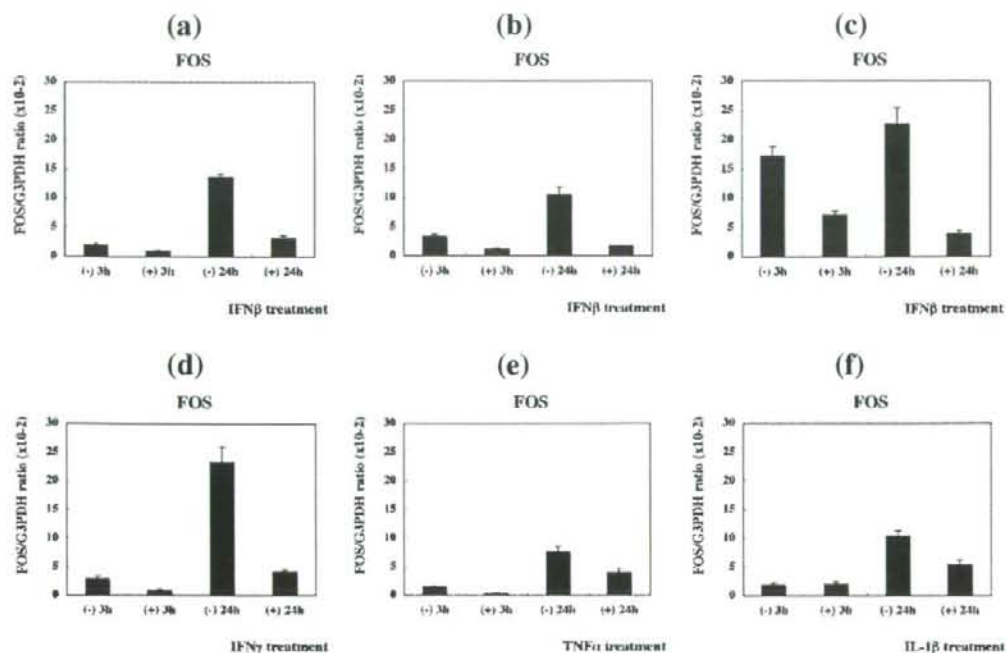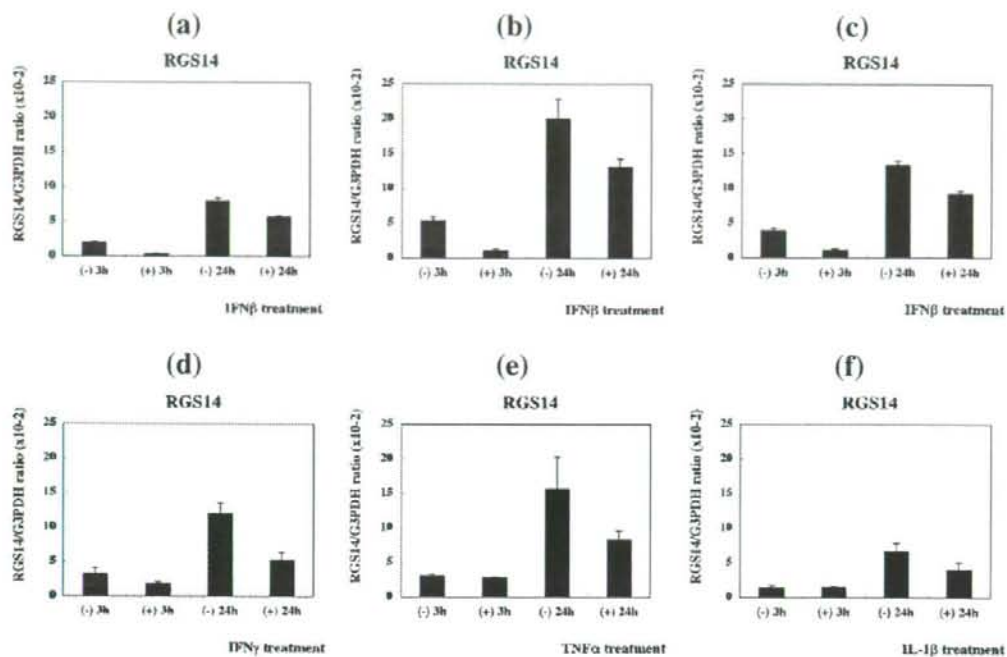**Figure 4**
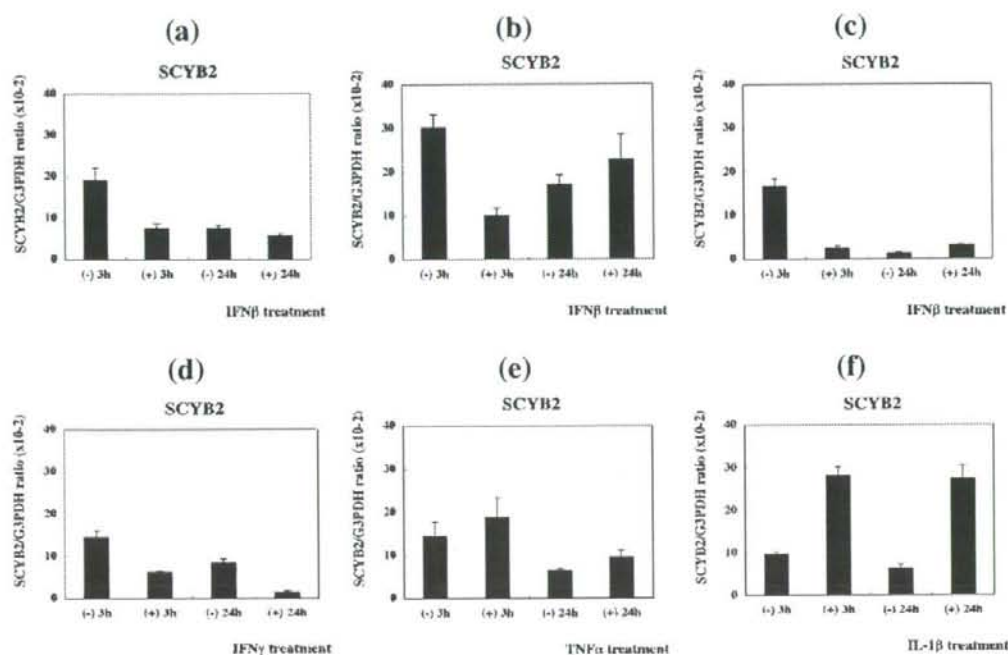*Real-time RT-PCR analysis of SCYA2 expression in PBMC.* See the footnote of Figure 1. The panels represent the expression of SCYA2 in (a) #1, IFNβ; (b) #2, IFNβ; (c) #3, IFNβ; (d) #1, IFNγ; (e) #1, TNFα; and (f) #1, IL-1β.

Figure 5
Real-time RT-PCR analysis of FOS expression in PBMC. See the footnote of Figure 1. The panels represent the expression of FOS in (a) #1, IFNβ; (b) #2, IFNβ; (c) #3, IFNβ; (d) #1, IFNγ; (e) #1, TNFα; and (f) #1, IL-1β.

Figure 6
Real-time RT-PCR analysis of RGS14 expression in PBMC. See the footnote of Figure 1. The panels represent the expression of RGS14 in (a) #1, IFNβ; (b) #2, IFNβ; (c) #3, IFNβ; (d) #1, IFNγ; (e) #1, TNFα; and (f) #1, IL-1β.

Figure 7
Real-time RT-PCR analysis of SCYB2 expression in PBMC. See the footnote of Figure 1. The panels represent the expression of SCYB2 in (a) #1, IFNβ; (b) #2, IFNβ; (c) #3, IFNβ; (d) #1, IFNγ; (e) #1, TNFα; and (f) #1, IL-1β.

