associated with dystrophin, maintains a polarized expression of AQP4 in astrocytes.[11] Although AQP4-deficient mice show no obvious neurological abnormalities, they have remarkably reduced brain swelling following the induction of cytotoxic edema after acute ischemic stroke, water intoxication, and bacterial infection, indicating a protective role of AQP4 in development of brain edema.[12,13] Primary cultures of astrocytes from AQP4-null mice have greatly reduced osmotic water permeability compared with astrocytes of wild-type mice.[14] AQP4 expression is enhanced in reactive astrocytes in demyelinating lesions of multiple sclerosis (MS) and ischemic lesions of cerebral infarction, suggesting compensatory upregulation of AQP4 against water imbalance under pathological conditions.[15,16] An IgG autoantibody reacts specifically with AQP4 in the patients with neuromyelitis optica (NMO), suggesting a primary role of humoral immunity against AQP4 in development of NMO.[17] AQP4-null mice show an increase in seizure threshold.[18] The perivascular expression of AQP4 is reduced in the hippocampal CA1 region of patients with mesial temporal lobe epilepsy.[19] These observations suggest that astroglial AQP4 modulates neuronal excitability by regulating osmotic and ionic environments surrounding neurons. AQP9 is also expressed in astrocytes where its expression is upregulated after ischemia.[20]

Although increasing evidence supports an active involvement of AQPs in the pathophysiology of human neurological diseases, either distinct or redundant roles of AQP1 and AQP4 in brain water homeostasis remain to be intensively investigated. In the present study, we investigated the expression of AQP1 and AQP4 in human astrocytes in culture and brain tissues of MS, cerebral infarction, and control cases. Our observations suggest that the expression not only of AQP4 but also of AQP1 was coordinately enhanced in astrocytes in MS and ischemic brain lesions.

MATERIALS AND METHODS

Human brain tissues

For immunohistochemistry, ten micron-thick serial sections were prepared from autopsied brains of four MS patients and ten non-MS subjects. The tissues were fixed with 4% paraformaldehyde or 10% neutral formalin and embedded in paraffin. MS cases included a 29 year-old woman with secondary progressive MS (SPMS) who died of asphyxia (MS#1), a 40 year-old woman with SPMS who died of respiratory failure (MS#2), a 43 year-old woman with primary progressive MS (PPMS) who died of hyperglycemia (MS#3), and a 33 year-old man with SPMS who died of sepsis (MS#4). Detailed clinical profiles of MS patients were described elsewhere.[21] Other neurological and psychiatric disease cases included a 47 year-old man with acute cerebral infarction who died of sepsis, an 84 year-old man with acute cerebral infarction who died of disseminated intravascular coagulation, a 62 year-old man with old cerebral infarction who died of pancreatic cancer, a 56 year-old man with old cerebral infarction who died of myocardial infarction, a 36 year-old woman with schizophrenia who died of lung tuberculosis, and a 61 year-old man with schizophrenia who died of asphyxia. Neurologically normal control cases included a 79 year-old woman who died of hepatic cancer, a 75 year-old woman who died of breast cancer, a 60 year-old woman who died of external auditory canal cancer, and a 74 year-old woman who died of gastric and hepatic cancers.

For Western blot analysis, MS cases included MS#1, #2, #3, and a 70 year-old woman with SPMS (MS#5) who died of pneumonia. Non-MS cases included a 76 year-old woman with Parkinson's disease (PD#1), a 61 year-old woman with amyotrophic lateral sclerosis (ALS#1), a 74 year-old woman with ALS (ALS#2), a 61 year-old man with ALS (ALS#3), a 66 year-old man with ALS (ALS#4), a 73 year-old man with schizophrenia (SCH#1), and a 77 year-old woman with depression (DEP#1). The postmortem interval of the cases ranges from 1.5 hours to 10 hours prior to freezing

the brain tissues.

Autopsies were performed either at the National Center Hospital for Mental, Nervous and Muscular Disorders, National Center of Neurology and Psychiatry (NCNP), Tokyo, Japan or at the Nishitaga National Hospital, Sendai, Japan. Written informed consent was obtained form all the cases examined. The present study was approved by the Ethics Committee of NCNP.

