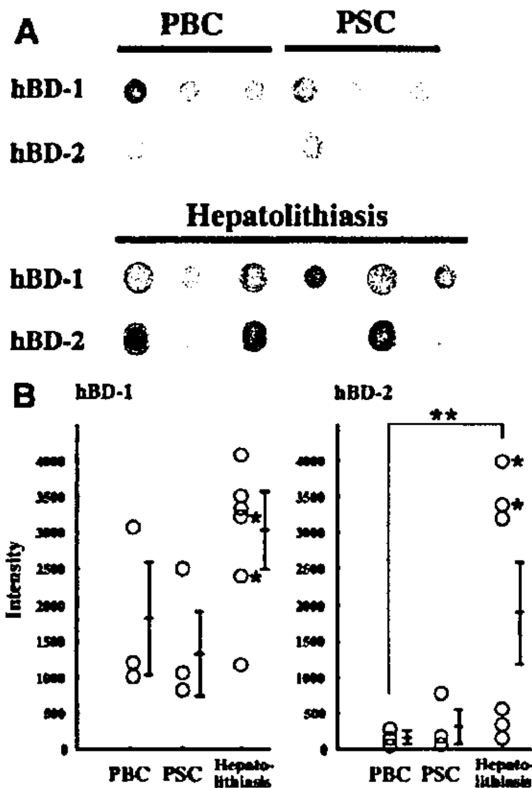

Fig. 4. Detection of human beta defensin (hBD)-1 and hBD-2 proteins in bile. (A) Dot bolt analysis revealed that hBD-1 is detectable in all bile samples, whereas hBD-2 is detected in 1 of 3 (33%) patients with primary biliary cirrhosis (PBC), 1 of 3 (33%) with primary sclerosing cholangitis (PSC), and each of 6 (100%) with hepatolithiasis. (B) Semiquantitative assessment of the intensity of immunoreactivity, using the National Institutes of Health Image Program, revealed that the average level of hBD-2 protein in hepatolithiasis is higher than that in PBC (**$P < .05$). **Bars** are SDs. **Open circles** indicate individual values. **Asterisk** is associated with cholestasis.

mRNA detected by real-time PCR. In addition, mononuclear cells positive for hBD-1 and hBD-2 mRNA were found in periductal zones.[30] These specific signals were not detectable when hybridized with the sense probe for these mRNA sequences.

Immunoblot Detection of hBD-1 and hBD-2 Proteins in Bile. hBD-1 was detectable to a variable extent in all bile samples; hBD-2 was detected in 1 of 3 bile samples from patients with PBC, 1 of 3 samples from patients with PSC, and all of 6 samples from patients with hepatolithiasis (Fig. 4A). Semiquantitative assessment of the intensity of the immunoreactivity revealed that 3 bile samples from patients with hepatolithiasis exhibited particularly high levels of hBD-2; the average level of hBD-2 protein in patients with hepatolithiasis was higher than that in patients with PBC (<0.05) (Fig. 4B). Three patients with hepatolithiasis and high hBD-2 levels were also positive for hBD-2 mRNA and hBD-2 protein in affected bile ducts and had high serum CRP levels (9.6, 7.0, and 5.3 mg/dL); the serum level of CRP in the remaining 3 patients with hepatolithiasis was not as high (1.2, 2.3, and 4.6 mg/dL).

Expression of hBDs in cultured cells. Amplification of hBD-1 mRNA occurred in all of the 5 cell lines that were cultured for 3 hours in the absence of a stimulant (medium only). Such expression was not affected by treatment with LPS or *E. coli* (Supplementary Fig. 3). In contrast, amplification of hBD-2 mRNA was not detected in any of the cell lines in the absence of a stimulant; *de novo* expression of hBD-2 occurred in all cell lines, except HepG2, after treatment with LPS or *E. coli* for 3 hrs (Supplementary Fig. 3). Real-time PCR revealed that after treatment of HuCCT1, CCKS1, hBEC1, and hBEC2 with LPS or *E. coli*, no significant increase of hBD-1 mRNA occurred, but compared to levels in unstimulated cells, there was appreciable upregulation of hBD-2 mRNA (Supplementary Fig. 3). In addition, immunoblotting for hBDs demonstrated hBD-1 protein and LPS-induced hBD-2 protein in supernatants of HuCCT1 and hBEC1 cells that had been cultured for 12 hours (Supplementary Fig. 3).