Discussion

IFNs are a family of cytokines that mediates antiviral, anti-proliferative and immunoregulatory activities. Type I IFNs, IFNα and β, are produced principally by virus-infected host cells, whereas type II IFN, IFNγ, is produced by activated T cells and natural killer (NK) cells. Type I IFNs activate JAK protein tyrosine kinases associated with the cell surface receptors for IFNs, leading to formation of the complex of signal transducer and activator of transcription (STAT) molecules with the IFN regulatory factor (IRF) family of transcription factors. The STAT/IRF complex translocates into the nucleus, and binds to the DNA sequences termed the IFN-stimulated response element (ISRE) or the IRF-recognition element (IRE). This binding subsequently activates transcription of a wide variety of IFN-responsive genes (IRGs) as well as the genes of type I and type II IFNs, leading to the biological responses triggered by the IFNs [24]. Both type I and type II IFNs enhance the expression of class I and class II MHC molecules [25]. Among nine distinct IRFs, IRF7 and IRF3 play a central role in induction of type I IFN genes via the virus-

activated MYD88-independent pathway or Toll-like receptor (TLR)7, 8 or 9-activated MYD88-dependent pathway [26], while IRF1 plays more active roles in induction of IFNγ-target genes essential for Th1-type immune response [25].

The present study by analyzing DNA microarray characterized a comprehensive list of immediate early IRGs in PBMC *in vitro*. Following a 3 to 24 hour-exposure to IFNβ, upregulated genes greatly outnumbered downregulated genes. All top 20 upregulated genes represent known IRGs previously identified in various cell types. The upregulated IRGs of PBMC were classified into several functional categories. The list included not only conventional IFN-response markers and components of IFN-signaling pathways, but also contained many proinflammatory chemokines and cytokines. This is surprising because IFNβ acts principally as an anti-Th1, anti-inflammatory cytokine [6,7]. By analyzing global gene expression profile, the present study for the first time showed that IFNβ induced a burst of gene expression of CXCR3 ligand chemokines

(SCYB11, SCYB10 and SCYB9) and CCR2 ligand chemokines (SCYA8 and SCYA2), which was verified by quantitative real-time RT-PCR analysis. The chemokine genes actually have ISRE or IRE in the promoter regions, indicating direct targets of IFNβ[27,28].

CXCR3 is expressed predominantly on activated Th1 T cells, while CCR2 is expressed chiefly on monocytes [29]. The number of CXCR3+T cells is increased in the blood of RRMS, and they accumulate in perivascular infiltrates in active MS lesions [30,31], while SCYB10 (IP-10) and SCYB9 (MIG) are detected in the cerebrospinal fluid (CSF) of RRMS at acute relapse and expressed in reactive astrocytes in active MS lesions [31,32]. SCYA2 (MCP1) and SCYA8 (MCP2) immunoreactivities are also identified in reactive astrocytes in active demyelinating lesions of MS [33,34]. These observations suggest that CXCR3, CCR2, and their ligand chemokines positively regulate active inflammation in MS. Although the precise cell types expressing CXCR3 ligand and CCR2 ligand chemokines in PBMC in response to IFNβ remain to be characterized, the chemokine burst plays a central role in rapid activation and systemic recruitment of Th1 T cells and monocytes immediately after initiation of IFNβ treatment. A recent study showed that IFNβ promotes trafficking of mouse leukocytes by regulating a specific set of chemokines [35]. However, concurrent upregulation of a set of CXCR3 and CCR2 ligand chemokines has not previously been reported in MS patients on a long-term IFNβ treatment [16,17,20-22], suggesting that this phenomenon is an immediate early but transient event in vivo. IFNβ immediately reduced the expression of RGS14 (the most significantly downregulated gene at 3 hours; see Table 3), a member of the regulator of G protein signaling (RGS) gene family that acts as a negative regulator of G protein-coupled receptor (GPCR) signaling. Since all chemokine receptors are GPCR, IFNβ-induced downregulation of RGS14 might facilitate chemokine responsiveness in the cells expressing RGS14 [36,37]. Much less is known about the mechanism for regulation of IFNβ-repressed genes [38]. We identified IL-8 as one of IFNβ-repressed genes in PBMC (Table 3). IFNβ inhibits the transcription of IL-8 gene, possibly by binding of NF-κB repressing factor (NRF) to a negative regulatory element of the IL-8 promoter [39]. Serum IL-8 levels and IL-8 secretion from PBMC are elevated in untreated MS, and then reduced following IFNβ therapy [40]. Downregulation of IL-8 expression in PBMC during IFNβ treatment provides a predictive indicator for the responders in RRMS [20].

IFNβ also promptly upregulated a variety of proinflammatory cytokines, such as IL-6, IL-15, osteopontin, TNFα, and IFNγ in PBMC (Table 4). IFNβ promotes production of TNFα and IFNγ in unstimulated PBMC but decreases their levels in preactivated PBMC [41-43]. IFNβ increases the number of IFNγ-secreting cells in vivo at the early period of the treatment [44]. Most importantly, proinflammatory cytokines and chemokines induced by IFNβ have relevance to treatment-related early adverse effects. There exists a close relationship between flu-like symptoms and increased levels of IL-6 [45]. A single injection of IFNβ induces a transient burst of SCYB10 (IP-10) in the plasma of RRMS patients, which correlates with an incidence of flu-like symptoms [46]. IFNβ enhances the expression of CD80, SCYB10 (IP-10) and SCYA2 (MCP1) in situ at sites of injection, leading to chemotaxis of lymphocytes and monocytes in the lesions of skin reaction [47-49]. We found that IFNβ aberrantly regulated the levels of expression of several cytochrome P450 (CYP) enzymes (see Additional files 3,4,5,6). Type I IFN reduces the activity of CYP enzymes that metabolize various endogenous and exogenous substrates, probably leading to an increase in the potential for IFN-related hepatotoxicity [50].

Finally, the list of IRGs included various apoptosis regulators and HSP family members. ISRE-like sequences are identified in the regulatory element of CASP1, CASP4, CASP8, TNFRSF6 (FAS), TNFSF6 (FASL) and TNFSF10 (TRAIL), suggesting that IFNβ acts as a proapototic cytokine [51,52]. A recent study showed that early and sustained induction of TRAIL provides a marker for IFNβ treatment response in MS [53]. Furthermore, IFNβ-inducible apoptosis regulators play an immunoregulatory role. TNFR1-associated via death domain (TRADD) inhibits IFNγ-induced STAT1α activation [54]. Receptor-interacting serine-theronine kinase 1 (RIPK1) regulates TLR3-independent viral double-stranded RNA-induced type I IFN production [55]. Because HSPs in general act as an anti-apoptotic defender, the induction of HSP gene expression might occur as a counterbalance against upregulation of proapoptotic regulators. Alternatively, IRGs could directly enhance HSP expression. IFNβ-induced STAT1, by interacting with heat shock factor-1 (HSF1), activates the HSP70 and HSP90β gene promoters [56].

Conclusion
Microarray analysis showed that IFNβ immediately induces a burst of gene expression of proinflammatory chemokines and cytokines in vitro that have potential relevance to IFNβ-related early adverse effects in MS patients in vivo.

