, MG-132 (Calbiochem), proteasome inhibitor I (PSI) (Calbiochem) and SN-50, were preincubated at various concentrations for 1 h before treatment with 5 ng/mL IL-1β. Dose–response experiments with

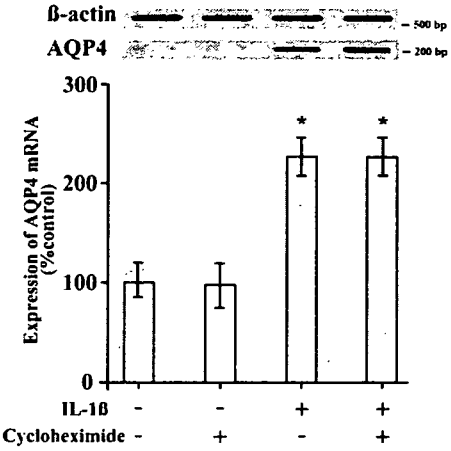

Fig. 4 Effect of cycloheximide on induction of AQP4 mRNA by IL-1β. Rat astrocytes were incubated with or without 5 ng/mL IL-1β or cycloheximide (20 μg/mL) for 6 h. Cells were processed for RT–PCR and semiquantification of AQP4 mRNA. Expression of mRNA (normalized with respect to β-actin) ($n = 4$ for each group) is shown as a mean ± SEM percentage of the control value. $^*p < 0.05$ versus control (Student's *t*-test with equal variance).

RT–PCR and western blotting analysis revealed that MG-132, PSI and SN-50 decreased the expression of AQP4 mRNA and protein in a concentration-dependent manner (Figs 6–8).

IL-1β induced degradation of IκB

NF-κB exists in a quiescent form in the cell cytoplasm, complexed with its inhibitory protein, IκB. When certain ligands bind to the cell surface, IκB is degraded, thus allowing NF-κB to translocate into the nucleus and influence transcription. Because our findings suggested the involvement of NF-κB in induction of AQP4 by IL-1β, experiments were conducted to determine whether IκB-α was degraded in response to IL-1β. Astrocytes were treated with 10 ng/mL IL-1β for 2–10 min before harvesting for western blot analysis. IκB-α protein levels were significantly decreased after 5 and 10 min of treatment with IL-1β (Fig. 9).

Transcriptional regulation of *AQP4* gene after IL-1β treatment in cultured rat astrocytes

Luciferase promoter gene assays were performed to determine the transcriptional activity of the *AQP4* gene in astrocytes in response to IL-1β. Nine luciferase promoter constructs of *AQP4* exon 0 and four of *AQP4* exon 1 were transfected into astrocytes, and luciferase activity was measured with or without IL-1β. In response to IL-1β, luciferase activities of the −1625/+28, −830/+28, −428/+28 and −224/+28 *AQP4* exon 0, and −1600/+240 *AQP4* exon 1 constructs were significantly induced (Fig. 10).