Human astrocytes in culture

Human astrocytes were established from neuronal progenitor (NP) cells in culture as described previously.[21] NP cells isolated from the brain of a human fetus at 18.5-week gestation were obtained from BioWhittaker (Walkersville, MD, USA). NP cells plated on a polyethyleneimine-coated surface were incubated in DMEM/F-12 medium (Invitrogen, Carlsbad, CA, USA) containing an insulin-transferrin-selenium (ITS) supplement (Invitrogen), 20 ng/ml recombinant human EGF (Higeta, Tokyo, Japan), 20 ng/ml recombinant human bFGF (PeproTech EC, London, UK), and 10 ng/ml recombinant human LIF (Chemicon, Temecula, CA, USA). For the induction of astrocyte differentiation, NP cells were incubated for several weeks in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin (feeding medium). This incubation induced vigorous proliferation and differentiation of astrocytes accompanied by a rapid reduction in non-astroglial cell types. Their purity exceeded 98% by GFAP immunolabeling. The cells were incubated for 48 hours in the feeding medium containing 50 ng/ml recombinant human TNFα, IFNγ or IL-1β (all from PeproTech). Then, they were processed for Western blot analysis. Human cell lines NTera2N, Y79, SK-N-SH, IMR-32, U-373MG, HeLa and HepG2 were maintained as described previously.[22]

Immunohistochemistry and immunocytochemistry

After deparaffination, tissue sections were heated in 10 mM citrate sodium buffer, pH

6.0 by autoclave at 125℃ for 30 sec in a temperature-controled pressure chamber (Dako, Tokyo, Japan). They were treated at room temperature (RT) for 15 min with 3% hydrogen peroxide-containing methanol to block the endogenous peroxidase activity. The tissue sections were then incubated with phosphate-buffered saline (PBS) containing 10% normal goat serum at RT for 15 min to block non-specific staining. They were incubated in a moist chamber at 4℃ overnight with primary antibodies listed in Table 1. After washing with PBS, the tissue sections were labeled at RT for 30 min with horseradish peroxidase (HRP)-conjugated secondary antibodies (Nichirei, Tokyo, Japan), followed by incubation with a colorizing solution containing diaminobenzidine tetrahydrochloride (DAB) and a counterstain with hematoxylin. For negative controls, the step of incubation with primary antibodies was omitted, or the tissue sections were incubated with a negative control reagent (Dako) instead of primary antibodies. Adjacent sections were immnolabeled with the antibodies against cell type-specific markers listed in Table 1.

For double labeling, tissue sections were initially stained with rabbit anti-AQP1 antibody (H-55; Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-AQP4 antibody (H-80; Santa Cruz Biotechnology) or mouse anti-AQP1 monoclonal antibody (1/22; Santa Cruz Biotechnology), then followed by incubation with alkaline phosphatase-conjugated secondary antibody (Nichirei) and colorized with New Fuchsin substrate. After inactivation of these antibodies by autoclaving the sections in 10 mM citrate sodium buffer, pH 6.0, they were relabeled with mouse anti-glial fibrillary acidic protein (GFAP) monoclonal antibody (GA5; Nichirei), or rabbit anti-AQP4 antibody (H-80), followed by incubation with HRP-conjugated secondary antibody (Nichirei) and colorized with DAB substrate.

For immunocytochemistry, human astrocytes in culture on cover glasses were fixed with 4% PFA in 0.1 M phosphate buffer, pH 7.4 at RT for 10 min, followed by incubation with PBS containing 0.5% Triton X-100 at RT for 20 min. For double immunolabeling, the cells were incubated at RT for 30 min with a mixture of GA5 and

H-55 or a mixture of GA5 and H-80. Then, they were incubated at RT for 30 min with a mixture of Alexa Fluor 568-conjugated anti-mouse IgG and Alexa Fluor 488-conjugated anti-rabbit IgG (both from Invitrogen). After several washes, they were mounted with glycerol-polyvinyl alcohol and examined under a Nikon ECLIPSE E800 universal microscope equipped with fluorescein and rhodamine optics. Negative controls were processed following all the steps except for exposure to primary antibody.