Abbreviations
MS = multiple sclerosis; IFNβ = interferon-beta; IRGs = IFNβ-responsive genes; PBMC = peripheral blood mononuclear cells; CNS = central nervous system; IFNγ = interferon-gamma; Th1 = T helper type 1; MHC = major histocompatibility complex; RRMS = relapsing-remitting multiple sclerosis; ISG = IFN stimulated gene; TLR = Toll-

like receptor; STAT = signal transducer and activator of transcription; IRF = interferon regulatory factor; ISRE = interferon-stimulated response element; IRE = interferon regulatory factor-recognition element; HSPs = heat shock proteins; CSF = cerebrospinal fluid; GPCR = G protein-coupled receptor; RGS = regulator of G protein signaling.

Competing interests

The author(s) declare that they have no competing interests.

Authors' contributions

JS, YN and HT carried out DNA microarray and real-time RT-PCR analysis, and JS drafted the manuscript. TY participated in the design of the study and helped to draft the manuscript. All authors read and approved the final manuscript.

Additional material

Additional File 1

The gene list of cDNA microarray utilized in the present study. The complete gene list of cDNA microarray utilized in the present study is shown. It includes 1,258 well-annotated genes, selected from cytokines, growth factors and their receptors, apoptosis regulators, oncogenes, transcription factors, cell cycle regulators and housekeeping genes.
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Additional File 2

Scatter plots of three distinct microarray experiments. The figure represents a scatter plot exhibiting the comparison between the fluorescence intensity (FI) of Cy5 signals in the longitudinal axis and FI of Cy3 signals in the horizontal axis. (a) the subject #1 (a 46 year-old healthy man), (b) the subject #2 (a 28 year-old healthy man), and (c) the subject #4 (a 27 year-old woman with RRMS who was a dropout of IFNβ treatment due to induction of frequent severe relapses).
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Additional File 3

The complete list of upregulated genes in PBMC following exposure to IFNβ for 3 hours. Upregulated genes in PBMC of the subject #1 (a 46 year-old healthy man) by a 3 hour-exposure to 50 ng/ml recombinant human IFNβ are listed with Cy5/Cy3 signal intensity ratio, gene symbol, GenBank accession number, and gene name. In vivo IRG in T cells and non-T cells of RRMS patients reported previously (Ref. 16) are underlined.
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Additional File 4

The complete list of downregulated genes in PBMC following exposure to IFNβ for 3 hours. Downregulated genes in PBMC of the subject #1 (a 46 year-old healthy man) by a 3 hour-exposure to 50 ng/ml recombinant human IFNβ are listed with Cy5/Cy3 signal intensity ratio, gene symbol, GenBank accession number, and gene name.
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Additional File 5

The complete list of upregulated genes in PBMC following exposure to IFNβ for 24 hours. Upregulated genes in PBMC of the subject #1 (a 46 year-old healthy man) by a 24 hour-exposure to 50 ng/ml recombinant human IFNβ are listed with Cy5/Cy3 signal intensity ratio, gene symbol, GenBank accession number, and gene name. In vivo IRG in T cells and non-T cells of RRMS patients reported previously (Ref. 16) are underlined.
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Additional File 6

The complete list of downregulated genes in PBMC following exposure to IFNβ for 24 hours. Downregulated genes in PBMC of the subject #1 (a 46 year-old healthy man) by a 24 hour-exposure to 50 ng/ml recombinant human IFNβ are listed with Cy5/Cy3 signal intensity ratio, gene symbol, GenBank accession number, and gene name.
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Additional File 7

Top 20 upregulated genes in PBMC following exposure to IFNβ for 3 hours: two additional subjects. Upregulated genes in PBMC of the subject #2 (a 28 year-old healthy man) and #4 (a 27 year-old woman with RRMS who was a dropout of IFNβ treatment due to induction of frequent severe relapses) following a 3 hour-exposure to 50 ng/ml recombinant human IFNβ are listed with Cy5/Cy3 signal intensity ratio, gene symbol, and gene name. Both CXCR3 ligand (yellow) and CCR2 ligand (blue) chemokines are highlighted.
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Aberrant transcriptional regulatory network in T cells of multiple sclerosis

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Abstract

To identify the molecular network of the genes deregulated in multiple sclerosis (MS), we studied gene expression profile of purified CD3+ T cells isolated from Hungarian monozygotic MS twins by DNA microarray analysis. By comparing three concordant and one discordant pairs, we identified 20 differentially expressed genes (DEG) between the MS patient and the genetically identical healthy subject. Molecular network of 20 DEG analyzed by KeyMolnet, a comprehensive information platform, indicated the close relationship with transcriptional regulation by the Ets transcription factor family and the nuclear factor NF-κB. This novel bioinformatic approach proposes the logical hypothesis that aberrant regulation of the complex transcriptional regulatory network contributes to development of pathogenic T cells in MS.
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Keywords: KeyMolnet; Microarray; Monozygotic twin; Multiple sclerosis; T cells; Transcriptional regulation

Multiple sclerosis (MS) is an inflammatory demyelinating disease mediated by an autoimmune process that is triggered by a complex interplay of both genetic and environmental factors. MS twin studies showed that the concordance rate for monozygotic (MZ) twins is approximately 30%, while it is less than 5% for dizygotic (DZ) twins, suggesting the possible involvement of not a single but multiple susceptibility genes in development of MS [13]. However, most of the candidate genes reported previously have not well been validated. Because regulation of gene expression is controlled through the combinatorial action of multiple transcription factors that activate or repress transcription via binding to cis-regulatory elements of target genes, the gene network analysis is more important to clarify the complex autoimmune process underlying the pathogenesis of MS.

DNA microarray technology is an innovative approach that allows us to systematically monitor the expression of thousands of genes in disease-affected tissues and cells. The comprehensive gene expression profiling has given new insights into molecular mechanisms promoting the autoimmune process in MS [19]. By using this technology, we recently showed that interferon-beta (IFNβ) treatment elevates the expression of a set of IFN-responsive genes in highly purified peripheral blood CD3+ T cells of relapsing-remitting MS patients [6]. IFNβ immediately induces a burst of gene expression of proinflammatory chemokines with potential relevance to IFNβ-related early adverse effects in MS [17]. T-cell gene expression profiling classifies a heterogeneous population of Japanese MS patients into four distinct subgroups that differ in the disease activity and therapeutic response to IFNβ [16]. The majority of differentially expressed genes in T cells between untreated MS patients and healthy subjects were categorized into apoptosis signaling regulators [15]. However, we could not exclude the possibility that the heterogeneous genetic backgrounds of the study population might affect gene expression data. In the present study, to identify the molecular network of the genes deregulated in MS, we performed microarray analysis of peripheral blood T cells isolated from the genetically homogeneous population of Hungarian MZ MS/MS and MS/healthy subject twins

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followed by gene network analysis using a novel bioinformatic tool. We focused highly purified CD3+ T cells because autoreactive pathogenic and regulatory cells might be enriched in this fraction.

Hungarian MZ MS twins were followed up for 2–15 years by certified neurologists (ZI and CR) at the MS Clinic of Pecs and Budapest. After written informed consent was taken, CD3+ T cells were isolated form heparinized peripheral blood of three pairs of concordant MZ MS twins (Families #1 to #3) and a pair of discordant MZ MS/healthy subject twin (Family #4), as described previously [6,16,15]. All subjects are women with the mean age of 33 ± 5 years and the mean disease duration of 6 ± 5 years. MS was diagnosed following the established criteria [8]. The patients were clinically active, showed variable lesion distributions on MRI, and exhibited a typical relapsing-remitting clinical course, except for one patient in Family #3 with clinically isolated syndrome (CIS). The patients showed the mean Expanded Disability Status Scale (EDSS) score of 1.3 ± 0.8. The clinical profile of the patients is shown in Supplementary Table 1 online. To minimize the influence of confounding factors on gene expression, none of the patients have received corticosteroids, interferons, glatiramer acetate, mitoxantrone or other immunosuppresive medications at least for 8 weeks before blood sampling. The present study was approved by the ethics committee of all institutes involved.