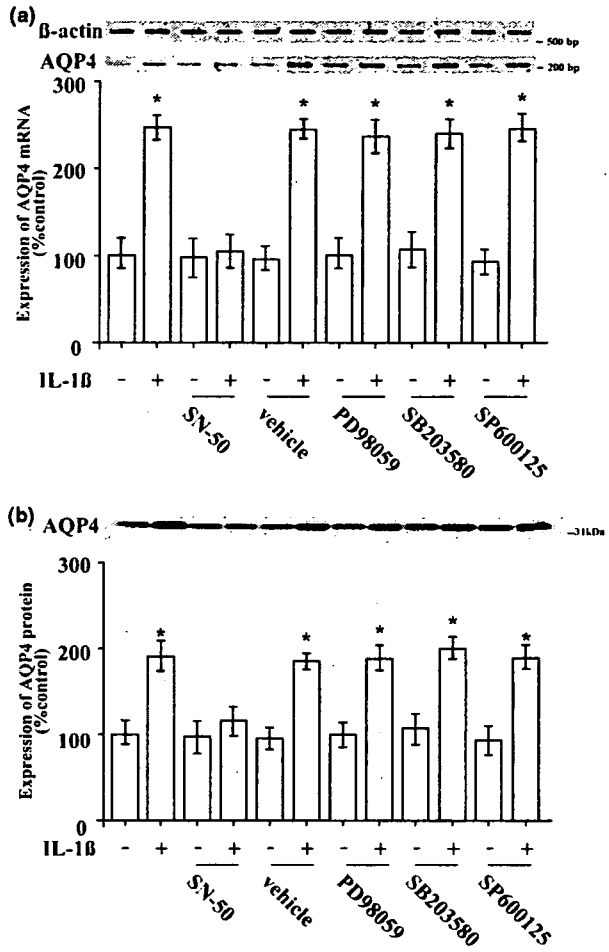

Fig. 5 Effects of MAPK and NF-κB inhibitors on induction of AQP4 mRNA and protein by IL-1β. (a) Rat astrocytes were incubated with or without 5 ng/mL IL-1β or MAPK or NF-κB inhibitor [p38 MAPK inhibitor SB203580 (10 μM), MEK1/2 inhibitor PD98059 (10 μM), JNK inhibitor SP600125 (10 μM) or NF-κB inhibitor SN-50 (10 μM)] for 12 h. Cells were processed for RT–PCR and semiquantification of AQP4 mRNA. Relative expression (normalized with respect to β-actin) ($n = 4$ for each group) is shown as a mean ± SEM percentage of the control value. $^*p < 0.05$ versus control. (b) Rat astrocytes were treated as in (a) and processed for western blot with affinity-purified AQP4 antibody. Western blots ($n = 4$ for each group) were analyzed by densitometry, and results expressed as a mean ± SEM percentage of the control value. $^*p < 0.05$ versus control (Student's *t*-test with equal variance).

Induction of AQP4 protein in rat brain by intracerebroventricular administration of IL-1β

We examined whether expression of AQP4 protein is induced in rat brain by intracerebroventricular administration of IL-1β. Both ipsilateral and contralateral cerebral hemispheres were taken as samples for western blot analyses of AQP4 at 6, 12, 24 and 48 h after administration of IL-1β, and for immunohistochemical staining 12 and 24 h after IL-1β administration.

