Identification and localization of aquaporin water channels in human salivary glands

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The secretion of fluid by the salivary glands is a two-stage process in which the secretory endpieces, or acini, generate an isotonic, plasmalike fluid that is modified as it flows through the ductal system (3). The acinar epithelial cells might therefore be expected to have a high membrane water permeability and are a likely site of aquaporin expression. In contrast, the ductal epithelial cells are known to have a low water permeability (33) and to modify the primary secretion, mainly by reabsorption of Na⁺ and Cl⁻, in the absence of any significant reabsorption of water. The emergent saliva is consequently hypotonic, particularly at low secretory rates.

Although fluid secretion by the acinar cells is believed to involve the osmotic coupling of water flow to active electrolyte transport, the role of aquaporin water channels in this process is not completely resolved. Of the known members of the aquaporin (AQP) family, AQP1, AQP4, AQP5, and AQP8 have so far been identified in mammalian salivary glands, mostly in studies of rat submandibular and parotid glands. AQP5, which was originally cloned from the rat submandibular gland (26), is localized in the luminal membrane of the serous acinar cells of rat submandibular and parotid glands (10, 22) and is believed to provide the main pathway for osmotic water flow from the acinar cells to the lumen. In support of this, knockout mice lacking AQP5 show depressed rates of salivary secretion and the small amount of saliva that is secreted is markedly hypertonic (17).

No aquaporin has yet been identified with certainty, however, in the basolateral membranes of salivary acinar cells. AQP1 appears to be expressed mainly in the capillary endothelial cells of rat parotid and submandibular glands (16, 23) and is therefore not directly involved in transepithelial fluid secretion. AQP4, surprisingly, has been detected in the excretory ducts of rat salivary glands, but its function there is unclear (6).

THE AQUAPORINS ARE A FAMILY OF SMALL, MEMBRANE-SPANNING PROTEINS THAT ACT AS HIGHLY SELECTIVE WATER CHANNELS (1, 31). SEVERAL MEMBERS OF THE FAMILY ARE EXPRESSED IN THE FLUID-TRANSPORTING EPITHELIA ASSOCIATED WITH THE GASTROINTESTINAL TRACT (18), AND THERE IS INCREASING EVIDENCE FOR THEIR INVOLVEMENT IN SALIVARY SECRETION (17).
Although there is evidence for the expression of AQP8 in the submandibular glands of rats (14) and mice (19), its localization has yet to be established.

Very little is known about the role of aquaporin water channels in human salivary glands. We have therefore undertaken a systematic investigation of aquaporin expression, not only in the major salivary glands (parotid, submandibular, and sublingual) but also in some of the minor salivary glands that are scattered throughout the mouth, particularly the labial glands. Here we report on the expression and localization of AQP1, AQP3, AQP4, and AQP5 in human parotid, submandibular, sublingual, and labial glands in studies using molecular and immunohistochemical techniques. As anticipated, we have found that AQP5 is expressed in the apical membranes of the acinar cells, but our most striking finding is that in humans, unlike rodents, AQP3 is generally present in the basolateral membranes.

MATERIALS AND METHODS

Tissue samples. Parotid, submandibular, sublingual, and labial gland tissue was taken from adult patients undergoing surgery for various primary maxillofacial interventions. Patients gave informed consent, and the procedures were approved by the Semmelweis University of Budapest Regional Committee of Science and Research Ethics. The tissues used did not contain atypical cells when assessed microscopically. For RT-PCR and Northern blot analysis, samples were frozen in liquid nitrogen immediately after surgery and stored at 

-Rose gel electrophoresis. Sequencing of RT-PCR products. Aliquots (20 μg) of total RNA from human parotid, submandibular, sublingual, and labial glands—and, for comparison, RNA from human brain—were resolved on a formaldehyde agarose gel and transferred onto nylon membranes (Hybond, Amersham). Probes for human AQP1, AQP3, AQP4, AQP5, and GAPDH were prepared using the RT-PCR products obtained from the human salivary gland samples as described in Sequencing of RT-PCR products. They were first purified from an agarose gel using a Geneclean II kit (Bio 101) and then labeled using a Multi-prime DNA labeling kit (Amersham) and [α-32P]dCTP. Northern hybridization was performed at high stringency (27) for both the aquaporins and GAPDH. A semiquantitative analysis was performed by densitometry of five different Northern blots using a digital imaging system (ImageQuant software version 3.3; Molecular Dynamics, Sunnyvale, CA). Expression was plotted as averaged AQP-to-averaged GAPDH density ratios.

