# Alterations in Tight Junctions Differ Between Primary Biliary Cirrhosis and Primary Sclerosing Cholangitis

SHOTARO SAKISAKA,[1] TAKUMI KAWAGUCHI,[2] EITARO TANIGUCHI,[2] SHINICHIRO HANADA,[2] KURUMI SASATOMI,[2] HIRONORI KOGA,[2] MASARU HARADA,[2] RINA KIMURA,[2] MICHIO SATA,[2] NORIMASA SAWADA,[3] MICHIO MORI,[3] SATORU TODO,[4] AND TOSHIHIKO KUROHIJI[5]

**Tight junctions (TJ) of biliary epithelial cells (BEC) and hepatocytes prevent bile regurgitation from the biliary tract. Alterations in these TJs may participate in chronic cholestatic liver diseases such as primary biliary cirrhosis (PBC) and primary sclerosing cholangitis (PSC). We examined the localization of 2 TJ proteins, ZO-1 and 7H6, in these diseases. Frozen sections from livers of PBC, PSC, extrahepatic cholestasis (Ex-C), and hepatitis C–associated cirrhosis (LC-C), as well as histologically normal livers, were processed for double-fluorescence immunohistochemistry. In controls and cirrhosis, 7H6 and ZO-1 colocalized surrounding the luminal space of the bile ducts and outlined the bile canalicular spaces between hepatocytes. In untreated PBC, immunostaining for ZO-1 in BEC of bile ducts 40 to 80 $\mu$m in diameter was preserved, but that for 7H6 was diminished to absent. In PBC treated with ursodeoxycholic acid (UDCA), immunostaining for 7H6 was well preserved. In PSC as well as in Ex-C, immunostaining for both 7H6 and ZO-1 was well preserved in bile ducts. In hepatocytes, ZO-1 showed preserved immunoreactivity, but immunostaining for 7H6 frequently disappeared. The percentage of bile ducts with immunostaining for 7H6 in all bile ducts with immunostaining for ZO-1 was significantly reduced in PBC compared with that in control, LC-C, Ex-C, and PSC (all $P <$ .0001). Substantial alteration in the TJ protein occurs predominantly in bile ducts in PBC and in hepatocytes in PSC, suggesting increased paracellular permeability along different paracellular routes for bile regurgitation in these chronic cholestatic liver diseases. (HEPATOLOGY 2001;33: 1460-1468.)**

Primary biliary cirrhosis (PBC), a chronic cholestatic liver disease characterized by immune-mediated bile duct destruction, shows elevated serum concentrations of bile acids, alkaline phosphatase, γ-glutamyl transpeptidase, and, in advanced stages, bilirubin.[1] In cholestasis, elevations of these substances in blood result at least in part from regurgitation of bile constituents into blood.[2]

Previous ultrastructural studies demonstrated not only degenerated or apoptotic biliary cells, but also separation of those cells associated with reduced intercellular digitation in PBC. Electron-dense deposits are often shown between the multilayered basement membrane and in lateral intercellular spaces of damaged bile ducts, which are surrounded by infiltrating macrophages containing neutral fats. These findings strongly suggested increased biliary epithelial permeability in PBC, resulting in bile leakage from the luminal space of bile ducts to the periductal area.[3-6]

Because the tight junction (TJ) of biliary epithelial cells (BEC) is the only intercellular barrier between the luminal space and the portal area, the functional integrity of TJ is crucial in preventing paracellular leakage of bile constituents. In several clinical forms of cholestasis and in cholestatic models, distinct functional and morphologic alterations of TJ have been described in hepatocytes.[3,7,8] Freeze-fracture electron microscopy is well suited to evaluation of hepatocyte TJ morphology.[9] However, this technique is not practical for examination of bile duct TJ, because the number of BEC is much smaller than that of hepatocytes in normal liver (3% to 5% vs. 60% of the liver cell population, respectively); the population of BEC is even smaller in liver with PBC.[5,10] Thus, previous reports concerning PBC have not addressed the morphology and function of TJ in bile ducts.

Primary sclerosing cholangitis (PSC), another chronic cholestatic liver disease, is characterized by fibrosing inflammation of extra- and intrahepatic bile ducts; like PBC, PSC results in chronic cholestatic biochemical changes in blood.[11] The bile regurgitation pathways resulting in elevated serum concentrations of bile-derived substances in PSC have not been demonstrated.

Recently, 10 TJ-associated proteins have been identified, including ZO-1 and 7H6.[12-16] ZO-1, a 220-kd phosphoprotein that couples occludin molecules, belongs to the membrane-associated guanylate kinase protein family and most likely has both structural and signaling functions.[17-19] A protein recently identified in a bile canaliculus–enriched membrane fraction, 7H6, is a 155-kd phosphoprotein.[20] Immunolocalization of 7H6 appears to correlate closely with paracellular permeability in several organs, including the rat liver.[21-24,25]

Abbreviations: PBC, primary biliary cirrhosis; TJ, tight junction; BEC, biliary epithelial cells; PSC, primary sclerosing cholangitis; UDCA, ursodeoxycholic acid; LC-C, hepatitis C–associated cirrhosis; Ex-C, extrahepatic cholestasis.

From the [1]Third Department of Medicine, Fukuoka University School of Medicine, Fukuoka, Japan; [2]Second Department of Medicine, Kurume University School of Medicine, Kurume, Japan; [3]Department of Pathology, Sapporo Medical University School of Medicine, Sapporo, Japan; [4]Department of Surgery, Hokkaido University School of Medicine, Sapporo, Japan; and [5]Shin Koga Hospital, Kurume, Japan.

Received July 24, 2000; accepted March 26, 2001.

Address reprint requests to: Shotaro Sakisaka, M.D., Ph.D., Third Department of Medicine, Fukuoka University School of Medicine, 7-45-1 Nanakuma, Johnan-ku, Fukuoka 814-0180, Japan. E-mail: sakisaka@fukuoka-u.ac.jp; fax: 81-92-874-2663.



0270-9139/01/3306-0016$35.00/0
doi:10.1053/jhep.2001.25086

In contrast, immunostaining for ZO-1 is not correlated with paracellular permeability.[21, 24,25]

In the present study, we assessed alterations in TJ of intrahepatic bile ducts and hepatocytes in livers involved by PBC and PSC. We performed immunohistochemistry using antibodies against 7H6 and ZO-1, followed by confocal laser scanning microscopy. We demonstrated that alterations in immunostaining for 7H6 occur predominantly in BEC in PBC and in hepatocytes in PSC, which suggests increased paracellular permeability for bile in relation to the 2 different cell types in these 2 chronic cholestatic diseases. In PBC after treatment with ursodeoxycholic acid (UDCA), the alteration of TJ in BEC was only slight or absent.

## PATIENTS AND METHODS

*Materials.* All reagents were purchased from Wako Pure Chemical Industries (Osaka, Japan) unless otherwise indicated.

