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Expression and Localization of Aqua-glyceroporins AQP3 and AQP9 in Rat Oral Epithelia

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Abstract

Aquaporins (AQP) are a family of small integral membrane proteins made up of 6 hydrophobic, α-helical, membrane-spanning domains surrounding a highly selective aqueous pore. AQP3, AQP7, and AQP9, termed aqua-glyceroporins, are known to be involved in the transport of water, glycerol, and other small molecules. In this study, we investigated the expression and localization of aqua-glyceroporins in rat oral stratified squamous epithelia of the palate, the buccal mucosa, the inferior aspect of the tongue, and the oral floor by using RT-PCR, immunofluorescence, and immunogold electron microscopy. AQP3 and AQP9 mRNAs were expressed in whole oral epithelium. Immunostaining for AQP3 was recognized in each type of epithelium. The results suggest that AQP3 synthesis begins predominantly in the cytoplasm of the basal cells. During the process of epithelial cell differentiation, AQP3 protein appears to accumulate and be transported to the plasma membrane, from where it is incorporated into the cornified or surface layers. The intracellular localization of AQP3 appears to correlate with the differentiation of keratinocytes, suggesting that it acts as an enhancer of the physiological permeability barrier together with membrane coating granules. The distribution pattern of AQP9 was limited to the marginal areas of the basal and suprabasal layers, which was different from that of AQP3. This difference in distribution between AQP3 and AQP9
suggestions that AQP9 in rat oral epithelia acts as a channel by facilitating glycerol uptake from the blood through the endothelial cells of the capillary vessels to the oral stratified squamous epithelium. AQP3 and AQP9 facilitate both transepithelial osmotic water flow and glycerol transport as pore-like passive transporters in the keratinocytes of oral epithelia, and may play a key role in not only hydration and the permeability barrier, but also cell proliferation, differentiation, migration, development, and wound healing by generating ATP.

Key words: AQP3 — AQP9 — Aqua-glyceroporin — Oral epithelium — Permeability barrier

Introduction

Due to their prominent physiological role in the cross-membrane channeling of water, the name aquaporin (AQP) was proposed for a group of integral membrane proteins first identified by Agre et al. More recently, the term aqua-glyceroporin has been applied to any AQP that functions as a water channel or glycerol facilitator. The AQP family of proteins is made up of 6 hydrophobic, α-helical, membrane-spanning domains surrounding a highly selective aqueous pore. Cloning has been used to produce homologues of these proteins, and has led to the recognition of at least 13 mammalian AQPs. Although all the members of this family contain structural motifs similar to those of AQP1, it is probable that each one also possesses the unique features required for its specific function or regulation. Correlation of the sequences and functional properties of this family has led to the identification of 3 distinct sub-groups: water-selective homologues, termed aquaporins, which include AQP0, AQP1, AQP2, AQP4, AQP5, AQP6 and AQP8; homologues, termed aqua-glyceroporins, that are permeated by water, glycerol, and other small molecules, and which include AQP3, AQP7 and AQP9; and AQP10. However, AQP10 seems to be an incompletely spliced form of the protein, having no conserved sixth transmembrane domain, only poor water permeability, and none for glycerol or urea. Functionally, however, it is still characterized as an aqua-glyceroporin. Recently, two new members of the AQP family reported in humans, termed super-aquaporins (AQP11 and AQP12), were reported to have unusual pore structures and functions. These channels have been identified and shown to play an essential role in diverse physiologic processes, including renal water re-absorption, exocrine gland secretion, and lacrimation. Furthermore, other studies have also shown evidence to support their role in cerebrospinal fluid secretion and re-absorption, generation of pulmonary secretion, and aqueous humour secretion.

AQP3 has been reported to be expressed in the kidney, urinary tract, digestive tract, and skin. It has been localized in the basal and intermediate cells of stratified squamous epithelia from the fauces to the forestomach and skin, and has also been detected in the suprabasal layer of the oral epithelium during human and mouse tooth development. Expression of AQP9 has been reported in a number of cell types, including leukocytes, osteoclasts, those of the ovaries and digestive tract, hepatocytes, and keratinocytes of the skin, but not in the developing tooth in mouse. In contrast, there is little evidence to support expression of AQP7 in the squamous epithelium of the skin, and it appears to be mainly expressed in the adipose tissue, muscle, and a variety of other tissues. However, no information is available regarding the expression and localization of aqua-glyceroporins in oral epithelia.

