Expression and localization of aquaporins in rat gastrointestinal tract

Koyama, Yu, Tadashi Yamamoto, Tatsuo Tani, Kouei Nihei, Daisuke Kondo, Haruko Funaki, Eishin Yaoita, Katsutoshi Kawasaki, Nobuaki Sato, Katsuyoshi Hatakeyama, and Itaru Kihara. Expression and localization of aquaporins in rat gastrointestinal tract. Am. J. Physiol. 276 (Cell Physiol. 45): C621–C627, 1999.—A family of water-selective channels, aquaporins (AQP), has been demonstrated in various organs and tissues. However, the localization and expression of the AQP family members in the gastrointestinal tract have not been entirely elucidated. This study aimed to demonstrate the expression and distribution of several types of the AQP family and to speculate on their role in water transport in the rat gastrointestinal tract. By RNase protection assay, expression of AQP1–5 and AQP8 was examined in various portions through the gastrointestinal tract. AQP1 and AQP3 mRNAs were diffusely expressed from esophagus to colon, and their expression was relatively intense in the small intestine and colon. In contrast, AQP4 mRNA was selectively expressed in the stomach and small intestine and AQP8 mRNA in the jejunum and colon. Immunohistochemistry and in situ hybridization demonstrated cellular localization of these AQP in these portions. AQP1 was localized on endothelial cells of lymphatic vessels in the submucosa and lamina propria throughout the gastrointestinal tract. AQP3 was detected on the circumferential plasma membranes of stratified squamous epithelial cells in the esophagus and basolateral membranes of cardiac gland epithelia in the lower stomach and of surface columnar epithelia in the colon. However, AQP3 was not apparently detected in the small intestine. AQP4 was present on the basolateral membrane of the parietal cells in the lower stomach and selectively in the basolateral membranes of deep intestinal gland cells in the small intestine. AQP8 mRNA expression was demonstrated in the absorptive columnar epithelial cells of the jejunum and colon by in situ hybridization. These findings may indicate that water crosses the epithelial layer through these water channels, suggesting a possible role of the transcellular route for water intake or outlet in the gastrointestinal tract.

RECENTLY, A FAMILY of water channels (aquaporin, AQP) has been identified as molecules that locate on plasma membrane of various cell types and increase its water permeability in mammals (12). AQP1 (CHIP28, channel-forming intrinsic protein of 28 kDa) was first found as a homologous protein to MIP (major intrinsic protein of bovine lens) in erythrocytes (12, 28) and the proximal tubule and thin descending limb of Henle in the kidney and then various epithelial and endothelial cells in systemic organs (23, 24). AQP2 is exclusively expressed in the apical membrane and intracellular vesicles of the collecting duct principal cells in the kidney and is demonstrated to be regulated by vasopressin (5, 22, 34). AQP3 (1, 10, 17) and AQP4 (6, 11) are present in many organs, but AQP5 and AQP6 are selectively localized in the salivary gland, eye, and lung (30) and in the kidney (18), respectively. Recently, AQP7 (7, 14), AQP8 (9, 13, 19), and AQP9 (8) were isolated from testis and liver libraries as new members of AQP. AQP7 is intensely expressed in the testis, kidney, and heart (7); AQP8 in the liver, pancreas, salivary gland, and testis (9, 13); and AQP9 in the liver and leukocytes (8).

The gastrointestinal tract, especially the small and large intestines, is a main organ for water entrance to the body. A large volume of water moves and is absorbed in the gastrointestinal tract every day, estimated as 7.5 liters in the small intestine and 1.5 liters in the large intestine in humans (25, 27). Although electrolytes have been demonstrated to cross the absorptive epithelial cells of these tissues by various transporters located on the cell membranes (transcellular route), the pathway of water transport in the gastrointestinal tract has not received much attention and water has been presumed to pass the epithelial cell layer by the paracellular route through tight junctions (26, 27). However, recent studies showing the existence of AQP on the epithelial cells in the gastrointestinal tract suggested their roles in the water absorption or secretion there (10–13), although the precise cellular localization and the portions of mRNA expression of the AQP family in the gastrointestinal tract have not been examined intensively to date. In the present study, we examined expression and localization of AQP1, AQP2, AQP3, AQP4, AQP5, and AQP8 in the rat gastrointestinal tract by RNase protection assay and by in situ hybridization and immunohistochemistry.