Amplification of hBD-1 mRNA occurred in 2 ICC cells, 2 hBECs, and HepG2 cells after culture for 3 hours; this expression was not affected by treatment with any cytokines of the stimulants (Supplementary Fig. 4). This finding was also demonstrated using real-time PCR (Supplementary Fig. 4). In contrast, amplification of hBD-2 mRNA occurred in 2 ICC cells and 2 hBECs after treatment with IL-1β or TNF-α for 3 hours, but not after treatment with PBS or any of the other 4 cytokines for 3 hours. hBD-2 amplification did not occur in HepG2 cells after any treatment. Real-time PCR showed that treatment with IL-1β or TNF-α for 3 hours significantly upregulated hBD-2 mRNA—approximately 32-fold and 17-fold, respectively (Supplementary Fig. 4). However, the other 4 cytokines did not affect expression of hBD-2.

Discussion

In this study, we have shown that hBD-1 protein is nonspecifically expressed in hepatocytes and BECs of intrahepatic bile ducts in all of the specimens of liver tissue that we examined. hBD-1 mRNA was also nonspecifically expressed in hepatocytes and BECs. hBD-1 mRNA and protein had a similar distribution within livers. These findings and the consistent detection of hBD-1 in bile in various diseases strongly suggest that hBD-1 plays a general antimicrobial role in the defense of the hepatobiliary system, similar to that documented in other epithelial organs.[11,15-18] Expression of hBD-1 in bile ducts may

contribute to the rarity of biliary tract infections. Bile is sterile under physiological conditions. However, the biliary tree is potentially exposed to enteric bacteria.

In contrast, expression of hBD-2 was restricted to large intrahepatic bile ducts and peribiliary glands when it was detectable; hepatocytes did not express hBD-2. hBD-2 expression was particularly frequent in bile ducts exhibiting features of cholangitis in patients with EBO and hepatolithiasis; such expression was infrequent in patients with PBC and PSC and was absent in liver from patients with CH-C and normal liver histology. In EBO and hepatolithiasis, enteric bacteria can usually be cultured from bile,[4,8,31] suggesting that bacteria-induced cholangitis may contribute to biliary epithelial expression of hBD-2. In patients with biliary epithelial expression of hBD-2, serum levels of CRP are higher than in patients with low expression of hBD-2; high serum CRP levels in this context may reflect active cholangitis involving large intrahepatic bile ducts.

Whether a systemic factor is involved in the expression of hBD-2 in intrahepatic bile ducts is an important issue. Absence of BEC expression of hBD-2 in the liver of patients with extrahepatic sepsis suggests that a systemic factor is unlikely to be involved in BEC expression of hBD-2 in chronic cholangitis. Furthermore, the absence of expression of hBD-2 in the surface epithelium of gallbladders from 3 patients with hepatolithiasis, in whom hBD-2 was expressed in stone-containing intrahepatic bile ducts, suggests that factors involved in hBD-2 expression in such patients are restricted to the affected bile ducts. Three patients with hepatolithiasis, high hBD-2 levels in bile, and high serum CRP levels were positive for hBD-2 mRNA and protein in the liver; 2 exhibited hBD-2 mRNA in bile ducts, suggesting that hBD-2 expressed in BECs of actively inflamed bile ducts might have been secreted into bile. hBD-2 is a secretory peptide antibiotic.[11] hBD-1 and hBD-2 were secreted by cultured HuCCT1 and hBEC1 after treatment with LPS. These findings suggest that in cholangitis associated with hepatolithiasis or EBO, biliary epithelial hBD-2 participates in the local defense of biliary tree against bacterial infection. This suggestion is supported by the finding that hBD-2 was predominantly expressed in segments of large bile ducts containing a polymorphonuclear inflammatory infiltrate but not in segments in which inflammation was minimal or absent. hBD-2 may act synergistically with other microbicidal molecules present in bile, such as hBD-1, lactoferrin, and lysozyme.[9,19]