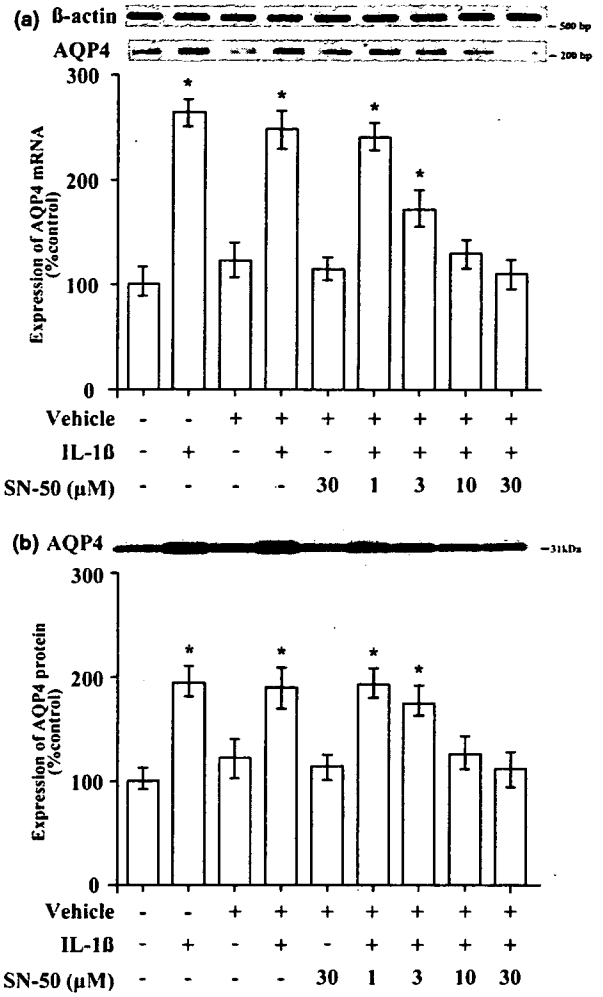

Fig. 6 Effect of inhibition of NF-κB on the induction of AQP4 mRNA and protein by IL-1β. (a) Rat astrocytes were incubated with or without 5 ng/mL IL-1β or various concentrations (1, 3, 10 or 30 μM) of the NF-κB inhibitor SN-50 for 6 h. SN-50 inhibits NF-κB activation by blocking translocation of NF-κB active complexes into the nucleus. To determine whether the reduction of AQP4 expression by SN-50 was a non-specific effect, astrocytes were incubated with vehicle (dimethyl sulfoxide [DMSO], 5 μM). Cells were processed for RT–PCR and semiquantification of AQP4 mRNA. Relative expression (normalized with respect to β-actin) ($n = 4$ for each group) is shown as a mean ± SEM percentage of the control value. *$p < 0.05$ versus control. (b) Rat astrocytes were treated as in (a) and processed for western blot with affinity-purified AQP4 antibody. Western blots ($n = 4$ for each group) were analyzed by densitometry, and results expressed as a mean ± SEM percentage of the control value. *$p < 0.05$ versus control (Student's *t*-test with equal variance).

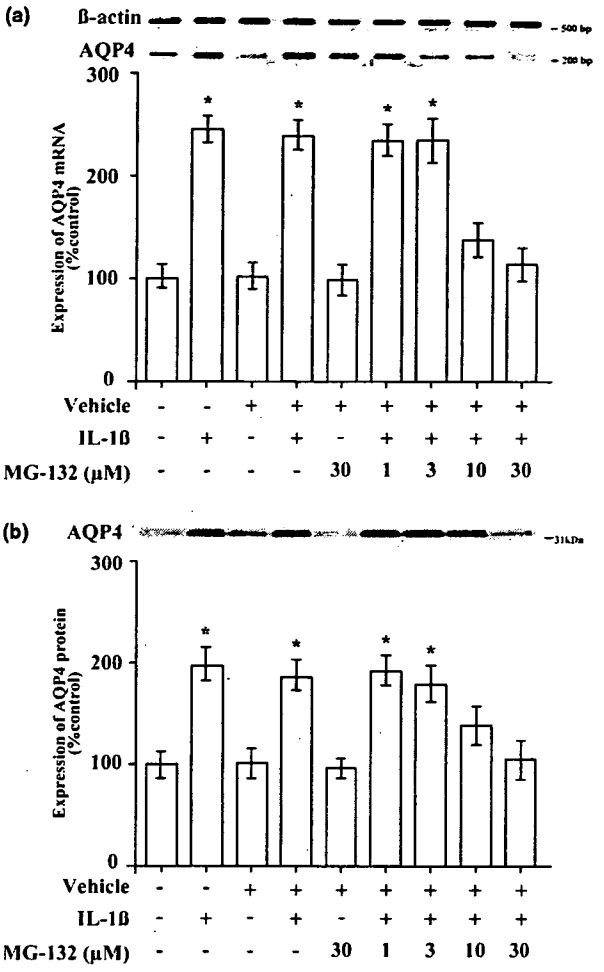

Fig. 7 Effect of blockade of NF-κB by the proteasome inhibitor MG-132 on induction of AQP4 mRNA and protein by IL-1β. (a) Rat astrocytes were incubated with or without 5 ng/mL IL-1β or various concentrations (1, 3, 10 or 30 μM) of the NF-κB inhibitor MG-132 for 6 h. MG-132, a proteasome inhibitor, inhibits NF-κB activation by reducing IκB degradation. To determine whether the reduction of AQP4 expression by MG-132 was a non-specific effect, astrocytes were incubated with vehicle (DMSO, 5 μM). Cells were processed for RT–PCR and semiquantification of AQP4 mRNA. Relative expression (normalized with respect to β-actin) ($n = 4$ for each group) is shown as a mean ± SEM percentage of the control value. *$p < 0.05$ versus control. (b) Rat astrocytes were treated as in (a) and processed for western blot with affinity-purified AQP4 antibody. Western blots ($n = 4$ for each group) were analyzed by densitometry, and results expressed as a mean ± SEM percentage of the control value. *$p < 0.05$ versus control (Student's *t*-test with equal variance).