*Liver Specimens.* Liver specimens used in this study were obtained by needle biopsy, surgical wedge biopsy, or at liver transplantation. These procedures were performed for diagnosis in 14 untreated patients who all were histologically confirmed to have PBC and all had demonstrable antimitochondrial antibody in serum. The histologic stage, based on Scheuer's classification,[26] was as follows: stage I in 4 cases, stage II in 6, stage III in 1, and stage IV in 3. Five liver specimens from PBC (all stage II) treated with UDCA for more than 1 year were also studied. Six liver specimens from patients with PSC were studied. The histologic stage of PSC, based on Ludwig's classification,[27] was stage II in 2 and stage IV in 4 (Table 1). Six histologically normal liver samples, 6 samples from patients with hepatitis C–associated cirrhosis (LC-C), and 4 with extrahepatic cholestasis (Ex-C) were also included in the study. Liver biopsy had been performed for diagnosis; only a few sections or a portion of the liver specimen were used in the study.

Informed consent was obtained from each patient, and the study protocol followed the ethical guidelines of the 1975 Declaration of Helsinki as confirmed by *a priori* approval by the Kurume University's Human Research Committee.

TABLE 1. Patient Characteristics

| | Age | Sex | Stage | TB (mg/dL) | ALT (IU/L) | ALP (IU/L) | GGT (IU/L) |
|---|---|---|---|---|---|---|---|
| PBC | | | | | | | |
| 1 | 62 | Male | I | 1.1 | 66 | 420 | 180 |
| 2 | 63 | Female | I | 0.5 | 55 | 893 | 288 |
| 3 | 64 | Female | I | 0.5 | 63 | 283 | 392 |
| 4 | 67 | Female | I | 0.8 | 16 | 686 | 223 |
| 5 | 58 | Female | II | 1.4 | 61 | 442 | 57 |
| 6 | 59 | Female | II | 0.9 | 82 | 660 | 280 |
| 7 | 67 | Female | II | 0.5 | 19 | 425 | 58 |
| 8 | 38 | Female | II | 0.7 | 25 | 1435 | 127 |
| 9 | 49 | Female | II | 0.6 | 49 | 696 | 225 |
| 10 | 59 | Male | II | 0.8 | 134 | 1204 | 447 |
| 11 | 44 | Female | III | 1.4 | 114 | 778 | 1082 |
| 12 | 49 | Female | IV | 10.9 | 122 | 1277 | 212 |
| 13 | 44 | Female | IV | 10.9 | 53 | 443 | 612 |
| 14 | 47 | Female | IV | 8.8 | 77 | 393 | 73 |
| PSC | | | | | | | |
| 1 | 24 | Male | II | 0.9 | 31 | 274 | 80 |
| 2 | 78 | Male | II | 0.8 | 45 | 309 | 66 |
| 3 | 16 | Female | IV | 10.8 | 155 | 985 | 120 |
| 4 | 28 | Male | IV | 21.6 | 84 | 311 | 58 |
| 5 | 47 | Female | IV | 33.0 | 20 | 237 | 70 |
| 6 | 25 | Male | IV | 0.7 | 74 | 505 | 89 |

Abbreviations: TB, total bilirubin; ALT, alanine-transaminase; ALP, alkaline phosphatase; GGT, γ-glutamyl transpeptidase.

*Immunohistochemistry.* Double-indirect immunofluorescent staining for 7H6 and ZO-1 was performed in liver specimens as previously described.[24,25] Briefly, 6-μm-thick nonfixed frozen sections were incubated with 100% cold acetone for 10 minutes. The sections were then incubated with a mixture of an anti-7H6 mouse monoclonal antibody[20] and an anti–ZO-1 rabbit polyclonal antibody (Zymed Laboratories, San Francisco, CA). Then, the sections were incubated with the secondary antibodies, a mixture of fluorescein isothiocyanate–conjugated goat anti-mouse IgM (Cappel, Aurora, OH) and tetramethylrhodamine isomer R–conjugated swine anti-rabbit immunoglobulins (Dako Japan, Kyoto, Japan). A confocal laser scanning microscope (FV500; Olympus, Tokyo, Japan) equipped with an argon/krypton laser capable of dual excitation and detection was used to visualize immunostaining for 7H6 and ZO-1.

*Quantitation of Immunostaining Intensity for 7H6 and ZO-1.* Immunostaining for 7H6 and ZO-1 in each group of samples was quantified in hepatocytes and in bile ducts 40 to 80 μm in diameter, the latter being the structures mainly affected in PBC.[28] In each group, confocal laser scanning microscopic images were obtained randomly from 3 liver specimens. All images were coded to avoid bias during quantitation. Immunostaining intensities for 7H6 and ZO-1 were assessed as individual pixel intensities using a Macintosh computer (Power Macintosh 7600/200; Apple Computer, Cupertino, CA) with the public-domain program NIH Image 1.62. Immunostaining intensity curves were constructed by averaging immunostaining intensity curves from 15 randomly selected hepatocyte and BEC TJ in 9 images.

*Percentage of Bile Ducts With Immunostaining for 7H6.* Liver specimens containing more than 6 bile ducts each measuring 40 to 80 μm in diameter were used for analysis. In double immunostaining for 7H6 and ZO-1, immunostaining for 7H6 was evaluated only in bile ducts retaining ZO-1 immunostaining. Bile ducts with more than two thirds of BEC showing 7H6 immunostaining were defined as bile ducts retaining immunostaining for 7H6, and the percentage of bile ducts with immunostaining for 7H6 in all bile ducts analyzed was calculated. A total number of bile ducts analyzed were 35 in controls, 37 in LC-C, 30 in Ex-C, 76 in PSC, 89 in PBC stages I and II, 31 in PBC stages III and IV, and 24 in PBC treated with UDCA.

*Statistical Analysis.* All data are expressed as the mean ± SE. Differences between 2 groups were analyzed by the Mann-Whitney *U* test. $P < .05$ was considered statistically significant.

## RESULTS

*Immunostaining Distribution.* In the BEC of control group livers, immunostaining for 7H6 and ZO-1 colocalized at the apical portion of the cell border that corresponds to the site of TJ. In contrast, immunostaining for 7H6 in BEC was greatly reduced in PBC. This change was recognized in bile ducts 40 to 80 μm in diameter in the early stages (stages I and II). This was also apparent in the late stages (stages III and IV), even though the numbers of bile ducts ranging from 40 to 80 μm in diameter were decreased in these stages. Immunostaining for 7H6 in BEC was well preserved in PBC treated with UDCA (Fig. 1). On the other hand, immunostaining for 7H6 was readily demonstrated in BEC in PSC as well as Ex-C (Fig. 2).

