Int J Legal Med (2011) 125:59–65
DOI 10.1007/s00414-010-0523-8

ORIGINAL ARTICLE

Immunohistochemical examination of intracerebral aquaporin-4 expression and its application for differential diagnosis between freshwater and saltwater drowning

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Received: 1 August 2010 / Accepted: 18 October 2010 / Published online: 11 November 2010
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Abstract Human brain samples were collected from 70 autopsy cases including 22 freshwater drowning (FWD), 26 saltwater drowning (SWD), and 22 non-drowning cases as controls. Then, immunohistochemical study combined with morphometry was carried out in order to examine the differential expression of AQP1 and AQP4 in the brain samples. Immunohistochemically, star-shaped cells bearing highly branched processes, often surrounding blood vessels, showed positive reactions for AQP1 and AQP4 in FWD, SWD, as well as control groups. Additionally, with double-color immunofluorescence analysis, AQP1- or AQP4-positive cells could be identified as GFAP-positive astrocytes. Moreover, AQP1-positive reaction was also observed in blood vessels. Morphometrically, there were no significant differences in AQP1 expression in astrocytes or in blood vessels among the three groups. In contrast, the average value of AQP4-positive astrocytes was significantly higher in FWD cases than in SWD and control groups. Moreover, AQP4 expression was significantly lower in SWD than in the control group ($p<0.05$). Moreover, there was no significant correlation between post-submerged interval and AQP expression in drowning cases. Therefore, immunohistochemical analysis of intracerebral AQP4 expression would be forensically useful for differentiation between FWD and SWD.

Keywords Drowning · Immunohistochemistry · Aquaporin-1 · Aquaporin-4 · Astrocytes

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Introduction

The examination of immersed bodies is one of the most important aspects of forensic practice, and forensic pathologists are often required to definitely diagnose drowning. At present, the combination of autopsy and histopathological examination, as well as diatom test and toxicological analyses, is routinely performed for diagnosis of drowning [1–3]. Furthermore, when drowning is diagnosed, further differentiation is also an indispensable aspect of forensic practice with respect to determination of freshwater drowning (FWD) or saltwater drowning (SWD). Therefore, many macroscopic, ultrastructural, and biochemical forensic studies on differentiation between FWD and SWD have been performed [4–10].

Recently, several water channel proteins that can regulate osmolarity throughout the body have been cloned [11, 12]. Aquaporins are small, integral membrane proteins (MW ~30,000) that provide a major pathway for water transport in many cell types in fluid-transporting tissues such as kidney, lung, and brain [12], and 13 members (AQP0–12) have so far been identified in mammals [11, 12]. AQP1 and AQP4 are the main water channel proteins in the brain [13], which are rapidly induced by various types of stimuli such as osmolarity [14, 15] and chemical or mechanical stress [16–18]. AQP1 and AQP4 are presumed to be involved in brain neuropsychiatric diseases such as Alzheimer's disease, multiple sclerosis, and schizophrenia [15, 19]. AQP1 and AQP4 appear to be part of mechanisms of cerebral volume regulation following ischemia, trauma, tumors, inflammation, and metabolic disturbances [20, 21]. Recently, we examined AQP expression in several human organs from a forensic perspective and found that AQPs are suitable as

markers for differentiation between FWD and SWD [22, 23]. In the present study, we immunohistochemically examined AQP1 and AQP4 expression in human brain and discussed its suitability for postmortem differentiation between FWD and SWD

Materials and methods

Antibodies

The following polyclonal antibodies (pAbs) were used for immunohistochemical and double-color immunofluorescence analyses in the present study: rabbit anti-human AQP1 pAbs, rabbit anti-human AQP4 pAbs and goat anti-human GFAP pAbs, and goat anti-human CD31 pAbs (Santa Cruz Biotechnology Inc., CA, USA).

Autopsy samples

A total of 70 human forensic autopsy cases (41 men and 29 women) with a postmortem interval of less than 72 h were selected from autopsy documents (Table 1). The post-submerged intervals ranged from 0.5 to 30 h. The individual ages ranged from 5 months to 90 years (mean age: 61.1 years). In each case, the cause of death was carefully diagnosed on the basis of complete autopsy, histology, toxicology, and diatom test. Cases were divided into three groups as follows: 22 FWD, 26 SWD, and 22 others, including six fire fatality cases, three traumatic shock cases, and one each of acute brain injury, sharp instrument injury, acute carbon monoxide poisoning, acute drug poisoning, hypothermia, asphyxia, subarachnoid hemorrhage, ischemic heart disease, sepsis, interstitial pneumonia, cervical vertebra fracture, aorta rupture, and cervical spinal cord injury. In accordance with the previous study [24], the brain samples were taken from the arterial boundary zone of the parieto-occipital lobe.

