Localization of Aquaporin Water Channels in the Airway of the Musk Shrew (Suncus murinus) and the Rat

Seishi MAEDA1, Hisao ITO2, Koichi TANAKA3, Tetsu HAYAKAWA1 and Makoto SEKI1

1)Department of Anatomy and 2)The Institute of Experimental Animal Center, Hyogo College of Medicine, 1–1 Mukogawa, Nishinomiya, Hyogo 663–8501, Japan

(Received 23 March 2005/Accepted 1 June 2005)

ABSTRACT. Aquaporins (AQPs) constitute a family of water channels that facilitate membrane water permeability in various tissues of animals. In this study, we compared the expression and localization of AQPs in the respiratory system of the musk shrew (Suncus murinus), which is an insectivore, and the rat by immunohistochemical methods. In both the musk shrew and the rat, AQP1 was expressed throughout the airway in endothelial cells of subepithelial blood vessels and in nasal submucosal fibroblasts. AQP3 and AQP4 were detected in neither the epithelium nor the subepithelial layer of the musk shrew airway, but were abundant in the rat airway epithelium. Musk shrew AQP5 was distributed in the superficial epithelial cells facing the airspaces and in submucosal glandular cells, but, unlike in the rat, not in lung alveolar cells. Additionally, the expression patterns of AQP4 and AQP5 of the musk shrew were partly similar to those of the human previously reported, absence of AQP4 and presence of AQP5 in the upper airway. The expression differences of AQPs between species in the airway indicate that the physiological importance of each AQP may be different in each species.

KEY WORDS: aquaporin, lung, nasal mucosa, trachea, water channel.

Aquaporins (AQPs) are water-selective channels that increase plasma membrane water permeability in secretory and absorptive cells that require rapid or regulated water movement [42, 43]. In mammals, at least eleven subtypes of AQPs have been identified (AQP0-10) and divided into two groups depending on their sequence homology and functions. Every AQP selectively transports water, but AQP3, -7, and -9 are also permeable with respect to neutral solutes such as urea and glycerol [55].

Several AQPs are also expressed in the respiratory system, specifically the nasal mucosa, trachea, bronchus, and lung. AQP1 is the first aquaporin that was identified in the lung of rat [16], and revealed to be localized predominantly to the blood microvascular endothelia adjacent to the airway, alveoli, and pleura [12, 13, 25, 38, 39, 46, 48]. AQP3 was found to be expressed in the basal cells of nasopharynx and trachea epithelia in both rat and human [14, 25, 28, 40]. Expression of AQP4 has been reported in the basolateral membrane of columnar cells of rat nasal, tracheal, and bronchial epithelia [14, 40], but not in the human airway [28]. AQP5, which was cloned from rat salivary gland [30, 44], has been identified in rat and human lung [26, 28, 40, 44], and AQP5 protein was seen to be localized in lung type 1 pneumocytes of rodents [40], whereas human AQP5 was distributed in the superficial epithelium of the upper airway [28]. However, most of these reports showed transcription levels of AQPs and/or protein localization within a small portion of the airway, and immunohistochemical localization of AQPs throughout the airway has only been reported once in the rat [40] and once in the human [28].

In the respiratory system, at least, there are many differences in the localization of AQP subfamily members among species. Because the environment influences the moistening of the airway, animals that inhabit a different environment may have different traits in their water movement system. It is thought that AQP expression patterns and functional importance of each AQP subfamily may be different among the indigenous animals of each environment. While functional differences in components of the airway are recognized among different species, it is difficult to estimate the essential roles played by AQP subfamily members in these tissues. In order to understand common functionalities of AQP subfamily members in the respiratory system, more reliable information on these subfamily members is needed.

The musk shrew (Suncus murinus) is a small animal that has attracted interest as an experimental insectivorous mammal and exhibits several primitive mammalian characteristics [11, 22, 47]. The musk shrew shares several similarities with humans in its morphological, physiological, and genetic features [17, 20, 22, 23] and is considered one available model for the study of human physiology and pathology [23, 53]. In this study, we examined the expression and distribution of AQPs throughout the airway of the musk shrew and rat by immunohistochemistry and immunoblotting. We discuss the results of our experiments to determine the localization of AQP subfamily members in the airways of the musk shrew, rat, and human [28]. We also discuss our ideas regarding the functions of each subfamily member in the different species.