In control specimens, hepatocytes showed immunostaining for both 7H6 and ZO-1 that was localized mainly at the outlines of the bile canaliculi. In contrast, immunostaining for 7H6 was discontinuous and less uniformly present in hepatocytes in early-stage (I and II) PBC. Immunostaining for 7H6 in hepatocytes was even more discontinuous or lost in late-stage (III and IV) PBC. Immunostaining for 7H6 was well preserved in hepatocytes of PBC treated with UDCA (Fig. 3). The PSC

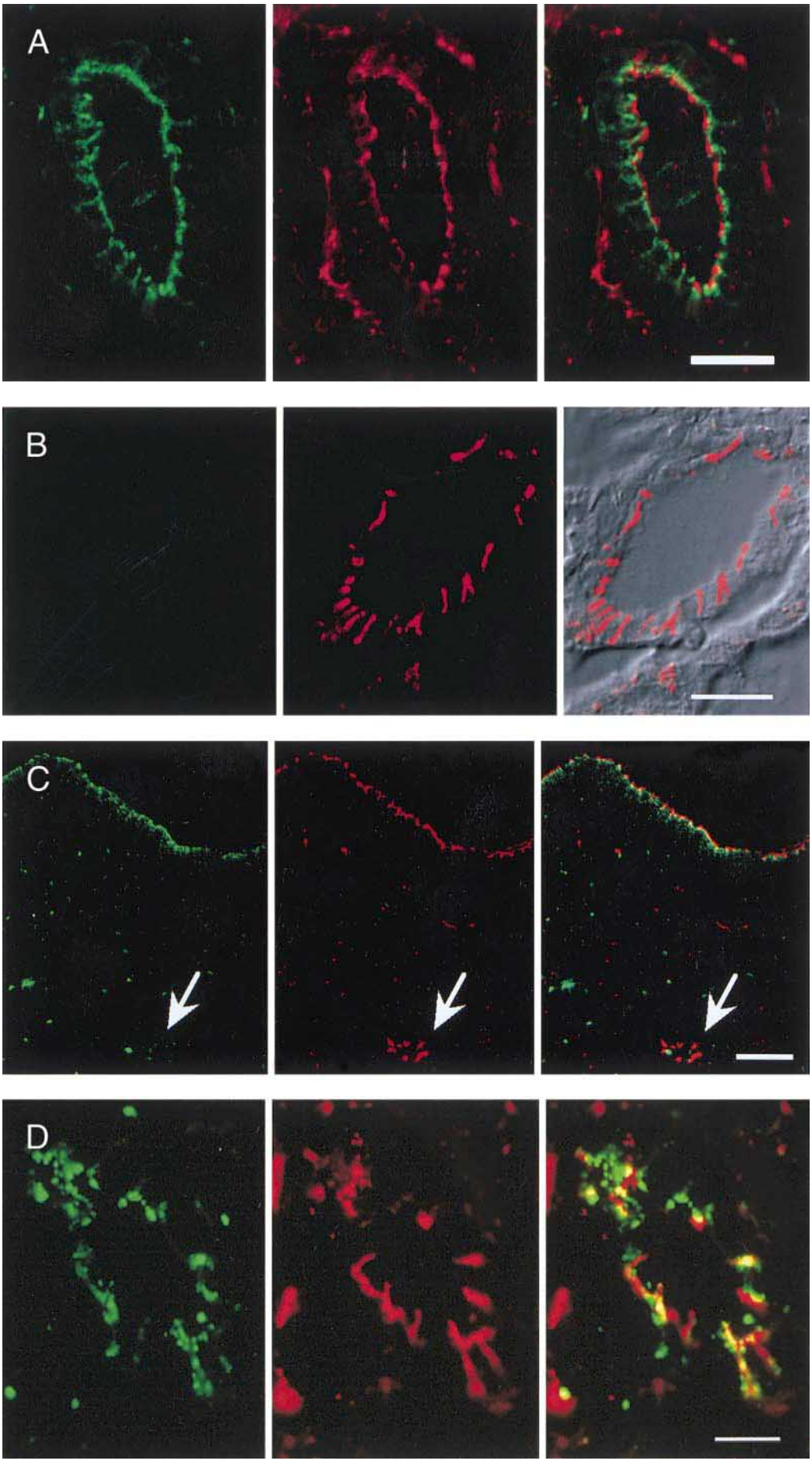


FIG. 1. Immunostaining for 2 proteins, 7H6 (*green* signal) and ZO-1 (*red* signal), localizes at the apical and lateral membranes of BEC in a control liver specimen. A merged image shows colocalization of immunostaining for 7H6 and ZO-1 (*yellow* signal) (A). In PBC (B and C), while the middle-sized bile duct (40 to 80 $\mu$m in diameter) (B and *arrow* in [C]) shows faint or absent immunostaining for 7H6 and retains staining for ZO-1, the large bile duct retains immunostaining for both 7H6 and ZO-1, resembling the pattern in the control (C). A merged image clearly shows disappearance of immunostaining for 7H6 protein in BEC of middle-sized bile ducts (B and C). A Nomarski image is overlaid on the merged image in PBC (B). In PBC treated with UDCA, immunostaining for 7H6 in BEC is well preserved (D). (A and B), *Bar* = 30 $\mu$m; (C), *bar* = 60 $\mu$m; (D), *bar* = 10 $\mu$m.

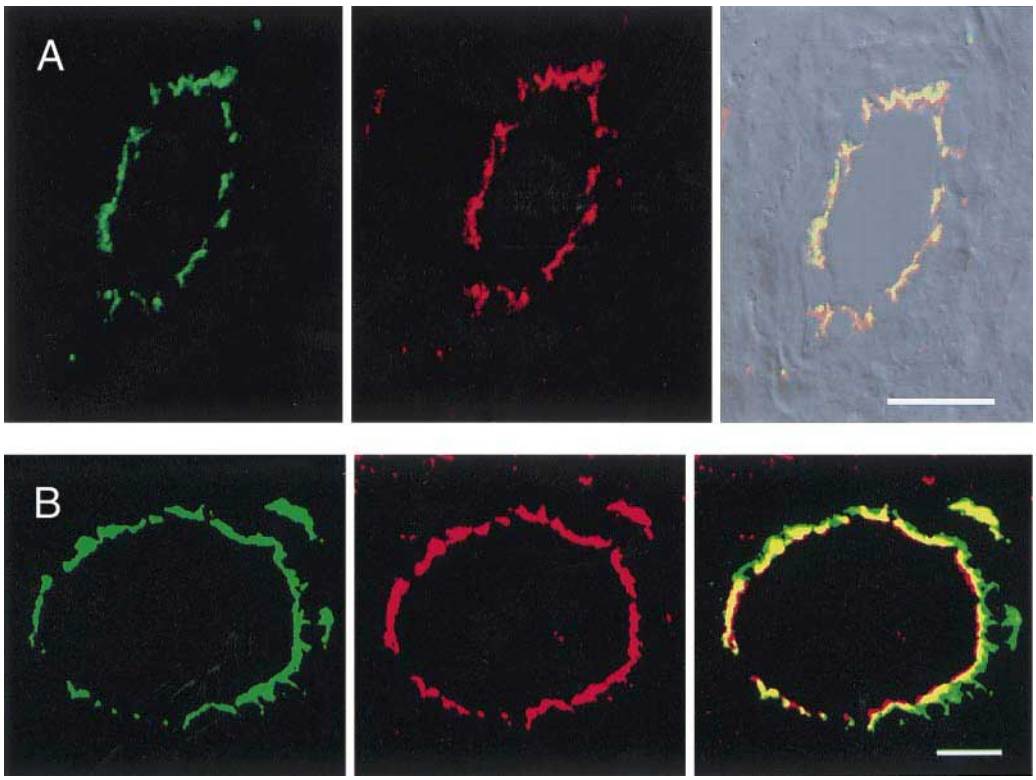


FIG. 2. Immunofluorescence localization of 7H6 (*green* signal) and ZO-1 (*red* signal) in bile ducts in PSC (A) and Ex-C (B). Immunostaining for 7H6 and ZO-1 is present at the apical and lateral membranes of BEC in both groups. A merged image (*yellow* signal) shows colocalization of immunostaining for 7H6 and ZO-1. A Nomarski image is overlaid on the merged image in PSC (A). (A), *Bar* = 30 $\mu$m; (B), *bar* = 10 $\mu$m.

and Ex-C groups showed markedly decreased immunostaining for 7H6 in hepatocytes (Fig. 4).