We utilized a custom microarray containing duplicate cDNA spots of 1258 well annotated genes of various functional classes, including cytokines/growth factors and their receptors, apoptosis regulators, oncogenes, transcription factors, signal transducers, cell cycle regulators and housekeeping genes (Hitachi Life Science, Saitama, Japan) [6,16,15]. Five micrograms of total RNA isolated from CD3+ T cells by using RNeasy Mini Kit (Qiagen, Valencia, CA) was *in vitro* amplified, and the antisense RNA (aRNA) was labeled with a fluorescent dye Cy5, while universal reference aRNA was labeled with Cy3. The arrays were hybridized at 62 °C for 10 h in the hybridization buffer containing equal amounts of Cy3- or Cy5-labeled cDNA, and they were then scanned by the ScanArray 5000 scanner (GSI Lumonics, Boston, MA). The data were analyzed by using the QuantArray software (GSI Lumonics). The average of fluorescence intensities of duplicate spots was obtained after global normalization between Cy3 and Cy5 signals.

Because microarray analysis generally produces a large amount of gene expression data at one time, it is often difficult to identify the meaningful relationship between gene expression profile and biological implications from available data. To overcome this difficulty, we conducted a novel bioinformatic approach to extract the molecular network that is the most relevant to the microarray data. The molecular network of the genes identified by microarray was analyzed by using the software named KeyMolnet originally developed by the Institute of Medical Molecular Design Inc., Tokyo, Japan (IMMD) [14]. KeyMolnet constitutes a comprehensive content database, composed of information on relationships among human genes, molecules, diseases, pathways and drugs, which have been carefully curated from selected review articles, literature, and public databases by expert biologists of IMMD. The contents are quarterly updated, composed of approximately 12,000 molecules in the version of April 1, 2007, and categorized into either the core contents collected from selected review articles with the highest reliability or the secondary contents extracted from abstracts of the PubMed database.

When DNA microarray data, i.e. the lists of either GenBank accession number or probe ID, were imported into KeyMolnet, it automatically provided corresponding molecules as a node on networks [7,14]. Among four different modes of search, the common upstream search method enables us to extract the most relevant molecular network composed of the genes coordinately regulated by putative common upstream transcription factors. The generated network was compared side by side with total 346 established canonical pathways of human cells pre-installed in KeyMolnet. The algorithm counting the number of overlapping molecular relations between the extracted network and the canonical pathway makes it possible to identify the canonical pathway showing the most significant contribution to the extracted network. The significance in the similarity between both is scored following the formula, where O = the number of overlapping molecular relations between the extracted network and the canonical pathway, V = the number of molecular relations located in the extracted network, C = the number of molecular relations located in the canonical pathway, T = the number of total molecular relations, composed of approximately 90,000 different sets pre-installed in KeyMolnet (the version of 1 April 2007), and X = the sigma variable that defines incidental agreement [3].

$$\text{score} = -\log_2 \left(\sum_{x=O}^{\text{Min}(C,V)} f(x) \right)$$

$$f(x) = \frac{{}_C C_x \cdot {}_{T-C} C_{V-x}}{{}_T C_V}$$

By microarray analysis, we identified top 50 and 100 differentially expressed genes (DEG) in T cells between each MS twin pair numbered #1 to #4 described above. Then, we selected a panel of 34 genes that were listed in 50 DEG of the discordant twin (MS/healthy subject) of Family #4 but were not included in 100 DEG of the concordant (MS/MS) twin pairs of Families #1 to #3. Among the 34 genes, we further extracted a set of 20 DEG by a cut-off point greater or equal to two-fold difference between the Family #4 pair. Then, to identify the molecular network with the most close relationship with 20 DEG, the gene list was imported into KeyMolnet (the version of 1 January 2007). This automatically extracted 43 genes directly linked to 20 DEG. The common upstream search of 43 genes illustrated a complex molecular network composed of 39 nodes (Fig. 1). By statistical analysis described above, the generated network showed the most significant relationship with gene regulation by the Ets transcription factor family presenting with the score of 10.2. This was followed by gene regulation by the nuclear factor NF-κB in the second rank (the score 9.0), the Myc/Mad family in the third rank (7.0), the IFN-regulatory factor (IRF) family in the fourth rank (6.0), and the estrogen receptor (ER) family in the fifth rank (4.7).
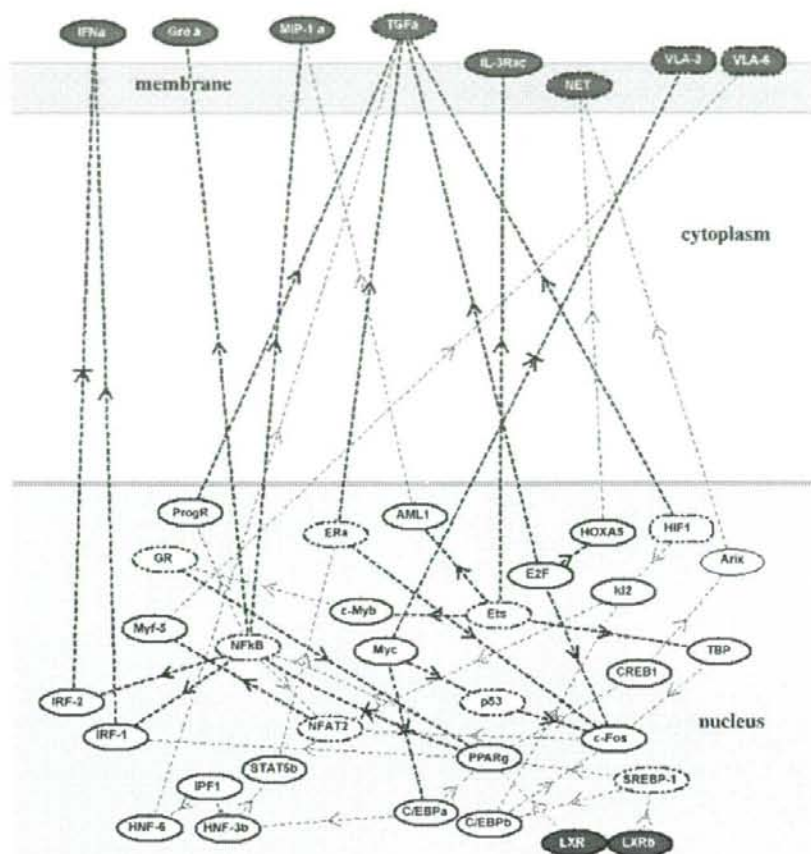
Fig. 1. The common upstream search of 20 differentially expressed genes in T cells of the discordant twin. The microarray data of 20 DEG in T cells differentilally expressed between the discordant MZ twin pair (Family #4) were imported into KeyMolnet that extracted 43 genes directly linked to 20 DEG. The common upstream search of 43 genes generated a molecular network composed of 39 nodes arranged according to the subcellular distribution. Red nodes represent upregulated genes, whereas blue nodes represent downregulated genes in MS. White nodes exhibit the genes automatically settled by KeyMolnet to establish molecular connections. The direction of molecular relation is indicated by dash line with arrow (transcriptional activation) or dash line with arrow and stop (transcriptional repression). Thick lines indicate the core contents, while thin lines indicate the secondary contents of KeyMolnet.