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

DNase-treated total cellular RNA was processed for cDNA synthesis using oligo$(dT)_{12-18}$ primers and SuperScript II reverse transcriptase (Invitrogen). Then, cDNA was amplified by PCR using HotStar Taq DNA polymerase (Qiagen, Valencia, CA, USA) and a panel of sense and antisense primer sets following: 5'caggtggaggagtatgacctggat3' and 5'gaccatgcaggtctggcagatctt3' for a 205 bp product of AQP1; 5'cctcgctggtggcctttatgagta3' and 5'gtctttccccttcttctcctctcc3' for a 218 bp product of AQP4; and 5'ccatgttcgtcatgggtgtgaacca3' and 5'gccagtagaggcagggatgatgttc3' for a 251 bp product of the glyceraldehyde-3-phosphate dehydrogenase (G3PDH) gene as an internal control. The amplification program consisted of an initial denaturing step at 95℃ for 15 min, followed by a denaturing step at 94℃ for 1 min, an annealing step at 60℃ for 40 sec and an extension step at 72.9℃ for 50 sec for 38 cycles, except for G3PDH amplified for 32 cycles. For the positive control, total RNA of the human frontal cerebral cortex (Clontech, Mountain View, CA, USA) was processed in parallel for RT-PCR.

Transient expression of AQP1 and AQP4 in HEK293 cells

Open reading flames (ORFs) of the human AQP1 gene (GenBank accession no. NM_198098) and the human AQP4 gene (GenBank accession no. NM_001650) were amplified by PCR using PfuTurbo DNA polymerase (Stratagene, La Jolla, CA) from human astrocyte cDNA using sense and antisense primer sets following:

5'gccagcgagttcaagaagaagctc3' and 5'ctatttgggcttcatctccaccct3' for AQP1 and 5'agtgacagacccacagcaaggcgg3' and 5'tcatactgaagacaatacctctcc3' for AQP4. Then, they were cloned in an expression vector pcDNA4/HisMax-TOPO (Invitrogen). For transient expression, the vectors were transfected into HEK293 cells by using Lipofectamine 2000 reagent (Invitrogen). At 72 hours after transfection, the cells were processed for Western blot analysis.

Western blot analysis

To prepare total protein extract, the cells and tissues were homogenized in RIPA lysis buffer composed of 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P40, 0.5% sodium deoxycholate, 0.1% SDS, and a cocktail of protease inhibitors (Sigma, St. Louis, MO, USA), followed by centrifugation at 12,000 rpm for 20 min at RT. The supernatant was collected, further solved in the urea-containing buffer, and separated on a 12% SDS-PAGE gel. The protein concentration was determined by a Bradford assay kit (BioRad, Hercules, CA, USA). After gel electrophoresis, the protein was transferred onto nitrocellulose membranes, and immunolabeled at RT overnight with the antibodies listed in Table 1. Then, the membranes were incubated at RT for 30 min with HRP-conjugated secondary antibodies (Santa Cruz Biotechnology). The specific reaction was visualized by using a chemiluminescent substrate (Pierce, Rockford, IL, USA). After the antibodies were stripped by incubating the membranes at 50℃ for 30 min in stripping buffer composed of 62.5 mM Tris-HCl, pH 6.7, 2% SDS and 100 mM 2-mercaptoethanol, the membranes were processed for relabeling several times with different antibodies.

RESULTS

The consititutive expression of AQP1 and AQP4 mRNA in human astrocytes

First, the expression of AQP1 and AQP4 mRNA was studied in human neural cells by RT-PCR analysis. Both AQP1 and AQP4 transcripts were identified in human astrocytes (AS) in culture, neuronal progenitor (NP) cells, and cell lines of NTera2 teratocarcinoma-derived neurons (NTera2N), Y79 retinoblastoma, SK-N-SH neuroblastoma, IMR-32 neuroblastoma, and U-373MG astrocytoma, along with in the human cerebral cortex (CBR) (Fig. 1, panels a and b, lanes 1, 3-9). AQP1 mRNA was detected in HeLa cervical carcinoma but not at substantial levels in HepG2 hepatoma, whereas high levels of AQP4 mRNA were expressed in HepG2 but not in HeLa cells (Fig. 1, panels a and b, lanes 10, 11). The levels of G3PDH mRNA, a housekeeping gene, were almost constant among the cells examined (Fig. 1, panel c, lanes 1, 3-11), while no products were amplified when total RNA was processed for PCR without inclusion of the reverse transcription (RT) step, confirming that a contamination of genomic DNA was excluded (Fig. 1, panels a-c, lane 2). These results indicate that human astrocytes as well as various human neural cell lines coexpress AQP1 and AQP4 at least at mRNA levels.

