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Aquaporin 4 is increased in association with human immunodeficiency virus dementia: Implications for disease pathogenesis

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Changes in astrocyte shape and function are known to occur in association with human immunodeficiency virus (HIV) dementia (HIVD). However, the causes and consequences of such changes are not completely understood. *In vitro* data suggest that changes in the expression of aquaporin 4 (AQP4), the aquaporin subtype expressed by astrocytes, can significantly influence cell shape and physiology. In the present study, the authors therefore investigated the possibility that AQP4 levels may be altered in HIVD. Using Western blot, the authors show that immunoreactivity for AQP4 is elevated in brain homogenates from the mid frontal gyrus of patients who died with HIVD ($P < .005$ HIV seronegative versus HIVD). Of interest, a significant increase was also observed in homogenates from HIV-infected individuals without dementia ($P < .05$ HIV seronegative versus neurologically normal HIV seropositive). In the present study the authors also examined the stimulated expression of AQP4 in cultured cells. Previous *in vitro* studies have shown that AQP4 expression may be increased by stimuli that induce cytoskeletal changes and/or the activation of p38 mitogen-activated protein (MAP) kinase. The authors therefore focused on tumor necrosis factor (TNF)-α, which has been linked to p38 MAP kinase activation, and thrombin, which may also induce changes in the actin cytoskeleton. Both may be elevated with HIVD. Again using Western blot, the authors show an increase in both AQP4 and phosphorylated p38 MAP kinase in homogenates from TNF-α- and thrombin-stimulated organotypic cerebellar and spinal cord cultures. Together, these studies suggest that AQP4 expression may be altered in HIVD and/or in response to exogenous proteinases. Additional studies may be warranted to determine whether altered AQP4 expression represents a protective and/or maladaptive response to central nervous system (CNS) inflammation. *Journal of NeuroVirology* (2005) **11,** 535–543.

Keywords: aquaporin; astrocytes; dementia; HIV; thrombin

Introduction

Our understanding of those events that are critical to the development of human immunodeficiency virus (HIV)–1–associated dementia (HIVD) is limited, but inflammation within the central nervous system (CNS) is thought to play an important role. HIVD typically occurs late in the course of HIV disease, when the specific immune response is impaired and the nonspecific is relatively activated.

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Because monocytes may indeed be relatively activated at this stage, these cells may more easily cross the blood brain barrier. The CNS entry of monocytes may be further facilitated by changes in the blood brain barrier itself and by changes in chemoattractant cytokine gradients that occur with HIV infection (Conant *et al*, 1998; Kelder *et al*, 1998; Langford *et al*, 2002; Nottet, 1999; Pereira *et al*, 2001; Persidsky *et al*, 1999; Sasseville *et al*, 1996; Schmidtmayerova *et al*, 1996; Westmoreland *et al*, 1998).

Activation of astrocytes is also observed in association with HIVD (Wesselingh and Thompson, 2001). Such activation may be a consequence of increases in circulating cytokine levels, which in rodent models can quickly affect gene expression in this cell type (Herkenham *et al*, 1998; Thibeault *et al*, 2001). In addition, monocytes which have migrated to the CNS are thought to release proinflammatory cytokines that can activate astrocytes.

In other pathological conditions that are marked by astrocyte activation and/or blood-brain barrier changes, expression of the water channel aquaporin 4 (AQP4) may be altered (Warth *et al*, 2004). For example, AQP4 is known to be increased in association with both glioma and stroke (Papadopoulos *et al*, 2002; Taniguchi *et al*, 2000). Pathologically increased expression of AQP4 may in turn have functional consequences, including edema and possibly, an altered seizure threshold (Amiry-Moghaddam *et al*, 2003; Binder*et al*, 2004; Papadopoulos *et al*, 2002). Of interest with respect to AQP4 in CNS infection, a recent study has shown reduced mortality in AQP4 null animals with *Pneumococcal* meningitis (Papadopoulos and Verkman, 2005).