The purpose of this study was to investigate the expression and intracellular localization of aqua-glyceroporins AQP3 and AQP9 in rat
oral epithelia by using RT-PCR, immunofluorescence, and high resolution immunogold electron microscopy of ultra-thin cryosections to characterize their involvement in the permeability barrier of oral keratinized squamous epithelium.

**Materials and Methods**

1. Isolation of oral epithelium

Oral mucosa was obtained from 30 male Sprague-Dawley rats (6 weeks old), each weighing approximately 200 g. Under anesthesia induced by intraperitoneal injection of sodium thiopental (Ravonal®, Mitsubishi Tanabe Pharma, Osaka, Japan), the whole oral mucosa was excised using surgical scissors. The epithelial layer was removed from the underlying connective tissues by digestion with dispase® I (25 U/ml; Roche, Mannheim, Germany) for 3 hrs at 37°C and then separated according to source (palate, buccal mucosa, tongue, or oral floor). The kidney medullae and liver were used as positive controls for AQP3 and AQP9, respectively. All experiments were performed in accordance with the Guidelines for Experimental Laboratory Animals at the Animal Facility of Tokyo Dental College (No.220501).

2. Reverse transcription-polymerase chain reaction (RT-PCR) analysis

Each type of epithelial tissue was homogenized in TRIZOL® Reagent (Invitrogen Corp., Carlsbad, CA, USA) by using a Teflon-pestle homogenizer (As One, Tokyo, Japan) and total RNA extracted. The chloroform suspension was centrifuged at 16,000 g for 20 min at 4°C. Isopropyl alcohol was added to the supernatant, which was then frozen at −80°C overnight. Subsequently, each sample was centrifuged at 16,000 g for 20 min and the total RNA pellets washed with 70% ethanol. Finally, the total RNA pellets were dissolved in RNase-free water and total RNA concentrations measured by absorbance using an ND-1000 spectrophotometer (LMS Co., Ltd., Tokyo, Japan). Oligo dT primer (0.5 ml), dNTP (1 ml), RNase inhibitor (0.25 ml), reverse transcriptase (0.5 ml), 10× buffer (1 ml) and MgCl₂ (2 ml) were added to total RNA (0.5 ml) and the volume adjusted to 10 ml with RNase-free water. The mixture was used to synthesize cDNA by reverse transcription (42°C for 1 hr, 94°C for 5 min, 5°C for 5 min). For PCR, the cDNA reaction mixtures were then diluted with 10 ml PCR buffer and mixed with 0.5 ml primers for:

- AQP3 (sense primer; 5′-ACCATCAACTTG GCTTTTGG-3′, antisense primer; 5′-AGGC CCAGATTGGCATCAG-3′),
- AQP9 (sense primer; 5′-TGGTAGTGAAGG CAGGGAAAC-3′, antisense primer; 5′-CGCT TGGGCAATAGAGCTAC-3′) or
- β-actin (sense primer; 5′-CCTGTATGCCTC TGTCGTA-3′, antisense primer; 5′-CCAT CTCTTGCTCGAGTCT-3′).

PCR reactions were carried out in the PTC-100™ programmable Thermal Controller (MJ Research, Inc., Watertown, MA, USA). The PCR conditions for AQP3 and β-actin were 30 cycles (94°C for 30 sec, 60°C for 30 sec, and 72°C for 1 min); for AQP9 they were 30 cycles (94°C for 30 sec, 65°C for 30 sec, and 72°C for 1 min). The amplification product sizes of AQP3, AQP9, and β-actin were 217, 210, and 260 base pairs, respectively. The PCR products were then electrophoresed on 2% agarose gels and the bands visualized by ethidium bromide staining.