The specificity of antibodies against AQP1 and AQP4

To verify the specificity of rabbit anti-AQP1 antibody (H-55) and anti-AQP4 antibody (H-80) utilized in the present study, the ORF of either the human AQP1 gene or the human AQP4 gene was introduced and transiently expressed in HEK293 cells. H-55 reacted specifically with the recombinant protein of AQP1 but not with AQP4 (Fig. 2A, panel a, lanes 1-3), whereas H-80 identified the recombinant protein of AQP4 but did not react with AQP1 (Fig. 2A, panel c, lanes 4-6), supporting the specificity of these antibodies.

The identification of AQP1 and AQP4 proteins in MS and other neurological and psychiatric disease brain tissues

In the next step, the expression of AQP1 and AQP4 proteins was studied in brain tissues of four MS and seven non-MS cases by Western blot analysis using H-55 and H-80 antibodies. The AQP1 protein consisting of two distinct bands of 28-kDa and 36-kDa, respectively, the latter of which represents the glycosylated form of the former,[23] were identified at variable levels in all brain tissues examined (Fig. 3, panel a, lanes 1-19). A single 34-kDa band of the AQP4 protein was also expressed at variable levels in all brain tissues examined (Fig. 3, panel b, lanes 1-19). The levels of AQP1 and AQP4 protein expression were positively correlated with total amounts of GFAP protein composed of multiple isoforms,[24] identified in the corresponding brain samples (Fig. 3, panel c, lanes 1-19). In contrast, the levels of HSP60, a housekeeping gene product for an internal control, were almost constant among the brain tissues examined (Fig. 3, panel d, lanes 1-19).

The differential regulation of AQP1 and AQP4 protein expression in human astrocytes in culture

The expression of AQP1 and AQP4 proteins was studied in cultured human astrocytes by Western blot analysis and immunocytochemistry using H-55 and H-80 antibodies. On Western blot, human astrocytes constitutively expressed both AQP1 and AQP4 proteins (Fig. 2B, panels e and f, lane 7). A 48 hour-exposure to IFNγ markedly elevated the levels of AQP4 and interferon gamma-inducible protein 30 (IFI30), the latter of which is the prototype of IFNγ-responsive proteins, but did not increase the levels of AQP1 in astrocytes (Fig. 2B, panels e, f and h, lane 9). Treatment with TNFα or IL-1β did not alter the levels of AQP1 or AQP4 in astrocytes (Fig. 2B, panels e and f, lanes 8 and 10). None of these cytokines affected the expression of GFAP and HSP60 (Fig. 2B, panels g and i, lanes 7-10).

By immunocytochemistry, AQP1 was identified not only on the plasma membrane

but also in the cytoplasm and the nucleus, possibly on the nuclear membrane of cultured human astrocytes (Fig. 4, panel a), while AQP4 was located exclusively on the cell-surface membrane (Fig. 4, panel d).

The enhanced expression of AQP1 and AQP4 in astrocytes bearing highly-branched processes in MS and ischemic brain lesions

Finally, the expression of AQP1 and AQP4 was studied in MS and other neurological and psychiatric disease brain lesions by immmunohistochemistry using H-55 and H-80 antibodies. Numerous multipolar fibrillary astrocytes bearing highly-branched processes, often surrounding blood vessels and neurons, expressed intensely AQP1 and AQP4 not only in chronic active demyelinating lesions but also in normal appearing white matter and adjacent cerebral cortex of MS brains (Fig. 5, panels a, c, and f). Double immunolabeling verified that the cells expressing AQP1 or AQP4 immunoreactivity were $GFAP^+$ (Fig. 5, panels c and f). In contrast, protoplasmic and hypertrophic astrocytes with poorly-branched processes were less intensely labeled with antibodies against AQP1 and AQP4.

An accumulation of astrocytes expressing AQP1 or AQP4 was also observed in ischemic lesions of cerebral infarction (Fig. 5, panels b, d and e) and in the cerebral cortex and white matter of schizophrenia brains (Fig. 5, panel i), but a fairly small population of cortical astrocytes showed an immunoreactivity for AQP1 or AQP4 in the brains of neurologically normal subjects, except for the surface of pia mater and subpial astrocytes which were constantly stained with anti-AQP4 antibody but not with anti-AQP1 antibody (Fig. 5, panels g and h). AQP1- or AQP4-immunoreactive astrocytes were much dense in the rim of necrotic core of ischemia than unaffected regions (Fig. 5, panel d). The choroids plexus epithelium (CPE) expressed both AQP1 and AQP4 (not shown). $GFAP^+$ gliotic tissues in chronic demyelinaing lesions in the optic nerve, spinal cord, and the cerebrum of MS were intensely immunolabeled with antibodies against AQP1 and AQP4 (Fig. 6, panels a-f). An accumulation of