The Ets family transcription factor, by interacting with various co-regulatory factors, controls the expression of a wide range of target genes essential for cell proliferation, differentiation, transformation, and apoptosis [18]. Ets-1, the founder member of the Ets family, is pivotal for survival and differentiation of T cells [10]. The Ets family transcription factors regulate the gene expression of autoimmune regulator (AIRE) that directs the expression of self-antigens in thymus potentially related to the autoimmune process of MS [9]. The NF-κB family, consisting of NF-κB1 (p50/p105), NF-κB2 (p52/p100), RelA (p65), RelB, and c-Rel, acts as a central regulator of innate and adaptive immune responses, cell proliferation, and apoptosis [1]. More than 150 target genes for NF-κB are currently identified [12]. A significant subset of NF-κB target genes, including proinflammatory cytokines TNFα and IL-1β, activate the expression

of NF-κB, providing a positive regulatory loop that amplifies and perpetuates inflammatory responses [1]. Importantly, RelA, c-Rel, and p50 are overexpressed in macrophages in active demyelinating lesions of MS [4]. Furthermore, RelA expression is enhanced on oligodendrocytes that survive in the lesion edge [2]. Recently, we found that the expression of the orphan nuclear receptor NR4A2, a direct target gene of NF-κB, is elevated at the highest level in CD3+ T cells of untreated MS patients [16,15]. Furthermore, targeted disruption of the NFKB1 gene confers resistance to development of experimental autoimmune encephalomyelitis (EAE), an animal model of MS [5]. *In vivo* administration of selective inhibitors of NF-κB activation protects mice from EAE [11]. All of these observations suggest the principal involvement of aberrant regulation of NF-κB in the pathogenesis of MS.

We have recently identified 286 genes differentially expressed in purified CD3$^+$ T cells between 72 untreated Japanese MS patients and 22 age- and sex-matched healthy subjects of heterogeneous genetic backgrounds [16]. When the list of 286 DEG was imported into KeyMolnet (the version of 1 April 2007), it extracted 456 genes directly linked to 286 DEG. The common upstream search of 456 genes illustrated a complex molecular network composed of 335 nodes (Supplementary Fig. 1 online). The generated network showed the most significant relationship with gene regulation by NF-κB (the score 13.0), IRF (12.3), LRH-1 (12.0), BLIMP-1 (11.4), and Ets (7.4) transcription factor families. Thus, these observations suggested again the central role of aberrant transcriptional regulatory network that involves NF-κB, Ets, IRF and other transcription factors in development of pathogenic T cells in the genetically heterogeneous population of Japanese MS patients.

Although the sample size in the present study is fairly small because of limited availability of the samples of MZ MS twins, the novel bioinformatic approach proposes the logical hypothesis that aberrant regulation of the complex transcriptional regulatory network might contribute to development of pathogenic T cells in MS. This hypothesis warrants evaluation by using a large cohort of the genetically identical MS and healthy subject twins.

Acknowledgements

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.neulet.2007.05.056.

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Original Article

Human astrocytes express aquaporin-1 and aquaporin-4 *in vitro* and *in vivo*

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Aquaporins (AQP) constitute an evolutionarily conserved family of integral membrane water transport channel proteins. Previous studies indicate that AQP1 is expressed exclusively in the choroid plexus epithelium, while AQP4 is localized on the vascular foot of astrocytes in the central nervous system (CNS) under physiological conditions. To investigate a role of AQP in the pathophysiology of neurological diseases involving astrogliosis we studied the expression of AQP1 and AQP4 in cultured human astrocytes and brain tissues of multiple sclerosis (MS), cerebral infarction and control cases. By reverse transcriptase-polymerase chain reaction and western blot analysis, cultured human astrocytes co-expressed both AQP1 and AQP4 mRNA and proteins, where AQP4 levels were elevated by exposure to interferon-gamma but neither by tumor necrosis factor-alpha nor interleukin-1beta, whereas AQP1 levels were unaffected by any of the cytokines examined. By western blot analysis, AQP1 and AQP4 proteins were detected in the brain homogenates of the MS and non-MS cases, where both levels were correlated with those of glial fibrillary acid protein. By immunohistochemistry, astrocytes with highly branched processes surrounding blood vessels, along with glial scar, expressed intensely AQP1 and AQP4 in MS and ischemic brain lesions, whereas neither macrophages, neurons nor oligodendrocyte cell bodies were immunopositive. These immunohistochemical results indicate that the expression not only of AQP4 but also of AQP1 was enhanced in MS and ischemic brain lesions located predominantly in astrocytes, suggesting a pivotal role of astrocytic AQP in the maintenance of water homeostasis in the CNS under pathological conditions.

INTRODUCTION

The aquaporins (AQP) constitute a family of integral channel proteins that facilitate the rapid transport of water across cell membranes in response to osmotic gradients.[1,2] Presently, at least 13 mammalian members of this family have been identified that are widely expressed in various fluid-transporting epithelial and endothelial cells. AQP1, AQP2, AQP4, AQP5 and AQP8 are chiefly water selective, while AQP3, AQP7, AQP9 and AQP10, named aquaglyceroporins, transport glycerol in addition to water. Among these, AQP1, AQP4 and AQP9 are expressed in the central nervous system (CNS).[1,2] AQP1, initially identified in red blood cells and renal proximal tubular epithelium, is found to be selectively and constitutively expressed in the apical surface of choroid plexus epithelium (CPE) in the CNS.[3] The AQP1 water channel is composed of a dumbbell-shaped homotetramer of 28-kDa subunits, each of which contains six transmembrane domains and an extracellular epitope that defines the Colton blood group antigen on red blood cells. AQP1 serves as both a water channel and a cGMP-gated ion channel on CPE.[4] AQP1-null individuals negative for the Colton antigens, who have homozygous mutations in the AQP1 gene, exhibit a defective urinary concentrating ability and reduced pulmonary vascular permeability following fluid overload, suggesting a major role of AQP1 in kidney and pulmonary physiology.[5,6] In mice, a targeted disruption of the AQP1 gene caused a decrease in cerebrospinal fluid (CSF) production and intracranial pressure, indicating a primary role of AQP1 in the secretion of CSF in the CNS.[7] Although AQP1 is expressed widely in vascular endothelial cells outside the CNS, its levels are very low in brain endothelial cells and no expression has been identified in primary cultures of rodent astrocytes.[8]

AQP4 is the predominant form of AQP enriched in the CNS.[9] It is constitutively expressed in the astroglial endfeet at the perivascular and the subpial membranes where it regulates brain water homeostasis by acting as a key constituent of the blood–brain barrier (BBB) and the blood–CSF barrier.[10] Alpha-syntrophin, an adapter protein 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 that AQP4 has a protective role in the 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 the compensatory up-regulation of AQP4 against water imbalance under pathological conditions.[15,16] An immunoglobulin G (IgG) auto-antibody reacts specifically with AQP4 in 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 up-regulated after ischemia.[20]

Although increasing evidence supports the active involvement of AQP 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, 10 micron-thick serial sections were prepared from autopsied brains of four MS patients and 10 non-MS brains. The tissues were fixed with 4% paraformaldehyde or 10% neutral formalin and embedded in paraffin. The MS cases included a 29-year-old woman with secondary progressive MS (SPMS) who died of asphyxia (MS1), a 40-year-old woman with SPMS who died of respiratory failure (MS2), a 43-year-old woman with primary progressive MS (PPMS) who died of hyperglycemia (MS3), and a 33-year-old man with SPMS who died of sepsis (MS4). Detailed clinical profiles of the 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 an old cerebral infarction who died of pancreatic cancer, a 56-year-old man with an 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, the MS cases included MS1, MS2, MS3 and a 70-year-old woman with SPMS (MS5) who died of pneumonia. Non-MS cases included a 76-year-old woman with Parkinson's disease (PD1), a 61-year-old woman with amyotrophic lateral sclerosis (ALS) (ALS1), a 74-year-old woman with ALS (ALS2), a 61-year-old man with ALS (ALS3), a 66-year-old man with ALS (ALS4), a 73-year-old man with schizophrenia (SCH1) and a 77-year-old woman with depression (DEP1). The post-mortem interval of the cases ranges from 1.5 h to 10 h 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, 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 the National Center of Neurology and Psychiatry.