Few *in vitro* studies have examined factors that control the expression of the AQPs. For example, the reasons for increased expression of this protein in glioma and stroke are not completely understood. At this point, however, expression of AQP4 is known to be influenced by osmotic stimuli (Arima *et al*, 2003; Hoffert *et al*, 2000). Such stimuli may activate AQP expression through the activation of kinases linked to cytoskeletal changes. For example, osmotic stress can induce changes in cell shape and thus the actin cytoskeleton (Komis *et al*, 2002; Lunn and Rozengurt, 2004), and such changes may in turn lead to the activation of p38 (Nemeth *et al*, 2004; Xu *et al*, 2001). In support of a role for such signaling, sorbitol has been shown to increase the expression of AQP5 in mouse lung epithelial cells through a p38 mitogen-activated protein (MAP) kinase–dependent mechanism (Hoffert *et al*, 2000). Moreover, mannitol may increase the expression of AQPs (AQP4 and -9) in rat astrocytes via a p38 MAP kinase-dependent mechanism (Arima *et al*, 2003).

In simian immunodeficiency virus (SIV) encephalitis, an animal model for HIVD, p38 MAP kinase levels are significantly elevated (Barber *et al*, 2004). Moreover, HIVD is associated with an increase in proinflammatory substances that might p38 MAP kinase activity and/or the actin cytoskeleton (Wesselingh *et al*, 1994). These include tumor necrosis factor (TNF)-α and thrombin (Boven *et al*, 2003; Wesselingh *et al*, 1994). We have therefore examined the expression of AQP4 in brains from control, neurologically normal HIV-seropositive, and HIV dementia patients. We have also tested TNF-α and thrombin for their ability to influence AQP4 expression in cultured cells.

Results

AQP4 is increased in mid-frontal gyrus brain tissue homogenates from patients with HIV dementia

Because AQP4 expression may be altered in pathological situations involving the activation of astrocytes, and astrocyte activaton is a common feature of HIV dementia, we examined the expression of AQP4 in brains from control (HIV−), neurologically normal HIV-seropositive (HIV+), and HIV demented (HIVD) patients. The immunoblots of brain homogenates from the mid-frontal gyrus (MFG) are shown in Figure 1. The results indicate that AQP4 expression is increased in the presence of both HIV infection and HIV dementia. Densitometric analysis (Figure 1b) done with Image J software, followed by statistical analysis of the data, confirms that there was significantly more intense immunoreactivity in the HIV+ group ($^{*}P < .05$) and HIVD group ($+P < .005$) as compared to the HIV− group. Note: Specificity of the AQP4 band was supported by subsequent studies that used an antibody to a different region of the protein (not shown). This antibody was provided to us by Drs. Daniel Gorelick and Peter Agre at Johns Hopkins University.

AQP4 levels in deep white matter tissue homogenates do not show a statistically significant increase in association with HIVD

Because the deep white matter may also be affected in HIVD, we examined expression of AQP4 in control and HIVD tissues from this region as well. Figure 2a shows the results of such studies. A similar trend towards increased AQP4 expression in HIV+ and HIVD groups can be appreciated, but statistical significance was not obtained (Figure 2b).

AQP4 expression is localized to astrocytes and increased in perivascular astroglial processes

To investigate the localization of AQP4 in the brain, we performed immunohistochemical studies using tissue sections obtained from HIV-seronegative, HIV-seropositive, and HIVD patients. In sections from the mid-frontal gyrus of HIV-infected patients with and without dementia, prominent AQP4 immunoreactivity was observed in cells having the morphological appearance of astrocytes. Representative HIVD tissue specimens, following staining for AQP4, are shown in Figure 3 A to C. Immunoreactivity of the perivascular area can be appreciated. Although AQP4