Immunohistochemical analyses

Brain samples were fixed in 4% paraformaldehyde solution with phosphate-buffered solution (PBS; pH 7.2), embedded in paraffin and sectioned (4–6 μm thick). After deparaffinization, sections were immersed in 0.3% H_2O_2–PBS for 30 min to block endogenous peroxidase activity and incubated overnight with rabbit anti-AQP1 pAbs (1:1,000) or rabbit anti-AQP4 pAbs (1:1,500) in PBS containing 1% normal serum corresponding to the secondary IgG and 1% bovine serum albumin (BSA) at 4°C. Thereafter, Envision$^+$ (Dako Cytomation, Kyoto, Japan) for rabbit immunoglobulin was added and incubated at room temperature for 1 h, and positive reactions were visualized with 3,3′-diamino benzidine tetrahydrochloride, followed by counter-staining with hematoxylin.

Double-color immunofluorescence analysis

To determine the cell types with AQP1 and AQP4 expression, double-color immunofluorescence analysis was performed. As described previously [25, 26], deparaffinized sections were blocked with PBS containing 1% normal donkey serum and 1% BSA to diminish nonspecific reactions. Thereafter, the sections were further incubated with pairs of anti-GFAP pAbs (1:25) and anti-AQP1 pAbs (1:100), anti-GFAP pAbs (1:25) and anti-AQP4 pAbs (1:100), anti-CD31pAbs (1:100) and anti-AQP1 pAbs (1:100), or anti-CD31pAbs (1:100) and anti-AQP4 pAbs (1:100) at 4°C overnight. After incubation with FITC-conjugated anti-goat IgG pAbs (1:50) and Cy3-conjugated anti-rabbit IgG pAbs (1:100) at room temperature for 1 h, the slides were observed under a fluorescence microscope. Fluorescent images were digitally merged.

Morphometrical analysis

According to the methods of previous studies [25, 26], morphometrical analysis was performed for semi-quantitative evaluation of immunohistochemical findings. Briefly, in each section, ten high-power fields (×400) were randomly selected. AQP1- or AQP4-positive star-shaped cells, of which nuclei were distinctly stained with hematoxylin, were evaluated under the high-power magnification (×400), and the number of AQP1- or AQP4-positive astrocytes was counted in each field. The average of

Table 1 Human forensic autopsy cases (41 men and 29 women) with a postmortem interval of less than 72 h

Cause of death	*N*	M/F	Age (years)		PMI (h)		PSI (h)	
			Range	Mean	Range	Mean	Range	Mean
FWD	22	13/9	0.5–90	65.6	5–72	26.8	0.5–24	8.1
SWD	26	13/13	23–80	57.5	11–72	31.9	0.5–30	10.8
Control	22	15/7	26–87	60.7	6–60	21.1		
Total	70	41/29	0.5–90	61.1	5–72	27.8	0.5–30	9.5