3. Immunofluorescence microscopy

Each type of epithelial tissue was quickly removed, immediately embedded in Tissue-Tek® OCT™ Compound (Sakura Finetechnical Co., Ltd., Tokyo, Japan) and rapidly frozen in liquid nitrogen. Frozen sections approximately 6 μm in thickness were cut and, after washing with phosphate-buffered saline (PBS), incubated with rabbit polyclonal antibodies to AQP3 (1:100; Chemicon International, Inc., Temecula, CA, USA) and AQP9 (1:100; Chemicon International, Inc.) for 2 hrs at room temperature. After washing with PBS, the tissues were incubated with a secondary antibody, goat anti-rabbit IgG, conjugated with Alexa Fluor® 488 (Molecular Probes,
Inc., Eugene, OR, USA) for 60 min at room temperature. As a control, specimens were treated with normal goat serum instead of each AQP antibody. Specimens were examined and photographed using a conventional fluorescence microscope (Axioskop 2: Carl Zeiss Co., Munchen-Hallbergmoos, Germany).

4. Immunoelectron microscopy

For ultra-thin immunogold labeling, the tissues were fixed with 4% paraformaldehyde and 0.2% glutaraldehyde in 0.1 M cacodylate buffer for 30 min by transcardial perfusion fixation. Epithelial tissues were removed and embedded in 2.3 M sucrose. Small pieces of each tissue were mounted and rapidly frozen with liquid nitrogen. Ultra-thin cryosections were cut using an ultra-cryo-microtome at −100°C (FC-4S, Reichert Ultracut-S; Reichert, Co., Heidelberg, Germany). The cryosections were mounted on carbon-coated formvar nickel grids and incubated with rabbit polyclonal antibodies to AQP3 and AQP9 (1:100; Chemicon) for 2 hrs at 37°C. After rinsing as described above, the sections were incubated with an anti-rabbit IgG secondary antibody conjugated with colloidal gold (10 nm: British BioCell International, Ltd., Cardiff, UK) for 60 min at 37°C. The sections were fixed with 1% glutaraldehyde and then rinsed and post-fixed with 1% osmium tetraoxide. After dehydration with ethanol and embedding in LR-white resin (London Resin Co., London, UK) by heating at 60°C for 12 hrs, the sections were post-stained with uranyl acetate and lead citrate. Specimens were examined and photographed using a transmission electron microscope (H7100: Hitachi, Co., Tokyo, Japan).

Expression of AQP mRNAs was normalized using β-actin (260 base pairs) as a standard loading control. The results of RT-PCR and sequence verification are shown in Fig. 1. AQP3 (217 base pairs) and AQP9 (210 base pairs) were expressed in all oral epithelia. Kidney medullae and liver epithelial cells were used as positive controls for AQP3 and AQP9, respectively (a, b).

Fig. 1 RT-PCR analysis of AQP3 and AQP9 in oral epithelia

217- and 210-base pair bands corresponding to AQP3 and AQP9 were expressed in all oral epithelia. Kidney medullae and liver epithelial cells were used as positive controls for AQP3 and AQP9, respectively (a, b).

Results

1. Expression of AQP3 and AQP9 mRNA/protein in rat oral epithelia

Oral epithelia from rat palate, the buccal mucosa, the inferior aspect of the tongue, and the oral floor were isolated and RT-PCR performed to evaluate the expression and distribution of AQP3 and AQP9 mRNAs.

2. Immunolocalization of AQP3 and AQP9 in rat oral epithelia

1) Immunofluorescence microscopy

To characterize the immunolocalization of AQP3 and AQP9 in oral epithelia, immunofluorescence staining was carried out with frozen sections. Conventional immunofluorescence microscopy revealed a strong fluorescence reaction for AQP3 in the epithelial layer of the oral epithelium, but not in the cornified or superficial layers. In the palatal epithelium, AQP3 reactivity was recognized in the cytoplasm of basal layer cells and on the periphery of spinous and granular layer cells, but not in cornified layer cells (Fig. 2a). In the buccal epithelium (Fig. 2c), the inferior
In palate, AQP3 reactivity was recognized in cytoplasm of basal layer (BL) and on periphery of spinous (SL) and granular layers (GL), but not in cornified layer (CL) (a). In buccal mucosa (c), inferior aspect of tongue (e), and oral floor (g), AQP3 was observed in cytoplasm of BL and periphery of SL and intermediate layer (IL), but not in superficial layer (SfL). AQP9 was observed on periphery of basal and suprabasal cells in palate (b), buccal mucosa (d), tongue (f), and oral floor (h). (×260, Bar; 100 µm)