MATERIALS AND METHODS

Animals: Adult male musk shrews (Suncus Murinus, 50–60 g) and Sprague-Dawley rats (280–350 g) used in this study were originally purchased from Clea Japan and bred at the Institute of Experimental Animal Sciences of Hyogo College of Medicine. The Animal Care and Use Committee
at Hyogo College of Medicine approved the procedures used on the animals. The animals were kept in separate cages and maintained under a 12 hr light-dark cycle at constant temperature (25°C) and humidity (65–75%). Food and drinking water were given *ad libitum* until sacrifice.

**Antibodies:** The following anti-AQP antibodies were used: rabbit anti-rat AQP1 (Chemicon, CA, U.S.A.), goat anti-human AQP3 (Santa Cruz biotechnology, CA, U.S.A.), goat anti-human AQP4 (Santa Cruz biotechnology, CA, U.S.A.), and rabbit anti-rat AQP5 (Calbiochem, CA, U.S.A.). Alternatively, rabbit anti-rat AQP3 (Alomone labs, Jerusalem, Israel) and rabbit anti-rat AQP4 (Chemicon, CA, U.S.A.) were used. Because the same results were obtained using anti-rat AQP3 and AQP4 antibodies as with anti-human AQP3 and AQP4 antibodies, we selected to use the anti-human AQP antibodies in this study. The following secondary antibodies were used: biotinylated or peroxidase conjugated goat anti-rabbit antibody (Vector, CA, U.S.A.) and donkey anti-goat antibody (Zymed, CA, U.S.A.).

**Immunohistochemistry:** The musk shrews and the rats were deeply anesthetized with diethyl ether and sodium pentobarbital (40 mg/kg, i.p.), then intracardially perfused with phosphate buffered saline (PBS, pH 7.2) followed by Bouin’s fixative. Tissues were removed and immersed for 2 hr in the same fixative, then dehydrated and embedded in paraffin. Six-µm thick sections were placed on silane-coated glass slides and rehydrated. The sections were heat-treated by autoclaving for 10 min at 116°C in citrate buffer (20 mM citrate, pH 6.0). After cooling down to room temperature, the sections were immersed in ice-cold methanol containing 0.3% H2O2 for 20 min to block endogenous peroxidase. After washing with PBS containing 0.05% Tween 20 (PBS/T), the sections were blocked with Block Ace™ (Dainippon Pharmaceuticals, Osaka, Japan) for 1 hr at room temperature, followed by incubation with each anti-AQP antibody (5–10 µg/ml) overnight at 4°C. After washing with PBS/T, the sections were incubated with secondary antibodies. Signal detection was performed by using an avidin-biotin complex system (ABC Elite, Vector, CA, U.S.A.) and diaminobenzidine. Negative controls for immunohistochemistry experiments were performed by omitting the primary antibodies or using non-immune rabbit or goat IgG instead of anti-AQP antibodies, with no positive bands being observed.

**SDS-PAGE and Immunoblotting:** Total membrane proteins were isolated from the nasal mucosa, trachea, and lung (the bronchus was removed) in musk shrews and rats. Kidney, colon, and the submandibular gland of musk shrews and rats were used to validate the binding ability of antibodies to the musk shrew AQP.s. Adult male animals (rats of weight 300 to 350 g, musk shrews of weight 50 to 65 g) were deeply anesthetized with diethyl ether and sodium pentobarbital. The animals were perfused with physiological saline followed by homogenizing buffer, 10 mM triethanolamine, 0.25 M sucrose (pH 7.4). Each tissue was removed and homogenized in homogenizing buffer containing 2 mM EDTA and a protease-inhibitor cocktail (Complete™, Roche Diagnostics, Mannheim, Germany). The samples were centrifuged at 4,000 × g for 10 min and the supernatants were re-centrifuged at 200,000 × g for 1 hr with a 90Ti angle rotor (Beckman, CA, U.S.A.). The precipitants were homogenized in 1% sodium dodecylsulfate (SDS) and protein concentrations were determined by BCA protein assay (Pierce, IL, U.S.A.). After denaturing with Laemmli’s sample buffer [29], samples (30 µg/lane) were separated by electrophoresis in a 12.5% SDS polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Boehringer Mannheim, Mannheim, Germany). The membrane was blocked with Block Ace™ followed by incubation with anti-AQP antibodies (1–5 µg/ml) for 2 hr at room temperature. After washing with PBS/T, the membrane was incubated with peroxidase-conjugated secondary antibodies for 2 hr at room temperature. Negative controls for immunoblotting experiments were performed by using non-immune rabbit or goat IgG instead of anti-AQP antibodies, with no positive bands being observed. The signal was detected using a chemiluminescent detection system (ECL™, Amersham Pharmacia Biotech, Buckinghamshire, U.K.) and visualized with the lumino-image analyzer LAS-1000 plus (Fuji photo film, Kanagawa, Japan). To exclude the possibility of samples leaking into the next lane, separate examinations for each sample were carried out and the same results were obtained.

