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ORIGINAL ARTICLE

Differential diagnosis between freshwater drowning and saltwater drowning based on intrapulmonary aquaporin-5 expression

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Abstract The intrapulmonary expression of aquaporin-5 (AQP5) was examined in an experimental drowning model and forensic autopsy cases to discuss the possibility for differentiation between freshwater drowning (FWD) and saltwater drowning (SWD). In animal experiments, mice were classified into four groups: (group I: FWD; group II: SWD; group III: postmortem immersion (PI); and group IV: cervical dislocation as controls. In group I, intrapulmonary AQP5 expression was significantly suppressed at both gene and protein levels, compared with the other three groups, and there was no significant difference in AQP5 expression among groups II to IV. In the next series, we examined AQP5 gene expression in human lung samples obtained from forensic autopsies at less than 48 h postmortem (nine FWD cases, five SWD cases, and 14 other cases). Although AQP5 mRNA could be detected in all lung samples under the employed experimental conditions, the intrapulmonary gene expression of AQP5 in FWD was significantly attenuated compared with the other groups. These observations imply that AQP5 expression in type I alveolar epithelial cells was suppressed by hypotonic water to prevent hemodilution from the physiological aspect. Moreover, the analysis of intrapulmonary AQP5 expression would be forensically useful for differentiation between FWD and SWD, or between FWD and PI.

Keywords Forensic molecular pathology · Drowning · Aquaporin-5

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Introduction

In forensic autopsy examination of immersed bodies, forensic pathologists have to carefully diagnose the cause of death because immersed bodies have not always died from drowning. At present, postmortem diagnosis of drowning is based on autopsy findings such as froth in the air passages, ballooning of the lungs, and pleural effusion, along with the detection of diatoms from multiple organs [1]. However, those findings and the diatom test are just supportive but not definitive for diagnosing drowning [1–3]. Moreover, it is also important to make a differential diagnosis between freshwater drowning (FWD) and saltwater drowning (SWD) [3]. Conventionally, there are macromorphological differences in the amounts of pleural fluid and lung weights (saltwater > freshwater) between FWD and SWD [4, 5]. Ultrastructural alterations of the lungs in FWD and SWD cases were also analyzed in experimental drowning models [6–8]. The changes in FWD seemed to be more predominantly osmotic in nature, as evidenced by severe cellular disruption, mitochondrial swelling, and endothelial destruction. On the contrary, vacuole formation, and discontinuity of alveolar lining cells were found in SWD, but not FWD. Moreover, several lines of accumulating evidence have indicated the evaluation of blood serum electrolyte concentrations, atrial natriuretic peptide, iron (Fe), or strontium [9–13], or the use of immunohistochemical techniques [14, 15]; however, there is no reliable technique for forensic practice.

Recently, several water channel proteins that can regulate osmolarity in the whole body have been cloned [16–20]. Aquaporins (AQPs) are a family of small (~30 kDa/monomer), homologous water-transporting proteins [18–20], and 13 members having been identified so far in mammals. In mammals, they are expressed in many

epithelial and endothelial cells involved in fluid transport, as well as in other cells that are thought not to carry out fluid transport, such as keratinocytes, adipocytes, and astroglias. AQP5, a major water channel in the lungs, is expressed in alveolar, tracheal, and upper bronchial epithelium, and regulates lung water homeostasis. A recent study demonstrated that hypertonic stress induced AQP5 expression in murine lung epithelial cell lines in vitro [21]. This led us to assume that the aspiration of hypertonic/hypotonic water during drowning might affect intrapulmonary AQP5 expression. In the present study, we investigated intrapulmonary AQP5 expression in an experimental drowning model and forensic autopsy cases and discussed its suitability for differentiation between freshwater drowning and saltwater drowning.

Materials and methods

Animal experiments

Animals

Specific pathogen-free 8- to 10-week-old male BALB/c mice were obtained from Sankyo Laboratories (Tokyo, Japan). All mice were bred and housed in a temperature-controlled (23±2°C) environment with a 12-h light/dark cycle. They were fed with standard feed and given water ad libitum. All animal experiments were approved by the Committee on Animal Care and Use of Wakayama Medical University.