MATERIALS AND METHODS

Tissue and RNA preparation. Tissues (esophagus, upper and lower portions of stomach, jejunum, middle portion of small intestine, ileum, and proximal and distal colons) were removed from Wistar-Kyoto rats (3 mo old) and were frozen at −80°C in n-hexane. Total cellular RNA was also isolated from these tissues by a modified acid guanidinium thiocyanate phenol-chloroform extraction method (TRIZol, GIBCO BRL, Life Technologies, Rockville, MD).
RNAse protection assay. AQP1 (356 bp, +226 to +581) and AQP3 (377 bp, +256 to +632) cDNA fragments were cloned from rat colon RNA, AQP4 (330 bp, +302 to +631) cDNA fragments were cloned from rat ileum RNA, and AQP5 cDNA (328 bp, +324 to +651) fragments were cloned from rat salivary gland RNA by a PCR-based cloning method using the nested, degenerate oligonucleotide primers for AQP family as reported previously (30). The PCR products were subcloned into pGEM T vectors (Promega, Japan, Tokyo, J apan), and their sequences were verified by an automated DNA sequencer (Perkin Elmer, Foster City, CA). Partial fragments of rat AQP2 cDNA (309 bp, +1 to +309), AQP8 cDNA (315 bp, +701 to +1015), and rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA were inserted in pSPORT1 vector (GIBCo BRL), pGEM11Z (Promega), and pGEM3Z, respectively, as reported previously (13, 33). These plasmids were linearized with appropriate restriction enzymes and used as templates for in vitro transcription of α-32P-labeled antisense cRNA probes.

The RNAse protection assay was carried out as follows (13, 33): 10 µg of each RNA sample were hybridized with 1 × 106 counts/min each of the AQP probe combined with the GAPDH probe in 10 µl of hybridization buffer (80% formamide, 40 mM PIPES, 0.1 M NaCl, 1 mM EDTA) overnight at 48°C. Then, unhybridized probes were digested with RNAse A and RNAse T1 at 30°C for 1 h, and the ribonucleases were digested with proteinase K at 37°C for 30 min. After phenol-chloroform extraction, the hybridized probes were precipitated with ethanol, denatured at 85°C, and electrophoresed on 6% polyacrylamide gels. The dried gels were exposed to X-ray films for 3 days (Fujifilm Photo Film, Kanagawa, J apan).

For quantitation of the autoradiography bands, the RNAse protection assay was repeated five times using different RNA samples. The X-ray films were optically scanned (HP Scanjet et 3C, Hewlett-Packard, Greeley, CO), and the density of each band was analyzed by a computerized densitometry (Power Macintosh 9500/132, Apple Computer, Cupertino, CA) using Macintosh software (version 1.59, NIH Division of Computer Research and Technology, Bethesda, MD). The data were represented as ratios (AQP mRNA/GAPDH mRNA band density) as reported previously (20, 33).

Immunohistochemistry and in situ hybridization. For immunohistochemistry, the tissues were fixed with methyl Carnoy’s fixative (60% methanol, 30% chloroform, 10% acetic acid), embedded in paraffin, and sectioned at 4 µm. They were sequentially incubated with rabbit anti-rat AQP1 antibody (Chemicon, Temecula, CA), anti-rat AQP3 antibody purified by an affinity chromatography (10), anti-rat AQP4 antibody (Chemicon), or normal rabbit serum, and then incubated with goat anti-rabbit immunoglobulins conjugated to peroxidase labeled polymer (EnVision, DAKO, Tokyo, J apan), colored by diaminobenzidine reaction and counterstained with hematoxylin.

Localization of AQP3, AQP4, and AQP8 mRNA-expressing cells was examined by in situ hybridization using digoxigenin-labeled cRNA probes for the rat AQP3, AQP4, and AQP8 or 35S-labeled cDNA probes for the rat AQP8 transcripted in vitro from the linearized rat AQP3 (377 bp, +256 to +632), AQP4 (330 bp, +302 to +631), and AQP8 cDNA template (809 bp, +81 to +889). Sense and antisense digoxigenin- or 35S-labeled probes were prepared according to the manufacturer’s protocol (Boehringer Mannheim, Mannheim, Germany) and as reported previously (13). The tissues of the rat gastrointestinal tract were cryosectioned at 10-µm thickness, and the sections were fixed in 4% paraformaldehyde in PBS and treated with 3 µg/ml of proteinase K (Promega) in 0.5 M NaCl, 10 mM Tris·HCl, pH 8.0, for 10 min at room temperature. Then they were hybridized with digoxigenin-labeled sense or antisense probe (1 ng/ml) or 35S-labeled sense and antisense AQP8 cRNA probes (1 × 106 counts per min per section) overnight at 55°C. After a washing in 2× saline-sodium citrate (SSC), 1 mM EDTA at room temperature, the sections were treated with 20 µg/ml of RNaseA for 30 min at room temperature, followed by washing in 0.1× SSC, 1 mM EDTA at 55°C for 2 h and washing in 0.5× SSC at room temperature. Thereafter, they were incubated at 4°C overnight with anti-digoxigenin antibody (Boehringer Mannheim, 1:200 dilution) and colored with nitro blue tetrazolium-5-bromo-4-chloro-3-indolyl phosphate. After color development, the sections were counterstained with Kernechtrot stain solution (Muto Pure Chemicals, Tokyo, J apan) for 20 min at room temperature, dehydrated with ethanol, and then mounted with ENTellan (Merck, Darmstadt, Germany).