**RESULTS**

Expression of AQPs in the musk shrew was confirmed in various tissues by immunoblotting and immunohistochemistry. AQP1 was found to be expressed throughout the airway of the musk shrew using immunoblotting and immunohistochemistry (Figs.1a and 2). In both the musk shrew and rat, AQP1 in the nasal mucosa was distributed in endothelial cells and interstitial fibroblasts of subepithelial blood vessels (Fig. 2a, 2b). No positive reaction was detected in nasal epithelial cells, both the respiratory and olfactory mucosal regions of the nasal cavity. In the trachea of the musk shrew, AQP1 was detected in the endothelial cells of the submucosal blood vessels but not in fibroblasts (Fig. 2c). In the rat, tracheal fibroblasts were positive for AQP1, as were blood vessels under the tracheal mucosa (Fig. 2d). Tracheal epithelial cells were negative for AQP1 in both animals. Expression of the lung AQP1 was observed primarily in epithelial cells of the pleura (Fig. 2e and 2f) and endothelial cells of the blood vessels (Fig. 2g and 2h), but the alveolar epithelium was negative for AQP1 in both the musk shrew and rat. The binding ability assay for the positive control of AQP1 in the kidney of the musk shrews, as well as the AQP1 in the rat, showed an abundant 28 kDa band (Fig. 1e). In addition, heavier bands were somewhat smaller in size in the musk shrew (32 to 35 kDa) than in the rat (35 to 45 kDa, Fig.1a and 1e).

AQP3 was expressed at an extremely low level in the whole airway of the musk shrew, but was abundantly expressed in the upper airway of the rat (Fig. 1b). In the rat,
the localization of AQP3 was observed in basal cells of the epithelium of the nasal mucosa and trachea (Fig. 3). No positive reactions were observed in the lungs of either animal. The binding ability assay by immunoblotting showed that the membrane fraction from the musk shrew colon reacted with AQP3 antibody (Fig. 1f).

AQP4 in the musk shrew was detected at a low level throughout the airway while expression of AQP4 in the rat was found abundantly in the nasal mucosa, at low levels in the trachea, and to be absent in the lung (Fig. 1c). Immunohistochemistry showed that AQP4 localization was found in the nasal epithelial cells in the rat. In addition, there was detection in submucosal glandular cells, and the signal was especially intense on the basolateral side of the cells (Fig. 1b). In the musk shrew, positive reactions were found neither in the epithelium nor in the submucosal glandular cells (Fig. 4a and 4b). In the trachea, a positive reaction of AQP4 was observed in the rat, while not in the musk shrew (Fig. 4c and 4d). In the lung, no cells reactive to AQP4 were observed in the musk shrew or the rat. The binding ability experiments using AQP4 antibody to the kidney of musk shrew and rat showed that this antibody was reactive to AQP4 in both musk shrew and rat (Fig. 1g).

In the musk shrew airway, bands representing AQP5 were detected in the nasal mucosa and the trachea, but not in the lung by immunoblotting (Fig. 1d). In the rat, a AQP5 positive band was detected in the lung and very little in the nasal mucosa. Using immunohistochemistry for AQP5,
positive reactions were seen on the apical side of the epithelium of the musk shrew’s nasal mucosa, but the signal was extremely weak in the same tissue of the rat (Fig. 5a and 5b). The localization of AQP5 in the nasal glands was different in the two animals: the entire rim of the nasal glandular cells was reactive in the musk shrew, but only the luminal side of the cells was positive to AQP5 in the rat (Fig. 5c and 5d). In the trachea, AQP5 was localized to the apical surface of the epithelium in the musk shrew but was not expressed in the rat (Fig. 5e and 5f). In the lung, no positive reactions were observed in the musk shrew, but type I alveolar cells were positive in the rat (Fig. 5g and 5h). Immunoblotting with an anti-AQP5 antibody showed that the antibody reacts with both musk shrew and rat AQP5 in the submandibular glands.
No positive reactions were observed using negative controls in which primary antibodies were omitted or non-immune goat or rabbit IgG was used. The results of the immunohistochemical analyses in this study are summarized in Table 1.

Fig. 3. AQP3 localization in the nasal mucosa (a and b) and trachea (c and d) of the musk shrew (a and c) and the rat (b and d). AQP3 is not detected in the nasal and tracheal mucosae in the musk shrew, whereas strong labeling can be seen in ciliated cells (arrowheads) and basal cells (arrows) of the rat epithelium. Abbreviations: Ep, epithelium. Bar = 20 µm.

