Aquaporins in complex tissues. I. Developmental patterns in respiratory and glandular tissues of rat

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Aquaporins in complex tissues. I. Developmental patterns in respiratory and glandular tissues of rat. Am. J. Physiol. 273 (Cell Physiol. 42): C1541–C1548, 1997.—Developmental expression of aquaporin water transport proteins is not well understood in respiratory tract or secretory glands; here we define aquaporin protein ontogeny in rat. Expression of aquaporin-3 (AQP3), AQP4, and AQP5 proteins occurs within 2 wk after birth, whereas AQP1 first appears before birth. In most tissues, aquaporin protein expression increases progressively, although transient high-level expression is noted in distal lung (AQP4 at postnatal day +2) and trachea (AQP5 at postnatal day +21 and AQP3 at postnatal day +42). In mature animals, AQP5 is abundant in distal lung and salivary glands, AQP3 and AQP4 are present in trachea, and AQP1 is present in all of these tissues except salivary glands. Surprisingly, all four aquaporin proteins are highly abundant in nasopharynx. Unlike AQP1, corticosteroids did not induce expression of AQP3, AQP4, or AQP5 in lung. Our results seemingly implicate aquaporins in proximal airway humidification, glandular secretion, and perinatal clearance of fluid from distal airways. However, the studies underscore a need for detailed immunohistochemical characterizations and definitive functional studies.

water channels; perinatal lung; trachea; nasopharynx; secretory glands

MATERIALS AND METHODS

Materials. Electrophoresis reagents were from Bio-Rad (Mellville, NY). Reagents for enhanced chemiluminescence were from Amersham (Arlington Heights, IL). Betamethasone was from Schering (Kenilworth, NJ). Bicinchoninic acid (BCA) protein assay and ImmunoPure Ag/Ab immobilization kit II were from Pierce (Rockford, IL). Other reagents and supplies were from Sigma Immunochemicals (St. Louis, MO), J. T. Baker (Phillipsburg, NJ), or Eastman Kodak (Rochester, NY).

Antibodies. All studies were undertaken with affinity-purified antibodies. The anti-peptide antibody specific for AQP5 was prepared as described (27). A synthetic peptide corresponding to the COOH-terminal of rat AQP5 (NH2-CEPEEDWEDHREERKKTIETLAH-COOH) was cross-linked to keyhole limpet hemocyanin and was used to immunize New Zealand Whiterabbis (Lafstrand Laboratories, Gaithersburg, MD). Polygonal anti-AQP5 immunoglobulin G was affinity purified from serum using Sulfolink coupling gel (Pierce) conjugated with 2–4 mg of the synthetic peptide. As a negative labeling control, anti-AQP5 was preincubated with a 100-fold excess of the immunizing peptide at 4°C for 24 h.

DISCOVERY OF THE AQUAPORIN family of water channel proteins has provided a molecular explanation for the rapid transport of water across the plasma membranes of many cells (17). Aquaporin-1 (AQP1), the first characterized water channel protein (23), was identified in erythrocyte membranes, renal proximal tubule (8), choroid plexus, eye, lung, vascular endothelium, and hepatobiliary epithelium (21). The absence of AQP1 from certain water-permeable tissues spurred the search for new homologues. Lack of AQP1 in renal collecting duct (8) predicted the existence of AQP2 and AQP3 at that site (10, 12, 15, 20). Similarly, the Aqp4 cDNA was isolated from rat brain (16) and lung (13), and the Aqp5 cDNA was isolated from rat salivary gland (24).

Regulation of water movement is known to be complex at all levels of the respiratory tract and probably may be altered in diverse clinical disorders such as pulmonary edema, cystic fibrosis, asthma, freshwater drownings, and allergic rhinitis. Although aquaporins presumably participate in these processes, the present understanding of aquaporin expression at various sites in the lung and upper airway is both incomplete and confusing. AQP1 is present in the peribronchial capillary endothelium and visceral pleura, and its expression is induced by corticosteroids (18). AQP3 and AQP4 have been described in basolateral membranes of tracheal epithelia (11). Although Aqp5 mRNA is strongly expressed in rat lung (24), the distribution of the protein has not been defined. Moreover, expression of aquaporins has not been assessed in the nasopharynx.