In contrast to the dramatic alteration of immunostaining for 7H6 in PBC and PSC, no marked change in localization of immunostaining for ZO-1 was seen in BEC or hepatocytes in PBC or PSC.

***Immunostaining Intensity.*** The distribution and intensity of immunostaining for 7H6 and ZO-1 were analyzed (Fig. 5). In control BEC, immunostaining for 7H6 and ZO-1 showed similar intensity and colocalization at TJ. In contrast, intensity of immunostaining for 7H6 in BEC was markedly reduced in early stages (I and II) of PBC compared with that in controls. The change in intensity of immunostaining for 7H6 was more evident in BEC in late stages (III and IV) in PBC. Immunostaining for 7H6 was well preserved in BEC of PBC treated with UDCA. In PSC and Ex-C, the intensity of immunostaining for 7H6 was relatively increased in BEC. The intensity of immunostaining for ZO-1 was increased in BEC of both PBC and PSC specimens compared with intensity in controls.

In hepatocytes of control specimens, immunostaining for 7H6 and ZO-1 colocalized at TJ with similar intensity. In hepatocytes of PBC, immunostaining for 7H6 was slightly decreased in intensity at TJ. Those changes were most evident in hepatocyte in late stages (III and IV) of PBC. In both early and late stages of PBC, changes in immunostaining for 7H6 were more evident in BEC than in hepatocytes. In PBC treated with UDCA, immunostaining for 7H6 was well preserved in hepatocytes. Immunostaining for 7H6 in hepatocytes was extremely diminished in PSC and in Ex-C. Intensity of immunostaining for ZO-1 was greater in hepatocytes in PBC, PSC, and Ex-C specimens than in controls. Changes in distribution in immunostaining for ZO-1 were not evident in PBC and PSC.

***Percentage of Bile Ducts With Immunostaining for 7H6.*** In controls, more than 90% of bile ducts showed retaining immunostaining for 7H6. This immunostaining was retained in bile ducts in LC-C, Ex-C, and PSC. However, only 34.6% $\pm$ 13.1% and 14.9% $\pm$ 11.3% of bile ducts showed immunostaining for 7H6 in early-stage (I and II) PBC and in late-stage (III and IV) PBC, respectively. The percentage of bile ducts with immunostaining for 7H6 was significantly lower than in control, LC-C, Ex-C, or PSC specimens (all $P < .001$). In PBC treated with UDCA, the percentage was 72.0% $\pm$ 5.2%, which was significantly higher than that in untreated PBC ($P < .05$) (Fig. 6).

## DISCUSSION

In this study, we demonstrated that immunostaining for 7H6 in BEC was significantly decreased or disappeared in PBC. This change was never seen in BEC of PSC, Ex-C, or LC-C cases, although immunostaining for 7H6 was decreased in hepatocytes in late stages of PBC, PSC, and Ex-C. Alteration of TJ in BEC, then, is a characteristic feature of PBC.

Ultrastructural changes in intrahepatic bile ducts have been well documented in PBC. Lateral intercellular spaces of BEC are irregularly dilated and contain granular material that appears similar to that found in duct lumens and in the cytoplasm of periductal macrophages.[3,4] Although these findings suggest alteration of paracellular permeability in BEC, to date, these have not directly addressed bile duct permeability or changes in molecular architecture of tight junctions in PBC.

TJ creates a regulated barrier in the paracellular pathway.[16] A growing number of TJ-associated proteins have been identified and characterized.[16] ZO-1, the best-characterized TJ-associated protein, belongs to a membrane-associated guanylate kinase homologue protein family.[16] On the other hand, 7H6 was isolated from the bile canaliculus–rich membrane