AQP1-expressing astrocytes was prominent in the subcortical white matter, while AQP4-immunoreactive astrocytes were distributed chiefly in the cerebral cortex in MS brains. Double immunolabeling indicated that a substantial population (approximately less than 10% of astrocytes immunoreactive with AQP1 or AQP4) of astrocytes coexpressed AQP1 and AQP4 (Fig. 5, panel i).

In contrast, neither macrophages, microglia, neurons, oligodendrocyte cell bodies were immunolabeled with antibodies against AQP1 and AQP4 in any cases examined (Fig. 7, panels a-f), indicating that the principal localization of these aquaporins is astrocytes, except for CPE which expresses both AQP1 and AQP4 and ependymal cells which express predominantly AQP4 (not shown).

DISCUSSION

By using RT-PCR, Western blot, and immunohistochemistry, we studied the expression of AQP1 and AQP4 in cultured human astrocytes and brain tissues of MS, cerebral infarction, and control cases. We showed that the expression not only of AQP4 but also of AQP1 was enhanced in MS and ischemic brain lesions, where they were located predominantly in astrocytes and glial scar. Thus, AQP1 expression is not CPE-specific in the human CNS under pathological conditions. The following observations supported this conclusion. First, cultured human astrocytes coexpressed AQP1 and AQP4 at both mRNA and protein levels. Second, AQP1 and AQP4 proteins were identified in brain homogenates of MS and other neurological and psychiatric disease cases, where the levels of AQP1 and AQP4 were correlated with those of GFAP. Finally, double-labeling immunohistochemistry showed that $GFAP^+$ fibrillary astrocytes with highly-branched processes surrounding blood vessels expressed intensely AQP1 and AQP4 in MS and non-MS brain lesions, whereas neither macrophages, neurons nor oligodendrocyte cell bodies expressed AQP1 and AQP4, and the population of

astrocytes immunoreactive with AQP1 or AQP4 is much smaller in control brains of neurologically normal subjects. Our observations suggest a pivotal role of AQP1 and AQP4 expressed on astrocytes in maintenance of water homeostasis in the CNS under pathological conditions. Recent studies showing that the expression of both AQP1 and AQP4 is greatly enhanced in reactive astrocytes in the brains of subarachnoid hemorrhage,[25] contusion,[26] and Creutzfeldt-Jakob disease[27], support our observations.

Several previous studies showed that both neoplastic and reactive astrocytes in the lesions of high-grade astrocytomas express AQP4 as well as AQP1, where AQP1 immunoreactivity is not evident in the areas of microvascular proliferation.[28-30] The robust expression of AQPs on astroglial lineage cells suggests their involvement in development of tumor-associated brain edema caused by a disruption of local water transport. Importantly, AQP1-null mice show reduced tumor vascularity and tumor cell migration after subcutaneous or intracranial implantation of tumor cells.[31] By contrast, overexpression of AQP1 in NIH-3T3 cells accelerates anchorage-independent cell growth characteristic of malignant transformation.[32] Mammary gland tumor cells expressing the AQP1 transgene show increased tumor cell extravasation.[33] Glial scar formation following cortical stab injury is remarkably impaired in AQP4-null mice associated with a reduced migration capacity of reactive astrocytes towards the site of injury.[34] All of these observations suggest that both AQP1 and AQP4 play a fundamental role in cell growth and migration, possibly by a mechanism that involves cytoskeletal reorganization triggered by AQP-mediated water transport at the leading edge of lamellipodia of proliferating and migrating cells. Furthermore, AQP1 plays a role in apoptotic cell shrinkage and affects downstream apoptotic events by regulating the cell volume.[35] By immunohistochemistry, we found that AQP1 was expressed not only on the plasma membrane but also in the cytoplasm and the nuclear membrane of cultured human astrocytes. The intracellular location of AQP1 has been reported previously. AQP1 integrates into the ER membrane when overexpressed in HEK293 cells.[36] Secretin induces a redistribution of AQP1 protein from intracellular vesicles to

the cell plasma membrane in cholangiocytes.[37]