Perinatal development has been extensively characterized in the rat. The final days of the 21-day gestation are marked by major increases in fetal body weight and development. Nevertheless, compared with other species, newborn rat pups are relatively immature and must rapidly adapt to terrestrial life. Immediately at birth, their respiratory tracts are cleared of fluid, and within the next few days, their eyes open, their kidneys attain the ability to concentrate urine, and their digestive systems mature in preparation for weaning. Although perinatal expression of aquaporin mRNAs has been described in total lung (28), a comprehensive delineation of aquaporin protein expressions has not been established in respiratory and secretory tissues during development. In this study, we define the ontogeny of AQP1, AQP3, AQP4, and AQP5 proteins in the distal lung, trachea, nasopharynx, and salivary glands of rat to provide insight into the potential roles of aquaporins in the complex physiology of these developing tissues. Cellular and subcellular localizations of these proteins are being established in these tissues from adult rats and are reported in an accompanying study (20a).
Polyclonal, affinity-purified rabbit antibodies to AQP1 (anti-AQP1), which react with the 4-kDa COOH-terminal domain of the protein were previously described (26). Affinity-purified rabbit antibodies to AQP3 (9) and AQP4 (27) were the gift of Dr. Mark Knepper (National Institutes of Health). Polyclonal rabbit antiserum to rat thrombomodulin was the gift of Drs. David Stern (Columbia University) and Dennis Brown (Massachusetts General Hospital).

Animals. Timed pregnant or adult Sprague-Dawley rats were allowed free access to food and water until they were killed. All rats were anesthetized with CO2 inhalation followed by cervical dislocation; fetal rats were decapitated after the mother was killed. Animals were perfused through the right and left ventricle with chilled 5 mM sodium phosphate (pH 8.0), 150 mM NaCl, and 1 mM EDTA (phosphate-buffered saline-EDTA) until free of blood. Tissues were removed, frozen on dry ice, and stored at -85°C for subsequent isolation of membranes. Tissues from animals <4 days old were pooled by litter to have adequate sample for processing. Animals from at least four separate litters were studied at each time point, and the data shown are representative of all observations.

Tissue membrane preparations. Tissues were homogenized in a Potter-Elvehjem homogenizer on ice in buffer containing 0.25 M sucrose, 1 mM phenylmethylsulfonyl fluoride, 0.5 mg/ml diisopropyl fluorophosphate, and 4 µg/ml leupeptin. Crude membrane pellets containing plasma membranes were isolated by low-speed (800 g) centrifugation of the homogenate followed by high-speed (200,000 g) centrifugation of the supernatant and solubilization in 1.5% sodium dodecyl sulfate (SDS; wt/vol). Total protein concentration was measured by BCA protein assay (Pierce), using bovine serum albumin as standard. Erythrocyte membranes from adult rats were prepared by five cycles of hypotonic lysis (5 mM sodium phosphate) in the presence of the above-listed protease inhibitors, followed by centrifugation (39,000 g).

Electrophoresis and immunoblots. SDS-polyacrylamide gel electrophoresis (PAGE) was performed using the buffer system of Laemmli (19) and 0.1 × 9 × 6 cm 12% acrylamide slabs. Total membranes (10–15 µg protein) were loaded for each sample; SDS-PAGE gels were stained with Coomassie brilliant blue (Bio-Rad) to confirm equivalence of samples. Immunoblotting was performed as described (6), using enhanced chemiluminescence and autoradiography to visualize immunoblots. Contact prints of autoradiographs were made for presentation of data. Autoradiographs of immunoblots incubated with antibodies to AQP1, AQP3, AQP4, or AQP5 were scanned by densitometry (Molecular Dynamics, Sunnyvale, CA), and relative abundances of the proteins were derived from the signals at 27–32 kDa.

Statistics. Densitometric analysis of immunoblots is expressed as means ± SE for each group. Each group was an independent data set; unpaired t-tests were performed to assess corticosterone effects on aquaporin expression.