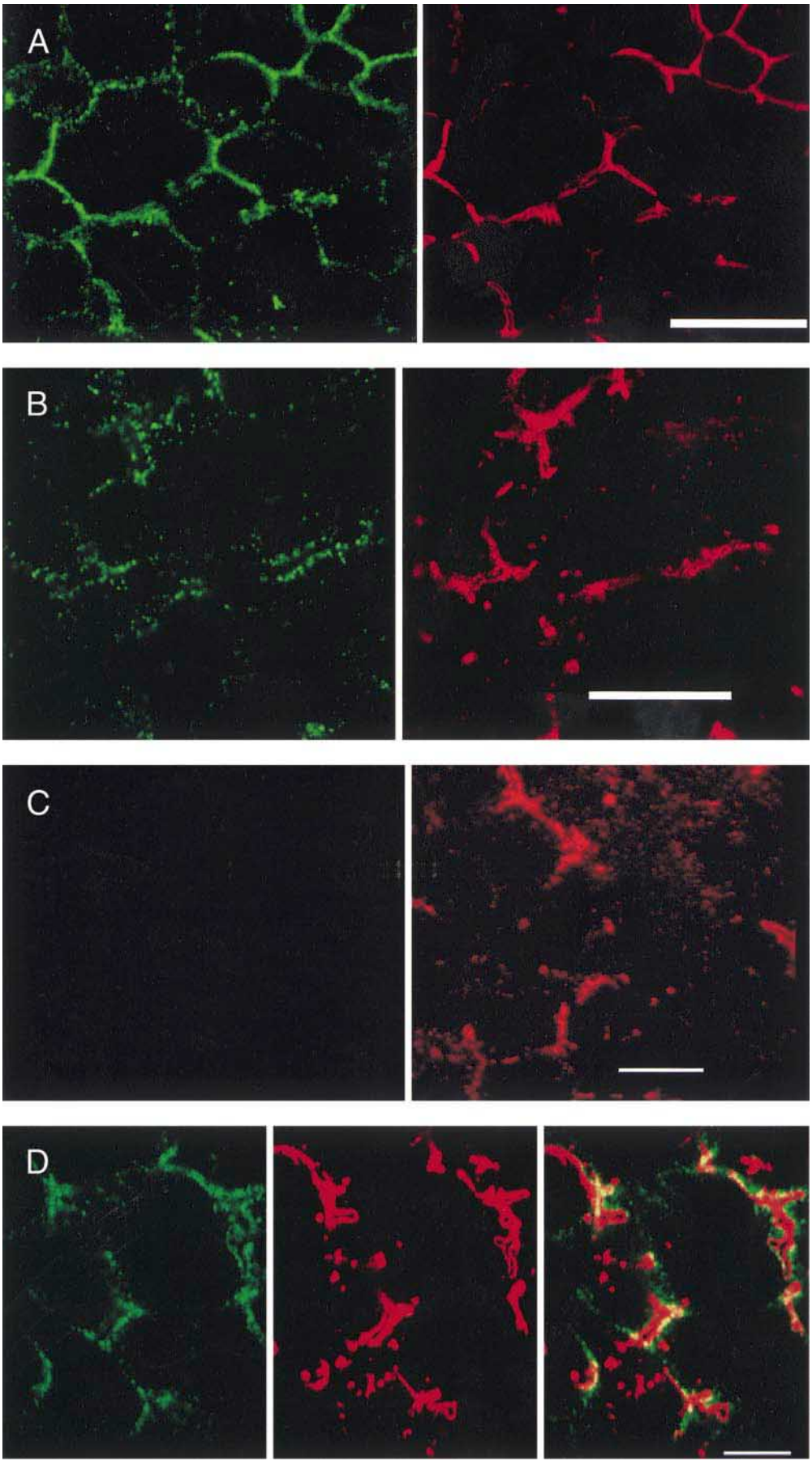


FIG. 3. Immunostaining for 7H6 (*green* signal) and ZO-1 (*red* signal) in hepatocytes of a control liver specimen (A). Immunostaining for both 7H6 and ZO-1 is colocalized along the bile canaliculi in a control liver. In an early stage (stage II) of PBC (B), immunostaining for 7H6 and ZO-1 shows discontinuous lines with slightly decreased intensity of immunostaining for 7H6, but not for ZO-1. In a late stage (stage IV) of PBC (C), immunostaining for 7H6 has disappeared, while immunostaining for ZO-1 persists along bile canaliculi. In PBC treated with UDCA (D), immunofluorescence for both 7H6 and ZO-1 is well presented. (A-C), *Bar* = 30 $\mu$m; (D), *bar* = 10 $\mu$m.

fraction in rat liver and is characterized as a TJ-associated phosphorylated protein.[20] Immunolocalization of 7H6 correlates closely with paracellular permeability, and 7H6 serves as a reliable immunocytochemical marker for monitoring epithelial barrier function.[21-23] Therefore, we performed immunostaining for 7H6 to evaluate paracellular permeability, and also stained for ZO-1, a protein whose immunolocalization does not correlate with paracellular permeability.[21]

In the preliminary study, when we stained both ZO-1 and CK-19, a marker for bile ducts, using livers of PBC, more than 80% of the remaining CK-19–positive bile ducts presented immunostaining for ZO-1 even in PBC livers (85.4% ± 14.5%). Thus, in double immunostaining for 7H6 and ZO-1, immunostaining for 7H6 was evaluated only in bile ducts retaining ZO-1 immunostaining. In the present study, immunostaining for 7H6 showed alteration or disappearance in BEC even in early PBC (stages I and II). These alterations occurred mainly in small to middle-sized bile ducts ($< 80\ \mu$m in diameter), the predominant lesion site in PBC.[27] While immunostaining for 7H6 was lost in these bile ducts, immunostaining for ZO-1 still was preserved in many bile ducts in PBC, suggesting that the BEC had retained their cellular contacts. On the other hand, these changes were not recognized in BEC in PSC, Ex-C, or LC-C.

Considering the characteristics of 7H6 noted above, we speculate the following pathogenetic sequence. Paracellular permeability of BEC is increased in PBC, allowing toxic bile acids or various antigens to enter the periductal area from the lumen of bile ducts. This spillover may promote infiltration of lymphocytes, macrophages, and plasma cells around bile ducts. These cells then secrete various cytokines, including tumor necrosis factor-$\alpha$, interferon-$\gamma$, interleukin-1, and interleukin-6, which in turn would aggravate bile duct injury in PBC.[29] The 7H6 changes are fairly specific: no changes of immunostaining for 7H6 occur in BEC of PSC, Ex-C, and in rats with bile duct ligation.[24] We also demonstrated that immunostaining for 7H6 was altered in both BEC and hepatocytes in the late stages of PBC (stages III and IV). Bile regurgitation is considered to occur through damaged BEC or through hepatocytes in these late stages, when the numbers of remaining bile ducts are decreased and severe hepatocytic cholestasis is present. However, it is not still clear that immunolocalization of 7H6 is closely related with paracellular permeability in BEC, although this was already demonstrated in many other cells, including hepatocytes.[21,22,24,25] Thus, further studies on the relationship between TJ permeability and immunolocalization of 7H6 in BEC should be performed. It is important to determine whether the changes in 7H6 localization are causally related phenomena or epiphenomenona. It has, however, many difficulties in evaluating the paracellular permeability in patients with PBC and following both structural and functional changes of TJ. 7H6 regulates paracellular permeability through protein phosphoryla-

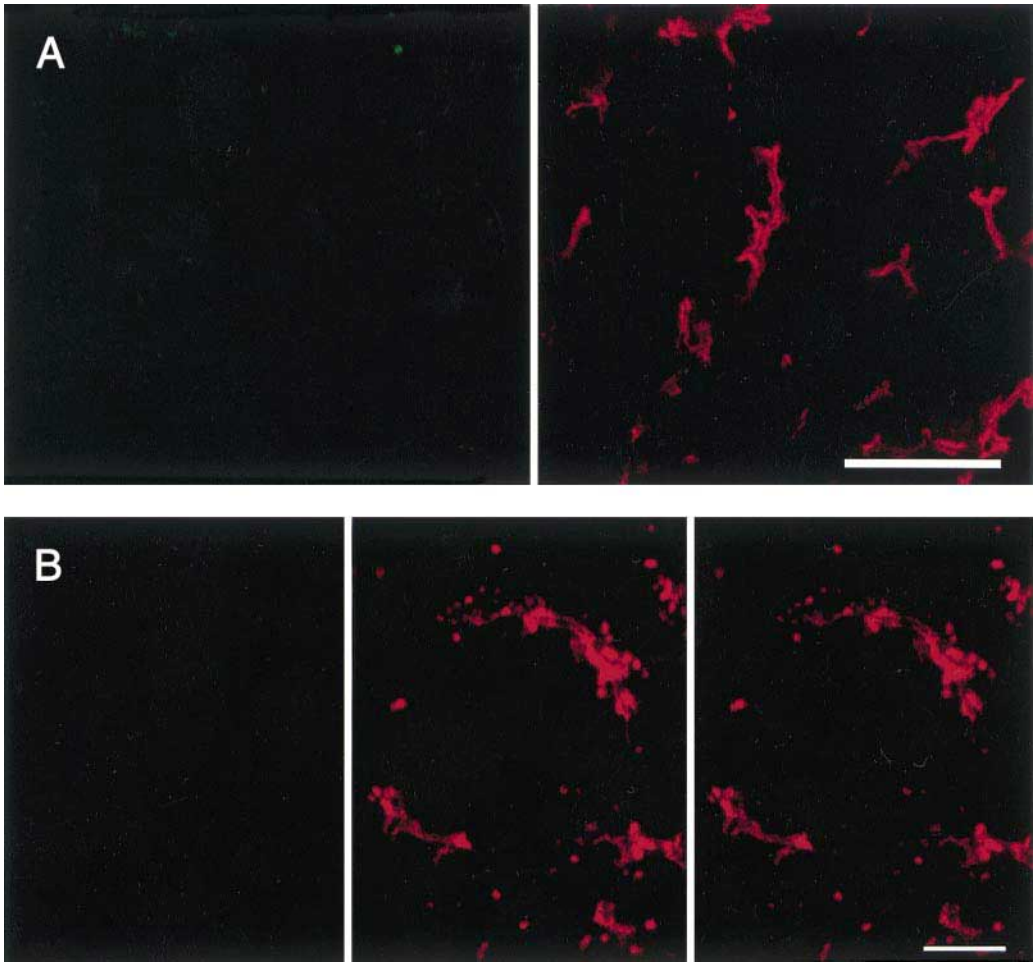


FIG. 4. Immunostaining of hepatocytes in both PSC (A) and Ex-C (B) shows faint or absent fluorescence for 7H6 (*green* signal), but preserves fluorescence for ZO-1 (*red* signal). (A), Bar = 30 $\mu$m; (B), *bar* = 10 $\mu$m.