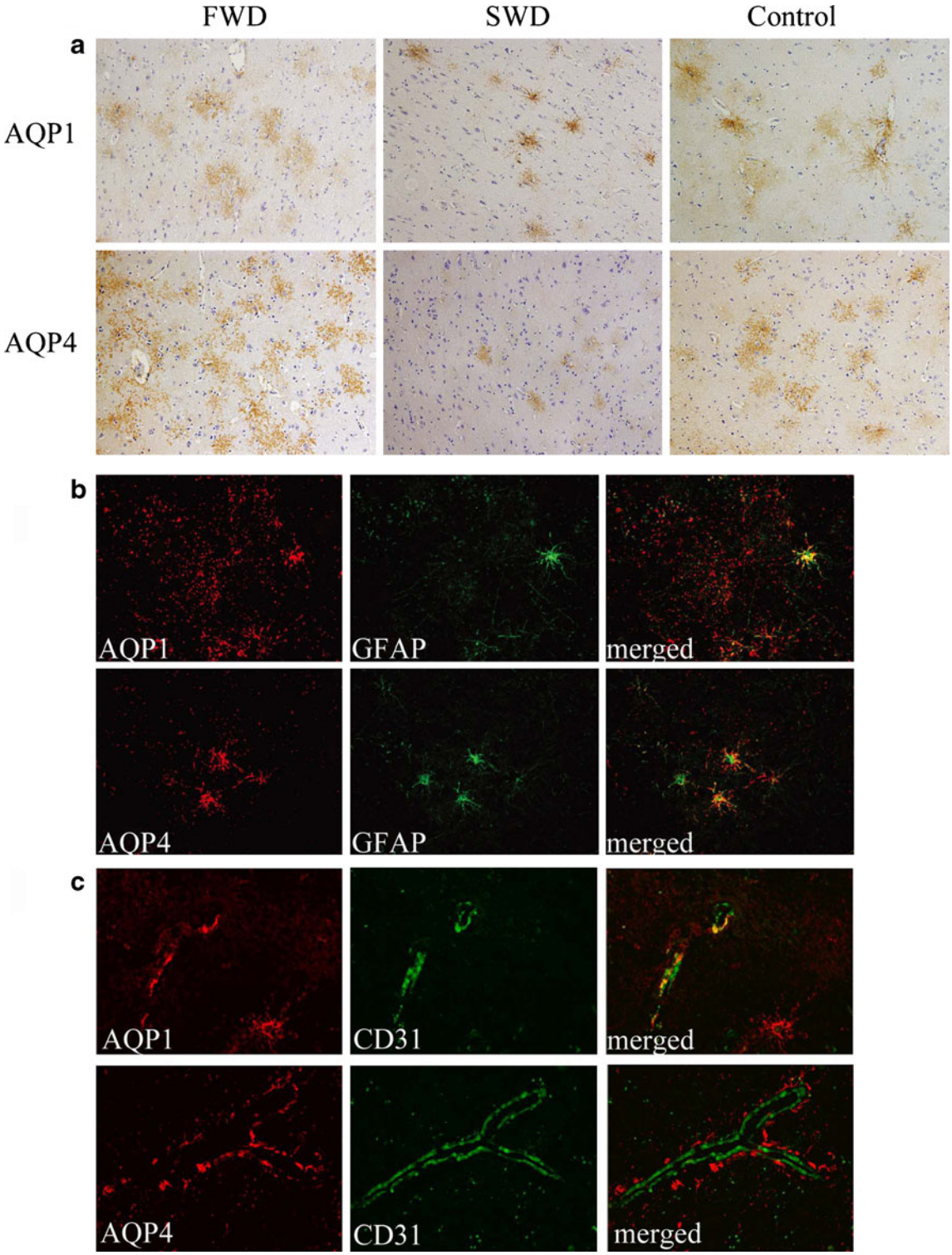

Fig. 1 **a** Immunohistochemical analyses of AQP1 and AQP4 expression in the human brain (original magnification, ×200). Representative results from 22 FWD, 26 SWD, and 22 control cases are shown. **b**, **c** Double-color immunofluorescence analyses indicated cell types with AQP1 or AQP4 expression. Images are shown using anti-AQP1, anti-AQP4, anti-GFAP, and anti-CD31 and labeled at the *bottom left of each panel*. Signals were digitally merged in the *right panel of each row* (original magnification, ×400). *FWD* freshwater drowning, *SWD* saltwater drowning

ten selected fields was evaluated as AQP1 or AQP4 expression. Moreover, the average of $AQP1^+$ and $CD31^+$ blood vessels was also counted in ten selected fields (×400). These analyses were performed by two investigators with no prior knowledge of the samples.

Statistical analyses

The means and standard errors of means (SEM) were calculated for all parameters determined in this study. Comparison of means was performed using one-way analysis of variance to determine whether differences existed among the group means, followed by Scheffé's F test to identify significantly different means. Correlation analysis was performed using nonparametric Spearman's correlation coefficient; $p<0.05$ was considered as significant.