As Fig. 11(a) shows, AQP4 protein was significantly increased in rats by intracerebroventricular administration of IL-1β. Expression of AQP4 protein was induced in both the ipsilateral and contralateral cerebral hemispheres.

AQP4 immunoreactivity is observed mainly around the vessels and under the pial surface in normal brain parenchyma (Badaut *et al.* 2002; Papadopoulos *et al.* 2002), and a normal immunostaining pattern of AQP4 was found after administration of normal saline, consistent with previous

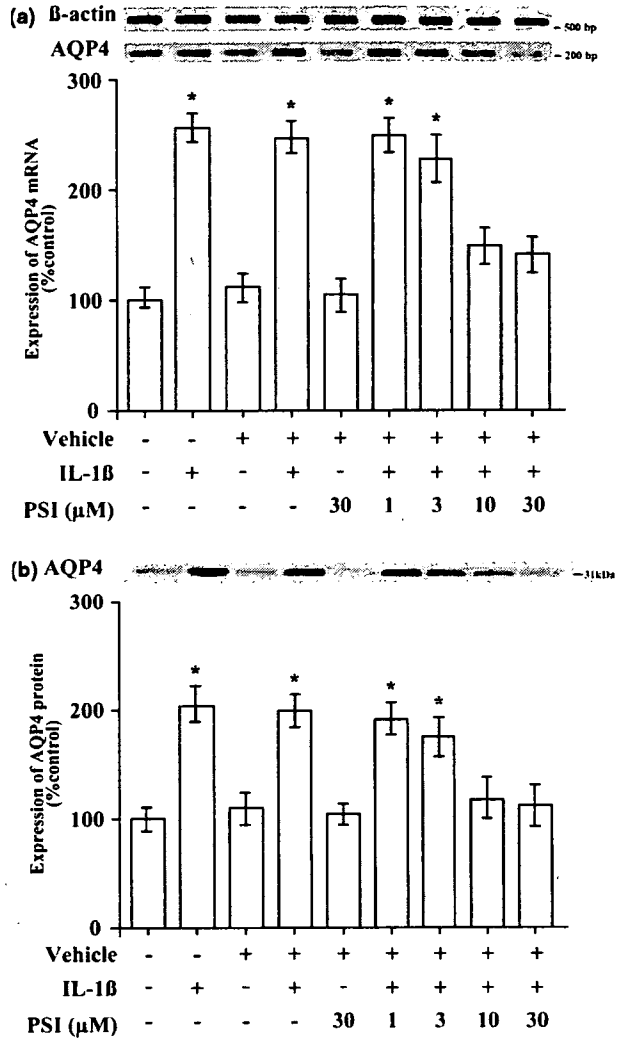

Fig. 8 Effect of blockade of NF-κB by another proteasome inhibitor, PSI, on induction of AQP4 mRNA and protein by IL-1β. (a) Rat astrocytes were incubated with or without 5 ng/mL IL-1β or various concentrations (1, 3, 10 or 30 μM) of the NF-κB inhibitor PSI for 6 h. PSI, a proteasome inhibitor, inhibits NF-κB activation by reducing IκB degradation. To determine whether the reduction of AQP4 expression by NF-κB inhibitors was a non-specific effect, astrocytes were incubated with vehicle (methanol, approximately 0.05%). Cells were processed for RT–PCR and semiquantification of AQP4 mRNA. Relative expression (normalized with respect to β-actin) ($n = 4$ for each group) is shown as a mean ± SEM percentage of the control value. $^*p < 0.05$ versus control. (b) Rat astrocytes were treated as in (a) and processed for western blot with affinity-purified AQP4 antibody. Western blots ($n = 4$ for each group) were analyzed by densitometry, and results expressed as a mean ± SEM percentage of the control value. $^*p < 0.05$ versus control (Student's *t*-test with equal variance).