Fig. 4. Localization of AQP4 in the nasal mucosa (a and b) and trachea (c and d) of the musk shrew (a and c) and rat (b and d). AQP4 is not detected in the nasal and tracheal epithelia in the musk shrew (a and c). In the rat, ciliated and basal cells of the nasal epithelium (b, arrows), as well as submucosal glandular cells (b, arrowheads), show labeling of AQP4. In the trachea, epithelial and basal cells are positive in the rat (d, arrows). Abbreviations: Ep, epithelium; G, submucosal gland. Bar = 20 µm.

DISCUSSION

In this study, we examined and compared the expression and distribution of AQPs in the airway tissues of the musk shrew, and the rat, demonstrating that there are AQP expression differences in the airways of these two animals.
The AQP1 distribution was observed in the endothelium of blood vessels and fibroblasts in the subepithelial layer, indicating that it is possible to contribute to the rapid water movement between the blood vessels and the epithelial cells via fibroblasts. Therefore, the subepithelial layer may work as a water pool through which water could be transported rapidly towards the nasal epithelium. It is important for the epithelium, which is in contact with the air interface, to be supplied with water, and AQP1 may contribute as a major interstitial water channel in mammals. A recent report showed that in human tissues AQP1 was detected only in the capillary endothelium of the lung and bronchus, and not in nasal mucosa [38]. While there is variation among animals, AQP1 is expressed in the capillary endothelial cells in various tissues [1, 2, 4, 27, 37, 38, 52, 56–58], and these observations may support our result. Immunoblotting of AQP1 showed bands of larger molecular weight corresponding to the glycosylated AQP1 [39] in both animals, but smaller

Fig. 5. Immunolocalization of AQP5 in the nasal epithelium (a and b) and submucosal glands (c and d), trachea (e and f), and lung (g and h) in the musk shrew (a, c, e, and g) and the rat (b, d, f and h). A strong reaction is seen in the surface membrane and cilia of the nasal epithelial cells facing the lumen in the musk shrew (a, arrows), whereas there is a weak reaction in the rat (b, arrows). The entire rim of the glandular cells is positive in the musk shrew (c, arrowheads) and only the luminal side of the glandular cells is positive in the rat (d, arrowheads). The superficial cell surface of the tracheal epithelium is positive for AQP5 in the musk shrew (e, arrows) but not in the rat (f). The lung cells are negative in the musk shrew (g), but type1 pneumocytes are positive in the rat (h, arrows). Abbreviations: A, alveolus; Br, bronchiole; Ep, epithelium; G, submucosal gland. Bar=20 μm.
bands were observed in the musk shrew than in the rat. While the essential role of the glycosylation of AQP1 is still not clear, this additional complexity of glycosylation may parallel the complexity of AQP1 function.

AQP3 and 4 were not detected in the musk shrew airway. Nielsen et al. showed that AQP3 and 4 are the major water channels of the nasal epithelium in the rat [40]. We tested two kinds of polyclonal antibodies raised against the human and rat AQP3 C-terminal 18 amino acid region. As the region of homology of the AQP3 C-terminal amino acid sequence was highly conserved in mammals [7, 18, 19, 21, 32], it can be thought that the low-level of reactivity to the musk shrew colon and airway was due to the relatively low level of expression of AQP3 in this animal. Contrary to the results of AQP3, AQP4 was determined to be abundant in the musk shrew kidney indicating that the anti-AQP4 antibody used in this study has a relatively high-binding affinity for the musk shrew AQP4. Because no positive reactions were detected in the nasal epithelial cells, this indicates that AQP4 may not be necessary in the nasal epithelium of the musk shrew. Kreda et al. [28] showed that AQP3 was expressed, like in the rat, in basal cells of the human nasal epithelium, but AQP4 was, like in the musk shrew, not detected in the airway epithelium.

We also showed that the expression of AQP5 in the musk shrew nasal epithelium is similar to the expression pattern found in humans. Interestingly, AQP5 was localized in the upper airway epithelium of the musk shrew as well as in the distal lung alveolar cells in the rat. AQP5 in humans was expressed in both superficial epithelium and in alveolar cells of the lung [28]. These results indicate that there are similar localizations of AQP5 in the human airway as well as within the airway of both the musk shrew and rat. It is difficult to explain in the musk shrew how the epithelium regulates the water balance of the cells. Uneven distribution of water channels, especially in the nasal epithelium of the musk shrew, may not adequately represent the state of humidification of the nasal cavity. There is the possibility that abundant expression of AQP5 in the nasal glands is enough to regulate humidity to the nasal surface, and may buffer drying effects that occur at the surface of the nasal epithelium. It is also thought that unknown factors may stimulate the expression of the other water channels, or there may be different kinds of moistening components that regulate the surface of the nasal epithelium in the musk shrew.