There have been several reports, including our own,[2,4,31,32] that bacteria and their products, such as LPS, are consistently demonstrable in bile in certain pathological states, particularly in hepatolithiasis and EBO. Accordingly, we examined the influence of local factors, such as bacteria and LPS, on the synthesis and expression of hBD-1 and hBD-2 by cultured human BECs. While spontaneous hBD-1 mRNA expression was consistently detected in the cultured ICCs, hBECs and HepG2 cells, hBD-2 mRNA expression was detected only in cultured ICCs and hBECs after stimulation—*e.g.*, by LPS or *E. coli*. These findings suggest that enteric bacteria and their products may be local factors involved in the expression of hBD-2 of BECs *in situ*. We have recently reported that murine BECs possess Toll-like receptors, which recognize pathogen-associated molecular patterns, such as LPS. Thus, BECs may respond directly to bacterial components.[31] Interestingly, no expression of hBD-2 mRNA by HepG2 cells was detectable after treatment with LPS, *E. coli*, or inflammatory cytokines; these cells did consistently express hBD-1 mRNA.

hBD-2 mRNA expression was induced by treatment of 2 ICC and 2 hBEC lines with IL-1β or TNF-α; such expression was not induced by IL-4, IL-5, IL-6, or IFN-γ. However, hBD-1 mRNA was spontaneously expressed by all of the cultured BECs and HepG2 cells. These findings imply that different mechanisms are involved in the synthesis of hBD-1 and hBD-2 in BECs. They also suggest different roles for these peptides in biliary pathophysiology. In addition to BEC lines, hBD-2 is also induced by IL-1β and LPS in gingival keratinocytes and bronchial epithelial cells.[16,22,33] Moreover, astrocytes and skin keratinocytes have been reported to produce hBD-2 after exposure to TNF-α..[20,34] These findings suggest that hBD-1 is a normal constituent of BECs that plays a role in the antimicrobial defense of bile ducts. In contrast, hBD-2 expression occurs in response to local infection and/or active inflammation; such phenomena are associated with local release of TNF-α and IL-1β. When expressed hBD-2 may also play a role in the antimicrobial defense of bile ducts.

In hepatolithiasis, bacterial infection of the intrahepatic biliary tree, particularly by enteric bacteria, seems to be a common event and is associated with the development of chronic proliferative cholangitis and expression of aberrant mucin.[35] Our results have shown that, in this disease, hBD-1 and hBD-2 were expressed on large intrahepatic bile ducts and proliferated peribiliary glands and suggest that they may mediate antimicrobial mechanisms in the biliary tree. However, that hepatolithiasis may become an intractable disease, if surgical or endoscopic treatment is unsuccessful, suggests that expression of hBDs on bile ducts alone is insufficient to completely eliminate bacteria from bile. Both hBD-1 and hBD-2 are salt-sensitive antimicrobial peptides; their actions have been reported to be compromised by salt-dependent inactivation.[19,21] Such inactivation may occur in the bile

ducts of patients with cystic fibrosis and may consequently predispose to chronic biliary infection and hepatolithiasis.

In conclusion, hBD-1 appears to be present in BECs under physiological conditions and to constitute a component of the antimicrobial defense of the hepatobiliary system. In active cholangitis due to bacterial infection, as may occur in association with hepatolithiasis, hBD-2 may be induced in large intrahepatic bile ducts, and when induced it may also participate in the antimicrobial defense of the hepatobiliary system. Further analyses of the bactericidal roles of hBD-1 and hBD-2 may provide support for the concept of using hBDs as "peptide antibiotics" to enhance biliary innate immunity in the treatment of infections of the biliary tract.

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Fractalkine and CX3CR1 Are Involved in the Recruitment of Intraepithelial Lymphocytes of Intrahepatic Bile Ducts

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Fractalkine is a chemokine with both chemoattractant and cell-adhesive functions, and in the intestine it is involved with its receptor CX3CR1 in the chemoattraction and recruitment of intraepithelial lymphocytes. We examined the pathophysiological roles of fractalkine and CX3CR1 in normal and diseased bile ducts. Expression of fractalkine and CX3CR1 were examined in liver tissues from patients with primary biliary cirrhosis (17 cases) and controls (9 cases of primary sclerosing cholangitis, 10 cases of extrahepatic biliary obstruction, 20 cases of chronic viral hepatitis C, and 18 cases of histologically normal livers). Expression of fractalkine in biliary epithelial cells (BECs) in response to cytokine treatments was examined using a human cholangiocarcinoma cell line (HuCC-T1) and human intrahepatic BEC line. The chemotaxis of CX3CR1-expressing monocytes (THP-1) toward fractalkine was assayed using chemotaxis chambers. Fractalkine messenger RNA/protein were expressed on BECs of normal and diseased bile ducts, and their expression was upregulated in injured bile ducts of primary biliary cirrhosis. CX3CR1 was expressed on infiltrating mononuclear cells in portal tracts and on $CD3^+$, $CD4^+$, and $CD8^+$ intraepithelial lymphocytes of injured bile ducts in primary biliary cirrhosis. Fractalkine messenger RNA expression was upregulated in two cultured BECs on treatment with lipopolysaccharide and Th1-cytokines (interleukin 1β, interferon gamma, and tumor necrosis factor α). THP-1 cells showed chemotaxis toward fractalkine secreted by cultured cells. In conclusion, Th1-cytokine predominance and lipopolysaccharide in the microenvironment of injured bile ducts resulting from primary biliary cirrhosis induce the upregulation of fractalkine expression in BECs, followed by the chemoattraction of CX3CR1-expressing mononuclear cells, including $CD4^+$ and $CD8^+$ T cells, and their adhesion to BECs and the accumulation of biliary intraepithelial lymphocytes. (HEPATOLOGY 2005;41:506-516.)