Experimental drowning model

An experimental drowning model was established as described previously [22]. After mice were anesthetized with an intraperitoneal injection of pentobarbital (50 μg/g body weight), a cervical midline incision was made and the trachea was exposed. Thereafter, freshwater or saltwater was administered intratracheally with a 26-gauge needle. The mice were classified into four groups (n=8 in each group):

Group I (FWD model): administration of distilled water (30 ml/kg) at 1 ml/min.

Group II (SWD model): administration of 3.5% NaCl (30 ml/kg) at 1 ml/min.

Group III (Postmortem immersion model, PI): mice were killed by cervical dislocation followed by the administration of 3.5% NaCl.

Group IV: mice were killed by cervical dislocation as a control.

The survival period, the time between the beginning of fluid instillation and cardiac arrest, was shorter than 3 min, 30 min after death, the lungs were removed and stored at −80°C until RNA or protein extraction.

Autopsy samples

Lung samples were obtained from 28 human forensic autopsy cases at less than 48 h postmortem (18 men and 10 women). The post-submerged intervals ranged from a few to 24 h. The age of the autopsy cases ranged from 26 to 83 years old (median 58.3). In each case, the cause of death was carefully diagnosed based on macroscopic, histopathological, and toxicological findings and a diatom test. Cases were divided into three groups as follows: nine FWD, five SWD, and 14 others including four traumatic shock, three hemorrhagic shock/exsanguination, three acute carbon monoxide poisoning, and one each of strangulation, brain stem laceration, acute chloroform poisoning, and subarachnoid hemorrhage. Tissue samples were taken from different lung lobes during autopsy, immediately frozen in liquid nitrogen, and stored at −80°C until use.

Immunohistochemical analyses

Lung samples obtained from group IV were fixed in 4% formaldehyde buffered with phosphate-buffered saline (PBS; pH 7.2), and embedded in paraffin, followed by sectioning (4–6 μm thick). After deparaffinization, the sections were immersed in 0.3% H_2O_2-PBS for 30 min and incubated with PBS containing 1% normal goat serum and 5% BSA to reduce nonspecific reactions and with rabbit anti-AQP1 or rabbit anti-AQP5 polyclonal antibodies (pAbs) (Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C, overnight. Thereafter, sections were incubated with Envision+ (DAKO, Kyoto, Japan) for rabbit immunoglobulin at room temperature for 1 h, and positive signals were visualized with diaminobenzidine, followed by counter-staining with methyl green.

Extraction of total RNAs and semi-quantitative RT-PCR

To examine the gene expressions of AQP1 and AQP5 in both murine and human lung samples, semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) analyses were performed as described previously [23]. Total RNA was extracted from lung samples using ISOGENE (Nippon Gene, Toyama, Japan) according to the manufacturer's instructions: 5 μg of total RNA were reverse-transcribed at 42°C for 1 h in 20 μl reaction mixture containing mouse Moloney leukemia virus reverse transcriptase (Toyobo, Osaka, Japan) with oligo (dT) primers (American-Pharmacia Biotech Japan, Tokyo, Japan). Thereafter, cDNA was amplified together *Taq* polymerase (Nippon Gene) using specific sets of primers for AQP1,

AQP5, and β-actin (Table 1). PCR amplification of each gene was conducted with optimal cycles consisting of 94°C for 1 min, optimal annealing temperature for 1 min, and 72°C for 1 min, followed by incubation at 72°C for 3 min. PCR products were fractionated on a 2% agarose gel and visualized by ethidium bromide staining. Band intensities were measured using Image Analysis software (Scion Image, Fredrick, MD, USA) and the ratios of AQP1 and AQP5 to β-actin calculated.