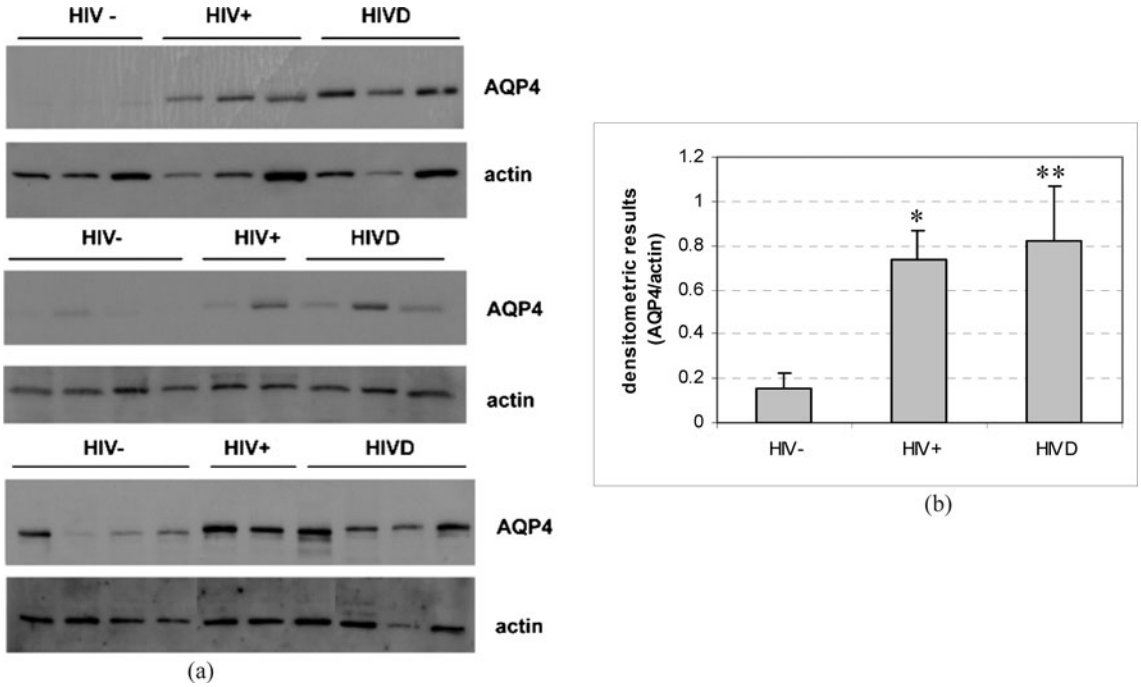

Figure 1 Immunoblots of brain homogenates from the mid-frontal gyrus (MFG). Western blot analysis (**a**) of AQP4 expression in MFG brain homogenates from HIV-seronegative controls (HIV−), HIV-seropositive individuals (HIV+), and HIVD patients (HIVD). Densitometric analysis (**b**) done with NIH Image J software, followed by statistical analysis of the data, confirms that there was significantly more intense immunoreactivity in the HIV+ ($^{*}P < .05$) and HIVD groups ($^{**}P < .005$) as compared to the HIV−. Data are presented as mean plus standard error, and pairwise comparisons (HIV+ or HIVD to control) were made using Student's *t* test.