Results

Expression of AQP1 and AQP4 in astrocytes

In the autopsy brain samples, AQP1 and AQP4 were intensely expressed on star-shaped cells bearing highly branched processes (probably astrocytes), often surrounding blood vessels in FWD, SWD, and control groups (Fig. 1a). Next, to determine the cell types of AQP1 or AQP4 expression, we performed double-color immunofluorescence analysis using anti-GFAP, a specific marker for astrocytes, and anti-AQP1 or anti-AQP4 antibodies. As shown in Fig. 1b, AQP1-positive star-shaped cells could be identified as GFAP-positive astrocytes. Similarly, most AQP4-positive star-shaped cells also showed a positive signal for GFAP. Taken together, these findings demonstrated that AQP1- and AQP4-expressing cells could be

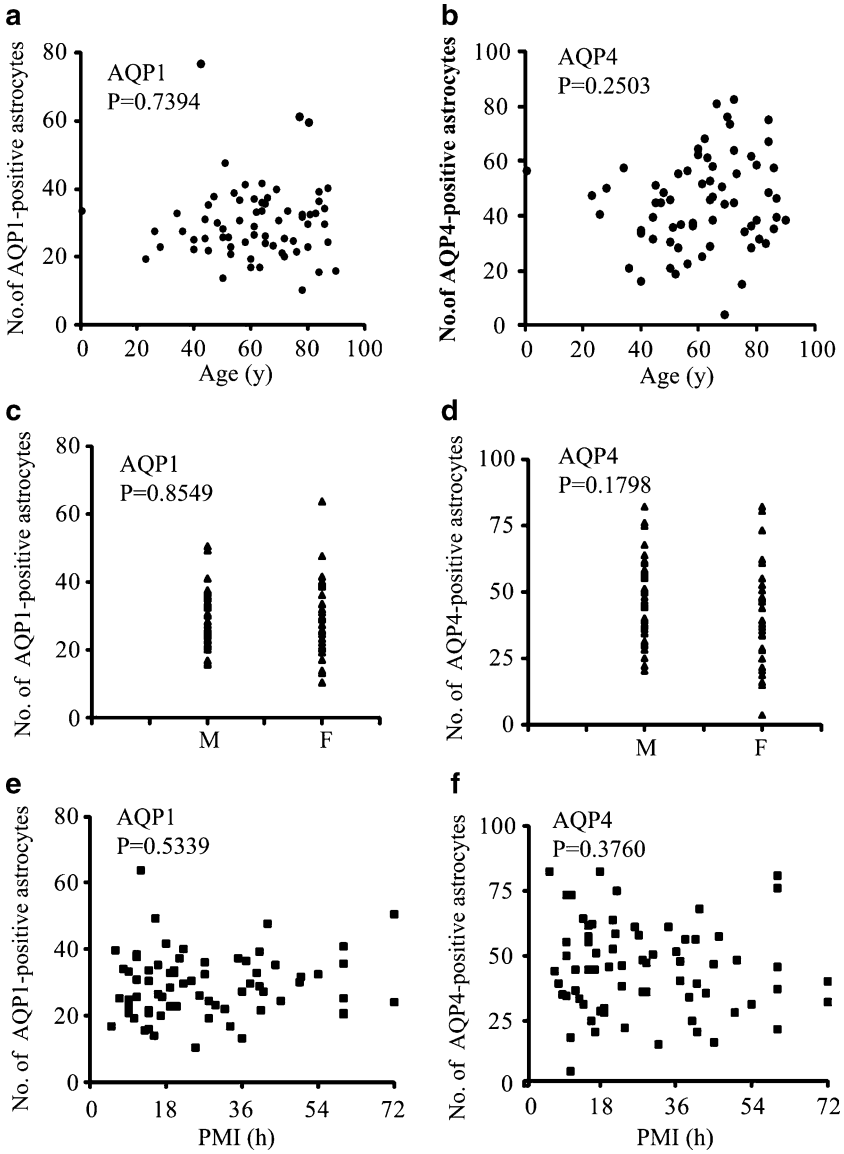

Fig. 2 **a**, **b** The relationship between age and AQP expression in all cases. **c**, **d** The relationship between gender and AQP expression in all cases. **e**, **f** The relationship between postmortem interval and AQP expression in all cases. These results were obtained with nonparametric Spearman's correlation analysis. *M* male, *F* female, *PMI* postmortem interval

identified as GFAP-positive astrocytes. Moreover, AQP1 expression was also observed in CD31-positive blood vessels in all samples (Fig. 1c).