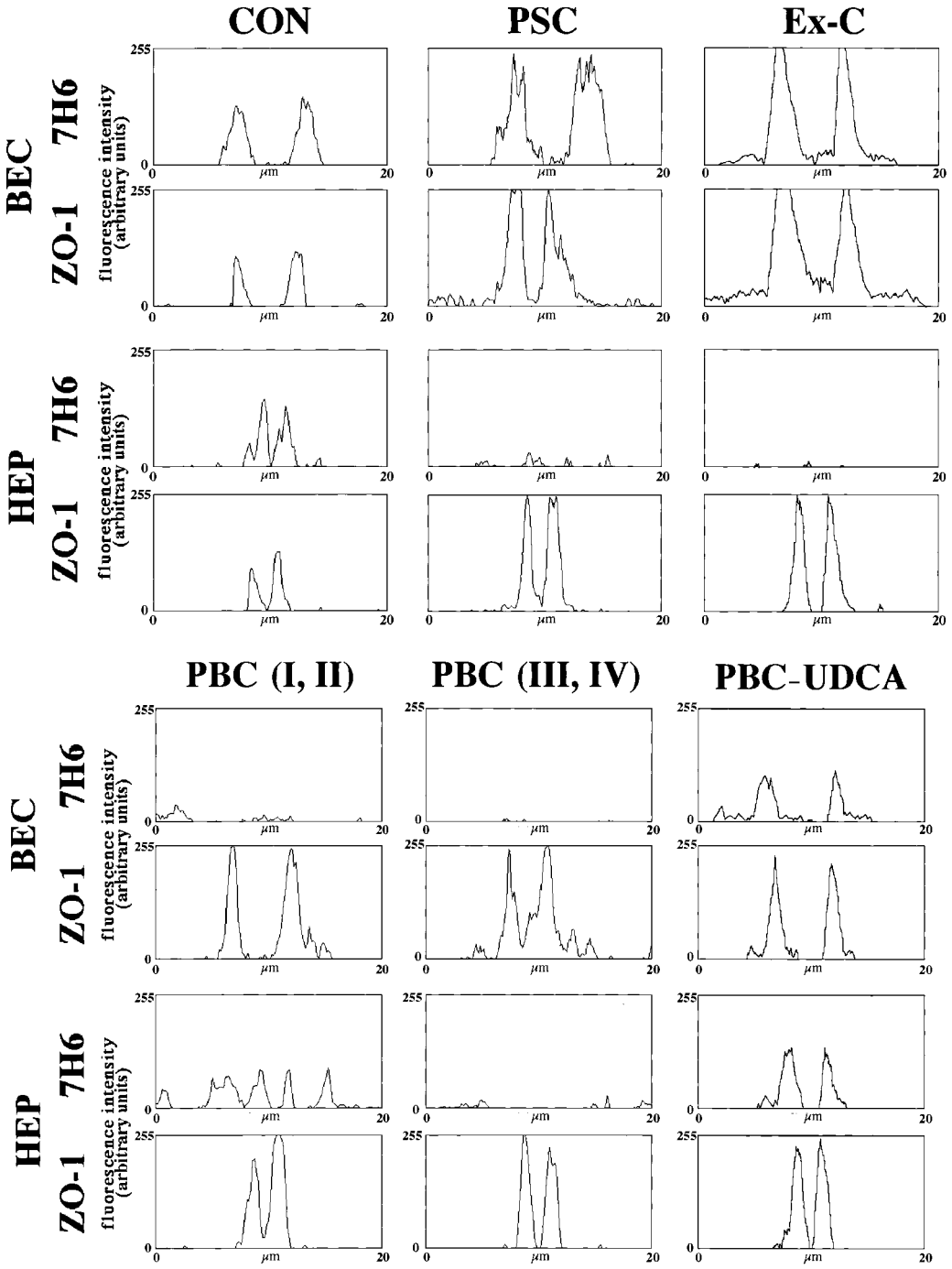


FIG. 5. Analysis of immunostaining intensity in confocal laser scanning microscopic images in control liver specimens (CON), PSC, Ex-C, early stages (I and II) and late stages (III and IV) of PBC, and PBC treated with ursodeoxycholic acid (PBC-UDCA). In BEC, immunostaining for 7H6 and ZO-1 normally are colocalized with similar intensity at TJ. Immunostaining for 7H6 is decreased at TJ in BEC in early stages of PBC (stages I and II) and more evidently in late stages of PBC (stages III and IV). In PBC-UDCA, immunostaining for 7H6 in BEC is fairly preserved. In PSC and Ex-C, immunostaining for 7H6 in BEC shows wider and greater intensity than in controls. Immunostaining for ZO-1 in BEC shows wider and more intensity in both PBC and PSC than in control specimens. In hepatocytes (HEP), immunostaining for 7H6 and ZO-1 colocalizes with similar intensity at TJ in controls. Hepatocytic immunostaining for 7H6 is relatively diminished in PBC of stages III and IV, as well as in PSC and Ex-C. Hepatocytic immunostaining for 7H6 is well preserved in PBC-UDCA. Immunostaining for ZO-1 in HEP increases with wider distribution in both PBC and PSC. Each curve shown indicates an average immunostaining intensity obtained by averaging 15 immunostaining intensity curves representing 15 sections from 5 specimens.

tion,[21] and can have various effects. In our previous study using rat livers,[25] there is an obvious relationship between the changes in 7H6 localization and paracellular permeability. The changes in 7H6 are seen in the early stage of cholestatic rat livers, which show normal liver-function tests and liver tissue. Similarly, the changes in 7H6 localization were seen even in patients with early-stage PBC in this study. Thus, the changes in 7H6 localization may be causally related phenomena.

Why immunostaining for 7H6 is altered in BEC in PBC is not clear, and several mechanisms could be suggested. First, cytotoxic bile acids may induce alterations of TJ in BEC. Bile acids are reabsorbed by BEC, where they can affect mitochondria to result in impaired adenosine triphosphate production.[30-32] With such adenosine triphosphate reduction, 7H6 could disappear, and paracellular permeability therefore would increase.[21-23] However, in PSC or Ex-C, the alteration of immunostaining for 7H6 in BEC was not observed. Therefore, there could be other factors besides toxic bile acids to produce these changes in BEC of PBC. The second possibility is accumulation of endotoxin in BEC in PBC.[33] Endotoxin is known to increase expression of uncoupling protein-2, which decreases the intracellular adenosine triphosphate level.[34] As the third possible mechanism, cytotoxic T cells, which frequently are present surrounding bile ducts in PBC,[35] could release proinflammatory cytokines. Sev-

eral of these cytokines are known to increase paracellular permeability.[36,37]

We also investigated changes in immunostaining for 7H6 in PSC, another chronic cholestatic liver disease. Here, a decrease or disappearance of immunostaining for 7H6 was seen in hepatocytes, but not BEC, which was quite similar to the result in Ex-C. These findings differ from those in PBC, indicating that, in PSC, bile regurgitates through TJ as a result of increased paracellular permeability between hepatocytes, not BEC. This distinction held even in the terminal stage of PSC. Similar changes of hepatocytic immunostaining for 7H6 could be seen in Ex-C and in rat bile duct ligation.[24] Altered hepatocyte TJ in PSC may be a secondary change resulting from impaired bile flow caused by narrowing or obliteration of bile ducts.