Regulatory mechanisms underlying the AQP gene expression are apparently complex. Systemic hyponatremia elevates the levels of expression of AQP1 on CPE.[38] Induction of severe hydrocephalus upregulates AQP4 but not AQP1 in perivascular astrocytes.[39] Pregnancy upregulates AQP4 expression in rat brain tissues.[40] Treatment with corticosteroids increases AQP1 expression in the capillary endothelium of the peritoneal membrane and the lung.[41,42] Hypertonic stress induces the expression of AQP1 in rodent renal medullary cells by activating extracellular signal-regulated kinase (ERK), p38 MAP kinase, and c-Jun N-terminal kinase (JNK), all of which regulate a hypertonicity-responsive element (HRE) located in the AQP1 promoter.[43] Testosterone upregulates but phorbol esters downregulate AQP4 mRNA and protein levels in rodent astrocytes in culture.[44,45] The levels of AQP4 mRNA expression are elevated during astrocytic differentiation of P19 embryonal carcinoma cells.[46] Interferon-alpha (IFNα) upregulates AQP5 gene expression in human parotid acinar cells.[47] We for the first time showed that AQP4 levels were elevated markedly in human astrocytes by exposure to IFNγ, the prototype of T helper type-1 (Th1) cytokines, but neither by TNFα nor IL-1β, whereas AQP1 levels were unaffected by all these cytokines, although the IFN-responsive elements have not been identified in the promoter of human AQP4 gene.[48]

It is worthy to note that various human cell lines of neuronal origin, such as NTera2N, Y79, SK-N-SH, and IMR-32, express substantial levels of AQP1 and AQP4 mRNA. Several previous studies showed that defined populations of neurons, along with neural stem cells, express AQP4 and/or AQP1 *in vivo*,[9,49-52] suggesting the possibility that these AQPs play an active role in maintenance of water balance in a much broader spectrum of cell types in the CNS under physiological and pathological conditions.

In conclusion, the expression not only of AQP4 but also of AQP1 was identified in

human astrocytes *in vitro*, and *in vivo* in various neurological diseases including MS, suggesting a pivotal role of astrocytic AQPs in regulation of water homeostasis in the human CNS under pathological conditions.

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FIGURE LEGENDS

Fig. 1. AQP1 and AQP4 mRNA expression in human neural cells. The expression of (a) AQP1, (b) AQP4, and (c) G3PDH (an internal control) mRNA was studied in human neural cells by RT-PCR analysis. The lanes (1-11) represent (1) the frontal cerebral cortex (CBR), (2) cultured astrocytes (AS) without inclusion of the reverse transcription step (RT-), (3) cultured astrocytes (AS) with inclusion of the reverse transcription step (RT+), (4) cultured neuronal progenitor (NP) cells, (5) NTera2 teratocarcinoma-derived neurons (NTera2N), (6) Y79 retinoblastoma, (7) SK-N-SH neuroblastoma, (8) IMR-32 neuroblastoma, (9) U-373MG astrocytoma, (10) HeLa cervical carcinoma, and (11) HepG2 hepatocellular carcinoma. The DNA size marker (100 bp ladder) is shown on the left.

Fig. 2. AQP1 and AQP4 protein expression in HEK293 cells and human astrocytes in culture. (A) HEK293 cells. To verify the antibody specificity, ORF of the human AQP1 gene or the human AQP4 gene cloned in pcDNA4 vector was expressed in HEK293 cells, followed by processing for Western blot analysis using (a) anti-AQP1 antibody (H-55), (c) anti-AQP4 antibody (H-80), or (b, d) anti-HSP60 antibody. The lanes (1-6) represent (1,4) non-transfected HEK293 cells, (2,5) AQP1-expressing HEK293 cells, and (3,6) AQP4-expressing HEK293 cells. **(B) Astrocytes.** Human astrocytes were exposed for 48 hours to three distinct cytokines, followed by processing for Western blot analysis using (e) H-55, (f) H-80, (g) anti-GFAP antibody (GA5), (h) anti-IFI30 antibody, or (i) anti-HSP60 antibody. The lanes (7-10) represent (7) untreated cells, and the cells treated with (8) TNFα, (9) IFNγ and (10) IL-1β. Twenty microgram of protein was loaded on each lane. The identical blots were processed for relabeling several times with different antibodies. HSP60 indicates an internal control.