Immunolocalization of Aquaporin-8 in Rat Digestive Organs and Testis*

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Summary. Expression of aquaporin-8 mRNA has previously been shown in hepatocytes, pancreatic acinar cells, colon epithelial cells and seminiferous tubules of the testis in the rat by in situ hybridization technique. However, immunolocalization of this water channel has not yet been demonstrated. In the present study, the localization of immunoreactive aquaporin-8 and expression of the mRNA were examined in rat organs (cerebrum, cerebellum, eye, salivary gland, heart, lung, liver, pancreas, esophagus, stomach, jejunum, ileum, colon, testis, ovary, kidney, spleen and lymphnode) by immunohistochemistry using an antibody against aquaporin-8 and ribonuclease protection assay. Aquaporin-8 was distinctly immunolocalized on the apical membranes of pancreatic acinar cells and mucosal epithelium of the colon and jejunum. In the liver, the bile canalicular membrane of hepatocytes was immunostained. In the testis, immunoreactive aquaporin-8 was demonstrated on the luminal side of the seminiferous tubules. At high magnification, the peroxidase reaction products appeared on the ramified cytoplasmic membrane of Sertoli cells surrounding the residual bodies or spermatogenic cells. Specificity of the antibody was verified by Western blot analysis showing a minor ~28 kDa band (deduced deglycosylated form of aquaporin-8) and a major ~30 kDa band (glycosylated form) in these organs. The intensity of aquaporin-8 immunoreactivity was approximately comparable to that of aquaporin-8 mRNA expression in the liver, pancreas, colon, jejunum and testis. The aquaporin-8 mRNA expression in the hepatocytes was presumed to be closely associated with the structure of bile canaliculi since the message was detected in hepatocytes immediately after isolation from the liver but not in cells following cultivation for three days.

The localization of immunoreactive aquaporin-8 indicated functions for this water channel in the secretion of bile and pancreatic juice, and the secretion or absorption of water in the colon and jejunum, and the maturation or liberation of spermatogenic cells in the testis.

Aquaporins are a family of water channels located on the plasma membrane of various cell types and have been shown to transfer water molecules through the membrane (Preston et al., 1992; Agre et al., 1998). Ten members (aquaporin-0 to aquaporin-9) of the aquaporin family have been identified in mammals. Several of them have been determined in regard to their roles in the extensive water absorption in the kidney (Sabolic et al., 1992; Fushimi et al., 1993; Nielsen et al., 1993a) and in water transit from one compartment to another in other organs (Denker et al., 1988).

The kidney is a primary organ for regulating the volume of body fluid by controlling the filtration of plasma in the glomerulus and reabsorption of water in several nephron segments. Several aquaporin members have been demonstrated in these nephron segments, and accumulating evidence suggests a pivotal role of these channels in water reabsorption in the kidney (Yamamoto and Sasaki, 1998; Nielsen et al., 1999). On the other hand, the gastrointestinal tract is a primary site of water intake into the body, but little is known about the presence or localization of aquaporin members.

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The aquaporin-8 gene was recently cloned as a new member of the water channel family from rat liver and testis, and expression of its mRNA was demonstrated in hepatocytes, pancreatic acinar cells, colon epithelium, and in the seminiferous tubules of the testis (Koyama et al., 1997; Ishibashi et al., 1997). Since the subcellular localization of this water channel has not been demonstrated, we examined the localization of aquaporin-8 by immunohistochemistry in the present study to speculate on the roles of this water channel in these cells.

MATERIALS AND METHODS

Tissues and RNA preparations
Several kinds of organs (cerebrum, cerebellum, eye, salivary gland, heart, lung, liver, pancreas, esophagus, stomach, jejunum, ileum, colon, testis, ovary, kidney, spleen, lymphnode) were removed from adult Wistar Kyoto rats. A part of each organ was fixed in methyl-Carnoy’s fixative overnight and embedded in paraffin for immunohistochemistry, while another part was homogenized in acid guanidinium thiocyanate solution (TRIZol, Life Oriental Technologies Inc., Tokyo, Japan) for isolation of the total cellular RNA.