Recent studies showed that a newly identified CX3C-chemokine (CX3CL1), fractalkine, plays an important role in the migration of leukocytes to target sites under physiological as well as pathological conditions.[1-5] Unlike other chemokines, fractalkine is expressed as a membrane-bound form on cells and also can be shed as a soluble chemotactic form. The latter is known to be a potent chemoattractant for $CD8^+$ and $CD4^+$ T cells, natural killer cells, and monocytes expressing its receptor (CX3CR1), whereas the former promotes strong adhesion of these leukocytes in an integrin-independent manner.[6,7] Muehlhoefer et al.[8] reported fractalkine protein and messenger RNA (mRNA) expression in epithelial cells and endothelial cells in small intestine under normal conditions, and their upregulation during inflammatory bowel disease.[8] They also disclosed that fractalkine expressed on the intestinal epithelium may regulate a subpopulation of $CD8^+$ T cells in the epithelial layer (intestinal intraepithelial lymphocytes [IELs]).[8]

Biliary epithelial cells (BECs) are known as immunologically potent cells, and BECs of inflamed bile ducts actively participate in inflammation by secreting cytokines and expressing immune receptors.[9,10] Chemokines also are expressed on pathological bile ducts.[10,11] For ex-

Abbreviations: mRNA, messenger RNA; IEL, intraepithelial lymphocyte; BECs, biliary epithelial cells; PBC, primary biliary cirrhosis; CVH, chronic viral hepatitis; CVH-C, hepatitis C virus–related chronic viral hepatitis; PSC, primary sclerosing cholangitis; EBO, extrahepatic biliary obstruction; IgG, immunoglobulin G; RT-PCR, reverse-transcriptase polymerase chain reaction; HIBEC, human intrahepatic biliary epithelial cell; LPS, lipopolysaccharide; IL, interleukin; IFN-γ, interferon γ; TNF-α, tumor necrosis factor α; PBS, phosphate-buffered saline; PDC-E2, pyruvate dehydrogenase E2 component.

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ample, Terada et al.[12] reported that stromal-derived factor 1 expressed on bile ducts is responsible for the migration and recruitment of lymphoid cells in portal tracts and around bile ducts in primary biliary cirrhosis (PBC) as well as in chronic viral hepatitis (CVH). Although the upregulation of fractalkine and CX3CR1 expression has been reported in hepatic parenchyma and bile ductules of hepatitis C virus–infected livers,[13] their expression and immunopathological significance remain unexplored in normal and pathological bile ducts.

Our recent study showed that biliary IELs are present at intrahepatic bile ducts under physiological conditions and mainly belong to $CD8^+$ T cells, as seen in the intestine. Interestingly, numbers of $CD4^+$, $CD8^+$, and $CD20^+$ biliary IELs were increased significantly at injured bile ducts of patients with PBC (K. Isse et al., submitted for publication), and this characterizes chronic nonsuppurative destructive cholangitis.[14-19] However, it is not clear how biliary IELs are recruited and regulated in the bile ducts.

In this study, we examined the expression of fractalkine and CX3CR1 in portal tracts, with an emphasis on small bile ducts and recruitment of biliary IELs, using human liver tissue specimens and cultured BECs.