Western blotting analysis of the membrane-rich fraction

To examine the protein content of AQP1 and AQP5 in murine lung samples, Western blotting analyses were performed. Samples were homogenized in ice-cold buffer (300 mM mannitol, 12 mM Hepes-Tris, Ph 7.4) containing complete protease inhibitor cocktails (Roche, Tokyo, Japan). After centrifugation at 1,000×*g* for 10 min, a pellet was prepared by centrifugation at 17,000×*g* for 45 min. The membrane pellet was then resuspended in fresh ice-cold buffer containing 0.5% CHAPS. Samples (40 µg/lane) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrotransferred onto nitrocellulose membranes. After immersion in the blocking buffer (10 mM Tris–HCl, pH 7.4 containing 0.5 M NaCl, 5% skimmed milk, and 0.5% Tween-20), the membranes were incubated with rabbit anti-AQP1, rabbit anti-AQP5, or -β-actin pAbs at 4°C overnight. After incubation with Envision anti-rabbit antibody for 30 min at room temperature, antigen–antibody complexes were detected using the ECL-Western blotting detection system (Amersham Bioscience Japan, Tokyo, Japan), according to the manufacturer's instructions. Band intensities were measured using Image Analysis software (Scion Image, Fredrick, MD, USA) and the ratios of AQP1 and AQP5 to β-actin calculated.

Statistical analysis

The means and SEMs were calculated for all parameters determined in this study. Statistical significance was evaluated by one-way analysis of variance (ANOVA) followed by Scheffe's *F* test. A value of $p<0.05$ was accepted as significant.

Results

Immunohistochemical analysis of AQP in murine lung

First, we examined the distribution of AQP1 and AQP5 in the untreated murine lung. In line with previous observations [16–18], AQP1 was expressed in vascular endothelial cells (Fig. 1a) and AQP5 could be detected in type I alveolar epithelial cells and bronchial epithelial cells (Fig. 1b). In human lung samples, similar results could be observed.

Freshwater drowning suppressed intrapulmonary AQP5 expression in mice

In group IV, gene expressions of AQP1 and AQP5 could be detected under the experimental conditions (Fig. 2a). There was no significant difference in the AQP1 gene between the four groups (Fig. 2b). Although AQP5 mRNA was detected to similar extents in groups II–IV, the gene expression of

Table 1 Sequences of the primers used for RT-PCR

Transcript	Sequence	Annealing temperature(°C)	Cycles	Product size(bp)
Mouse				
AQP1	(F) 5'-TGCGTTCTGGCCACCACTGAC-3' (R) 5'-GATGTCGTCAGCACATCCAGGTC-3'	62	34	327
AQP5	(F) 5'-CTCTGCATCTTCTCCTCCAC-3' (R) 5'-TCCTCTCTATGATCTTCCCAG-3'	62	34	334
β-actin	(F) 5'-TTCTACAATGAGCTGCGTGTGGC-3' (R) 5'-CTCATAGCTCTTCTCCAGGGAGGA-3'	60	32	456
Human				
AQP1	(F) 5'-CCCTCTTTGTCTTCATCAGCATCGGTTC-3' (R) 5'-ATGTCGTCGGCATCCAGGTCATACTC-3'	55	38	715
AQP5	(F) 5'-CTTCCTCAAGGCCGTGTTC-3' (R) 5'-GCTGGAAGGTCAGAATCAGC-3'	55	38	398
β-actin	(F) 5'-TTCTACAATGAGCTGCGTGTGGC-3' (R) 5'-CTCGTAGCTCTTCTCCAGGGAGGA-3'	60	32	456