aspect of the tongue (Fig. 2e) and the oral floor epithelium (Fig. 2g), positive AQP3 staining was observed in the cytoplasm of the basal layer cells, and an intense positive reaction was recognized on the periphery of the spinous layer and intermediate layer cells;
no reaction was observed in superficial layer cells, however.

In contrast, clear positive fluorescence indicating AQP9 expression was observed on the periphery of the basal and suprabasal cells of palatal epithelium (Fig. 2b), buccal epithelium (Fig. 2d), the inferior aspect of the tongue (Fig. 2f), and the oral floor epithelium (Fig. 2h). Furthermore, AQP9 staining was observed on the periphery of the upper spinous-to-granular layer cells of the palate and intermediate-to-superficial layer cells of the buccal mucosa, the inferior aspect of the tongue, and the oral floor epithelium.

2) Immunoelectron microscopy

To confirm the immunolocalization and subcellular distribution of AQP3 and AQP9 in oral epithelial cells, morphological observations were carried out using colloidal gold immunoelectron microscopy with ultra-thin cryosections. Figure 3 shows the ultrastructural localization of AQP3 and AQP9 in the epithelium of the oral floor. In the spinous layer cells, 10-nm gold particles indicating localization of AQP3 accumulated in the peripheral area close to the plasma membrane, and gold particles were also recognized in the cytoplasm of those cells. Only a few particles
were found in the nuclei and mitochondria as background, non-specific reactions (Fig. 3a). Meanwhile, on the upper side of the intermediate layer cells, AQP3-reactive gold particles were localized predominantly at the cell surface, forming a line on the plasma membrane (Fig. 3b). Gold particles indicating AQP9 were localized in the cytoplasm and also on the plasma membrane of the basal and spinous layer cells (Fig. 3c), and were recognized exclusively on the plasma membrane of intermediate layer cells (Fig. 3d; Des; desmosomes, ICS; intercellular space).

**Discussion**

It is well known that AQP3 and AQP9 play a major role as aqua-glyceroporins not only in the transport of water, but also of glycerol and other small molecules. Previous studies have shown that AQP3 is expressed in a wide variety of organs, such as the kidney and the urinary tract, digestive tract, respiratory tract, eye, brain, and skin. In renal collecting ducts, AQP3 is localized at the basolateral membrane, where epithelial cells would encounter possible osmotic water loss due to the difference in tonicity between luminal urine and intracellular fluid. AQP3 is also present in the squamous epithelium of skin, where it serves as a barrier against water loss and improves hydration below the cornified layer. AQP9 expression has been reported in several tissues, including those of the digestive tract, liver, and skin. At the boundary between the granular and cornified layers, MCGs are transported to the surface of keratinocytes, where their membranes fuse with the plasma membrane, after which the lipid lamellae are released into the extracellular spaces. Thus, MCGs are thought to be responsible for the physiological permeability barrier in stratified squamous epithelia, and AQP seems to be involved, acting as an enhancer for this function.

In stratified squamous epithelium, during the process of differentiation, lipids are accumulated and packaged into small vesicular organelles known as membrane coating granules (MCG) that become evident by electron microscopy in the upper part of the spinous layer. At the boundary between the granular and cornified layers, MCGs are transported to the surface of keratinocytes, where their membranes fuse with the plasma membrane, after which the lipid lamellae are released into the extracellular spaces. This suggests that AQPs facilitate water flow by osmosis across the plasma membrane via the transcellular pathway; that they play a role in paracellular fluid transport (the major pathway across stratified epithelium for large molecules); and that they are involved in the physiological permeability barrier.

