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BASIC AND TRANSLATIONAL—BILIARY

Claudin 2 Deficiency Reduces Bile Flow and Increases Susceptibility to Cholesterol Gallstone Disease in Mice

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BACKGROUND & AIMS: Bile formation and secretion are essential functions of the hepatobiliary system. Bile flow is generated by transepithelial transport of water and ionic/nonionic solutes via transcellular and paracellular pathways that is mainly driven by osmotic pressure. We examined the role of tight junction–based paracellular transport in bile secretion. Claudins are cell–cell adhesion molecules in tight junctions that create the paracellular barrier. The claudin family has 27 reported members, some of which have paracellular ion- and/or water-channel–like functions. Claudin 2 is a paracellular channel-forming protein that is highly expressed in hepatocytes and cholangiocytes; we examined the hepatobiliary system of claudin 2 knockout (*Cldn2*$^{-/-}$) mice. **METHODS:** We collected liver and biliary tissues from *Cldn2*$^{-/-}$ and *Cldn2*$^{+/+}$ mice and performed histologic, biochemical, and electrophysiologic analyses. We measured osmotic movement of water and/or ions in *Cldn2*$^{-/-}$ and *Cldn2*$^{+/+}$ hepatocytes and bile ducts. Mice were placed on lithogenic diets for 4 weeks and development of gallstone disease was assessed. **RESULTS:** The rate of bile flow in *Cldn2*$^{-/-}$ mice was half that of *Cldn2*$^{+/+}$ mice, resulting in significantly more concentrated bile in livers of *Cldn2*$^{-/-}$ mice. Consistent with these findings, osmotic gradient-driven water flow was significantly reduced in hepatocyte bile canaliculi and bile ducts isolated from *Cldn2*$^{-/-}$ mice, compared with *Cldn2*$^{+/+}$ mice. After 4 weeks on lithogenic diets, all *Cldn2*$^{-/-}$ mice developed macroscopically visible gallstones; the main component of the gallstones was cholesterol (>98%). In contrast, none of the *Cldn2*$^{+/+}$ mice placed on lithogenic diets developed gallstones. **CONCLUSIONS:** Based on studies of *Cldn2*$^{-/-}$ mice, claudin 2 regulates paracellular ion and water flow required for proper regulation of bile composition and flow. Dysregulation of this process increases susceptibility to cholesterol gallstone disease in mice.

Keywords: Mouse Model; Claudin 2; Hepatic Microcirculation; TJ.

The hepatobiliary system plays critical roles in lipid and cholesterol homeostasis.[1–4] Dysregulation of this system causes various diseases; impaired bile secretion leads to cholestasis[5] and an imbalance in bile composition leads to gallstone disease.[6–8] Cholesterol, phospholipids, and bile acids are the major components of bile. They are originally hydrophobic and their conjugated hydrophilic forms exist in a micellous state,[2] which can aggregate and form stones in the aqueous bile in any condition.[6,7] Therefore, the bile formation and bile flow are critical for hepatobiliary function.

The incidence of gallstones is high in the Western world,[7,8] and the pathogenesis of cholesterol gallstone disease is relatively well studied. The cholesterol saturation index (CSI), the ratio of the cholesterol concentration to that of bile acids and phospholipids, is a well-known indicator for cholesterol crystal nucleation. On the other hand, the water content of bile and bile flow are key factors in gallstone disease that have not been fully examined.[8,9]

In the biliary system, hepatic bile, which is 98% water, is secreted at a rate of 30–40 mL/h in humans. Bile formation is preceded by the active secretion of solutes from hepatocytes, such as bile acids by bile acid–dependent secretion, and glutathione, bilirubin, and/or HCO_3^- by bile acid–independent secretion. Water and electrolytes are then secreted.[2,10] Water molecules transverse the epithelium through paracellular and/or transcellular routes. The identification and analysis of aquaporins (AQPs) has extended our understanding of how the transcellular water movement through epithelial cell sheets is regulated.[11,12] On the other hand, it is still unsettled how and to what extent water moves paracellularly between the cells in epithelial sheets, mainly because it is difficult to separate the transcellular and paracellular pathways experimentally.

The tight junction (TJ) is an adhesion apparatus that resides in the most apical part of the lateral membrane. TJs surround epithelial cells and attach them together. The TJ is also fundamentally responsible for the paracellular barrier of epithelia, including their charge- and size-dependent permeabilities; these properties enable the selective movement of solutes and water for biologic functions.[13–15] On the

Abbreviations used in this paper: **AQP, aquaporin; BW, body weight; CSI, cholesterol saturation index; IBDU, intrahepatic bile duct unit; TJ, tight junction.**

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freeze-fracture electron microscopy, TJ strands appear as a continuous meshwork in the apical intercellular space. These strands are thought to be derived from polymerized claudins forming the paracellular barrier, although the polymerization mechanism is unknown. The claudins are a large family of tetraspanning transmembrane proteins, with at least 27 members in human/mouse, which are thought to have various functions in claudin-based paracellular systems.