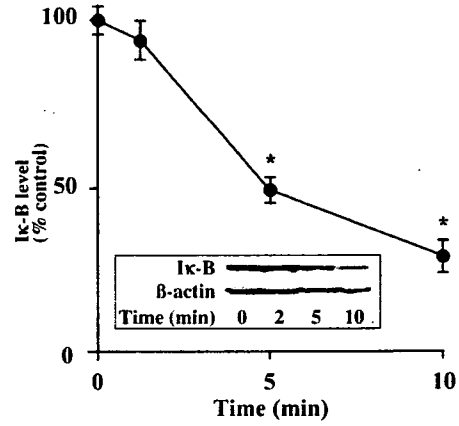

Fig. 9 Effect of IL-1β on IκB degradation in astrocytes. Rat astrocytes was incubated in medium containing 5 ng/mL IL-1β. At the designated times, cells were processed for western blot with affinity-purified IκB antibody. Western blots ($n = 4$ for each group) were analyzed by densitometry, and results expressed as a mean ± SEM percentage of the control value. $^*p < 0.05$ versus control (one-way factorial ANOVA combined with Scheffe's test).

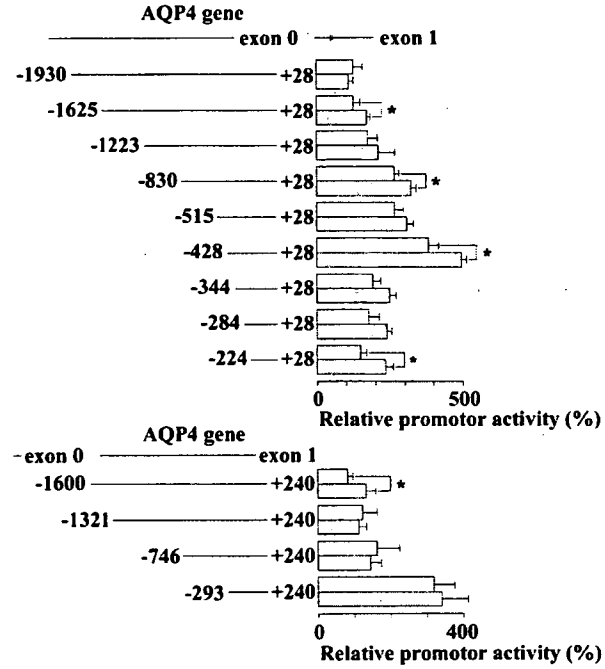

Fig. 10 Promoter activity analysis of the 5′ flanking regions of the *AQP4* gene. (a) *AQP4* promoter luciferase plasmid constructs were transfected into rat astrocytes. The transfected cells were incubated in medium with or without 5 ng/mL IL-1β for 24 h. Luciferase activity was analyzed using the dual-luciferase reporter assay system (Promega). Results are expressed as a mean ± SEM percentage of *AQP4* exon 0 and 1 construct activities in the absence (open bars) or presence (filled bars) of 5 ng/ml of IL-1β, $^*p < 0.05$ versus control ($n = 3$), (Student's *t*-test with equal variance).

reports (Vizuete *et al.* 1999). It was clear at higher magnification that intracerebroventricular administration of IL-1β increased staining for AQP4 around blood vessels, but not at lower magnification (Fig. 11b). Water content in brain

