Biliary Secretion of Endotoxin and Pathogenesis of Primary Biliary Cirrhosis

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Previous studies suggested endotoxin, derived from the intestine through the portal blood to the liver, was predominantly metabolized by Kupffer cells. In the present study, fluorescent-labeled endotoxin injected into the rat portal vein was demonstrated not only in Kupffer cells but also in hepatocytes. Furthermore, a great amount of labeled endotoxin was recovered in bile. In the livers of patients with primary biliary cirrhosis (PBC), immunohistochemistry demonstrated significant retention of endotoxin in the biliary epithelial cells, and treatment with ursodeoxycholic acid significantly reduced the retention in those cells. The study for detection of apoptosis demonstrated increased rates of apoptosis in hepatocytes and biliary epithelial cells in PBC liver, and the rate of apoptosis in biliary epithelial cells was significantly reduced after treatment with ursodeoxycholic acid. Immunohistochemistry in PBC liver demonstrated significant reduction of fluorescence intensity for a 7H6 antigen in biliary epithelial cells, indicating the increased paracellular permeability of bile ducts, because cellular immunolocalization of that antigen has been shown to be inversely correlated with the paracellular permeability of the tight junction. These results suggest that, in biliary epithelial cells, retention of endotoxin, increased apoptosis, and increased permeability of tight junctions may be involved in the pathogenesis of PBC.

BILIARY SECRETION OF ENDOTOXIN

Endotoxin, localized in an outer leaflet of Gram-negative bacterial membrane, produces various actions in liver diseases, such as multi-organ failure, endotoxin shock, and hepatorenal syndrome. The liver is the major organ metabolizing endotoxin in the portal blood derived from the intestine. Therefore, several liver diseases including alcoholic liver disease, fulminant hepatitis, and cholestatic liver disease, are frequently associated with endotoxemia. Previous studies indicated that Kupffer cells and hepatic macrophages predominantly participate in clearance of endotoxin from the sinusoidal blood [1-3]. The present study was aimed to clarify the role of hepatocytes in clearance of endotoxin.

Fluorescein isothiocyanate (FITC)-labeled LPS$^b$ (25 mg/100 g body weight, Sigma) was injected into the rat portal vein. Bile was collected from the cannulated common bile duct. Fluorescence intensity of collected bile was measured by fluorescence spectrophotometry. Both FITC-LPS of preinjected form and that in bile were analyzed by HPLC. The livers were removed, fixed in 10 percent formaldehyde, and embedded in paraffin. One μm thick liver sections were deparaffinized and fluorescence in sections was observed by a confocal laser scanning microscope (Olympus GB-200).

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$^b$ Abbreviations: LPS, lipopolysaccharide; FITC, fluorescein isothiocyanate; HPLC, high pressure liquid chromatography; PBC, primary biliary cirrhosis; BEC, biliary epithelial cells; UDCA, ursodeoxycholic acid; TJ, tight junction.
Ten min after injection of FITC-LPS, significant fluorescence was detected in bile and reached its peak at 20 min. Twenty-five percent of injected fluorescence was recovered in bile within 60 min, while less than 5 percent of injected fluorescence was recovered in bile after injection of FITC-albumin. HPLC demonstrated the same retention time for preinjected FITC-LPS and FITC-LPS recovered from bile at 20 min after injection of FITC-LPS, suggesting that FITC-LPS is not metabolized in hepatocytes and secreted into bile. Confocal laser scanning microscopy demonstrated significant fluorescence not only in Kupffer cells but also in hepatocytes. Fluorescence intensity was higher in periportal hepatocytes than in porivenous hepatocytes (Figure 1). Colchicine (a microtubule inhibitor) significantly reduced secretion of FITC-LPS in bile. Pretreatment with Gadrinium chloride (GdCl₃, an inhibitor of macrophage function) prevented uptake of FITC-LPS into Kupffer cells but not uptake into hepatocytes, and recovery of FITC-LPS in bile was not changed.

These findings indicate:
1) LPS is taken up not only by Kupffer cells but also significantly by hepatocytes.
2) An enormous amount of LPS is secreted into bile.
3) LPS is transcellularly transported in hepatocytes depending on microtubules (probably via tubulo-vesicular pathway).
4) Biliary secretion of endotoxin is independent from its uptake by Kupffer cells. LPS in sinusoidal blood, therefore, is directly taken up by hepatocytes and secreted into bile independent from endotoxin metabolism in Kupffer cells.

Thus, it is suggested that in several liver diseases such as fulminant hepatitis, alcoholic liver disease, and cholestasis, dysfunction of hepatocytes in uptake and biliary secretion of LPS may result in reduced metabolism of LPS in liver, leading to systemic endotoxemia (Figure 2) [4].

Figure 1. Confocal laser scanning micrograph of a rat liver 30 min after injection of FITC-labeled LPS. While bright fluorescence was noted in Kupffer cells (arrow), a significant level was also seen in hepatocytes. Fluorescence intensity for FITC-LPS was higher in periportal hepatocytes (fluorescence intensity, Fl = 112) than in perivenular hepatocytes (Fl = 51).
Figure 2. Schematic picture of endotoxin metabolism in the liver. Endotoxin derived from the colon is taken up by Kupffer cells and hepatocytes that preferentially secrete endotoxin into bile.

PATHOGENESIS OF PRIMARY BILIARY CIRRHOSIS

a) Endotoxin localization in PBC liver

Although PBC is characterized by the destruction of intrahepatic bile ducts, neither pathogenesis nor primary events have been clarified. We have studied PBC livers immunohistochemically using an anti-LPS antibody (E5, Pfizer) to investigate the immunolocalization of endotoxin in the patient's liver, since as we described, large amount of endotoxin is secreted into bile, and PBC is characterized as chronic cholestasis.