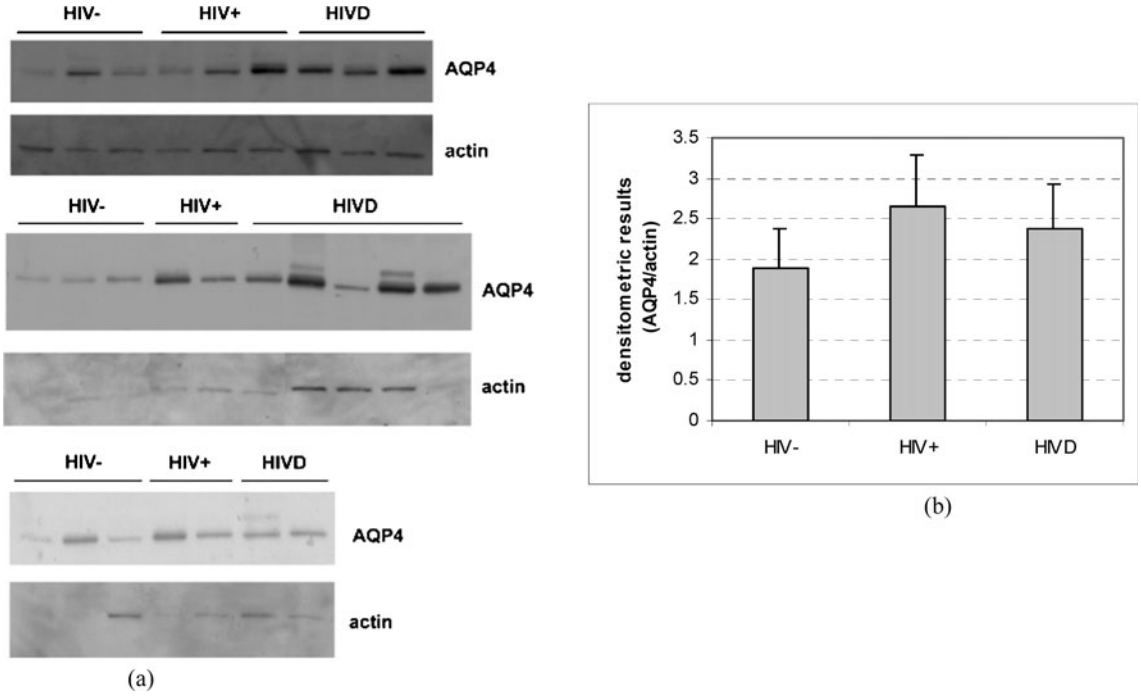

Figure 2 Immunoblots of brain homogenates from the deep white matter (DWM). Western blot analysis (**a**) of AQP4 expression in DWM brain homogenates from HIV-seronegative controls (HIV−), HIV-seropositive individuals (HIV+), and HIVD patients (HIVD). Densitometric analysis (**b**) done with NIH Image J software, followed by statistical analysis of the data, did not show a statistically significant difference between groups. Data are presented as mean plus standard error, and pairwise comparisons (HIV+ or HIVD to control) were made using Student's *t* test.

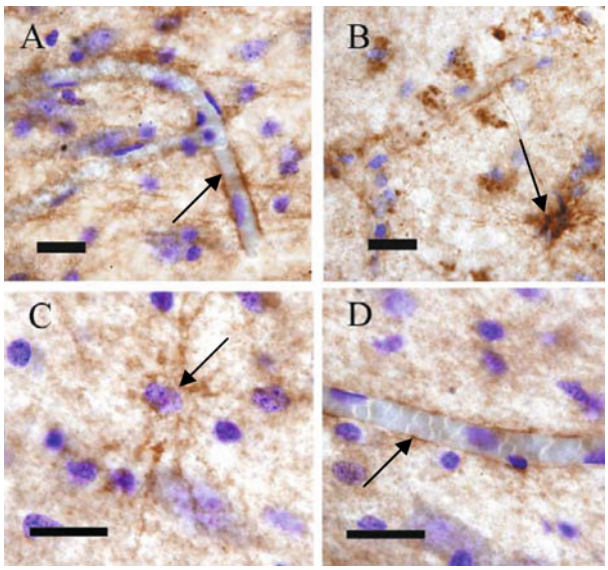

Figure 3 Immunolocalization of AQP4 in human brain. AQP4 expression in perivascular processes (arrow in **A**) and cytoplasm (arrows in **B** and **C**) of activated astrocytes, as inferred by morphology, in the cerebral cortex of a patient with a history of HIVD. (**D**) AQP4 immunoreactivity is present but less intense in (*arrow*), as seen in this section from cerebral cortex obtained from a control patient without HIV infection or dementia. Note: The bar in **A** and **B** represents 50 μm, whereas that in **C** and **D** represents 20 μm.

immunoreactivity could also be appreciated in brain tissue specimens from non-HIV-infected controls, the immunoreactivity appeared to be less intense. A representative HIV− control tissue specimen, following staining for AQP4, is shown in Figure 3D.

Treatment of organotypic cultures with thrombin or TNF-α is associated with an increase in AQP4 protein expression

Because thrombin can stimulate G protein–coupled receptors, which are linked to changes in the actin cytoskeleton, and AQP expression may be influenced by changes in the same, we tested thrombin for its ability to increase the expression of AQP4. In addition, because TNF-α has previously been shown to increase AQP4 expression, it was used as a positive control. Organotypic cultures were serum-starved for 6 hours and then stimulated with 10 U/ml thrombin or 10 ng/ml TNF-α for 16 h. Cell lysates were then prepared and examined by Western blot for AQP4. Figure 4 shows an overall increase in AQP4 band intensity from lysates of thrombin- and TNF-α-stimulated cells. Data are shown for mouse cerebellar (Figure 4, upper panel) and rat spinal cord (Figure 4, lower panel) culture extracts.