The bile-regurgitation route in cholestatic liver diseases, including PBC and PSC, has not been studied previously, although biochemical evidence of bile regurgitation has been described. The present results, demonstrating marked alteration or disappearance of immunolocalization of 7H6 of BEC in PBC, support the hypothesis that increased paracellular permeability between BEC may be central to pathogenesis of PBC. However, further studies are required to identify the primary events in PBC.

Administration of UDCA improves clinical and biochemical cholestatic parameters; the alteration in the immunolocalization of 7H6 was greatly corrected as well. It is possible that the normalized immunolocalization of 7H6 protein at TJ produced by treatment with UDCA indicates preserved function of TJ in BEC and contributes to biochemical and histologic improvement in PBC. However, whether UDCA has direct effects to correct the alteration of TJ in PBC should be investigated further.

Although PBC and PSC are chronic cholestatic diseases of unknown etiology and both result in increased serum concentrations of bile constituents, we conclude that the paracellular bile-regurgitation route differs greatly between these diseases (Fig. 7).

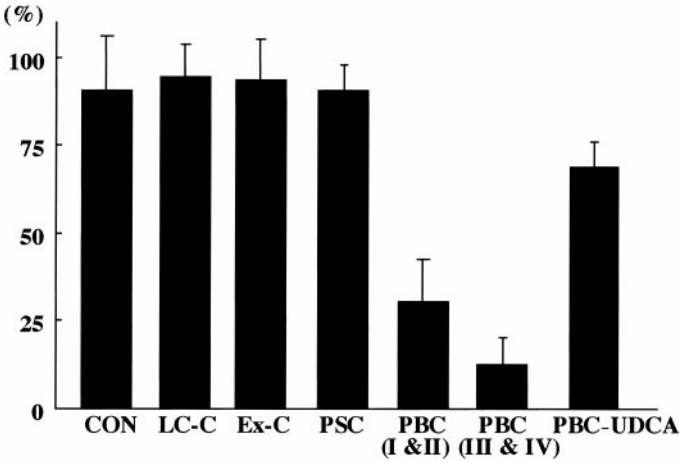


FIG. 6. Percentages of bile ducts with retained 7H6 staining among all bile ducts with immunostaining for ZO-1 is significantly decreased in both early (I and II) and late (III and IV) stages of PBC compared with controls, LC-C, Ex-C, and PSC (all $P < .001$). The percentages are significantly higher in PBC-UDCA than those in untreated PBC ($P < .05$). CON, control; PBC-UDCA, PBC treated with UDCA.

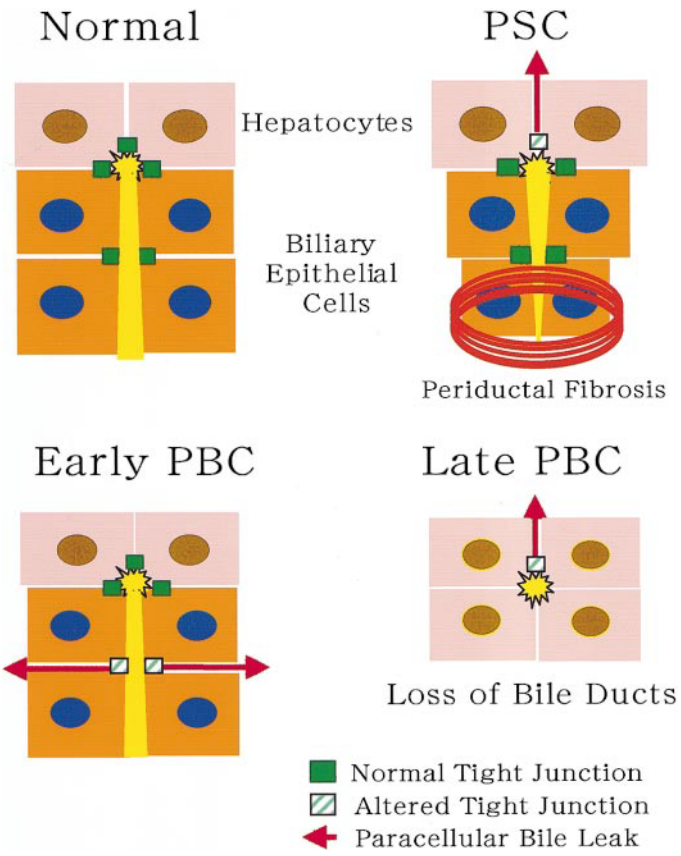


FIG. 7. Schematic representation of mechanisms for paracellular bile regurgitation in PBC and PSC. In the normal state, bile canaliculi of hepatocytes and the lumen of bile ducts are sealed by TJ, preventing paracellular bile leakage. In the early stages of PBC, the TJ of BEC are altered, promoting bile leakage. Because many middle-sized bile ducts already have disappeared in the late stage of PBC, elevated intracanalicular pressure produces alteration of TJ proteins in hepatocytes as well, promoting bile leakage through the TJ of hepatocytes. In PSC, bile may leak preferentially through hepatocyte TJ, which are believed to be less tight than BEC TJ, predisposing the former to damage from elevated intrabiliary pressure as a result of constriction of bile ducts by periductal fibrosis.

*Acknowledgment:* The authors thank Hiromi Ishibashi, Takashi Nishizaki, Keizo Sugimachi, and Hidetoshi Matsunami for providing liver specimens from transplant recipients.

## REFERENCES

1. Poupon RE, Lindor KD, Cauch-Dudek K, Dickson ER, Poupon R, Heathcote EJ. Combined analysis of randomized controlled trials of ursodeoxycholic acid in primary biliary cirrhosis. Gastroenterology 1997;113:884-890.
2. Reichen J, Francis R. Cholestasis. In: Arias IM, Boyer JL, Fausto N, Jakoby WB, Schachter D, Shafritz DA, eds. The Liver: Biology and Pathobiology. 3rd ed. New York: Raven, 1994:1291-1326.
3. Nakanuma Y, Kono N, Ohta G, Kato Y, Kobayashi K. Ultrastructural changes of bile duct epithelium in primary biliary cirrhosis in relation to progression of bile duct loss. Virchows Arch A Pathol Anat Pistol 1982; 398:149-161.
4. Tanikawa K, Sakisaka S. Hepatic ultrastructure of jaundice. Medicina 1983;20:1972-1975.
5. Phillips MJ, Poucell S, Patterson J, Valencia P. The Liver: An Atlas and Text of Ultrastructural Pathology. New York: Raven, 1987:101-158.
6. Nakanuma Y, Tsuneyama K, Gershwin ME, Yasoshima M. Pathology and immunopathology of primary biliary cirrhosis with emphasis on bile duct lesions: recent progress. Semin Liver Dis 1995;15:313-328.