Alternatively, the sections hybridized with 35S-labeled sense and antisense AQP8 cRNA probes were exposed to autoradiographic emulsion (NR-M2, Konica, Tokyo, J apan) for 5–7 days in the dark at 4°C, developed, and counterstained with hematoxylin.

RESULTS

AQP mRNA expression in gastrointestinal tract. RNAse protection assay showed ubiquitous expression of AQP1 and AQP3 mRNA along the gastrointestinal tract, although the expression intensity varied; AQP1 mRNA expression was abundant in the middle portion of the small intestine, ileum, and the proximal colon and was much less in other portions of the rat gastrointestinal tract (Fig. 1A). AQP3 mRNA expression was intense in the lower portion of the stomach, middle portion of the small intestine, ileum, and both proximal and distal colon, whereas the expression was less in the esophagus, upper portion of stomach, and jejunum (Fig. 1B). In contrast to AQP1 and AQP3 mRNA expression, AQP4 mRNA expression was relatively selective: abundant in the lower portion of the stomach and the ileum, but faint in the esophagus and the jejunum, and negligible in the upper portion of the stomach or colon (Fig. 1C). AQP8 mRNA was exclusively expressed in the jejunum and colon (Fig. 1D). No AQP2 or AQP5 mRNA was detected in the rat gastrointestinal tract (data not shown). The quantitative data on the AQP mRNA expression are summarized in Table 1.

Localization of AQP mRNA and protein. By immunohistochemistry, AQP1 was demonstrated on the endothelial cells of the lymphatic vessels in the submucosa and lamina propria and capillary endothelial cells in the smooth muscle layer throughout the gastrointestinal tract (Fig. 2A). AQP3 was localized on the circumferential plasma membranes of stratified squamous epithelial cells of the esophagus (Fig. 2, B and C) and those of the upper portion of the stomach (data not shown). In the lower portion of the stomach, the localization of AQP3 was restricted in the basolateral membrane of columnar epithelia of cardiac glands in the vicinity of the junction to the upper portion (Fig. 2D). In the small intestine from jejunum to ileum, AQP3 immunostaining was not evident, although minimal or trace immunoreactive AQP3 was present in the columnar epithelia in the villi and crypt (Fig. 2E). In contrast, AQP3
mRNA expression was apparently localized in the crypt epithelia of ileum and the middle of small intestine by in situ hybridization (Fig. 2, F and G). In the colon, the basolateral membrane of surface columnar epithelial cells was apparently immunostained with the anti-AQP3 antibody (Fig. 2H).

By immunohistochemistry, AQP4 was localized on the basolateral membranes of the parietal cells in oxyntic glands of the lower stomach, but not in the chief cells (Fig. 3A). The basolateral membranes of the crypt cells in the small intestine (from jejunum to ileum), which were present at the bottom of the crypts, were labeled with the anti-AQP4 antibody (Fig. 3B). In situ hybridization also showed AQP4 mRNA expression at the comparable sites; gastric glands and deep crypt cells of the small intestine (Fig. 3, C and D). However, no or negligible immunostaining for AQP4 was detectable in the esophagus, the upper portion of the stomach, and the colon (data not shown).

The localization of AQP8 mRNA-expressing cells was examined in the rat gastrointestinal tract by in situ hybridization. The absorptive columnar epithelial cells were prominently labeled in the jejunum (Fig. 4, A, B, E, and F) and colon (Fig. 4, C, D, G, and H) with both digoxigenin- and 35S-incorporated antisense cRNA probes for AQP8 mRNA but not with the sense probes. No signals were detected in the esophagus, stomach, and ileum with the antisense AQP8 probes (data not shown).