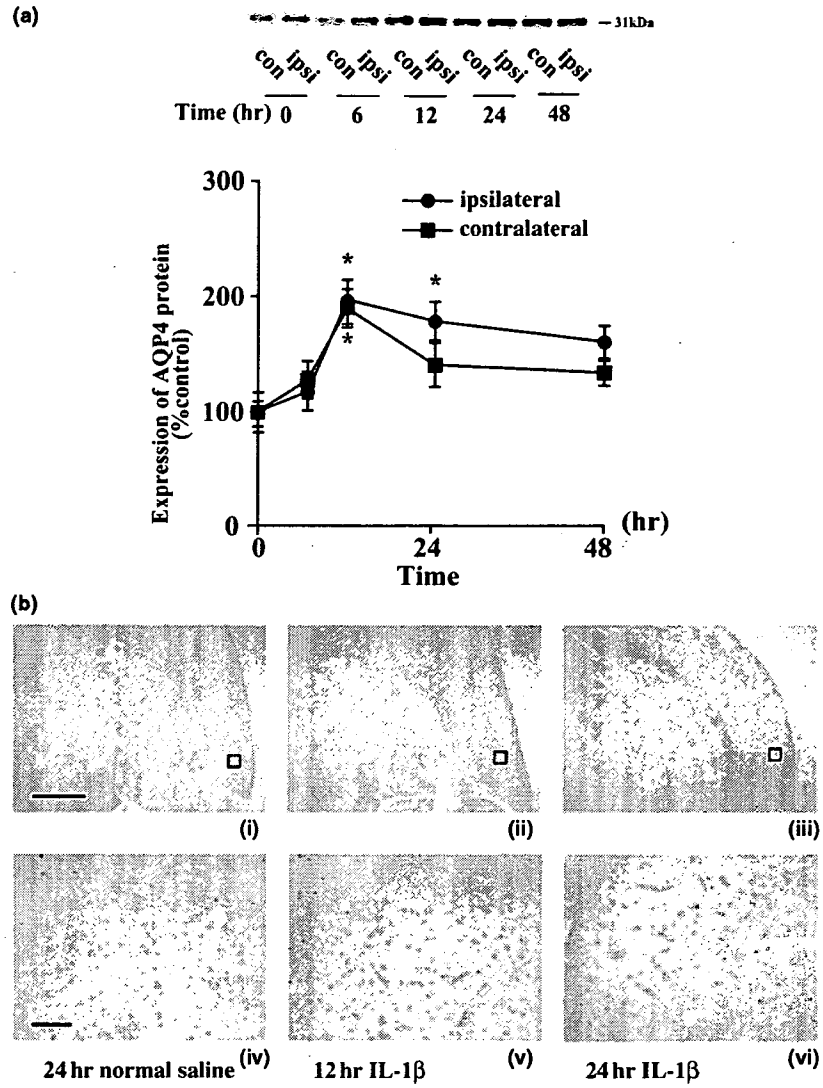

Fig. 11 Effect of intracerebroventricular administration of IL-1β on expression of AQP4. (a) Rats were intracerebroventricularly cannulated under anesthesia, and 100 ng IL-1β in 5 μL or an identical volume of carrier solution was administered. After the indicated time periods, the animals were killed, and their brains were divided into right (ipsilateral, ipsi) and left (contralateral, con) cerebral hemispheres. They were used as samples for western blot analyses of AQP4, as described above. Western blots ($n = 4$ for each group) were analyzed by densitometry, with results expressed as a mean ± SEM percentage of the value for the sham group contralateral cerebral hemisphere. $^*p < 0.05$ versus sham. (b) Photomicrographs showing the patterns of immunostaining of AQP4 12 and 24 h after intracerebroventricular administration of normal saline or IL-1β in ipsilateral cerebral hemispheres. At higher magnification, it is clear that staining of AQP4 increased after intracerebroventricular administration of IL-1β, but not at lower magnification (b). Scale bar 1 mm in (i, ii, iii) and 100 μm in (iv, v, vi). (one-way factorial ANOVA combined with Scheffe's test)

was not, however, significantly affected by intracerebroventricular administration of IL-1β (data not shown).