Discussion

In the present study, we have shown that immunoreactivity for AQP4, as detected by Western blot, is increased in association with HIV infection and HIVD.

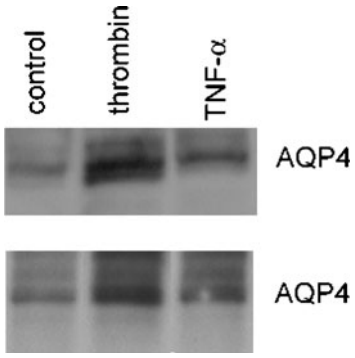

Figure 4 AQP4 in homogenates from control, thrombin-, and TNF-α-stimulated organotypic slice cultures. Western blot analysis of AQP4 protein levels in control, thrombin-, and TNF-α-stimulated slice culture extracts shows increased AQP4 immunoreactivity in association with both thrombin and TNF-α. The upper blot shows results from mouse cerebellar slice cultures and the lower from rat spinal cord.

Moreover, we have shown that increased immunoreactivity can be detected in tissue sections from the mid-frontal gyrus, a region that has been identified as an area of productive viral replication. With respect to other CNS regions that show neuropathological changes in association with HIVD, the mid-frontal gyrus also shows prominent astrocyte activation and expression of Fas ligand (Vargas *et al*, 2002).

In this study we have also examined AQP4 expression in HIVD brain in terms of cellular localization. Consistent with its known distribution (Papadopoulos *et al*, 2002; Warth*et al*, 2004), AQP4 is expressed by cells with the morphological appearance of astrocytes. A recent study has, however, suggested that AQP4 may also be expressed by micgroglial cells (Tomas-Camardiel*et al*, 2004) and based on morpholocial criteria alone, we cannot rule out staining in this cell type. Staining is nonetheless observed along blood vessels, consistent with expression at the astrocyte foot process. Staining is also observed more diffusely in cells that have the appearance of activated astrocytes. Although immunoreactivity for AQP4 at the foot process is often observed in normal brain, diffuse immunoreactivity is typically observed in conditions associated with glial activation (Warth *et al*, 2004).

As to the potential causes of increased AQP4 expression in HIVD, we have presented data that thrombin and TNF-α may participate. Both may be increased in association with CNS inflammation (Boven *et al*, 2003; Wesselingh *et al*, 1994). As to potential mechanisms involved in thrombin- and TNF-α-stimulated AQP4 expression, both may influence the activity of p38 MAP kinase (Eto *et al*, 2002; Nwariaku *et al*, 2002). Although p38 MAP kinase may affect cytoskeletal dynamics (Petrache*et al*, 2003), changes in the cytoskeleton may also influence the activity p38. It has been shown, for example, that cell detachment and volume changes can induce activation of p38 MAP kinase (Rosen *et al*, 2002). In one

study, exposure of cells to hyperosmolar (600 mOsm) phosphate-buffered saline was associated with cell shrinkage and an osmolality-dependent increase (518%) in p38 activity (Xu *et al*, 2001). Depolymerization of actin, as induced by cytochalasin B, has also been linked to the activation of p38 MAP kinase (Nemeth *et al*, 2004). Because thrombin can induce Rho-dependent changes in the actin cytoskeleton, and TNF-α has been linked to changes in both actin and microtubule stability (Petrache *et al*, 2003), it is thus intriguing to speculate that these agents may affect AQP4 expression at least in part through mechanisms that are triggered by changes in cell shape.

Despite the focus of the present manuscript, thrombin and TNF-α are not likely to be the only HIVD-related proinflammatory stimulus that might increase the expression of AQP4. A number of proinflammatory cytokines may influence cell shape and/or p38 MAP kinase activity. And another class of proteins known to be elevated in HIVD, the matrix metalloproteinases (MMPs) (Conant *et al*, 1999; Yong *et al*, 2001), may do the same. MMPs may influence cell shape via matrix destruction and consequent changes in adhesive interactions (Yong *et al*, 1998), via direct interactions with integrins (Conant *et al*, 2004), and/or via the recently described ability of at least one of these enzymes to signal through the G protein-coupled proteinase activated receptor-1(Boire *et al*, 2005).