**DISCUSSION**

The epithelial layer of the gastrointestinal tract system serves as entrance and barrier for water and nutrients from outside to inside the body. Two routes, paracellular and transcellular, are speculated for solutes across the barrier (26, 27). On the other hand, water has been presumed to move between epithelial cells (paracellular route) by interpretation of electrophysiological studies, although transcellular water movement was not completely denied (26, 27). The histological characteristics of the interepithelial junction are leaky in the small intestine and moderately leaky in the colon, supporting this presumption. Thus the structural stability or rigidity of the tight junction sealing adjacent epithelial cells has been said to correspond to the structure that determines the permeability of water through the paracellular route. In the

### Table 1. Quantitative evaluation of AQP family mRNA expression in rat gastrointestinal tract

<table>
<thead>
<tr>
<th>Sample</th>
<th>AQP1</th>
<th>AQP3</th>
<th>AQP4</th>
<th>AQP8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Esophagus</td>
<td>2.4±0.9</td>
<td>5.7±2.5</td>
<td>0.5±0.2</td>
<td></td>
</tr>
<tr>
<td>Stomach (upper portion)</td>
<td>5.4±1.6</td>
<td>7.4±1.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stomach (lower portion)</td>
<td>3.2±0.9</td>
<td>12.7±1.4</td>
<td>10.7±1.4</td>
<td></td>
</tr>
<tr>
<td>Jejunum</td>
<td>2.2±1.1</td>
<td>2.3±0.4</td>
<td>1.2±0.4</td>
<td>10.2±2.1</td>
</tr>
<tr>
<td>Small intestine (middle)</td>
<td>16.6±2.2</td>
<td>14.4±3.9</td>
<td>3.2±1.3</td>
<td></td>
</tr>
<tr>
<td>Ileum</td>
<td>16.4±1.3</td>
<td>14.2±1.4</td>
<td>7.3±2.4</td>
<td></td>
</tr>
<tr>
<td>Proximal colon</td>
<td>10.3±2.0</td>
<td>15.5±2.1</td>
<td>5.2±2.6</td>
<td></td>
</tr>
<tr>
<td>Distal colon</td>
<td>7.4±1.2</td>
<td>21.7±3.2</td>
<td>8.7±2.9</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SD of ratios (AQP/GAPDH mRNA densitometric unit) × 100; n = 5 samples. AQP, aquaporins; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
Fig. 2. Immunohistochemistry and in situ hybridization for AQP1 and AQP3 in rat gastrointestinal tract. AQP1 colored brown by immunoperoxidase staining is localized on endothelial cells of lymphatic vessels in esophagus (A, ×120). Squamous epithelia in esophagus are also labeled with anti-AQP3 antibody (B, ×360) and not stained with control rabbit serum (C, ×360). AQP 3 is demonstrated on basolateral membranes of cardiac gland epithelia in lower stomach (D, ×240), whereas AQP 3 is questionable in small intestine (E, ×240). In situ hybridization using antisense probe for AQP 3 shows AQP 3 mRNA expression in villous epithelia in ileum (F, ×360) but hybridization with sense probe for AQP 3 to same site is negligible (G, ×360). Basolateral membranes of surface columnar epithelial cells are immunostained with anti-AQP3 antibody in colon (H, ×120).

Fig. 3. Immunohistochemistry and in situ hybridization for AQP4 in rat gastrointestinal tract. Immunoreactive AQP4 is present on basolateral membranes of parietal cells in lower portion of stomach (A, ×240) and deep crypt epithelia in ileum (B, ×240). Antisense probe for AQP4 hybridizes to AQP4 mRNA expression in deep crypt epithelia of ileum (C, ×240) but no significant signals are seen in ileum with sense AQP4 probe hybridization (D, ×240).
kidney, 99% of the volume of water filtered from the glomerulus is reabsorbed at the proximal tubules, thin descending limbs of Henle, and the distal tubules. The tight junction of proximal tubular epithelia and the epithelia of thin descending limbs of Henle are shallow and discontinuous, which is characteristic for leaky epithelia, suggesting that water moves between the intercellular junction (12). However, AQP1 was localized on the apical and basolateral membranes of the epithelial cells along these nephron segments (28), and a transcellular route for water reabsorption at these nephron segments is now highly predicted (12).