RESULTS

Tissue distribution of AQP5 protein. Membranes from multiple different adult rat tissues were electrophoresed into SDS-PAGE gels and stained with Coomassie blue (Fig. 1A, top) or analyzed by immunoblot with anti-AQP5 (Fig. 1A, bottom). A 27-kDa band representing the AQP5 monomer is strongly expressed in lung and submandibular gland and is also present in trachea, nasopharynx, lacrimal gland, eye, and mammary gland. Longer exposures of the immunoblot revealed weak expression in parotid and sublingual glands (not shown). Similar to AQP1 (8, 26), the higher-molecular-mass bands in lung probably represent incompletely solubilized oligomers of AQP5. Brain, colon, pancreas, erythrocytes, prostate, and stomach do not express AQP5. Incubation of anti-AQP5 antibody with the peptide immunogen completely eliminated binding to lung and submandibular gland membranes (Fig. 1B).

Expression of aquaporins in lung and upper airway. To assess the distribution of AQP5 and other aquaporins in adult rat lung and upper airway, tissues were isolated from several regions (Fig. 2A): distal lung, right main-stem bronchus and perihilar lung tissue, trachea, nasopharynx, and submandibular gland. Membranes from each of these sections were electrophoresed into SDS-PAGE gels and analyzed by immunoblotting with antibodies to AQP1, AQP3, AQP4, and AQP5.

The AQP1-immunoblot (Fig. 2A) reveals a 48-kDa band in distal lung which is not present in submandibular gland. AQP1 is also present in trachea, nasopharynx, and submandibular gland membranes but is absent from brain, colon, pancreas, and stomach. AQP3 is expressed in brain, colon, pancreas, and stomach (Fig. 2B), as well as in lung and submandibular gland. AQP4 is detectable in brain, colon, prostrate, and stomach (Fig. 2C). AQP5 is particularly abundant in lung and submandibular gland (Fig. 2D) and is also present in trachea, nasopharynx, and submandibular gland. The molecular-mass bands in lung probably represent incompletely solubilized oligomers of AQP5. Brain, colon, pancreas, erythrocytes, prostate, and stomach do not express AQP5. Incubation of anti-AQP5 antibody with the peptide immunogen completely eliminated binding to lung and submandibular gland membranes (Fig. 1B).
AQP5 is strongly expressed in distal lung, perihilar region, nasopharynx, and submandibular gland but is only weakly expressed in trachea. AQP1 is strongly expressed in all four regions of the respiratory tract but expression is faintly detected in submandibular gland. AQP4 is strongly expressed in nasopharynx but is less strongly expressed in trachea and is not detectable in distal lung. Neither AQP3 nor AQP4 is expressed in submandibular gland (Fig. 2B).

Ontogeny of aquaporins in distal lung. AQP5 and AQP1 are expressed in the distal lung of perinatal and adult rats. Total membranes from distal lung of fetal, postnatal, and adult rats were electrophoresed into SDS-PAGE gels. Loading equivalence was confirmed by Coomassie blue staining (Fig. 3, top), and the gels were analyzed by immunoblot using aquaporin antibodies (Fig. 3, bottom). AQP5 is reproducibly expressed +2 days after birth in rat lung but is barely detected at embryonic day E21 or postnatal day +1. AQP5 expression in lung increases 20-fold from postnatal day +2 to +14, with a further 10-fold increase from postnatal day +14 to adult (densitometric analyses not shown). As

Fig. 2. Distribution of aquaporins in lung, upper airway, and salivary gland of adult rat. A: schematic representation of rat lung and upper airway demonstrating sites of tissue harvest for immunoblots: 1, distal lung; 2, main-stem bronchus and proximal lung; 3, trachea; 4, nasopharynx; 5, submandibular salivary gland. B: total membranes (15 µg protein) from each of indicated areas (A) were electrophoresed into 12% SDS-PAGE gels; separate immunoblots were incubated with affinity-purified antibodies to AQP5, AQP1, AQP4, or AQP3. Exposure times for autoradiographs: AQP5, 10 min; AQP1, 5 min; AQP4, lane 4 5 min, other lanes 2 h; AQP3, 5 min.