F forward primer, *R* reverse primer

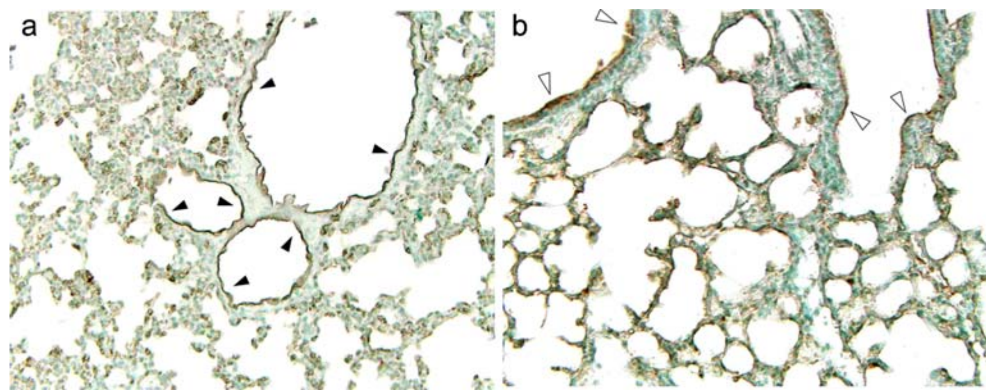

Fig. 1 Immunohistochemical analyses of AQP1 (**a**) and AQP5 (**b**) in the murine lung (original magnification, ×200). Representative results from eight individual animals are shown. Black arrowheads indicate AQP1-positive vascular endothelial cells and white arrowheads indicate AQP5-positive bronchial epithelial cells

AQP5 in group I was significantly attenuated compared with groups II–IV (Fig. 2a). Semi-quantitative analysis revealed that the ratio of AQP5 to β-actin in group I was significantly reduced, compared with the other three groups (Fig. 2c). Consistently, intrapulmonary protein expression of AQP5 but not AQP1 was significantly suppressed in FWD compared with the other three groups (Fig. 3a–c). These observations implied that hypotonic stress would suppress AQP5 but not AQP1 expression.

Intrapulmonary AQP5 gene expression was attenuated in FWD cases

In the next series, we examined the gene expression of AQP1 and AQP5 in lung samples obtained from forensic autopsy cases. The mRNA of AQP1 and AQP5 could be detected in all lung samples examined (data not shown). There was no significant dependency on age, gender, or postmortem interval within 48 h for intrapulmonary AQP gene expression (data not shown). Consistent with animal experiments, the ratios of AQP5 to β-actin in FWD cases was significantly suppressed compared with SWD and other cases (Fig. 4).

Discussion

Several lines of accumulating evidence have demonstrated that six different AQPs (AQP1, 3, 4, 5, 8, and 9) are expressed in the airways and lung of both humans and mice. In particular, AQP1 and AQP5 are expressed in the apical membrane of the microvascular endothelium, and type I alveolar epithelial cells, respectively [16–18], consistent with our results. AQP3 and AQP4 are expressed in the basolateral membrane of basal cells of the tracheal epithelium, and in the basolateral membrane of columnar cells in bronchi and trachea, respectively [16–18]. Although the expressions of both AQP8 and AQP9 could be observed, their precise location and functional role remain unclear [24, 25]. It is easily accepted that water aspiration may affect AQP expression in the lungs. In our study, intrapulmonary expression of AQP5, but not AQP1, was affected; thus, we focused on the expression of AQP5 in drowning cases.

AQP5 is localized in submucosal glands of the respiratory tract, salivary and lacrimal gland epithelia, and corneal epithelium, as well as in the apical membrane of type I alveolar epithelial cells [16–18]. Recently, mice lacking