Materials and Methods

Liver Tissue Specimens and Serum

Wedge-biopsied or surgically resected liver specimens fixed in 10% formalin and embedded in paraffin were obtained from 17 patients with PBC, 9 patients with primary sclerosing cholangitis (PSC), 10 patients with extrahepatic biliary obstruction (EBO) of short duration, 20 patients with hepatitis C virus–related CVH (CVH-C), and 18 patients with histologically normal livers (Table 1). The diagnosis in each case was made based on a combination of clinicolaboratory and histopathological findings. PBC was staged histologically,[20] and the grading and staging of CVH-C were carried out according to international criteria.[21] Samples obtained by wedged liver biopsies in cases of uncertain diagnosis and nonneoplastic parts obtained from surgically resected livers for liver tumor(s) or for traumatic liver rupture were used. Three-micrometer–thick sections were cut from each paraffin block. Several were processed routinely; the remainder were processed for immunohistochemistry.

Fresh tissue samples from seven livers (two PBC cases, three CVH-C cases, and two normal livers) also were available. They were embedded in optimum cutting temperature compound (Miles Inc., Elkhart, IN) and 5-μm–thick frozen sections were prepared for microdissection and *in situ* hybridization examination.

Table 1. Liver Tissue Specimens and Sera From the Patients Examined

Diseases	Patient No.	Average Age (yr)	Sex	
			Male	Female
Liver tissue specimens				
PBC (stage 1/2)	17	61.7	2	15
PSC	9	62.2	7	2
EBO	10	73.6	5	5
CVH-C	20	63.8	12	8
Normal liver	18	59.2	8	10
Sera				
PBC (stage 1/2/3/4 = 9/5/4/2)	20	55.2	7	13
EBO	20	66.8	10	10
CVH-C (stage F0/1/2/3 = 2/5/4/5)	16	66.8	9	7
Normal liver	12	59.2	6	6

Abbreviations: PBC, primary biliary cirrhosis; PSC, primary sclerosing cholangitis; EBO, extrahepatic biliary obstruction; CVH-C, HCV-related chronic viral hepatitis.

Both the formalin-fixed and fresh liver specimens were obtained from the liver disease files of our department.

The intrahepatic biliary tree is classified into intrahepatic large and small bile ducts and bile ductules.[15,22] The large bile ducts are characterized by the presence of accompanying peribiliary glands and roughly correspond to the first to fourth branches of the right and left hepatic ducts. The small bile ducts are classified into septal and interlobular bile ducts according to their size and location within portal tracts. In this study, mainly the small bile ducts were examined.

Serum samples were obtained from 20 PBC patients, 20 EBO patients, including 10 with extrahepatic biliary carcinoma, 16 CVH-C patients, and 12 healthy volunteers (Table 1) and were used for the measurement of soluble fractalkine by an enzyme-linked immunosorbent assay kit (R&D Systems, Minneapolis, MN). Informed consent was obtained from all the subjects.

Immunohistochemistry for Fractalkine and CX3CR1

Deparaffinized sections were incubated with rabbit polyclonal antibodies against fractalkine (2 μg/mL; IBL, Fujioka, Japan) or CX3CR1 (5 μg/mL; IBL). The sections then were treated with goat antirabbit immunoglobulins conjugated with peroxidase-labeled dextran polymer (Envision$^+$; DAKO, Tokyo, Japan). After a benzidine reaction, the sections were counterstained lightly with hematoxylin. As negative controls, nonimmunized rabbit IgG was used as primary antibody. Evaluation of immunostaining (positive or negative, and scoring of staining intensity) always was performed blindly and was discussed by two pathologists (K.I. and Y.N.).

Expression of Fractalkine in Bile Ducts. Seventy-five small bile ducts from normal livers, 50 from EBO livers, 100 from CVH-C livers, 45 from PSC livers, and