Recently AQP1, AQP3, and AQP4 mRNA expression was demonstrated in the gastrointestinal tract, and the transcellular route of water for absorption and secretion was suggested (10–13). However, AQP1 was localized on the apical and basolateral membranes of the epithelial cells along these nephron segments (28), and a transcellular route for water reabsorption at these nephron segments is now highly predicted (12).

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Interestingly, the present study demonstrated novel expression of AQP3 mRNA and immunolocalization of AQP3 on the circumferential plasma membrane of stratified squamous epithelial cells in the esophagus. The esophagus has not been regarded as a water-
absorptive portion in the digestive tract, and therefore the presence of AQP3 may indicate its role for maintenance of wetness on the luminal surface of the esophagus as speculated for AQP5 in corneal squamous epithelial cells in the eye (4). Although transport of solutes through esophageal epithelia has not been examined intensively, the presence of Na-H transporter has been shown in the esophageal epithelial cells and its regulation of intracellular pH has been suggested (15). AQP3 on the esophageal epithelium may cooperate with the transporter for maintenance of intracellular solute-water balance.

AQP4 has been shown selectively on the basolateral membrane of the gastric parietal cells of the rat (3). The current study confirmed the observation that AQP4 was present in the parietal cells of the stomach. In humans, AQP4 was reported to locate in both the chief cells and the parietal cells (21); however, distinctive identification of chief cells and parietal cells may be necessary to confirm the presence of AQP4 in the chief cells. AQP4 was also detected on the basolateral membrane of basal intestinal crypt gland cells in the small intestine by immunostaining as suggested by the RNase protection assay revealing a distinct AQP4 mRNA expression in this portion of the gastrointestinal tract. Although AQP4 has been demonstrated in the colon epithelium by a previous study (2), immunoreactive AQP4 or AQP4 mRNA expression was negligible in the colon in the present study. AQP4 mRNA expression is assumed to be markedly faint if any in colon because the RNase protection assay employed in this study is highly sensitive for detection of mRNA expression. The presence of AQP4 in the colon needs to be studied further in the future.

AQP8 mRNA expression was demonstrated in the colon in recent studies (13, 17) and also in the jejunum in the present study. In situ hybridization study further localized the mRNA sites in the columnar epithelia of jejunum and colon. Although precise subcellular localization of AQP8 remains to be identified by immunostaining, it may be feasible to speculate a significant involvement of AQP8 in the enormous water movement in jejunum and colon.

The presence of AQP3, AQP4, and AQP8 in the epithelial cells in the gastrointestinal tract may suggest an involvement of water channels in water intake or outlet in this organ through a transcellular route, as a crucial role of AQP has been presumed in water reabsorption in the kidney. The redundant mRNA expression of several AQP members in each portion of the gastrointestinal tract suggested their roles in water transport in this organ. As AQP4 and AQP3 were localized on basolateral membranes of deep crypt epithelia in jejunum and surface epithelia in colon, respectively, other AQP should be present in the apical membrane for the intracellular water transport through these epithelia. AQP8 may be the one or one of AQP members that are present in the apical membranes of these epithelia and may be involved in water transport in a cooperative manner with AQP4 and AQP3 in the basolateral membranes, although subcellular localization of AQP8 has not been defined. Gastric cardiac gland cells and parietal cells also possess AQP3 and AQP4, respectively, only on the basolateral membranes. The possible presence of other AQP on the apical membranes of these cells needs to be searched. No prominent phenotypic abnormalities in intake of water or nutrient have been observed in the AQP1-deficient human subjects (29) and AQP1 or AQP4 knockout mice in physiological conditions (16). These observations may indicate compensatory redundancy of AQP in the gastrointestinal tract at a single cell level if water channels play a pivotal role in water intake and food digestion.

Although a possible role of water channels in water absorption or secretion in the gastrointestinal tract is predicted by the present study, the major route, transcellular or paracellular, for water to cross the gastrointestinal epithelium is still obscure. Transcellular water movement has been denied in the small intestine because no significant water permeability was observed in the membrane fraction of small intestinal epithelia (31, 32). This observation may be partly comparable to our present findings that immunoreactive AQP3 and AQP4 were restricted in the deep gland cells in small intestine and AQP8 mRNA expression was also restricted in the jejunum. The role of AQP in water permeability in each portion of the small intestine needs to be examined intensively in the future. In addition, pathological conditions related to water intake and outlet such as diarrhea or malabsorption may be determined in part by the expression or the amounts of AQP family members in the gastrointestinal tract. The possible role(s) and involvement of AQP in physiological and pathological conditions of the gastrointestinal tract remain to be studied.

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