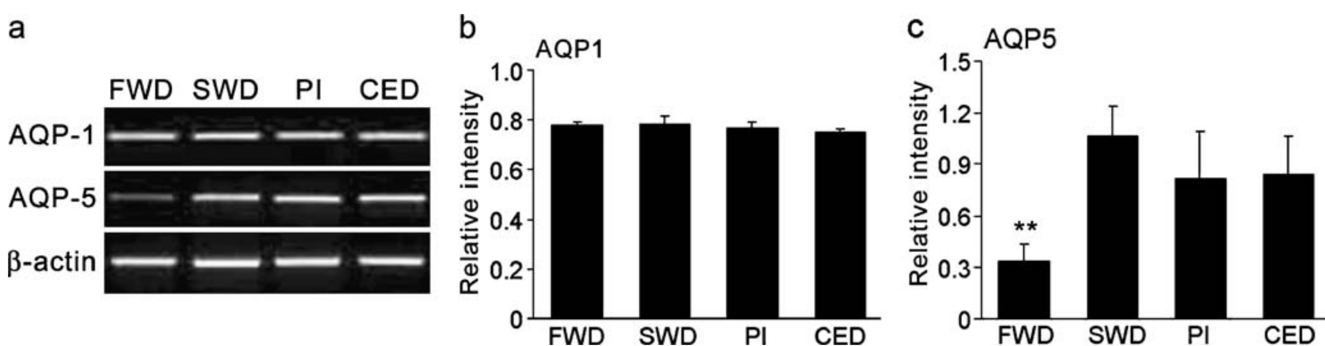

Fig. 2 **a**–**c** RT-PCR analyses of gene expressions of AQP1 and AQP5 in murine lungs. Representative results from eight individual animals are shown in **a**. Ratios of AQP1 (**b**) and AQP5 (**c**) to β-actin were calculated and shown here. All values represent the means ± SEM (n= 8). ** p<0.01 vs other groups. *FWD* freshwater drowning, *SWD* saltwater drowning, *PI*: postmortem immersion, *CED* cervical dislocation (control)

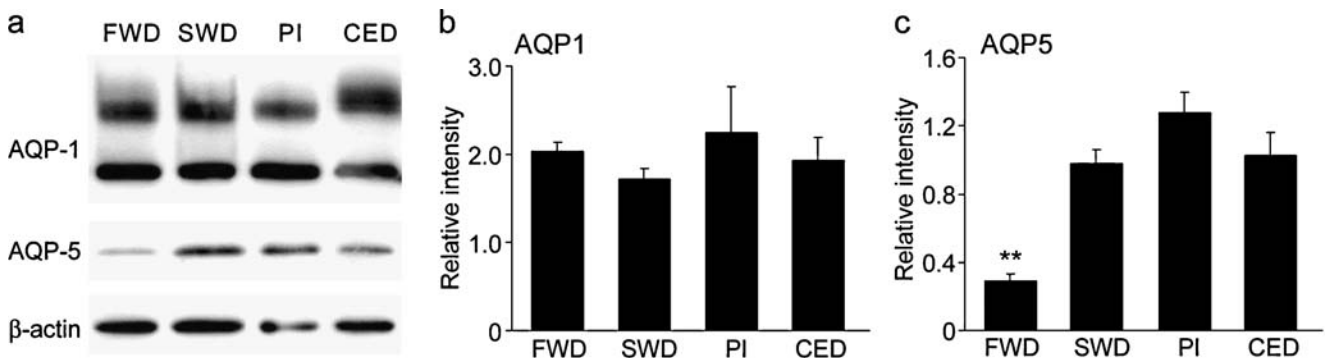

Fig. 3 **a**–**c** Western blotting analyses of AQP1 and AQP5 in the murine lung. Representative results from eight individual animals are shown in **a**. Ratios of AQP1 (**b**) and AQP5 (**c**) to β-actin were calculated and shown here. All values represent the means±SEM ($n=8$). $^{**}p<0.01$ vs other groups. *FWD* freshwater drowning, *SWD* saltwater drowning, *PI* postmortem immersion, *CED* cervical dislocation (control)

AQP5 have been developed to investigate its pathophysiological function. The absence of AQP5 significantly reduced saliva fluid secretion in mice [26]. AQP5-deficient mice exhibited a marked reduction of osmotic water permeability across the alveolar epithelium (passive water transport) [27]. FWD causes hypervolemia, marked hemodilution, hemolysis, and decrease of serum electrolytes, except for potassium, by transportation of hypotonic water into microvessels through type I alveolar epithelial cells [28, 29]. From the localization and functional role of AQP5, hypotonic water was apparently transported through AQP5 in an osmolarity-dependent manner in FWD. Thus, it was considered that AQP5 expression in type I alveolar epithelial cells was suppressed by hypotonic water to prevent hemodilution as a vital reaction in FWD.