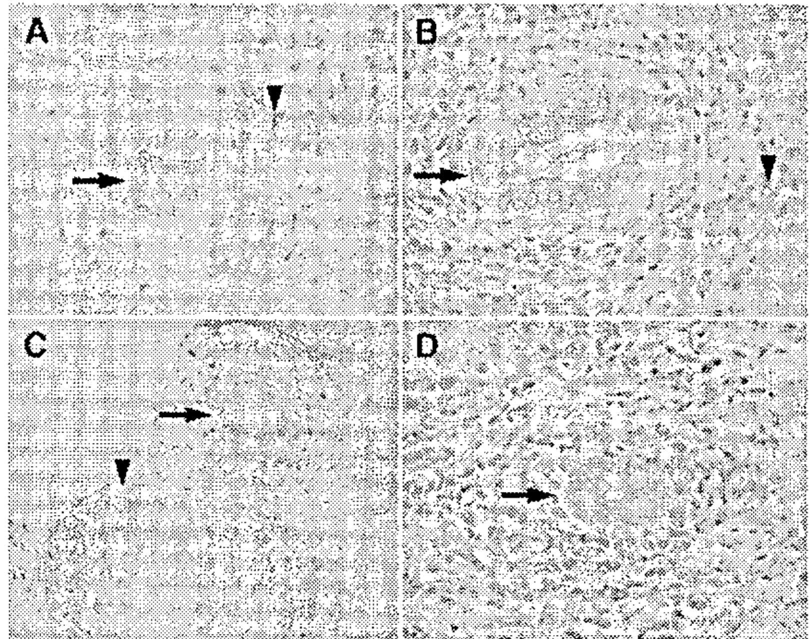

Fig 1. Immunohistochemistry for fractalkine and CX3CR1 in the liver tissue. (A) Normal liver. Fractalkine is expressed in endothelial cells of small vessels (**arrowhead**), whereas small bile duct is faintly positive (**arrow**). Hepatocytes are negative for fractalkine. (B) Primary biliary cirrhosis. Fractalkine is evidently expressed in pathological bile ducts (**arrow**). Small vessels in portal tract also express fractalkine (**arrowhead**). (C) Normal liver. CX3CR1 expression is faint in the normal small bile duct (**arrow**) and weak in the smooth muscle of hepatic artery (**arrowhead**). (D) Primary biliary cirrhosis. CX3CR1 is evidently expressed in injured bile ducts in the membranous and cytoplasmic parts (**arrow**). CX3CR1-positive mononuclear cells are accumulated around the damaged bile ducts and portal area. (A-D) Visualized by benzidine reaction and counterstained by hematoxylin (original magnification, ×200).

85 from PBC livers were chosen for evaluation in each case. In comparison with the intensity in endothelial cells (strongly positive) and parenchymal hepatocytes (negative), fractalkine expression in bile ducts was evaluated as either absent/faint (±), slightly positive (+), or strongly positive (++; Fig. 1A-B).

CX3CR1-Positive Mononuclear Cells. With the National Institutes of Health (Bethesda, MD) image program, CX3CR1-positive mononuclear cells were counted in portal tracts excluding the portal vein, bile ducts, and arteries, and their number per area (mm^2) was calculated.

Expression of CX3CR1 in Bile Ducts. The immunohistochemical expression of CX3CR1 in small bile ducts was classified as negative (no or faint staining) or positive (Fig. 1C-D). Furthermore, to confirm the intracellular distribution of CX3CR1, alkaline phosphatase-labeled Envision (DAKO) with Fast Red (Vector Laboratories, Burlingame, CA) was used and the samples were viewed under a confocal laser scanning microscope (Zeiss LSM5 Pascal; Carl Zeiss Japan, Tokyo, Japan).

Double Immunostaining of CX3CR1/CD3, CD4, or CD8. To characterize infiltrating lymphocytes, double-immunostaining for CX3CR1 and either CD3, CD4, or CD8 was performed in normal and diseased livers. Sections were incubated with polyclonal rabbit anti-CX3CR1 antibody and monoclonal mouse anti-CD3 antibody (clone PS1, neat; Nichirei, Tokyo, Japan), anti-CD4 antibody (clone 1F6, neat; Nichirei), or anti-CD8 antibody (clone C8/44B, ×200; DAKO). After treatment with Alexa Fluor 488 goat antirabbit IgG and Alexa Fluor 594 goat antimouse IgG (10 μg/mL; Molecular Probes, Eugene, OR), fluorescence was observed under a fluorescent microscope.

In Situ *Hybridization Study*

Polymerase chain reaction (PCR)-amplified products containing the human fractalkine mRNA sequence and T7-RNA and Sp6-RNA polymerase promoters were obtained. Then, single-stranded RNA probes complementary (antisense) and anticomplementary (sense probe) to the corresponding gene transcripts were obtained using *in vitro* transcription according to the standard protocol of the digoxigenin RNA Transcription Kit (Roche, Indianapolis, IN). Paraformaldehyde-fixed frozen sections (from one normal and two PBC livers) were incubated with hybridization solution (DAKO) mixed with digoxigenin-labeled fractalkine antisense and sense probes at 50°C for 16 hours. Slides were washed finally in 0.2× sodium chloride–sodium citrate. After blocking reagent (Roche) was applied, the sections were incubated with alkaline phosphatase–conjugated antidigoxigenin antibody (Roche) for 1 hour. Color development was performed with a nitroblue tetrazolium salt/5-bromo-4-chloro-3-indolyl phosphate toluidine salt solution (Roche) for 3 to 6 hours in a dark room.