As to the possible consequences of dysregulated AQP4, one study has shown that knock down of this protein through the use of small interfering RNA duplexes (siRNA) was associated with changes in astrocyte shape, proliferation, and protein expression (Nicchia *et al*, 2003). Given that astrocyte functions include the secretion of neurotrophins, changes in protein expression could be physiologically important.

AQP4 has also been shown to play a role in cell migration (Saadoun *et al*, 2005). It is thus of interest that thrombin, which can stimulate cell migration, up-regulates AQP4 expression. The ability of AQPs to facilitate cell migration may, however, depend on their being localized in a polarized manner. Thus, if AQP4 is grossly up-regulated and expressed in a diffuse manner, its ability to facilitate cell or foot process migration might be impaired.

As to other potential consequences of dysregulated AQP4, it has been proposed that clearance of potassium is dependent on concomitant water flux through astrocyte membranes (Eid *et al*, 2005). Altered expression of AQP4 may thus influence seizure threshold. It has, for example, been shown that disruption of the gene encoding α-syntrophin was associated with decreased Kir 4.1 and AQP4 labeling in astrocyte end feet as well as with delayed potassium clearance after neuronal activation (Amiry-Moghaddam *et al*, 2003). In addition, hyperthermia induced seizures in the α-syntrophin knockouts were noted to be intensified (Amiry-Moghaddam *et al*, 2003). Effects of changes in AQP4 expression are likely to be complex however. For example, another study related to neuronal excitability showed that mice lacking AQP4 had an increased threshold to pentylenetetrazol induced seizures (Binder *et al*, 2004).

With respect to AQP4 expression in the context of CNS inflammation, however, a recent animal study is of particular interest (Papadopoulos and Verkman, 2005). In this study, AQP4-null mice showed reduced mortality and brain edema in acute *Pneumococcal* meningitis (Papadopoulos and Verkman, 2005). The authors of this study nonetheless pointed out that although AQP4 deletion seems have an overall beneficial effect in the setting of cytotoxic edema, it seems to have an overall detrimental effect in vasogenic. They thus proposed that AQP4, which is capable of bidirectional water transport, may facilitate water movement into brain astroglia during the cytotoxic edema and out of the brain with vasogenic (Papadopoulos and Verkman, 2005). The ultimate use of AQP4 antagonists will therefore need be considered with caution, especially in a chronic condition such as HIVD that may have elements of both cytotoxic and vasogenic edema.

In summary, we have shown that AQP4 levels may be increased in HIV infection and HIVD. We have also shown that thrombin, a proteinase that is elevated in association with HIVD (Boven *et al*, 2003), can stimulate an increase in AQP4 protein as detected by Western blot. These results are novel in that they show alterations in AQP4 in a chronic inflammatory condition whereas earlier studies have focused on more acute conditions. In addition, the finding that increased AQP4 expression may occur in HIV-infected patients without dementia is intriguing, and allows one to at least consider that the protein could play a role in changes such as the blood-brain barrier dysfunction that can predispose some to HIVD. Although increased expression of AQP4 may nonetheless be no more than a marker for HIV infection and HIVD, future studies are warranted to determine whether it may influence disease pathogenesis. Such studies should in turn impact decisions regarding therapeutics that may intentionally or otherwise affect AQP4 function.

Materials and methods

Diagnostic criteria and sample selection

Tissues were obtained from a prospectively followed, clinically characterized, population of patients. The diagnosis of HIVD was made using American Academy of Neurology diagnostic criteria (Janssen *et al*, 1991). The severity staging of HIVD was made according to Memorial Sloan Kettering criteria (Price, 1988). Patients having a score of $\geq$0.5 at death were included in the HIVD group. For additional patient characteristics, including age and CD4 count, see Table 1.