Fig. 3. Ontogeny of aquaporins in distal rat lung. Total membranes (15 µg protein) from distal lung parenchyma of fetal rats (embryonic days E17, E19, and E21) and postnatal rats (postnatal days +1 to +45; adult = more than +60 days) were electrophoresed into 12% SDS-PAGE gels and stained with Coomassie blue or analyzed by immunoblot with affinity-purified antibodies to AQP5, AQP1, AQP4, or AQP3.
previously reported (18), AQP1 is present in rat lung at embryonic day E19, increases severalfold at birth, and is sustained at high levels into adulthood. AQP4 protein is strongly detected in lung membranes only at postnatal day +2. AQP3 is not expressed in rat lung membranes. Although the crude membranes analyzed in these immunoblots are likely to contain fragments of intracellular organelles, none of these aquaporins has been identified in an intracellular distribution. Because the abundance of endothelium relative to epithelium will increase during development, the immunoblots were analyzed with an antibody to rat thrombomodulin; this marker for endothelium demonstrated strong presence of the thrombomodulin in prenatal tissue with only a gradual increase after birth (not shown). Thus the marked developmental inductions of AQP1 and AQP5 in distal lung do not reflect corresponding marked alterations in the abundance of capillary endothelium.

Ontogeny of aquaporins in trachea. AQP5, AQP1, AQP4, and AQP3 each exhibit a distinct pattern of expression in trachea during the perinatal period. Tracheal membranes from rats of different ages were analyzed by immunoblot using aquaporin antibodies (Fig. 4). AQP5 is expressed in rat trachea by postnatal day +12, exhibits a temporary increase at postnatal day +21, and declines to a very low level thereafter. AQP1 is present in trachea at all postnatal ages and as early as embryonic day E21 (data not shown). AQP4 is expressed only at very low levels in trachea, where it is first detected at postnatal day +12 (not visible in Fig. 4) and becomes more abundant by postnatal day +42. AQP3 is present at postnatal day +21, exhibits a marked increase at postnatal day +42, and declines to moderate levels thereafter.

Ontogeny of aquaporins in nasopharynx. All four aquaporins are strongly expressed in the nasopharynx of postnatal rats. Nasopharyngeal membranes from rats of different ages were analyzed by immunoblot using aquaporin antibodies (Fig. 5). AQP5 is expressed at postnatal day +5, but the level of expression rises markedly by postnatal day +21. AQP1 is already present in nasopharynx from embryonic day E20 and gradually increases thereafter. In contrast to trachea, AQP4 is strongly expressed in nasopharynx by postnatal day +21 but not earlier. AQP3 is very weakly expressed at embryonic day E20 (visible only with longer exposures; not shown), but increases soon after birth and rises to strong levels at postnatal day +21 and adulthood.

Corticosteroid-mediation of aquaporin expression in lung. To assess the effects of corticosteroids on aquaporin expression in lung, rats of various gestational and postnatal ages were injected intramuscularly with beta-
methasone (0.35 mg/kg) or 0.9% NaCl (0.02 ml) on consecutive days; whole lungs were harvested on the 3rd day, and membranes were analyzed by immunoblot (Fig. 6). Representative immunoblots from postnatal days +1, +4, and from adult animals (Fig. 6A) demonstrate that AQP5 is not induced in rat lung by corticosteroids. Immunoblots incubated with anti-AQP4 or AQP3 also failed to reveal increased expression by corticosteroids (not shown). As previously established (18), AQP1 is induced in lung by four- to eightfold at all ages (Fig. 6B). Thus corticosteroid induction is not a general feature of aquaporins in the respiratory tract.