The results of the present study using RT-PCR, immunofluorescence, and immunoelectron microscopy revealed expression of AQP3 and AQP9 water channels in rat oral stratified squamous epithelia of the palate, the buccal mucosa, the inferior aspect of the tongue, and the oral floor.

AQP3 was localized in the cytoplasm of the basal cells and cell membranes of all the epithelial layers investigated, apart from the cornified or superficial layers. Positive staining for AQP9 was observed in the basal layers of all the tissues investigated, where it appeared to be concentrated in the cellular membrane of the basal layer, in contrast to AQP3 staining, which was apparent in the cytoplasm of the basal layers. Although this study was not designed to elucidate the physiological role of AQP channels in oral epithelia, the differences found in their localization indicate that they play at least a contributing role in the regulation of cell volume and osmolality during the process of cell differentiation, which may vary depending on the underlying submucosal layer involved. This suggests that AQPs facilitate water flow by osmosis across the plasma membrane via the transcellular pathway; that they play a role in paracellular fluid transport (the major pathway across stratified epithelium for large molecules); and that they are involved in the physiological permeability barrier.
non-keratinized buccal mucosa, the inferior aspect of the tongue, or the oral floor epithelium.

This pattern of localization indicates that not only the cornified layer of keratinized epithelium, but also the superficial layer of non-keratinized epithelium comprises a principal component of the permeability barrier that protects against environmental influences such as physical damage, bacterial infection, desiccation, and heat loss, and plays a role in the maintenance of homeostasis. Vielhaber et al.\(^3\) have demonstrated that ceramide is localized in MCGs at the cornified envelope and within the intercellular spaces of the stratum corneum by immunogold electron microscopy. In contrast, only small amounts of ceramide were observed in the superficial layer of oral non-keratinized epithelium.\(^9,41\) Non-keratinized epithelium also contains small amounts of neutral polar lipids, mainly cholesterol sulfate and glucosylceramide. This type of epithelium is considerably more permeable to water than is keratinized epithelium.\(^33\)

The lipid content of each type of squamous epithelium determines its permeability to water, and the intracellular distribution of AQP3 and AQP9 appear to correlate with MCG localization and lipid secretion in the intercellular spaces of the stratum corneum.

Glycerol is a small degradation product of glucose, proteins, and other triacylglycerols. It is an important intermediary in energy metabolism and a substrate for the biosynthesis of various lipids.\(^6\) In addition, glycerol can control osmotic activity in the cytoplasm.\(^6\) A recent study on expression of AQP3 in skin has shown that AQP3-facilitated water transport is involved in cell migration and accelerating wound healing; furthermore, AQP3-facilitated glycerol transport is involved in generating ATP and facilitates epithelial cell proliferation and differentiation, and also tumorigenesis.\(^15,35,36,38\) On the other hand, immunohistological studies have detected strong expression of AQP9 in perivenous hepatocytes, suggesting a possible function in glycerol uptake from sinusoidal capillaries during gluconeogenesis in the liver.\(^30,31\)

In the present study, RT-PCR revealed clear expression of AQP9 in rat oral epithelium of the palate, the buccal mucosa, the inferior aspect of the tongue, and the oral floor. The distribution pattern of AQP9 was limited to the marginal areas of the basal and suprabasal layers, a pattern which differed from that of AQP3. This difference in distribution between AQP3 and AQP9 suggests that AQP9 in rat oral epithelia acts as a channel by facilitating glycerol uptake from the blood through the endothelial cells of the capillary vessels to the oral stratified squamous epithelium. Earlier studies using AQP9-knockout mice indicated that AQP9 played a central role in glycerol metabolism and maintaining skin hydration. No defects in wound healing were observed, however.\(^31\)

AQP3 and AQP9 facilitate both transcellular osmotic water flow and glycerol transport as pore-like passive transporters in the keratinocytes of oral epithelia, and may also play key roles in not only hydration and the permeability barrier, but also cell proliferation, differentiation, migration, development, and wound healing by generating ATP. Further study is needed, however, to fully determine the role of AQP3 and AQP9 in oral epithelial cells.

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