By confocal laser scanning microscopy, immunofluorescence for LPS was clearly demonstrated in the biliary epithelial cells (BEC) in PBC. Some immunofluorescence was also observed in infiltrating cells around bile ducts as well as in hepatocytes. In the livers of patients with viral liver cirrhosis type C and the histologically normal livers, immunofluorescence was faint or absent in BEC. Fluorescence intensity in BEC was measured using image analyzer software built in the confocal laser scanning microscope. Fluorescence intensity in BEC was significantly higher in PBC compared with that in liver cirrhosis and normal control. Second liver biopsy was obtained in 10 PBC patients after at least 1 year treatment with ursodeoxycholic acid (UDCA). After UDCA treatment, immunofluorescent intensity for LPS in BEC was significantly reduced.

These results indicated that in PBC endotoxin is retained in BEC, facilitating BEC to express adhesion molecules, such as ICAM-1, on the plasma membrane. Furthermore, endotoxin could be released to the portal areas from BEC, leading to the infiltration of inflammatory cells around bile ducts.

b) Apoptosis of biliary epithelial cells in PBC [5]

To assess the involvement of apoptosis in the pathologic process of PBC, sections of PBC livers were processed by the in situ nick-end labeling method using "Apop Tag Plus" (Oncor, Gaithersburg, MD). In brief, after proteinase K digestion and inactivation of endogenous peroxidase, liver specimens were incubated in a solution containing terminal
deoxynucleotidyl transferase and digoxigenin-labeled deoxyuridine triphosphate and deoxyadenosine triphosphate. Then, the specimens were reacted with peroxidase-labeled anti-digoxigenin antibody. Color development was performed with 3-3’-diamino-benzidine-tetrahydrochloride solution and then the specimens were counterstained with methyl green. All BECs and at least 1000 hepatocytes were evaluated in each specimen for DNA fragmentation. Positive rates for DNA fragmentation in hepatocytes and BEC were calculated. Liver specimens of the second liver biopsy of 10 PBC patients, liver tissues of 17 chronic viral hepatitis C, and 16 histologically normal liver tissues (controls), were also analyzed.

In PBC, the frequency of positive cells with DNA fragmentation was significantly increased in BEC compared with that in normal controls. Regarding hepatocytes, the positive frequency for DNA fragmentation both in PBC and chronic viral hepatitis was significantly higher than that in the normal controls (Table 1). After treatment with UDCA for at least 18 months, the frequency of BEC positive for DNA fragmentation was significantly reduced in PBC.

These results indicate that apoptosis is involved in cellular injury of BEC as well as hepatocytes in PBC. Recently, toxic bile salts have been shown to induce apoptosis in cultured hepatocytes [6], suggesting that intracellular retention of the salts may contribute to hepatocellular apoptosis during cholestasis in PBC. Mechanisms of cholangiocyte apoptosis is not fully understood, however, recent in vitro studies have demonstrated Fas and cytokines, such as TNF-α and IFN-γ, could induce cholangiocyte apoptosis [7, 8]. Thus, agents which prevent the apoptotic events including the activation of caspase proteases, Fas signaling, and the mitochondria permeability transition may prove clinically useful. Treatment with UDCA, the most promising drug for PBC, may reduce apoptosis in the PBC liver, since UDCA can prevent the mitochondria permeability transition [9] and suppress IFN-γ-induced major histocompatibility complex class II gene expression [10].

c) Increased paracellular permeability of biliary epithelial cells in PBC.

Recently, many tight junction (TJ) proteins and TJ-associated proteins, such as occludin, ZO-1, ZO-2, cingulin, and 7H6 antigen, were identified and characterized. 7H6 antigen is a 155 kd TJ-associated protein. Immunofluorescence localization of 7H6 antigen was demonstrated to closely correlate with paracellular permeability [11, 12]. We have studied 10 PBC livers by immunofluorescence cytochemistry using an anti-7H6 antigen antibody and a laser scanning microscope.

In PBC livers, immunofluorescence for 7H6 antigen was demonstrated to be discontinuous in hepatocytes, and reduced or disappeared in BEC, while it was continuously localized at the bile canaliculi of hepatocytes as well as BEC in control livers.

| Table 1. Positivity for DNA Fragmentation. |
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| Frequencies of DNA-fragment-positive cells |
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Fluorescence intensity for 7H6 antigen of BEC in PBC was significantly reduced in PBC compared with that in controls. These results indicate the TJ in PBC are impaired both in BEC and hepatocytes, although BEC have been more seriously involved. Paracellular permeability of BEC is increased in PBC, leading to allow toxic bile acids or various antigens leak to the periductal area from the lumen of bile ducts. The spillover may promote infiltration of lymphocytes, macrophage, and plasma cells around bile ducts, and these cells can secrete various cytokines, including TNF-α, IFN-γ, IL-1, and IL-6. These cytokines further aggravate the bile duct injury in PBC.

Taken all together, these results suggest the following as speculative pathogenesis in PBC. Stimulated cytokine production by infiltrated lymphocytes and macrophages around the bile ducts may increase the apoptosis of BEC. Impaired BEC increase paracellular leakage of bile constituents containing toxic bile acids and endotoxin into the portal area. In addition, impaired BEC cannot excrete the intracellular endotoxin absorbed from bile, promoting the cellular expression of ICAM-1 and major histocompatibility complex class II, which further accelerates the infiltration of inflammatory cells which attack BEC (Figure 3).

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