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, Carlsbad, CA, USA), 20 ng/mL recombinant

human epithelial growth factor (EGF) (Higeta, Tokyo, Japan), 20 ng/mL recombinant human bFGF (basic fibroblast growth factor, PeproTech EC, London, UK) and 10 ng/mL recombinant human LIF (leukemia inhibitory factor, Chemicon, Temecula, CA, USA). For the induction of astrocyte differentiation, the NP cells were incubated for several weeks in DMEM (Dulbecco's modified Eagle medium, Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin (feeding medium). This incubation induced the vigorous proliferation and differentiation of astrocytes accompanied by a rapid reduction in nonastroglial cell types. Their purity exceeded 98% by glial fibrillary acid protein (GFAP) immunolabeling. The cells were incubated for 48 h in the feeding medium containing 50 ng/mL recombinant human tumor necrosis factor-alpha (TNFα), IFNγ or interleukin-1beta (IL-1β) (all from PeproTech EC, London, UK). 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 mmol/L citrate sodium buffer, pH 6.0 by autoclave at 125°C for 30 s in a temperature-controlled 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°C overnight with the 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 HE. 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 immunolabeled with the antibodies against the cell type-specific markers listed in Table 1.

For double labeling, the 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, CA, USA) or mouse anti-AQP1 monoclonal antibody (1/22; Santa Cruz

Table 1 Primary antibodies utilized for immunohistochemistry and western blot analysis

Antibodies (ID)	Suppliers	Code	Origin	Antigen utilized for raising antibodies	Concentration used for immunohistochemistry	Concentration used for western blotting
AQP1 (H-55)	Santa Cruz Biotechnology	sc-20810	Rabbit	A peptide covering amino acid residues 215–269 of human AQP1	0.4 μg/mL	0.2 μg/mL
AQP1 (1/22)	Santa Cruz Biotechnology	sc-32737	Mouse	A peptide covering amino acid residues 249–269 of rat AQP1	8 μg/mL	NA
AQP4 (H-80)	Santa Cruz Biotechnology	sc-20812	Rabbit	A peptide covering amino acid residues 244–323 of human AQP4	0.4 μg/mL	0.2 μg/mL
GFAP	Dako	N1506	Rabbit	GFAP purified from bovine spinal cord	Prediluted	NA
GFAP (GA5)	Nichirei	422261	Mouse	GFAP purified from swine spinal cord	Prediluted	Further diluted at 1:1000
MBP	Dako	N1564	Rabbit	MBP purified from human brain	Prediluted	NA
NF (2F11)	Nichirei	412551	Mouse	NF purified from human brain	Prediluted	NA
CD68 (KP1)	Dako	N1577	Mouse	Lysosomal granules macrophages of human lung	Prediluted	NA
IFI30 (T-18)	Santa Cruz Biotechnology	sc-21827	Goat	A peptide mapping within an internal region of human IFI30 (GILT)	NA	0.2 μg/mL
HSP60 (N-20)	Santa Cruz Biotechnology	sc-1052	Goat	A peptide mapping at the N-terminus of human HSP60	NA	0.1 μg/mL

AQP1, aquaporin-1; AQP4, aquaporin-4; GFAP, glial fibrillary acidic protein; GILT, gamma interferon-inducible thiol reductase; HSP60, 60-kDa heat shock protein; IFI30, interferon gamma-inducible protein 30; MBP, myelin basic protein; NA, not applied; NF, neurofilament; CD68, macrophage antigen.

Biotechnology, CA, USA), then followed by incubation with alkaline phosphatase-conjugated secondary antibody (Nichirei) and colored with New Fuchsin substrate. After the inactivation of these antibodies by autoclaving the sections in 10 mmol/L citrate sodium buffer, pH 6.0, they were relabeled with mouse anti-GFAP monoclonal antibody (GA5; Nichirei), or rabbit anti-AQP4 antibody (H-80), followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibody (Nichirei) and colorized with DAB substrate.

For immunocytochemistry, human astrocytes in culture on cover glasses were fixed with 4% paraformaldehyde (PFA) in 0.1 mol/L 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 antimouse IgG and Alexa Fluor 488-conjugated antirabbit IgG (both from Invitrogen, Carlsbad, CA, USA). After several washes, they were mounted with glycerol-polyvinyl alcohol and examined under a Nikon ECLIPSE E800 universal microscope (Nikon, Tokyo, Japan) equipped with fluorescein and rhodamine optics. Negative controls were processed following all the steps described above, except for exposure to a primary antibody.

Reverse transcription polymerase chain reaction (RT-PCR) analysis

DNase-treated total cellular RNA was processed for cDNA synthesis using oligo(dT)$_{12-18}$ primers and SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Then, cDNA was amplified by PCR using HotStar Taq DNA polymerase (Qiagen, Valencia, CA, USA) and a panel of sense and antisense primer sets as follows: 5'-CAGGTGGAGGAGTATGACCTGGAT-3' and 5'-GAC CATGCAGGTCTGGCAGATCTT-3' for a 205 bp product of AQP1; 5'-CCTCGCTGGTGGCCTTTATGAGTA-3' and 5'-GTCTTTCCCCTTCTTCTCCTCTCC-3' for a 218 bp product of AQP4 and 5'-CCATGTTCGT CATGGGTGTGAACCA-3' and 5'-GCCAGTAGAG GCAGGGATGATGTTC-3' 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°C for 15 min, followed by a denaturing step at 94°C for 1 min, an annealing step at 60°C for 40 s and an extension step at 72.9°C for 50 s for 38 cycles, except for G3PDH amplified for 32 cycles. For the positive control, the 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 (ORF) 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, USA) from human astrocyte cDNA using the sense and antisense primer sets as follows: 5'-GCCAGCGAGTTCAAGAAGAAGCTC-3' and 5'-CTATTTGGGCTTCATCTCCACCCT-3' for AQP1, and 5'-AGTGACAGACCCACAGCAAGGCGG-3' and 5'-TCATACTGAAGACAATACCTCTCC-3' for AQP4. Then, they were cloned in an expression vector pcDNA4/HisMax-TOPO (Invitrogen Carlsbad, CA, USA). For transient expression, the vectors were transfected into HEK293 cells by using Lipofectamine 2000 reagent (Invitrogen Carlsbad, CA, USA). At 72 h after transfection, the cells were processed for western blot analysis.

Western blot analysis

To prepare for total protein extract, the cells and tissues were homogenized in radioimmunoprecipitation assay lysis buffer composed of 50 mmol/L Tris-HCl (hydrochloride), pH 7.5, 150 mmol/L sodium choride, 1% Nonidet P40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl chloride (SDS) and a cocktail of protease inhibitors (Sigma, St Louis, MO, USA), followed by centrifugation at 10 000 g 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 (Bio-Rad, 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. The membranes were then incubated at RT for 30 min with HRP-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA). 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°C for 30 min in stripping buffer composed of 62.5 mmol/L Tris-HCl, pH 6.7, 2% SDS and 100 mmol/L 2-mercaptoethanol, the membranes were processed for relabeling several times with different antibodies.