Several lines of evidence suggest that AQP is induced by hypertonic stress in vitro and in vivo [21, 30]. In particular, Hoffert et al. demonstrated that AQP5 expression was upregulated in murine lung epithelial cell lines by hypertonic stress [21]. Moreover, it is well-known that SWD can cause hypernatremia and hyperchloremia by an increase of water transportation from microvessels to interstitial spaces. In line with these observations, we hypothesized that intrapulmonary AQP5 expression would be upregulated in SWD. In contrast with our expectations, there was no change in the intrapulmonary AQP5 expression in the SWD model using mice. This discrepancy can be explained by evidence that hyperosmotic stress-inhibited total DNA synthesis, RNA transcription, and protein synthesis [31].

In forensic practice, differentiation between FWD and SWD is important to diagnose the death of immersed bodies because bodies found in the sea have not always died from saltwater drowning. There have been many macroscopic, ultrastructural, and biochemical forensic studies on the differentiation of FWD and SWD [4–15]. At present, molecular biological techniques have been developed, and widely distributed to the field of medical sciences. Thus, the application of molecular techniques may make forensic diagnosis more objective and corrective. Although the degradation of mRNA or DNA due to postmortem intervals has to be taken into consideration, several lines implied the practical availability of examination at

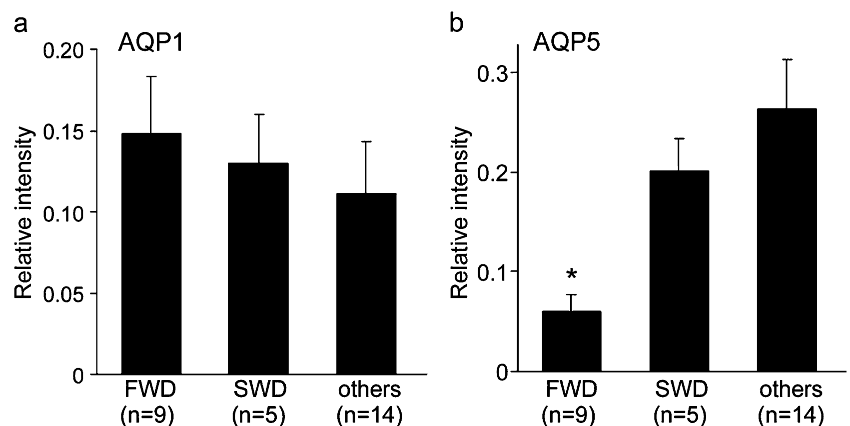

Fig. 4 RT-PCR analyses of gene expressions of AQP1 and AQP5 in lungs obtained from autopsy cases. Ratios of AQP1 (**a**) and AQP5 (**b**) to β-actin were calculated and shown here. All values represent the means± SEM. $^{*}p<0.05$ vs other groups. *FWD* freshwater drowning, *SWD* saltwater drowning

messenger RNA (mRNA) level [32–34]. Actually, Ishida et al. [35] examined intrapulmonary surfactant protein (SP)-A1 and SP-A2 mRNA in various autopsy cases. Although the ratio of SP-A1/SP-A2 could contribute to diagnosing acute asphyxia death, it was not valuable enough to differentiate FWD from SWD. However, from the viewpoint of forensic pathology, our results are presumed to be valuable to differentiate FWD and SWD based on the intrapulmonary AQP5 expression.

It is also necessary to distinguish vital drowning from postmortem immersion. In our experimental study, there was significant difference in intrapulmonary AQP5 expression between PI and FWD, but not SWD models, suggesting that the suppression of AQP5 expression in the lungs would be evaluated as a vital reaction in FWD. Recently, the number of sudden unexpected death cases during taking a bath increased. Thus, in such cases, the evaluation of intrapulmonary AQP5 expression may give useful information. Furthermore, other markers have to be found out for the differentiation between SWD and PI.

Our results are still of limited in the practical application to forensic casework. In the present study, the post-submerged intervals of cases examined ranged from a few to 24 h. It is well-known that longer interval after immersion can accelerate the degradation of mRNA and protein. Thus, further investigation is necessary using human lung samples with longer post-submerged intervals. Finally, the present study shows at least the possibility of forensic molecular diagnosis for the differentiation between FWD and SWD, or between FWD and PI.

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