Ontogeny of aquaporins in glandular tissues. Because Aqp5 mRNA was identified in lacrimal gland, eye, and salivary glands (24), multiple rat tissues were harvested and analyzed by immunoblot with aquaporin antibodies (Fig. 7). AQP5 protein is strongly expressed in submandibular and sublingual salivary glands but is more weakly expressed in parotid gland (Fig. 7A). AQP5 is also clearly expressed in lacrimal gland and eye. Submandibular glands from rats of different ages were analyzed similarly (Fig. 7B); AQP5 is weakly expressed by embryonic day E20, but expression increases postnatally. Weak expression of AQP1 may be detected postnatally but only after prolonged exposure of the immunoblot (data not shown). AQP4 and AQP3 are not expressed in submandibular gland.

DISCUSSION

Fluid management in alveoli, upper airways, and glandular epithelia is complex, since a myriad of anatomical and physiological factors influences the disposition of water. If we are to understand the regulation of water transport in the respiratory tract and glandular tissues, characterization of the relevant molecules is essential. In this study, we identify distinct tissue distributions and ontogenies for AQP1, AQP3, AQP4, and AQP5 proteins in the distal lung, trachea, nasopharynx, and secretory glands of rat (Table 1).

Ontogeny of the aquaporins is known to be complex. Our previous studies of Aqp1 mRNA (4) defined three
developmental patterns of expression in fetal and postnatal rat: 1) transient prenatal expression with marked decline in postnatal expression (periosteum, endocardium, and corneal endothelium); 2) perinatal expression with increased expression throughout later life (kidney and erythropoietic tissues); and 3) high-level expression throughout prenatal and postnatal life (choroid plexus). The studies reported here reveal significant complexity of aquaporin protein expression in respiratory tract and glandular epithelia. Similar to pattern 1, AQP4 protein was found to be transiently expressed at postnatal day +2, after which the protein was barely detectable in distal lung. Although less well pronounced, peak expressions were noted in trachea by AQP5 protein (postnatal day +21) and AQP3 protein (day +42), followed by declines in these proteins. Similar to pattern 2, expression of AQP1 protein begins in distal lung just before birth, whereas expression of AQP5 protein occurs just after birth, and expression of both proteins increases thereafter. Discrepancies exist in the previous studies of aquaporin mRNA expression during development. For example, two groups noted transient high expression of Aqp4 mRNA in the early postnatal period (25, 31), but this was not observed by a third group (28). The studies reported here document that AQP4 protein is transiently expressed in distal lung at this point in development.

The major function of the respiratory bronchioles and alveoli is to facilitate gas exchange in the distal lung. To accomplish this, the alveolar type I pneumocytes assume a thin, flattened surface with minimal volume separating blood from airspace. Just before birth, this region of lung is well known to switch from a secretory to an absorptive tissue, a process dependent on alveolar epithelial sodium channels, α-ENaC (22). Fetal lung water clearance begins before parturition and is completed within a day after birth (reviewed in Ref. 2), and α-ENaC gene disruption causes a lethal buildup of alveolar fluid (14). Although aquaporins are also believed to participate in this process, AQP5 first appears in distal lung at postnatal day +2. Thus, although alveolar AQP5 may play a role in perinatal lung water clearance, its developmental program and lack of steroid induction indicate that it is not a major determinant. Our previous study demonstrated that AQP1 resides in peribronchiolar capillary endothelium and is induced by corticosteroids (18). It was thus concluded that AQP1 may participate in the second phase of perinatal lung water clearance (removal of fluid from lung interstitium) but not the first phase. The physiological importance of the curious expression of AQP4 protein in distal lung only on postnatal day +2 is unexplained. Thus the mechanisms by which fluid leaves the alveolar space at birth are still incompletely defined.

Tight regulation of the airway surface liquid over the bronchi and trachea is critical to normal function of the mucociliary apparatus and host defense (reviewed in Ref. 30). Aquaporins in the tracheal epithelium and secretory glands may be participants in this process.