Table 1 Patient information

Case[a]	*Diagnosis*	*Age (years)*	*Sex (h)*	*PMD*	*Cause of death*	*MSK (0.5–3)*	*Dementia progression*	*Neuroimaging (MR unless indicated)*	*Neuropathology change*
229	HIVD	35	M	15	Vacuolar myelopathy	0.5	Medium	Increased T2 signal intensity (2 mo. premortem)	Focal gliosis, microglial nodules
332	HIVD	49	M	13	Brochopneumonia	3	Rapid	Increased long TR signal intensity (5 mo. premortem)	Mild to moderate cortical atrophy, cortical and DWM gliosis
362*	HIVD	46	M	16	Infection with sepsis	2	Rapid	Mild, diffuse atrophy (3 mo. premortem)	Satellitosis and perivascular cuffing
363	HIVD	52	M	12	HIV dementia	3	Rapid	Bilateral WM hyperintensities (4 mo. premortem)	Freeze-thaw artifact
391	HIVD	50	F	6	CMV, MAI, adrenal failure	3	Rapid	Atrophy (CT, 7 mo. premortem)	Microglial nodules, perivascular cuffing
405**	HIVD	39	M	37	CMV, MAI, renal failure	2	Medium		Gliosis, microgliosis, MNGC in BG
407*	HIVD	73	M	13	Respiratory failure	2	Rapid	Atrophy and low attenuation in periventricular DWM (CT, 3 mo. premortem)	Microgliosis, gliosis, vacuolation in cortex
476	HIVD	36	M	12	Extensive Pneumonia	1	Slow		Gliosis and microgliosis
506**	HIVD	34	M	6	*S. aureus* sepsis	3	Rapid	Decreased WM attenuation bifrontally (CT, 1 wk premortem)	Gliosis, MNGC, perivascular cuffing
512**	HIVD	47	M	9	HIV sensory neuropathy	1	Slow	Moderate parenchymal volume loss, no abnormal T1/T2 signal (1 mo. premortem)	No significant findings
527**	HIVD	38	M	12	Sepsis and peritonitis	3	Medium		MNGC, microglial nodules, gliosis
541	HIVD	41	M	21	Acute bronchoneumonia	0.5	Slow	Atrophy, no abnormal signal on T1/T2 (10 days premortem)	No significant findings
542*	HIVD	38	M	24	Acute pulmonary edema	0.5	Slow	Atrophy, small area of increased signal in right frontal lobe (10 mo. premortem)	Mild atrophy in temporal lobes
554*	HIVD	37	M	14	Acute pancreatitis	2	Slow	Diffuse WM hyperintensity bilaterally symmetric, prominent sulci (7 mo. premortem)	Focal gliosis, wm pallor
266*	HIV+	41	F	10	PNP and sepsis				
277*	HIV+	68	F	17	Pseudomonas sepsis			Atrophy, BG calcifications (CT)	
291*	HIV+	32	M	8	Severe sternal infection				
408**	HIV+	52	M	22	Chronic renal insufficiency			Atrophy, no significant T1/T2 changes (4 days premortem)	
489**	HIV+	49	M	20	Lung and adrenal CMV			No signal changes, mild prominence of sulci and ventricles (10 mo. premortem)	
491	HIV+	56	M	12	Pseudomonas pneumonia				
531**	HIV+	9	M	13	Diffuse alveolar damage				
540	HIV+	36	M	5	*S. aureus* pneumonia				
548	HIV+	44	F	28	CHF				
556	HIV+	53	M	17	Pneumonia				
256	HIV−	40	M	8	Respiratory failure			No abnormal attenuation (CT, 7 mo. premortem)	
347	HIV−	49	M	7	PCP and HS pneumonitis				
349*	HIV−	33	F	2	Prescribed medicine overdose				
355	HIV−	7 mo.	M	12	SIDS				
357	HIV−	18	M	12	Gunshot wound				
523*	HIV−	45	M	10	Asthma				
526	HIV−	59	F	18	CHF				
529	HIV−	38	M	11	AMI				
539	HIV−	42	F	22	Neurodegenerative disease				
543	HIV−	61	F	21	Cecal perforation				
544	HIV−	56	M	13	Multiple PE				

[a]HIV Brain Bank identifier.
HIVD: HIV dementia; HIV+: HIV positive nondemented; HIV−: HIV negative controls.
Mean age in HIVD group: 47 y; mean PMD: 13 h; mean CD4 count: 93 (5 unknown).
Mean age in HIV+ group: 49 y; mean PMD: 16 h; mean CD4 count: 48 (4 unknown).
Mean age HIV− group: 40 y; mean PMD: 13 h.
*Cases used only for MFG analysis.
**Cases used only for DWM analysis.