For Western blot analysis, pieces of the pancreas, liver, colon, ileum and testis were homogenized using a Potter type homogenizer. The homogenate was centrifuged for 30 min at 10,000 g at 4°C to remove cellular debris. The supernatant was re-centrifuged for 10 min at 40,000 g at 4°C to obtain the membrane fraction. The membrane fraction (~20 μg) prepared from the liver was further treated with glycosidase F (Takara Biochemicals, Tokyo) at a concentration of 1 U/ml at 37°C overnight.

Rat hepatocytes were isolated from the liver by the method described previously (Sugiyma et al., 1998). In brief, animals were anesthetized with pentobarbital (Nembutal, Dainippon Pharmaceutical Co., Ltd., Osaka, Japan) and their livers were perfused with 0.05% collagenase (Sigma Aldrich Japan, Tokyo) solution via the portal vein. After 15-min perfusion, the liver was removed and cut in pieces, which were gently suspended by pipetting in MEM medium (Nissui Pharmaceutical Co., Ltd., Tokyo). Hepatocytes thus liberated were cultured in William’s medium E (GIBCO BRL Life Technologies, Rockville, MD, USA) supplemented with 10% fetal calf serum, 10⁻⁸ M insulin, and 10⁻⁶ M dexamethasone in type I collagen-coated 100-mm plastic plates at 37°C for three days. Total cellular RNA was isolated from the cells by the acid guanidinium thiocyanate phenol-chloroform extraction method.

Western blot analysis and immunohistochemistry
An antibody against a synthetic rat aquaporin-8 peptide raised in rabbits was purchased from Alpha Diagnostic Intl. Inc. (San Antonio, TX, USA). Reactivity of the antibody to rat aquaporin-8 was examined by Western blot analysis using the plasma membrane fractions obtained from rat liver, pancreas, colon, ileum and testis, and the glycosidase F-treated liver membrane fraction. The specificity was examined after incubation of the antibody with excess (>10 : 1 molar ratio) of synthetic rat aquaporin-8 peptide (Alpha Diagnostic Intl. Inc.) at 4°C overnight. The samples (~20 μg of protein) diluted in the 2X sample buffer [100 mM Tris-HCl (pH 6.8), 4% SDS, 10% β-mercaptoethanol, and 20% glycerol], were electrophoresed on a 12.5% SDS-polyacrylamide gel, and the bands were transferred to a polyvinylidene difluoride membrane (Immobilon-P, Millipore Corp., Bedford, MA, USA). The membrane was immersed in a blocking buffer (10% nonfat milk, 0.05% Tween 20, and 0.5% NaN₃ in PBS) and incubated with the anti-rat aquaporin-8 antibody (final conc. 0.25 μg/ml) in a blocking buffer) or the antibody preabsorbed with the synthetic aquaporin-8 peptide. After several washings in 0.05% Tween 20 PBS, the membrane was incubated for 1 h with goat-anti-rabbit immunoglobulins conjugated to peroxidase-labeled dextran polymer (EnVision, DAKO Japan, Kyoto), and the reaction was visualized by ECL chemiluminescence (Amer sham Pharmacia Biotech, Tokyo).

For immunohistochemistry, the tissues fixed in methyl-Carnoy’s solution (see above) and embedded in paraffin were sectioned at a 4 μm thickness. The sections were then incubated with the anti-rat aquaporin-8 antibody (2 μg/ml) or the antibody preabsorbed with synthetic rat aquaporin-8 peptide, and the goat anti-rabbit immunoglobulins conjugated to peroxidase labeled dextran polymer. The peroxidase reaction products were visualized by 3’-diaminobenzidine tetrahydrochloride and 0.01% hydrogen peroxide reaction; the sections were counterstained with hematoxylin.

Ribonuclease protection assay
Partial fragments of rat aquaporin-8 cDNA (315 bp; +701 ~ +1015) and rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA were subcloned in pGEM 11Z and pGEM 3Z vectors (Promega Japan, Tokyo), respectively. These plasmids were linearized with appropriate restriction enzymes and used as templates for in vitro transcription of 32P-labeled antisense cRNA probes.