Table 1. Summary of aquaporin ontogeny in lung

<table>
<thead>
<tr>
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<th>AQP1</th>
<th>AQP3</th>
<th>AQP4</th>
<th>AQP5</th>
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</thead>
<tbody>
<tr>
<td>Distal lung</td>
<td>E19</td>
<td></td>
<td>+2*</td>
<td>+2</td>
</tr>
<tr>
<td>Trachea</td>
<td>E20</td>
<td>E20</td>
<td>+12</td>
<td>+12</td>
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<tr>
<td>Nasopharynx</td>
<td>E20</td>
<td>E20</td>
<td>+21</td>
<td>+5</td>
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<tr>
<td>Submandibular gland</td>
<td>E20</td>
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Age is given in days at first expression (E19 and E20, embryonic days; all others postnatal). AQP, aquaporin. *Detected only at postnatal day +2.
levels of expression

AQP3 and AQP5 reach their highest levels of expression 1 mo after birth and expression is reduced in older animals. As in trachea and distal lung, each of the aquaporins has a distinct ontogeny in nasopharynx, where these proteins are expressed abundantly. Because the expression of AQP1 and AQP3 in nasopharynx precedes that of AQP4 and AQP5, determination of the ontogeny of nasopharyngeal water permeability may reveal the functional role of each aquaporin expressed there. Both vascular and epithelial mechanisms have been proposed as the mechanism for humidifying inspired air (reviewed in Ref. 1), but the developmental program for this upper airway function has not yet been defined. The ontogeny of AQP5 in lacrimal (data not shown) and submandibular glands is similar to that of distal lung. Absence of other water channels in these glands suggests that regulated secretion of saliva or tears in these tissues may involve the presence of still-unidentified members of the aquaporin water channel family.

Although still speculative, considerable evidence suggests that aquaporins may play important roles in diseases of the human respiratory tract and secretory glands. Adequate gas exchange necessitates tight control of water in the distal airways and alveolar space, and altered expression or function of AQP5 or AQP1 in distal lung may contribute to the pathogenesis of pulmonary edema. Moreover, the normal abundance of AQP5 and AQP1 in distal lung implicates these proteins as the sites of vascular water entry during freshwater drownings. Changes in the characteristics of the airway surface liquid affect not only mucociliary transport but also play a role in the pathogenesis of diseases such as exercise- or cold-induced asthma (reviewed in Ref. 1) and cystic fibrosis (reviewed in Ref. 3). It is tantalizing to postulate that secondary alterations in water channel expression or function may contribute to the pathogenesis of cystic fibrosis or provide a therapeutic mode for altering the viscosity of airway secretions. Abundant expression of aquaporins in the nasopharynx strongly suggests their participation in normal physiological processes, such as humidification of inspired air, but also suggests that alterations in their function or expression will contribute to the pathogenesis of nasal congestion and allergic or virus-induced rhinorrhea. Salivary and lacrimal gland dysfunction is the hallmark of Sjögren’s syndrome and also results from head and neck radiotherapy. Recent experimental gene therapy studies suggest that at least partial restoration of glandular function may be accomplished by introduction of adenoviral vectors containing aquaporin cDNAs (7).

Despite the evidence implicating aquaporins in physiological functions of lung, the observation that humans with disruption of the AQP1 gene do not suffer clinically obvious inadequacies in respiratory or glandular functions argues that our insight into the biological needs for these molecules is still rudimentary. Thus redundancies in aquaporin expressions and the likelihood of multiple still-undiscovered aquaporins dictate the need for cautious interpretation of these data and provides a reminder that we must not overstate our current levels of understanding of these physiological processes.

The studies reported here demonstrate the need for elucidation of the specific cellular and subcellular sites of expression of these aquaporins in these tissues and the need for improved functional evaluations. Ongoing studies will define the sites of expression of AQP1, AQP3, AQP4, and AQP5 throughout the respiratory tract and glandular epithelium and may predict the locations of still-unidentified water channels in these tissues. It is anticipated that certain functions will involve multiple aquaporins acting in concert, similar to collecting duct principal cells in which AQP2, AQP3, and AQP4 are known to reside. Appropriate management of fluid in the vascular, interstitial, and airspace compartments is believed to be essential for normal function of the respiratory system and secretory glands. Thus, in parallel to extensive investigation of solute transporters (reviewed in Ref. 5), investigation of water channels will be needed to understand the physiology and pathophysiology of respiratory tract and glandular tissues.

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