Cell culture
Organotypic cultures were prepared and maintained as described previously (Rothstein *et al*, 1993). Cerebelli of 8-day-old mouse pups and spinal cords from 8-day-old rat pups were collected under sterile conditions and sectioned transversely into 350-μm slices. Slices were cultured in Millicell CM semipermeable culture inserts at a density of two to five slices per well in an incubator at 37°C (5% CO_2, 95% humidity). Under these conditions, >95% of cultures retained cellular organization. Culture medium was changed twice weekly.

Stimuli
Purified thrombin was purchased from Sigma Chemical (St. Louis, MO). TNF-α was purchased from Chemicon (Temecula, CA). The proteins were separated in aliquots and stored frozen.

Preparation of homogenates
Brain tissue homogenates were prepared from approximately 300 mg tissue in 300 μl homogenization buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% sodium dodecyl sulfate [SDS], 1% NP-40, 0.5% sodium deoxycholate, 0.2 mM phenylmethylsulfonyl fluoride [PMSF], 0.5 mM dithiothreitol [DTT]), using an electrical homogenizer for 15 s. The samples were then kept on ice for 20 min and subsequently spun in a desktop eppendorf centrifuge at 4°C and 14,000 rpm for 20 min. Supernatants were then saved, and stored at −80°C, for subsequent analysis. Homogenates from cultured cells were similarly prepared except that 40 μl homogenization buffer was used per organotypic slice and 15-s sonication was substituted for electrical homogenization.

Western blot
Western blot was performed using 30 μg of protein for brain homogenates and 60 μg for cell extracts. Samples were then mixed with 2× Laemmli sample buffer containing 5% 14.3 M β-mercaptoethanol and boiled for 5 min at 95°C. Electrophoresis was performed on a 4% to 15% Tris-glycine polyacrylamide gradient gel (BioRad, Hercules, CA). Following electrophoretic transfer of the protein to a polyvinylidene difluoride (PVDF) membrane (BioRad), the membrane was blocked in 5% nonfat dry milk in TBST (150 mM NaCl, 100 mM Tris base, 0.1% Tween 20, pH 7.6) buffer for 1 h. The blot was then probed with 1:1000 dilution of primary antibody (anti-AQP4, Alpha Diagnostics, San Antonio, TX) for 1.5 h at room temperature. After washing the membrane 3 times (15 min each) in TBST buffer, it was incubated with secondary antibody (peroxidase-conjugated goat anti-rabbit) for 1/2 hour at room temperature. The membrane was then washed again in TBST buffer and immunoreactive bands were visualized using electrochemiluminescence (Amersham). In the experiments using brain homogenates, membranes were stripped in buffer (2% SDS, 62.5 mM Tris-HCl, 100 mM β-mercaptoethanol) for 15 min at 55°C, washed in TBST, and reprobed using a primary antibody to β-actin (Abcam, Cambridge, MA).

Densitometry
Densitometric analysis was performed using NIH imaging software (Image J). Films were scanned, background was subtracted, and pixel densities for each lane were then obtained.

Tissue processing and immunocytochemistry
Tissues were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS), or buffered formalin, and dehydrated in a 30% sucrose/PBS solution for 3 days. Fixed brains were sectioned at 40 μm thickness with a freezing microtome. Sections were stored at −20°C in cryoprotectant solution. Prior to staining, brain sections were rinsed with Tris-buffered saline (TBS) and blocked for 60 min at room temperature in TBS with 5% donkey serum and 0.4% Triton X-100. Sections were then incubated at 4°C for 48 h with anti-AQP4 (Alpha Diagnostics), washed, and incubated at room temperature with a biotinilated secondary antibody for 3 h. Sections were again washed, and then incubated with ABC reagent according to the manufacturer's instructions (Vector). The immune reaction was then visualized following incubation with diaminobenzoate (DAB) (Vector).

Statistical analysis
Statistical evaluation was done using the Student's *t* test with $P < .05$ considered to be significant. All data presented are given as means ± SE.

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