Antibodies. Polyclonal antibodies were raised to synthetic peptides (18–24 amino acids) corresponding to regions in the carboxyl termini of the respective water channels and were affinity purified. The antibodies, which were raised against rat AQP1 (LL266; Ref. 29), rat AQP3 (LL178; Ref. 4), rat AQP4 (LL182; Ref. 28), human AQP4 (LL485; Ref. 8), and human AQP5 (kindly provided by Peter Agre, Johns Hopkins University, Baltimore, MD), have all been described and characterized previously.

Immunohistochemistry. Human salivary gland tissue was cut into small blocks and transferred immediately into cold fixative (4% paraformaldehyde in 0.1 M PBS, pH 7.4) and stored for at least 2–4 h. The tissue blocks were then rinsed in PBS, embedded in paraffin, and sectioned at 7- to 10-μm thickness. Paraffin was removed by soaking the sections overnight in xylene. After rehydrating in 99% ethanol (3 times) and 96% ethanol (twice, 10 min each), endogenous
peroxidase was blocked with 0.5% hydrogen peroxide in absolute methanol for 30 min and rehydration was finished in 70% ethanol for 10 min.

For optimal target retrieval, sections were boiled in 1 mM Tris solution (pH 9.0) supplemented with 0.5 mM EGTA in a microwave oven set at half power for 10 min. After cooling and being soaked in 50 mM NH4Cl in 0.01 M PBS (pH 7.4) for 30 min, sections were rinsed three times in PBS containing 1% BSA, 0.05% saponin, and 0.2% gelatin for 10 min. Sections were incubated overnight at 4°C and for 1 h at room temperature with affinity-purified primary antibody in PBS (containing 0.1% BSA and 0.3% Triton X-100) at the following dilutions: rat AQP1 1:800, rat AQP3 1:800, rat AQP4 1:10, human AQP4 1:10, and human AQP5 1:1,600. They were then rinsed three times in PBS containing 0.1% BSA, 0.05% saponin, and 0.2% gelatin for 10 min and incubated for 1 h at room temperature with a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (P448; DAKO, Glostrup, Denmark) diluted 1:100 in PBS containing 0.1% BSA and 0.3% Triton X-100. After rinsing with the same solution as before, labeling was visualized with 1 mg/ml diaminobenzidine containing 1 µl/ml 30% hydrogen peroxide. After being rinsed three times in PBS for 10 min, sections were counterstained with Meier stain (1 min), rinsed with running tap water for 20 min, dehydrated in a graded ethanol series (70%, 96%, 99%), cleared in xylene, and mounted with Eukitt mounting medium (O. Kindler). Sections of human kidney were used as positive controls (data not shown). Labeled sections were examined by light microscopy (DMRE, Leica), and images were captured with a video camera (DXX-950P, Sony) connected to a PC frame-grabber (CMA-D2, Sony) and Digital Still Recorder (DKR-700, Sony). Digitized images were processed for documentation using Adobe Photoshop D5.5 and CorelDraw 9.0 software.

RESULTS

RT-PCR analysis. RNA samples from human parotid, submandibular, sublingual, and labial glands and human brain were assessed for AQP1 (807 bp), AQP3 (373 bp), AQP4 (429 bp), AQP5 (760 bp), and GAPDH (605 bp) transcripts by RT-PCR using specific primers for each. RT-PCR yielded products that ran as single bands of the expected size as detected by ethidium bromide-stained agarose gel electrophoresis (a representative example is shown in Fig. 1). Sequencing of the products obtained in a further round of PCR using nested primers showed 100% identity with the published human AQP nucleotide sequences.

Northern blot analysis. The first-round PCR products obtained with the aquaporin and GAPDH primers were cleaned. 32P-labeled, and used as cDNA probes for a Northern blot analysis of total RNA. Expression levels of mRNA for AQP1, AQP3, AQP4, and AQP5 were compared in human parotid, submandibular, sublingual, and labial glands and in human brain as a positive control for AQP4 (a representative example is shown in Fig. 2). GAPDH was used as a reference for the amount of RNA in each sample. Hybridization with the specific probes demonstrated transcripts of the expected sizes: 3.1 kb for AQP1 (25), 2.2 kb for AQP3 (11), 5.5 kb for AQP4 (32), 1.6 kb for AQP5 (26), and 1.3 kb for GAPDH (5).