Ten micrograms of the RNA sample were hybrid-
ized with the aquaporin-8 and GAPDH probes at a specific radioactivity of 1 x 10^4 cpm/μl each in a hybridization buffer (80% formamide, 40 mM PIPES, 0.4 M NaCl, 1 mM EDTA) overnight at 48°C (SAMBROOK et al., 1989). Unhybridized probes were then digested with ribonuclease A and ribonuclease T1 mixture at 30°C for 1 h and the ribonucleases were digested with proteinase K at 37°C for 30 min (NIHEI et al., 2001). 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 (Fuji Photo Film Co., Kanagawa, Japan).

RESULTS

Western blot analysis

Specific reactivity of the anti-aquaporin-8 antibody was examined by Western blotting. As shown in Figure 1, two distinct bands, a major ~30 kDa and a minor ~28 kDa, were blotted intensely in the liver and testis plasma membrane fractions, and less intensely in the pancreas and colon samples. No bands were labeled in the ileum sample. These bands were not detected when the antibody was preabsorbed with the aquaporin-8 peptide. After treatment of the liver sample with glycosidase F, the major ~30 kDa band was down-sized to the ~28 kDa band.

Immunohistochemistry

Immunoreactive aquaporin-8 was demonstrated in the pancreas, liver, jejenum, colon and testis, but not in the other organs examined. In all preparations, the immunoreaction was abolished when the antibody was pre-absorbed with the synthetic aquaporin-8 peptide.

In the pancreas, an intense immunoreactivity for aquaporin-8 was further immunolocalized on the apical plasma membrane of acinar cells facing the intercellular canalculi (Fig. 2a). The immunoreactivity was also recognized on some vesicular membranes located close to the apical membrane (Fig. 2b). It was most likely that they corresponded to the limiting membranes of secretory granules located close to the apical membrane. Some of them seemed to correspond to O-shaped invaginations of the plasma membrane. The immunoreactivity was not recognizable in the pancreatic islets or pancreatic ducts.

In the liver, the immunoreactivity for aquaporin-8 was less intense, being restricted to hepatocytes.

None of the other cells were immunostained—including endothelial cells, Kupfer cells, bile ducts and peripheral leukocytes (Fig. 2c). In the hepatocytes immunoreactive aquaporin-8 was localized at the bile canalliculi and neither on the plasma membrane facing the space of Disse nor on the hepatocyte-hepatoocyte interface. At higher magnification, the immunoreaction was clearly localized on the plasma membrane forming the bile canalliculi (Fig. 2d).

In the jejunum and colon, the immunoreaction for aquaporin-8 was moderate in intensity but apparent in the absorptive epithelial cells (Fig. 3a, c). The apical part of the columnar epithelial cells was immunoreactive in these organs (Fig. 3b). Closer observation revealed that the apical plasma membrane beneath the microvilli of the striated border was most intensely immunostained; the microvilli were negative in the immunostaining. The lateral plasma membranes were partly and weakly immunoreactive. No other cells, including goblet cells, were immunolabeled with the anti-aquaporin-8 antibody.

In the testis, aquaporin-8 was immunoreactive in the seminiferous tubules; the staining intensity varied, probably in association with the stage of spermatogenesis (Fig. 4a). Intense staining was observed on the luminal side. Namely, the adluminal compartment of the germ epithelium was selectively immunoreactive, while the basal compartment of the epithelium was negative (Fig. 4b). At higher magnification, the immuno-
Fig. 2. Immunohistochemistry for aquaporin-8 in the pancreas and liver. a. In the pancreas, aquaporin-8 is immunolocalized on the apical membrane of acinar cells. The islet of Langerhans (L) lacks the peroxidase reaction products. b. At higher magnification, the immunoreactivity is localized on the apical plasma membrane and on the intercellular canaliculi (arrows) between adjoining acinar cells. Secretory vesicles (arrowheads) beneath the apical membrane appear immunostained. c. In the liver, immunoreactive aquaporin-8 is found between the hepatocytes as dots or lines (arrowheads) and not in the other cells (arrows). d. Higher magnification shows the immunostaining for aquaporin-8 on membranes limiting the bile canaliculi (arrowheads). Neither circulating lymphocytes (arrows) nor endothelial cells (E) are stained. a, c: ×360, b, d: ×1,800
Fig. 3. Immunohistochemistry for aquaporin-8 in the jejunum and colon. a. In the jejunum, the apical surface (arrowheads) of the absorptive epithelium is weakly stained. b. The immunostaining is apparent on the bases (large arrowheads) of microvilli and not on the microvilli (small arrowheads) of the absorptive epithelial cells. Goblet cells (G) are not stained. c. In the colon, the immunoreaction is found on the apical surface (arrowheads) of absorptive epithelial cells and not on the intestinal gland cells. d. The immunoreaction is intense on the apical surface of absorptive epithelium (arrowheads). a, c: ×360, b, d: ×1,800
Fig. 4. Immunohistochemistry for aquaporin-8 in the testis. 