Morphometrical analyses

In double-color immunofluorescence analyses, $GFAP^+$ cells with positive reactions for AQP1 and/or AQP4 looked like star-shaped cells, indicating that astrocytes expressed AQP1 and AQP4. Thus, in morphometrical analyses, $AQP1^+$ or $AQP4^+$ star-shaped cells, of which nuclei were distinctly stained with hematoxylin, were evaluated as astrocytes. There was no significant dependence on age, gender, or postmortem interval within 72 h for intracerebral AQP expression (Fig. 2a–f). Semi-quantitative analysis revealed that the average of AQP1-positive astrocytes showed no significant differences among FWD, SWD, and control groups (mean±SEM, 25.5±2.10 vs. 31.1±2.1 vs. 30.8±1.5, $p>0.05$; Fig. 3a). Similarly, both AQP1- and CD31-positive blood vessels had no significant differences among the three groups (FWD vs. SWD vs. control, 8.0±1.0 vs. 7.5±0.6 vs. 5.7±0.4, $p>0.05$; Fig. 3c). In contrast, the average of AQP4-positive astrocytes was significantly increased in FWD compared with those in SWD and control groups (FWD vs. SWD, 56.1±4.2 vs. 33.6±2.4, $p<0.005$; FWD vs. control, 56.1±4.2 vs. 45.2±1.8, $p<0.05$). On the other hand, the average value of AQP4-positive astrocytes was lower in SWD than in the control group ($p<0.05$; Fig. 3b). Moreover, there was no significant correlation between post-submerged interval and AQP1 or 4 expressions ($p>0.05$, Fig. 3d–e).

Discussion

In the brain, three different aquaporins (AQP1, 4, 9) have been identified [12, 27], of which AQP1 was found to be expressed at the apical membrane of choroid plexus, endothelial cells of blood vessels, under physiological conditions in several mammalian species and in humans; whereas, AQP1 was induced in astrocytes under several pathological conditions. In addition, AQP1 was implicated in cerebrospinal fluid production [27]. AQP4 is expressed on astrocyte cells and ependymal and pial surfaces and is responsible for brain water transport [12, 27]. AQP9 has been reported to be present on astrocytes and on sub-populations of neurons and is implicated in brain energy metabolism [27]. In this study, AQP1 was found to be expressed on astrocyte foot processes and blood vessels. AQP4 was expressed on astrocyte foot processes, ependymal cells, and pial surfaces that was consistent with previous observations. Moreover, in our study, the average value of intracerebral AQP4-, but not AQP1-positive astrocytes, was affected by osmotic stress; therefore, we focused on the expression of AQP4 in drowning cases.

Astrocytes were recognized as one of the cellular components in the brain and play a critical role in maintaining fluid and osmolarity of the brain extracellular space [28]. In the present study, an intensive positive reaction for AQP4 was found on the astrocyte foot processes and blood vessels. Several lines of accumulating evidence have demonstrated that AQP4 expression on astrocytes is responsible for osmotic water permeability in vivo and in vitro [19, 29, 30]. Thus, it is considered that