The results of a semiquantitative analysis of the Northern blot hybridization data obtained from several different patients are shown in Fig. 3. Averaged data for each of the aquaporins, expressed as ratios of the corresponding values obtained for GAPDH, are plotted for each of the glands with the exception of the sublingual glands, for which insufficient tissue was available. The data confirm that mRNAs for AQP1, AQP3, and AQP5 are all expressed at significant levels in the labial, parotid, and submandibular glands. The AQP4 signals from the glands were not significantly different from background, whereas the brain RNA provided a strong positive control result.

Immunohistochemical analysis. Immunohistochemistry was used to localize AQP1 (Fig. 4), AQP3 (Fig. 5), AQP4 (Fig. 6), and AQP5 (Fig. 7) in paraffin sections of human labial, sublingual, parotid, and submandibular glands. Each of the human salivary gland samples showed similar staining properties for all four of the...
aquaporins examined. There were some small differences in the staining pattern between serous acini, which are the predominant type in the parotid glands, and mucous acini, which are predominant in the sublingual and labial glands. The differences were also clearly visible in the submandibular glands, in which both types are present.

As expected from the rodent studies, AQP1 was detected in the endothelia of the capillaries and small blood vessels. More interestingly, AQP1 was also expressed in the myoepithelial cells (Fig. 4). These are contractile cells with a polygonal cell body, central nucleus, and long, tapering processes that form a basketlike framework around the acini and smaller ducts. The structure of these cells is seen most clearly in a tangential section of a serous submandibular gland acinus shown in Fig. 4E.

AQP3 stained strongly at basal and lateral locations in both serous and mucous acini in all of the glands (Fig. 5). However, in the submandibular gland it appeared to be more abundant in the serous acini than in the mucous acini (Fig. 5, D and E). AQP3 was not
detected at the apical membranes of the acinar cells or in the ductal cells.

Although an AQP4 cDNA fragment was readily amplified by RT-PCR (Fig. 1), AQP4 protein was not observed in any of the glands with immunohistochemistry (Fig. 6) using either the antibody to rat AQP4 (Fig. 6) or the human AQP4 (data not shown).

Very abundant AQP5 labeling was confined to the apical plasma membrane domains of the acinar cells in all of the glands (Fig. 7). In the largely serous parotid gland and in the serous acini and demilunes of the mixed submandibular gland, staining could also be seen in the secretory canaliculi radiating from the small luminal space (Fig. 7, E–G). The submandibular gland exhibited

![Fig. 5. Immunohistochemistry of AQP3 in human labial (A), sublingual (B), parotid (C), and submandibular (D, E) glands. AQP3 is seen at the basal (arrows) and lateral (arrowheads) surfaces of the acinar cells. The staining is generally stronger in the serous acini than in the mucous acini (M), especially when compared in the mixed submandibular gland (D, E). Salivary ducts are labeled with an asterisk. Magnifications: ×260 (A); ×330 (B); ×460 (C); ×150 (D); ×300 (E).](image)

![Fig. 6. Immunohistochemistry of AQP4 in human labial (A), sublingual (B), parotid (C), and submandibular (D) glands. No AQP4 staining was observed in either the acini or the ducts of any of the glands. Magnification ×100.](image)
much stronger labeling of the serous acini and demilunes than of the mucous acini. In the labial and sublingual glands there was strong apical labeling, in both their mucous acini and serous demilunes (Fig. 7, A–D).

DISCUSSION

Tissues and organs associated with large water fluxes, such as the kidney, airways, eye, and brain, show complex patterns of aquaporin expression, often involving several different members of the AQP family at different cellular locations within the same tissue. The same might therefore be expected to be true for exocrine glands, which transport comparably large volumes of isotonic or hypotonic fluid. Thus far, only AQP5 has been unequivocally identified as being associated with the fluid-secreting cells of salivary glands (10, 22), and such studies have been largely confined to rodent glands.