RESULTS

The constitutive 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
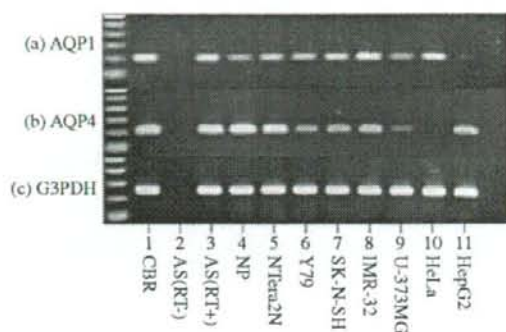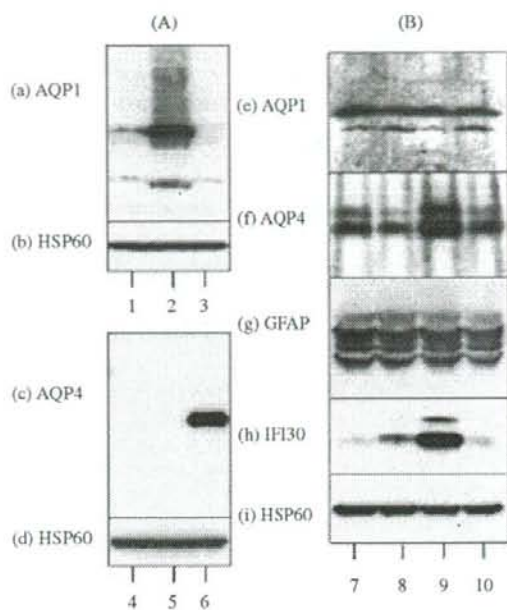
Fig. 1 Aquaporin (AQP1 and AQP4) mRNA expression in human neural cells. The expression of (a) AQP1 (b) AQP4 and (c) G3PDH (an internal control) mRNA was studied in human neural cells by reverse transcriptase-polymerase chain reaction analysis. The lanes (1–11) represent (1) the frontal cerebral cortex; (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.

AQP1 and AQP4 transcripts were identified in human astrocytes (AS) in the culture, neuronal progenitor (NP) cells and cell lines of NTera2 teratocarcinoma-derived neurons (NTera2N), Y79 retinoblastomas, SK-N-SH neuroblastomas, IMR-32 neuroblastomas and U-373MG astrocytomas, along with in the human cerebral cortex (Fig. 1, panels a and b, lanes 1, 3–9). AQP1 mRNA was detected in the 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 step, confirming that the 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 co-express AQP1 and AQP4, at least at mRNA levels.

The specificity of antibodies against AQP1 and AQP4

To verify the specificity of the H-55 and 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

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Fig. 2 Aquaporin (AQP1 and AQP4) protein expression in (A) HEK293 cells and (B) human astrocytes in culture. To verify the antibody specificity, the open reading frame of the human AQP1 gene and 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); and (b,d) anti-HSP60 antibody. Lanes 1–6 represent (1,4) non-transfected HEK293 cells; (2,5) AQP1-expressing HEK293 cells; and (3,6) AQP4-expressing HEK293 cells. (B) Human astrocytes were exposed for 48 h to three distinct cytokines, followed by processing for western blot analysis using: (e) H-55; (f) H-80; (g) anti-glial fibrillary acid protein antibody (GA5); (h) anti-interferon gamma-inducible protein 30 antibody; and (i) anti-HSP60 antibody. Lanes 7–10 represent (7) untreated cells; (8) cells treated with tumor necrosis factor-alpha (TNFα); (9) IFNγ and (10) interleukin-1beta (IL-1β). Twenty micrograms of protein was loaded on each lane. The identical blots were processed for relabeling several times with different antibodies. HSP60 indicates an internal control.

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-
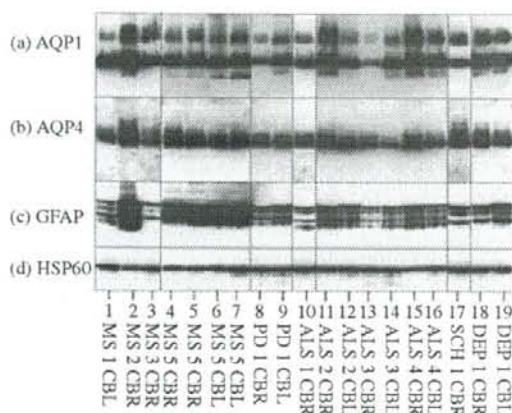
Fig. 3

Fig. 3 Aquaporin (AQP1 and AQP4) protein expression in multiple sclerosis (MS) and other neurological and psychiatric disease brain homogenates. The expression of (a) AQP1; (b) AQP4; (c) glial fibrillary acid protein; and (d) HSP60 proteins was studied by western blot analysis using H-55 and H-80 antibodies in brain homogenates prepared from either the frontal cerebral cortex or the cerebellar cortex of four MS (numbered MS1 to MS4) and seven non-MS cases, numbered according to their diseases. Lanes 1–19 represent; (1) MS1 CBL; (2) MS2 CBR; (3) MS3 CBR; (4) MS5 CBR; (5) a different specimen of MS5 CBR; (6) MS5 CBL; (7) a different specimen of MS5 CBL; (8) PD1 CBR; (9) PD1 CBL; (10) ALS1 CBR; (11) ALS2 CBR; (12) ALS2 CBL; (13) ALS3 CBR; (14) ALS3 CBL; (15) ALS4 CBR; (16) ALS4 CBL; (17) SCH1 CBR; (18) DEP1 CBR; and (19) DEP1 CBL. Sixty micrograms of protein was loaded on each lane. The identical blots were processed for relabeling several times with different antibodies. HSP60 indicates an internal control. ALS, amyotrophic lateral sclerosis; DEP, depression; PD, Parkinson's disease; SCH, schizophrenia.

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
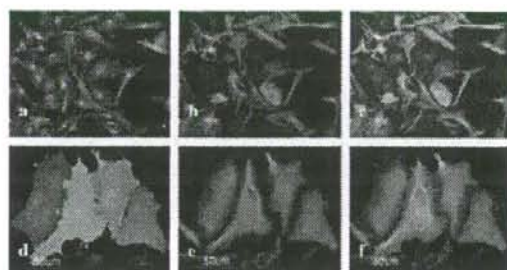
Fig. 4 Aquaporin (Aqp1 and AQP4) immunoreactivities in human astrocytes in culture. Human astrocytes in culture were immunolabeled with (a) anti-AQP1 antibody (H-55); or (d) anti-AQP4 antibody (H-80); in combination with (b,e) anti-glial fibrillary acid protein antibody (GA5). The panels (c,f) represent the merging of individual immunoreactions.

immunocytochemistry using H-55 and H-80 antibodies. On western blot, the human astrocytes constitutively expressed both AQP1 and AQP4 proteins (Fig. 2B, panels e and f, lane 7). A 48 h-exposure to IFNγ markedly elevated the levels of AQP4 and interferon gamma-inducible protein 30, the latter being 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 immunohistochemistry 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 the adjacent cerebral cortex of the 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.