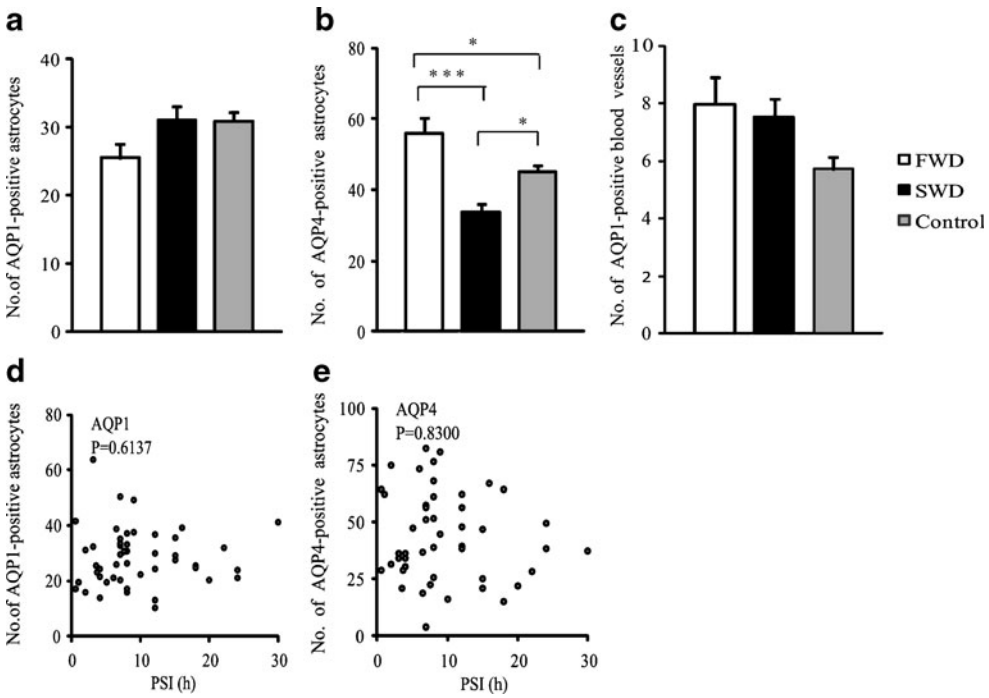

Fig. 3 The average values of **a** AQP1- and **b** AQP4-positive astrocytes in all brain samples were calculated and are shown here. **c** The average of AQP1-positive blood vessels in all cases. Morphometrical analysis was performed as described in the "Materials and methods" section. All values represent the means±SEM. *$p<0.05$, ***$p<0.005$. **d**, **e** The effects of post-submerged interval (PSI) on **d** AQP1 and **e** AQP4 expression in drowning cases. Statistical analyses were performed using nonparametric Spearman's correlation analysis

AQP4 expression in astrocytes is more bioactive during osmotic stress. Thus, in the present study, we evaluated AQP4-positive astrocytes.

In transgenic mice, increased AQP4 expression in glial cells increased osmotic water permeability and brain water content following acute water intoxication [29]. Moreover, increased AQP4 protein expression was observed in rat brain following systemic hyponatremia [14]. Consistently, in this study, we observed that AQP4 expression in astrocytes was increased in FWD. In addition, increased AQP4 expression was due to conformational changes or gating of the channel (passive water transport) [27, 31], but not due to increased mRNA or protein synthesis or recycling [14, 27]. It is well-known that FWD can cause hypervolemia, marked hemodilution, hemolysis, and the decrease of serum electrolytes, except potassium, by the transportation of hypotonic water into microvessels [32, 33]. From the location and functional role of AQP4, hypotonic water was apparently transported through AQP4 in an osmolarity-dependent manner in FWD. Thus, it is considered that AQP4 expression in astrocytes was significantly upregulated by hypotonic water to prevent hemodilution as a vital reaction in FWD.

In contrast, decreased AQP4 expression in astrocytes was observed in the SWD group. This observation may be supported by evidence that AQP4 expression in astrocytes was decreased in response to 10% hypertonic saline osmotherapy in ischemic cerebral edema in rat [34]. This finding further supports our observation of decreased AQP4 expression in astrocytes in SWD compared with that in FWD. Decreased expression of AQP4 in astrocytes may be a protective mechanism to prevent astrocyte shrinkage in SWD.

In forensic practice, the differentiation between FWD and SWD is important to evaluate immersed bodies. There have been many studies on the differentiation of FWD and SWD [4–10]. Our previous study demonstrated that intrapulmonary AQP5 or intrarenal AQP2 expression was suitable for the differentiation between FWD and SWD [22, 23]. In the present study, immunohistochemical detection of AQP4 expression in the brain was suitable for distinguishing FWD and SWD. In terms of forensic applicability, combined analysis of AQP expression from multiple fluid-transporting organs would give more accurate and objective information for differential diagnosis of drowning.

In the use of forensic samples, degradation due to postmortem interval is always taken into consideration. In the present study, there was no significant dependence of AQP expression on postmortem interval within 72 h, which was consistent with our previous studies with antibodies for VEGF, ubiquitin, IL-1α, IL-8, MCP-1, and MIP-1α in skin wound healing [35–38]. This may suggest that postmortem interval within 72 h had less influence on protein immunoreactivities in autopsied samples.

In summary, our results suggest that AQP4 at least has potential as a marker to differentiate between FWD and SWD. Thus, combined analysis of AQP expression from multiple organs and other reported markers should be carried out in order to more accurately differentiate between FWD and SWD.

Acknowledgments We sincerely thank Ms. Mariko Kawaguchi for her excellent assistance in preparing this manuscript. This study was financially supported in part by a Grant-in-Aid for Scientific Research (A) from the Ministry of Education, Culture, Sports, Science, and Technology of the Japanese Government.

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