In rat salivary glands, AQP1 expression is restricted to the endothelial cells of the microvasculature (16, 23). In this study, however, in addition to its expression in capillary endothelium, we have demonstrated that the pattern of AQP1 labeling in human salivary glands corresponds with the shape and distribution of the myoepithelial cells, whose long, thin processes occupy the interstices and sulci among the basal regions of the acini and smaller ducts. It is unclear why myoepithelial cells should express aquaporins, because their main function is presumably contractile rather than secretory. On the other hand, it could be argued that a high membrane water permeability would help to ensure that the “basket” of myoepithelial cell processes, wrapped around the acinus, does not constitute a significant barrier to osmotic water flow into the lateral intercellular spaces.

Several rat exocrine glands have been shown to express AQP5 at the luminal surface of the acinar cells. These include the rat lacrimal gland (12, 20) and the subepithelial glands of the upper airways (22) as well as the rat submandibular and parotid salivary glands (10, 22). In the human salivary glands, we have found that AQP5 is abundantly expressed in the luminal membranes of both serous and mucous acinar cells. In the serous acini of the parotid and submandibular glands, it is also present in the extensive secretory canaliculi that radiate from the luminal space and effectively increase the apical surface area of the cells. Assuming that water flows across this membrane in the formation of the primary secretion, the presence of aquaporins would help to compensate for the very small area of this membrane compared with the basolateral surface of the cells (24).

In some exocrine glands, such as the lacrimal glands (12, 21), upper airway glands (22), and gastric glands (7, 15), AQP3 and/or AQP4 are expressed in the basolateral membranes of the secretory cells. Neither of

Fig. 7. Immunohistochemistry of AQP5 in human labial (A–C), sublingual (D), parotid (E), and submandibular (F, G) glands. AQP5 is present in each of the human salivary glands. Labial and sublingual glands show strong apical labeling in their mucous acini (arrows) and in their serous demilunes (asterisk). In the serous acini of the parotid and submandibular glands AQP5 is very abundant in the apical membrane and in the secretory canaliculi between the cells (arrowhead). In the submandibular gland, staining is much stronger in the serous acini (arrowheads) and serous demilunes (asterisk) than in the mucous acini (arrows). Salivary ducts were not labeled with AQP5. SD, striated duct (E); ID, intercalated duct (G). Magnifications: ×150 (A, F); ×200 (B); ×400 (C); ×250 (D); ×180 (E, G).

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these, nor any other known aquaporins, have hitherto been identified in the basolateral membranes of salivary acinar cells. Given the apparent absence of AQP3 from rat salivary glands (13, 22, 30), it was surprising to find strong evidence for AQP3 expression at this location in the human salivary glands. We found levels of mRNA expression and intensities of immunostaining comparable with those of AQP5 in each of the major glands as well as in the labial glands, suggesting that this basolateral water channel plays a significant role in fluid secretion.

Although evidence of AQP4 (mercurial-insensitive water channel; MIWC) expression has been reported in RT-PCR studies of rat salivary glands (9), the only available information about its localization suggests that it is present in the excretory duct (6). This is surprising because the excretory duct has a very low permeability to water (33). In the human glands, although we were readily able to amplify a cDNA segment with the sequence of AQP4 by RT-PCR, our Northern blot analysis suggested that the mRNA expression level was virtually indistinguishable from baseline. Consistent with this, we were unable to detect AQP4 in any of the glands by immunohistochemistry. We therefore conclude that, if present, AQP4 is expressed either at low levels or in only a very small fraction of the total cell population.

In summary, we have found clear evidence for the expression of AQP1, AQP3, and AQP5 in all of the major human salivary glands and also in the labial glands. Although not playing a direct role in fluid secretion, AQP1 probably contributes to the water permeability of the microvasculature, and its presence in the myoepithelial cells may facilitate the access of water to the basal surfaces of the acinar cells. The principal pathway for osmotically coupled water transport across the secretory epithelium, however, seems likely to involve both AQP3 at the basolateral membrane of the acinar cells and AQP5 at the luminal membrane. Thus a network of aquaporins is likely to be involved in salivary secretion.

NOTE ADDED IN PROOF

Recently, it has been reported (Wellner RB, Hoque ATMS, Goldsmith CM, and Baum BJ. Evidence that aquaporin-8 is located in the basolateral membrane of rat submandibular gland acinar cells. Pflügers Arch 441: 49–56, 2000) that AQP8 protein may be present in basolateral membrane.

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REFERENCES