a. The luminal side of seminiferous tubules (S) is immunoreactive. The intensity of immunoreaction is variable among the tubules. 

b. In a seminiferous tubule peroxidase reaction products are observed between spermatozoa (arrowheads), residual bodies (☆) or spermatocytes (★) in the adluminal compartment. In the basal compartment, the cytoplasmic membrane of Sertoli cells and spermatocytes located in the basal portion are not immunolabeled. 

c. At higher magnification, the peroxidase reaction products are deposited in a honeycomb-like pattern (arrowheads) separating residual bodies (☆) which are vacuoles containing some debris. 

d. Spermatozoa (arrowheads) and spermatocytes (★) are not immunostained. Fine vesicular peroxidase reaction products are found between the residual bodies (☆) or spermatocytes (★). 

a: ×390, b: ×740, c, d: ×1,800
reactive aquaporin-8 was apparently found around the surface of vesicular residual bodies and was localized to the ramified cytoplasm of Sertoli cells extended between spermatogenic cells, i.e. spermatocytes, spermatids and residual bodies (Fig. 4c). Spermatozoa and other spermatogenic cells in the germ epithelium were free of the peroxidase reaction products (Fig. 4d). The interstitial tissue including Leidig cells was completely immunonegative.

**Ribonuclease protection assay**

Expression of aquaporin-8 mRNA was intense in the liver, pancreas and testis, and was weak in the

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**Fig. 5.** Ribonuclease protection assay showing the expression of aquaporin-8 mRNA in rat organs and isolated hepatocytes. a. AQP8 mRNA is clearly expressed in the liver, pancreas, jejunum, colon and testis. Lanes 1: cerebrum, 2: cerebellum, 3: eye, 4: salivary gland, 5: heart, 6: lung, 7: liver, 8: pancreas, 9: jejunum, 10: colon, 11: esophagus, 12: stomach, 13: ileum, 14: testis, 15: ovary, 16: kidney, 17: spleen, 18: lymphnode. b. The expression of aquaporin-8 mRNA is detected in RNA samples prepared from the liver (lanes 1 and 2), and from hepatocytes immediately after separation from the liver (lanes 3 and 4). The expression is undetectable in hepatocytes cultured for three days (lane 5).
jejenum and colon (Fig. 5a). No expression of aquaporin-8 mRNA was demonstrated in the other organs examined.

To confirm the association of the aquaporin-8 with bile canaliculi in hepatocytes, the expression of aquaporin-8 mRNA was examined in rat hepatocytes obtained immediately after isolation from the liver and those in culture for three days after the isolation. Although aquaporin-8 mRNA was detected in the hepatocytes immediately after isolation from the liver, it was not detected in those hepatocytes which had been cultured (Fig. 5b).

**DISCUSSION**

*In situ* hybridization studies have demonstrated the expression of aquaporin-8 mRNA in the hepatocytes, pancreatic acinar cells, absorptive epithelial cells of the jejunum and colon and the seminiferous tubules of the testis in the rat (Koyama et al., 1997; Ishibashi et al., 1997). The expression may be different in other species since in the mouse aquaporin-8 mRNA was also found—in addition to the liver and colon—in the placenta and heart, where it was not expressed in the rat as recorded in the present and previous studies (Ma et al., 1997).