In the trachea, almost the same expression pattern of AQPs found in the nasal mucosa was shown in both the musk shrew and rat, although tracheal fibroblasts in the musk shrew were negative for AQP1. This difference may be due to musk shrews not needing to actively facilitate water transport in the trachea or alternative water transport systems may be present in musk shrews. It may be thought that a regulation system similar to that operating in the nasal mucosa may contribute to humidification of the airway and protection of the epithelium against drying of the trachea.

In the present study, AQP1 was shown to be the only water channel expressed in the lung of the musk shrew. AQP1 distribution in the lung of the musk shrew was comparable to that found in the rat. That is, AQP1 was localized to pleura and to pulmonary blood vessels. It is indicated that AQP1 in the pleura may supply and/or withdraw moisture of the serosa in the thoracic cavity of the animals. It seems to be thought that hydrothorax may be caused by affects of the AQP1. In rats and humans, AQP1 and AQP5 are expressed in blood vessels and alveolar type I pneumocytes, respectively. It can thus be hypothesized that AQP1 and 5 may function to maintain moisture on the epithelial surface and to discharge the water from the air space after accidental or incidental aspiration of fluid [8, 9, 12, 15, 26, 40, 44, 46]. It is difficult to explain what kinds of water transport systems may compensate the absence of AQP5 in the type I pneumocytes in the musk shrew. It is thought that other membrane ion channels may permeate ions with water, and this may supply sufficient water in the airway in the musk shrew. Examination using knockout mice lacking AQPs to assess the role of AQPs in several organs including the lung.

Table 1. Summary of the results of immunohistochemistry of anti-AQPs antibody in the airway of the musk shrew and the rat.

<table>
<thead>
<tr>
<th>Abbreviations</th>
<th>S, musk shrew; R, rat; EPL, epithelium; SEL, subepithelial layer</th>
</tr>
</thead>
<tbody>
<tr>
<td>AQP1</td>
<td>AQP3</td>
</tr>
<tr>
<td>Nasal mucosa</td>
<td>EPL</td>
</tr>
<tr>
<td></td>
<td>SEL</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Trachea</td>
</tr>
<tr>
<td></td>
<td>SEL</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lung</td>
</tr>
<tr>
<td></td>
<td>SEL</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>
had been postulated [5, 6, 33–35, 41, 45, 51]. However, active fluid transport due to efficient water permeability in the lung was not affected in AQP1 or AQPS knockout mice, even though water permeability of airspace capillaries was decreased 10-fold by the deletion of these AQPs [3, 31, 48–50]. In addition to these studies, pharmacological blockade of Na+ channels evokes significant fluid transport inhibition in the mouse lung and the same phenotypes are seen in Na+ channel knockout mouse lung [36]. It is thought that AQPs in the lung may not have physiologically crucial functions but may augment and/or compensate water facilitation along with non-selective water transport systems [31, 34]. Thus, it seems that the low level expression of AQPs in alveolar cells in the musk shrew does not affect lung physiology to a great extent.

It is still uncertain whether these similarities and differences in AQP distributions between the species reflect functional similarities and differences. It is thought that AQP expression may be regulated at the transcriptional and/or translational levels [59, 60]. In fact, it has been shown that human AQP4 mRNA was expressed in the airway epithelium, while AQP4 is immunohistochemically undetectable [28]. While it is unknown whether there is mRNA expression of AQPs in the musk shrew airway epithelium, it is possible that post-transcriptional regulation exists in the musk shrew airway, just as in humans. In any case, it can be postulated that the genetic regulatory system and/or natural circumstances of the musk shrew and the rat may be the cause of the diversity in expression pattern of AQPs in the airway.

ACKNOWLEDGMENTS. The authors thank Ms. Megumi Hatta and Mr. Katsumi Gion (Department of Anatomy, Hyogo College of Medicine) for their expert care of keeping the animals. We also thank Mr. Mitsuru Nishibayashi (Institute of Experimental Animal Center of Hyogo College of Medicine) for his expert care of keeping the animals. This study was supported in part by a Grant-in-Aid for Researchers, Hyogo College of Medicine.

REFERENCES


21. Johnston, H., Koukoulas, I., Jeyaseelan, K., Armugam, A., Ear-