7. Lake JR, Licko V, Van Dyke RW, Scharschmidt BF. Biliary secretion of fluid-phase markers by the isolated perfused rat liver. Role of transcellular vesicular transport. J Clin Invest 1985;76:676-684.
8. Elias E, Hruban Z, Wade JB, Boyer JL. Phalloidin-induced cholestasis: a microfilament-mediated change in junctional complex permeability. Proc Natl Acad Sci U S A 1980;77:2229-2233.
9. De Vos R, Desmet VJ. Morphologic changes of the junctional complex of the hepatocytes in rat liver after bile duct ligation. Br J Exp Pathol 1978; 59:220-227.
10. Alpini G, Phillips JO, LaRusso NF. The biology of biliary epithelia In: Arias IM, Boyer JL, Fausto N, Jakoby WB, Schachter D, Shafritz DA, eds. The Liver: Biology and Pathobiology. 3rd ed. New York: Raven, 1994: 623-653.
11. Wiesner RH, LaRusso NF. Clinicopathologic features of the syndrome of primary sclerosing cholangitis. Gastroenterology 1980;79:200-206.
12. Stevenson BR, Siliciano JD, Mooseker MS, Goodenough DA. Identification of ZO-1: a high molecular weight polypeptide associated with the tight junction (zonula occludens) in a variety of epithelia. J Cell Biol 1986;103:755-766.
13. Gumbiner B, Lowenkopf T, Apatira D. Identification of a 160-kDa polypeptide that binds to the tight junction protein ZO-1. Proc Natl Acad Sci U S A 1991;88:3460-3464.
14. Balda MS, Anderson JM, Matter K. The SH3 domain of the tight junction protein ZO-1 binds to a serine protein kinase that phosphorylates a region C-terminal to this domain. FEBS Lett 1996;399:326-332.
15. Keon BH, Schafer S, Kuhn C, Grund C, Franke WW. Symplekin, a novel type of tight junction plaque protein. J Cell Biol 1996;134:1003-1018.
16. Mitic LL, Anderson JM. Molecular architecture of tight junction. Annu Rev Physiol 1998;60:121-141.
17. Willot E, Balda MS, Fanning A, Jameson B, Van Itallie CM, Anderson JM. The tight junction protein ZO-1 is homologous to the Drosophila discs-large tumor suppressor protein of septate junctions. Proc Natl Acad Sci U S A 1993;90:7834-7838.
18. Woods DF, Bryant PJ. The discs-large tumor suppressor gene of Drosophila encodes a guanylate kinase homolog localized at septate junctions. Cell 1991;66:451-464.
19. Furuse M, Itoh M, Hirase T, Nagafuchi A, Yonemura S, Tsukita S, Tsukita S. Direct association of occludin with ZO-1 and its possible involvement in the localization of occludin at tight junctions. J Cell Biol 1994;127: 1617-1626.
20. Zhong Y, Saitoh T, Minase T, Sawada N, Enomoto K, Mori M. Monoclonal antibody 7H6 reacts with a novel tight junction–associated protein distinct from ZO-1, cingulin and ZO-2. J Cell Biol 1993;120:477-483.
21. Zhong Y, Enomoto K, Isomura H, Sawada N, Minase T, Oyamada M, Konishi Y, et al. Localization of the 7H6 antigen at tight junctions correlates with the paracellular barrier function of MDCK cells. Exp Cell Res 1994;214:614-620.
22. Satoh H, Zhong Y, Isomura H, Saitoh M, Enomoto K, Sawada N, Mori M. Localization of 7H6 tight junction–associated antigen along the cell border of vascular endothelial cells correlates with paracellular barrier function against ions, large molecules, and cancer cells. Exp Cell Res 1996; 222:269-274.
23. Kimura H, Sawada N, Tobioka H, Isomura H, Kokai Y, Hirata K, Mori M. Bacterial lipopolysaccharide reduced intestinal barrier function and altered localization of 7H6 antigen in IEC-6 rat intestinal crypt cells. J Cell Physiol 1997;171:284-290.
24. Kawaguchi T, Sakisaka S, Sata M, Mori M, Tanikawa K. Different lobular distributions of altered hepatocyte tight junctions in rat models of intrahepatic and extrahepatic cholestasis. HEPATOLOGY 1999;29:205-216.
25. Kawaguchi T, Sakisaka S, Mitsuyama K, Harada M, Koga H, Sasatomi K, Kimura R, et al. Cholestasis with altered structure and function of hepatocyte tight junction and decreased expression of canalicular multispecific organic anion transporter in a rat model of colitis. HEPATOLOGY 2000;31:1285-1295.
26. Scheuer PJ, Lefkowitch JH. Liver Biopsy Interpretation. 5th ed. London: Saunders, 1994:38-61.
27. Ludwig J, Barham SS, LaRusso NF, Elveback LR, Wiesner RH, McCall JT. Morphologic features of chronic hepatitis associated with primary sclerosing cholangitis and chronic ulcerative colitis. HEPATOLOGY 1981;1: 632-640.
28. Nakanuma Y, Ohta G. Histometric and serial section observations of the intrahepatic bile ducts in primary biliary cirrhosis. Gastroenterology 1979;76:1326-1332.
29. Crawford JM, Boyer JL. Clinicopathology conferences: inflammation-induced cholestasis. HEPATOLOGY 1998;28:253-260.
30. Rodrigues CM, Fan G, Ma X, Kren BT, Steer CJ. A novel role for ursodeoxycholic acid in inhibiting apoptosis by modulating mitochondrial membrane perturbation. J Clin Invest 1998;101:2790-2799.
31. Patel T, Bronk SF, Gores GJ. Increases of intracellular magnesium promote glycodeoxycholate-induced apoptosis in rat hepatocytes. J Clin Invest 1994;94:2183-2192.
32. Lazaridis KN, Pham L, Tietz P, Marinelli RA, deGroen PC, Levine S, et al. Rat cholangiocytes absorb bile acids at their apical domain via the ileal sodium-dependent bile acid transporter. J Clin Invest 1997;100:2714-2721.
33. Sasatomi K, Noguchi K, Sakisaka S, Sata M, Tanikawa K. Abnormal accumulation of endotoxin in biliary epithelial cells in primary biliary cirrhosis and primary sclerosing cholangitis. J Hepatol 1998;29:409-416.
34. Cortez-Pinto H, Yang SQ, Lin HZ, Costa S, Hwang CS, Lane MD, Bagby G, et al. Bacterial lipopolysaccharide induces uncoupling protein-2 expression in hepatocytes by a tumor necrosis factor-alpha–dependent mechanism. Biochem Biophys Res Commun 1998;251:313-319.
35. Koga H, Sakisaka S, Kawaguchi T, Taniguchi E, Ohishi M, Sasatomi K, et al. Involvement of FAS ligand-expressing T lymphocytes in bile duct injury of primary biliary cirrhosis. HEPATOLOGY 1998;28:543A.
36. McRoberts JA, Riley NE. Modulation of growth factor and cytokine-induced increases in T84 cell monolayer permeability by media components. Am J Physiol 1994;267:C537-C543.
37. Mano Y, Ishii, Okamoto H, Igarashi T, Kobayashi K, Toyota Y. Effect of tumor necrosis factor alpha on intrahepatic bile duct epithelial cells of rat liver. HEPATOLOGY 1996;23:1602-1607.