Information on the subcellular localization of this water channel is valuable for clarification of its roles in the cells concerned. For this purpose, we preliminarily attempted immunoelectron microscopy, but unfortunately found that the tissues lost their immunoreactivity to the anti-aquaporin-8 antibody after they were fixed with 2% paraformaldehyde or periodate-lysine-paraformaldehyde for a few hours. Methyl-Carnoy fixation, which preserves the immunoreactivity, is not suitable for electron microscopy. Therefore, in this study we examined the subcellular localization of aquaporin-8 simply by light microscopic immunohistochemistry but viewed the preparations at high magnifications. This allowed us to determine for the first time the subcellular localization of aquaporin-8; i.e. on the apical membranes of hepatocytes, pancreatic acinar cells, colon and jejunum absorptive epithelial cells and Sertoli cells in the rat testis.

In the hepatocytes, immunoreactive aquaporin-8 was selectively demonstrated on the apical membrane constituting bile canaliculi. The association of aquaporin-8 with the bile canaliculus structure is also supported by the finding that aquaporin-8 mRNA was not expressed in hepatocytes after three days of cultivation when the bile canaliculus structure was lost; aquaporin-8 mRNA expression was distinct in the hepatocytes immediately after isolation from the liver. The observation suggests that water moves from the hepatocyte cytoplasm to the bile canaliculus through the aquaporin-8 water channel. In contrast to aquaporin-8, aquaporin-9 was recently immunolocalized on the basolateral membrane of hepatocytes facing the space of Disse (Elkjær et al., 2000; Niihe et al., 2001). Since aquaporin-9 was characterized as passing both water and neutral solutes (Ishibashi et al., 1998; Tsukaguchi et al., 1998), this water channel was suggested to play a role in the transfer of these between hepatocytes and the circulation.

The present study demonstrated the aquaporin-8 water channel on the apical membrane of pancreatic acinar cells, suggesting a role for the water channel in the secretion of pancreatic juice. The presence of aquaporin-8 on the limiting membrane of presumed secretory vesicles in the apical cytoplasm of pancreatic acinar cells may suggest that this water channel transfers water into the vesicles, resulting in expansion of the volume ready for secretion of the contents through fusion to the apical membrane.

A large volume of water may move everyday through the epithelium in the small and large intestine as sites for water entrance or secretion in the body (Phillips et al., 1972; Powell, 1987). However, the pathway of water transport through the epithelial cell layer of the gut has not received much attention. Although a pathway between epithelial cells (paracellular route) has been proposed (Powell, 1981), recent studies demonstrating the localization of several water channel members along the gastrointestinal tract call for a re-evaluation of the notion of how water enters the body (Koyama et al., 1999; Ma et al., 1999). In the digestive tract of rats, aquaporin-1, aquaporin-3, and aquaporin-4 have been localized in the capillary and lymphatic endothelial cells, colon epithelial cells, and stomach parietal cells, respectively (Nielsen et al., 1993b; Fujita et al., 1999). Since aquaporin-3 was found on the basolateral membrane in the colon epithelial cells (Koyama et al., 1999), the localization of aquaporin-8 on the apical membrane of these cells suggests a co-operative role of aquaporin-3 and aquaporin-8 in water transit from the lumen to the interstitium through a transcellular route.

In the testis, aquaporin-8 was localized in the adluminal compartments of the germ epithelium, where secondary spermatocytes, spermatids, and spermatozoa are maturing. It is also known that Sertoli cells extend their ramified cytoplasm from the basal lamina to the adluminal compartments, while their large cell bodies rest on the basement membrane of the seminiferous tubules. At the adluminal compartments, the cytoplasm of Sertoli cells ramifies.
throughout the germina1 epithelium enclosing all the
cells of the spermatogenetic series. Aquaporin-8 was
demonstrated on the ramified parts of Sertoli cells
by the present study. Since Sertoli cells have multiple
functions including the secretion of tubular fluid and
factors and the nursing and regulation of sper-
matogenetic cells, aquaporin-8 is presumed to be in-
volred in some of these functions. It may be worthy
to note that aquaporin-7 has been demonstrated in
spermatids and mature sperm cells (ISHIHASHI et al.
1997). These observations suggest that water move-
ment might be crucial in spermatogenesis and the
liberation of mature sperm cells in the seminiferous
tubules.

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