Discussion

The purpose of this study was to determine whether expression of AQP4 changes in response to a pro-inflammatory cytokine, IL-1β. The relationships between expression of AQPs and pro-inflammatory cytokines have been most extensively investigated in pulmonary edema (Towne *et al.* 2000, 2001). It was found that levels of pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α) were increased, and that expression of AQP5 mRNA and protein was decreased in the lungs of mice 7 and 14 days after adenoviral infection *in vivo*. Moreover, it has been shown that activation of NF-κB is probably required for the inhibition of AQP5 by TNF-α *in vitro* (Towne *et al.* 2001). Cher *et al.* suggested that phospholipase A2 alters the expression of genes related to pulmonary inflammation, such as those for IL-1β and TNF-α, and to pulmonary edema, such as those for Na^+/K^+-ATPase, AQP1 and AQP5. They noted that it is unclear whether reduced expression of water channels is directly regulated by phospholipase A2 or by an inflammatory mediator (Cher *et al.* 2003). These studies suggested that the ability of the pro-inflammatory cytokines to regulate the expression of AQPs might help explain the relationship between pulmonary inflammation and edema formation (Towne *et al.* 2001). However, little is known about the relationships between cytokines and AQP4 expression in brain. We therefore examined whether IL-1β regulates the expression of AQP4. In this study, it was demonstrated that IL-1β stimulates the expression of AQP4

mRNA and protein in cultured rat astrocytes and in cerebral tissues from rats. AQP4 mRNA and protein levels increased with dose of IL-1β. The AQP4 mRNA level was maximally increased after 6 h of IL-1β treatment, but returned nearly to baseline level by 24 h. The AQP4 protein level remained high for at least 48 h. IL-1ra blocked the induction of AQP4 expression in response to IL-1β. These findings suggest that IL-1β influences AQP4 expression through IL-1 receptor type 1 (IL-1R1)-mediated mechanisms. Pretreatment of cells with cycloheximide, a protein synthesis inhibitor, did not inhibit the effects of IL-1β, suggesting that IL-1β increased the expression of AQP4 without involvement of *de novo* protein synthesis.

We then investigated the mechanisms of induction of AQP4 in response to IL-1β. To characterize the specific contributions of signal transduction mechanisms in IL-1β-induced AQP4 expression, the effects of inhibitors of p38, ERK, JNK and NF-κB activity were examined. It has been established that IL-1β activates both MAPK cascades (Huwiler and Pfeilschifter 1994; Raingeaud *et al.* 1995; Parker *et al.* 2002) and the NF-κB pathway (Osborn *et al.* 1989; Beg *et al.* 1993; Kishimoto *et al.* 1994; Parker *et al.* 2002). Blockade of the NF-κB pathway significantly inhibited the induction of AQP4 mRNA and protein by IL-1β, whereas blockade of the MAPK cascades did not. To determine whether NF-κB inhibitors specifically prevented AQP4 induction by IL-1β, astrocytes were incubated with vehicle alone, but this had no effect on AQP4 induction, suggesting that prevention of induction of AQP4 by NF-κB inhibitors was not a non-specific effect. We thus showed that activation of NF-κB is involved in the induction of expression of AQP4 by IL-1β. Additionally, by observing the disappearance of IκB, we showed that NF-κB is activated by IL-1β in cultured rat astrocytes, consistent with findings of previous studies (Kuno and Matsushima 1994). We conclude that IL-1β induces the expression of AQP4 mRNA and protein through IL-1R1, and that NF-κB activation is involved in this induction. This is the first report of the regulation of expression of AQP4 by a pro-inflammatory cytokine in brain.