Evidence suggests that claudins generally function to create the paracellular barrier function of epithelial cell sheets. However, some of them increase the paracellular ion permeability when expressed in paracellular barrier–established cell sheets; these are described as "ion-channel forming" claudins,[14,16,17] although they also elicit a relatively nonspecific permeability for small nonionic solutes. Previous studies suggested that the combination of claudin species expressed in cells is important for determining the permselectivity of the TJs.[14,15] Consistent with this idea, physiologic studies suggest that epithelium types can be classified as leaky or tight, based on transepithelial conductance and/or paracellular solute flux measurements.[17,18]

Claudin 2 was the first channel-forming claudin to be identified,[13,19] with suggested roles in water and cation permselectivity.[20–22] In claudin 2 knockout (*Cldn2*$^{-/-}$) mice, deficiencies in paracellular permeability and cation selectiveness impair the reabsorption of Na^+, Cl^-, and water in renal proximal tubules.[20] In the small intestine, claudin 2 loss significantly decreases the Na^+ permeability.[23] In addition, double-knockout mice in the intestine of claudin 2 and claudin 15, another channel-forming claudin, are deficient in sodium-driven nutrient absorption due to the insufficiency of Na^+ in the intestinal lumen, which is usually supplied paracellularly from the submucosal space, which leads to infant death.[24,25] Thus, the ion channel–forming claudins appear to be important for regulating biologic functions, in addition to their paracellular barrier function.

In the liver, claudin 1, 2, and 3 are dominant. Among them, barrier-forming claudin 1 dysfunction is reported to lead to a rare autosomal recessive neonatal ichthyosis-sclerosing cholangitis syndrome, which is characterized by scalp hypotrichosis, scarring alopecia, ichthyosis, and sclerosing cholangitis, as described previously.[26] In cholangitis in neonatal ichthyosis-sclerosing cholangitis syndrome liver, a bile leakage through claudin 1–deficient TJs of hepatocellular and biliary cells is suggested to result in direct hepatocellular and/or biliary injuries and in cholestasis. Here we focused on the hepatobiliary system in *Cldn2*$^{-/-}$ mice. The concentrations of bile acids, phospholipids, and cholesterol were significantly increased in the *Cldn2*$^{-/-}$ bile. We found that *Cldn2*$^{-/-}$ mice showed decreased transepithelial electrical conductance and water permeability in the hepatobiliary system, resulting in decreased bile flow. In addition, after 4 weeks on a lithogenic diet, the prevalence of cholesterol gallstones was 100% in the *Cldn2*$^{-/-}$ mice vs 0% in *Cldn2*$^{+/+}$ mice.

Our findings indicate that the channel-forming claudin 2 is critical for bile homeostasis in the hepatobiliary system. We also provide the first evidence that this claudin plays a key role in bile flow as a type of water flow in the body.

Materials and Methods

Because of limitations in article length, the detailed Materials and Methods are presented in the Supplementary Material.

Results

Claudin Expression Patterns in Liver and Gallbladder

To determine which claudin subtypes are expressed in the TJs of the hepatobiliary system, we first examined the claudin gene expressions in liver and gallbladder by quantitative reverse transcription polymerase chain reaction. Claudins 1, 2, 3, 12, and 25 were the major claudins in the liver (Figure 1). Among these, claudin 2 is a channel-forming type, and was recently suggested to be a water-permeable claudin.[19,27] Therefore, to elucidate the roles of TJs in regulating bile formation and secretion in the hepatobiliary tract, we analyzed *Cldn2*$^{-/-}$ mice.

We next examined the localization of claudin subtypes in the *Cldn2*$^{+/+}$ liver by immunofluorescence analyses. Claudin 2 was expressed in the perivenous regions, bile ducts, and gallbladder epithelium. In contrast, the claudin 1 signals were strong in the periportal zone and slightly weaker in the perivenous zone. Claudin 3 showed clear signals in both the perivenous and periportal zones. In the *Cldn2*$^{+/+}$ bile duct and gallbladder epithelium, claudins 2, 3, and 7 were strongly expressed (Supplementary Figures 1 and 2).

In the *Cldn2*$^{-/-}$ mice, the claudin 2 signal was not detected, but the other claudin expression patterns and levels were not significantly different from the *Cldn2*$^{+/+}$ mice in the liver, bile duct, or gallbladder epithelium. There were no differences in the expression patterns of occludin, ZO-1, or E-cadherin between the *Cldn2*$^{+/+}$ and *Cldn2*$^{-/-}$ liver (Supplementary Figure 3).

Histologic Analysis of the Cldn2$^{-/-}$ *Liver and Gallbladder*

We next examined the liver and gallbladder in 8-week-old *Cldn2*$^{+/+}$ and *Cldn2*$^{-/-}$ mice by H&E staining and freeze fracture electron microscopy and found no obvious differences between the *Cldn2*$^{+/+}$ and *Cldn2*$^{-/-}$ samples (Figure 2*A*, *B*, and *C*). It was previously reported that claudin 2 knockdown prevents canalicular formation in WIF-B9 cells[28] and that β-catenin knockout mice show decreased claudin 2 expression and wider bile canaliculi.[29] We therefore examined the bile canaliculi more closely by scanning electron microscopy and immunofluorescence analyses. We found no obvious differences in the bile canaliculi width or shape between the *Cldn2*$^{+/+}$ and *Cldn2*$^{-/-}$ liver, although the bile canaliculi is slightly wider in periportal zone than in perivenous zone both in *Cldn2*$^{+/+}$ and *Cldn2*$^{-/-}$ bile canaliculi (perivenous zone: Cldn2$^{+/+}$ 0.79 ± 0.03 μm, Cldn2$^{-/-}$ 0.78 ± 0.04 μm; periportal zone: Cldn2$^{+/+}$ 0.92 ± 0.03 μm, Cldn2$^{-/-}$ 0.90 ± 0.02 μm) (Supplementary Figure 4*A* and *B* and Supplementary Table 1). Together, these data suggested that histologically, almost no changes were detected in the *Cldn2*$^{-/-}$ liver and gallbladder.

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