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An accumulation of astrocytes expressing AQP1 or AQP4 was also observed in the 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 the 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 more dense in the rim of the necrotic core of ischemia than in unaffected regions (Fig. 5, panel d). The CPE expressed both AQP1 and AQP4 (not shown). GFAP⁺ gliotic tissues in chronic demyelinating lesions in the optic nerve, spinal cord and the cerebrum of the MS brains were intensely immunolabeled with antibodies against AQP1 and AQP4 (Fig. 6, panels a–f). An accumulation of AQP1-expressing
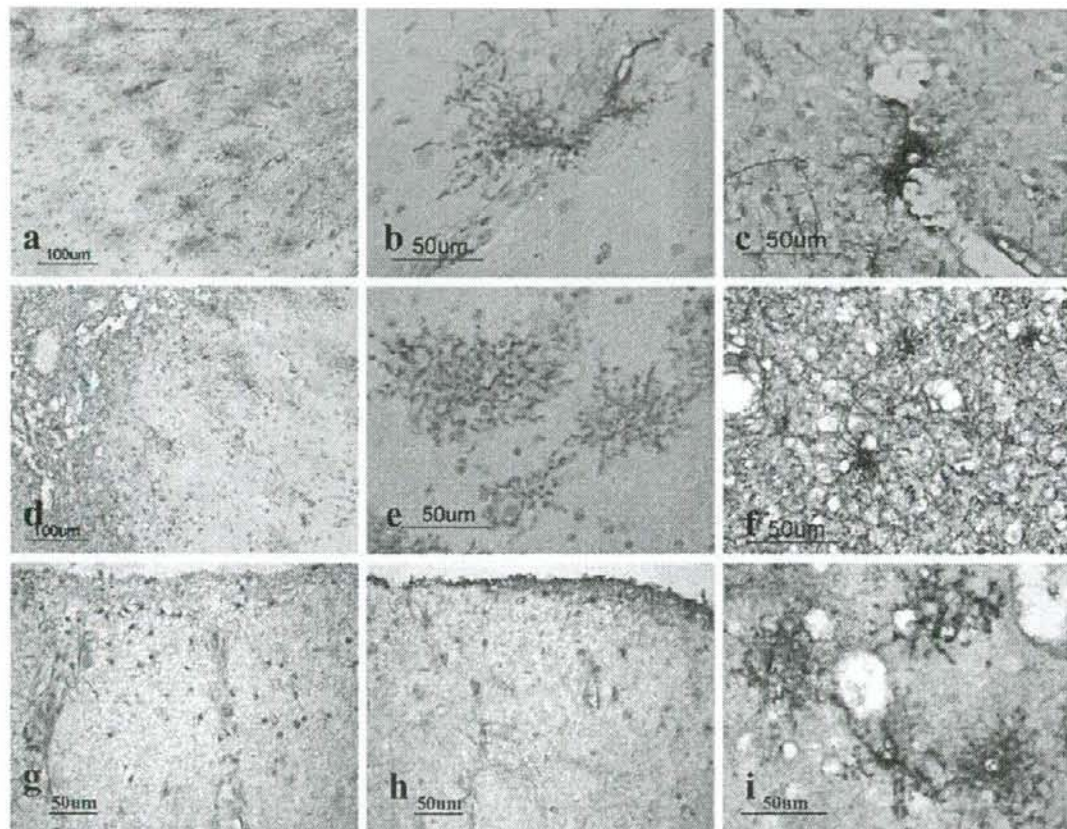
Fig. 5 Aquaporin (AQP1 and AQP4) immunoreactivities in fibrillary astrocytes with highly branched processes in multiple sclerosis (MS) and other neurological and psychiatric diseased brains. The expression of AQP1 and AQP4 proteins was studied in MS and non-MS brains by immunohistochemistry. (a) AQP1 (H-55), numerous astrocytes in chronic active demyelinating lesion in the frontal cerebral cortex of multiple sclerosis case no. MS1; (b) AQP1 (H-55), a fibrillary astrocyte in the unaffected cortex adjacent to an old infarct lesion in the frontal cerebral cortex; (c) double labeling of AQP1 (H-55, red) and glial fibrillary acid protein (GFAP) (GA5, brown), a fibrillary astrocyte in a chronic active demyelinating lesion in the occipital cerebral cortex of MS1; (d) AQP4 (H-80), astrogliosis in the rim of the necrotic core of an old infarct in the temporal cerebral cortex; (e) AQP4 (H-80), two fibrillary astrocytes in the unaffected cortex adjacent to an old infarct lesion in the temporal cerebral cortex; (f) Double labeling of AQP4 (H-80, red) and GFAP (GA5, brown), several fibrillary astrocytes in a chronic active demyelinating lesion in the occipital cerebral cortex of MS1; (g) AQP1 (H-55), no obvious immunoreactivity in the frontal cerebral cortex of a neurologically normal subject; (h) AQP4 (H-80), the pia and subpial astrocytes, the same region as (g); (i) Double labeling of AQP1 (1/22, red) and AQP4 (H-80, brown), several fibrillary astrocytes in the frontal cerebral cortex of a patient with schizophrenia.
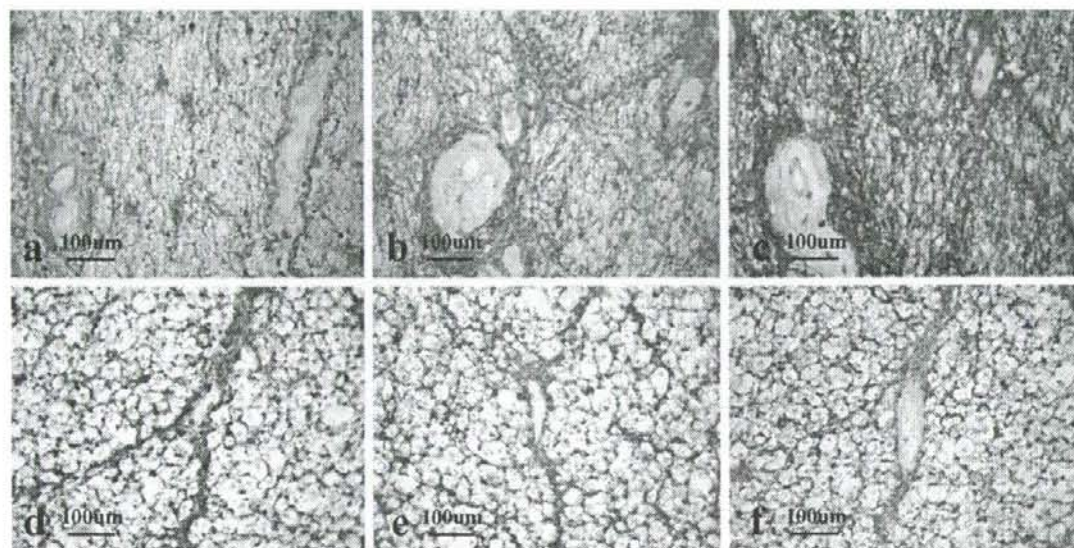
Fig. 6 Aquaporin (AQP1 and AQP4) immunoreactivities in gliotic tissues in multiple sclerosis (MS) brains. The expression of AQP1 and AQP4 proteins was studied in the optic nerve and the spinal cord by immunohistochemistry. The panels (a–f) represent the following: (a) rabbit polyclonal antibody (GFAP) gliotic tissues in a chronic active demyelinating lesion in the optic nerve of case no. MS2; (b) AQP1 (H-55), in the same region as (a); (c) AQP4 (H-80), the same region as (a); (d) glial fibrillary acid protein (rabbit polyclonal antibody), gliotic tissues in the posterior funiculus of the spinal cord of case no. MS3; (e) AQP1 (H-55), the same region as (d); (f) AQP4 (H-80), the same region as (d).

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 co-expressed 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 of the cases examined (Fig. 7, panels a–f), indicating that the principal localization of these aquaporins is in 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 scars. Thus, AQP1 expression is not CPE-specific in the human CNS under

pathological conditions. The following observations supported this conclusion. First, cultured human astrocytes co-expressed AQP1 and AQP4 at both mRNA and protein levels. Second, AQP1 and AQP4 proteins were identified in the 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 that immunoreact with AQP1 or AQP4 is much smaller in the control brains of neurologically normal subjects. Our observations suggest a pivotal role of AQP1 and AQP4 expressed on astrocytes in maintaining 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