Several papers on mechanisms of regulation of AQP4 expression have been published. We have reported a decrease in AQP4 mRNA expression by protein kinase C (PKC) in cultured rat astrocytes (Yamamoto *et al.* 2001). On the other hand, AQP4 mRNA and protein levels in astrocytes were unaffected by protein kinase A. It appears that inflammation might be related to the expression of AQP4, because PKC is activated in inflamed tissues. It is known that PKC is activated by IL-1β in astrocytes, and that PKC, in turn, activates ERK1/2, mediated by MEK1/2. However, in the present study, induction of AQP4 in response to IL-1β was affected by NF-κB inhibitors, but not by the MEK1/2 inhibitor PD98059. This finding is consistent with those of other studies showing that NF-κB is a crucial regulator of inflammatory responses in the brain (O'Neill and Kaltschmidt 1997). In addition to these findings, we recently reported that hypertonicity stimulates the expression of AQP4 through a p38 MAPK-dependent pathway in rat astrocytes (Arima *et al.* 2003). In the present study, we demonstrated that expression of AQP4 in response to IL-1β could be regulated by the NF-κB pathway, but not by the p38 MAPK pathway, and that the NF-κB pathway might be a previously unidentified regulatory system for AQP4 expression.

AQP4 is known to encode two distinct mRNAs with different translation-initiating methionines, M1 and M23. M1 is contained in the exon 0 (encoding the long isoform) promoter region of *AQP4*, and M23 in exon 1 (encoding the short isoform). IL-1β treatment resulted in a statistically significant increase in luciferase activity in five promoter constructs, including four constructs of exon 0 and one construct of exon 1. The significantly increased construct of exon 1 contained a putative NF-κB *cis*-acting binding site, whereas that of exon 0 did not. Almost all constructs of exon 0 were activated, as the shortest construct can probably be indirectly activated by IL-1β treatment. These findings suggest that transcriptional activation of the *AQP4* gene by IL-1β can be mediated either directly or indirectly. We detected a single band in western blot analysis, although two promoters of exon 0 and exon 1 were activated by IL-1β treatment in luciferase assays. The band we detected by western blotting appears to correspond to the short isoform of AQP4; the long isoform is quite scarce and can be detected only in membrane fractions (Neely *et al.* 1999). In addition, the 34-kDa band was undetectable under the present experimental conditions, although western blot analysis was performed with our own antibody specific for the N-terminus of the long isoform of AQP4 (data not shown).

IL-1β is one of the pivotal pro-inflammatory cytokines in the brain (Allan 2000; Rothwell and Luheshi 2000). It is involved in a wide range of non-specific immune and physiological responses, and contributes to neurodegeneration. The most direct evidence that IL-1β contributes to brain injury is as follows. First, the brain edema that occurs following various brain insults is exacerbated by the intracerebroventricular administration of IL-1β. Infarct volume in the brain is increased by exogenous application of IL-1β at the onset of ischemia. However, it has been reported that IL-1β alone is not neurotoxic (Stroemer and Rothwell 1998; Holmin and Mathiesen 2000), because brain edema is not exacerbated in normal brain by its intracerebroventricular administration. Second, inhibition of the effects of IL-1β *in vivo* by administration of an IL-1 receptor antibody, an IL-1β antibody and a caspase inhibitor, or deletion of the caspase 1 gene reduces the loss of neurons caused by ischemic or traumatic brain injury in rats (Relton and Rothwell 1992; Yamasaki *et al.* 1992; Hara *et al.* 1997). Third, expression of IL-1β in brain is dramatically increased in many neurode-

generative diseases (Minami *et al.* 1991; Ban *et al.* 1992) and IL-1β is produced mainly by glia (Sairanen *et al.* 1997). Finally, glia (astrocytes) have IL-1 receptors on their surface (Rubio 1994). It has been demonstrated that innate immune responses such as microglia activation, leukocyte infiltration and the expression of other pro-inflammatory cytokines are abrogated in IL-1R1 null mouse (Basu *et al.* 2002).

We have found a new pathway by which IL-1β induces the expression of AQP4 in brain. There is, however, no conclusive proof that AQP4, the expression of which is increased by IL-1β, is related to the mechanism of formation or that of resolution of brain edema. It remains to be determined how this new pathway is involved in the processes of formation and resolution of brain edema.

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