Microdissection and Reverse-Transcriptase PCR for Fractalkine mRNA

Frozen sections from one CVH-C and four PBC livers in which bile ducts were identifiable were fixed gently in 75% ethanol and then stained with hematoxylin–eosin.

Table 2. Primers for Human Fractalkine, CX3CR1, and CD45 mRNA

Gene	Primer Sequences	PCR Product	Annealing
Fractalkine	Forward 5'-GATGGCTCCGATATCTCTG-3' Reverse 5'-CTGCTGCATCGCGTCCTTG-3'	262 bp	60°C
CX3CR1	Forward 5'-CGTCCAGACCTTGTTCACAC-3' Reverse 5'-CTGGCAAGTAAATTGGGGTACA-3'	296 bp	55°C
CD45	Forward 5'-CAAAGGAACAGGCTGAAGGT-3' Reverse 5'-CAAATATTGGTTCGCTGCATT-3'	220 bp	55°C
β-actin	Forward 5'-CAAGAGATGGCCACGGCTGCT-3' Reverse 5'-TCCTTCTGCATCCTGTCGGCA-3'	275 bp	60°C

Abbreviation: bp, base pairs.

An Arcturus laser capture microscope (Laser Capture Microdissection System PixCell II; Arcturus, Mountain View, CA) was used to microdissect BECs. Briefly, intrahepatic bile ducts were identified, and approximately 500 BECs from several bile ducts in each case were microdissected and captured on a microcentrifuge cap. Total RNA was extracted from these microdissected materials using a PicoPure RNA Isolation kit (Arcturus).

For reverse-transcriptase PCR (RT-PCR), total RNA, RTase (ReverTra Ace; Toyobo, Tokyo, Japan), and random primers were used. The nucleotide sequences for fractalkine, CX3CR1, and CD45 were analyzed and primers were generated. RT-PCR for CD45 (leukocyte common antigen) was performed to check for possible contamination by leukocytes of the microdissected bile duct samples. PCR conditions were denaturation at 94°C for 1 minute, annealing for 1 minute, and elongation at 72°C for 2 minutes. The primer sequences and annealing temperature are shown in Table 2.

Cell Culture

One human intrahepatic cholangiocarcinoma cell line, HuCC-T1[23] (JCRB0425; Health Science Research Resources Bank, Osaka, Japan), and one nonneoplastic human intrahepatic BEC cell line (HIBEC), primary culture, originating from an explanted liver with hepatitis C virus–related cirrhosis[24] were used. These cells grew on culture dishes with standard medium, RPMI 1640 medium (Gibco, Rockville, MD) with 10% fetal bovine serum for HuCC-T1, and DMEM/F12 (Gibco) with 10% fetal calf serum for HIBEC. They were cultured at 37°C in 95% air and 5% CO_2.

Three-Dimensional Culture of HuCC-T1 Within Collagen Gel. As described in a previous study,[25] HuCC-T1 cells were embedded in collagen gel (Nitta Gelatin, Osaka, Japan) and cultured. Three days later they were fixed in formalin and embedded in paraffin. The expression of fractalkine and CX3CR1 was examined immunohistochemically (see Immunohistochemistry for Fractalkine and CX3CR1).

Treatment of Cultured Cells With Lipopolysaccharide and Inflammatory Cytokines

Lipopolysaccharide (LPS) and interleukin (IL)-1β, IL-4, IL-6, interferon gamma (IFN-γ), and tumor necrosis factor α (TNF-α) were purchased from PeproTech (Rocky Hill, NJ). Both HuCC-T1 and HIBEC possess receptors for LPS (Toll-like receptor 4) and for these cytokines (K. Harada et al., submitted for publication). When cultured cells reached a semiconfluent state, the medium was replaced with fresh medium and LPS 1 μg/mL or either of the cytokines at 1,000 U/mL was added for a 3-hour culture.

RT-PCR for Fractalkine mRNA Expression

Total RNA was isolated from cultured HuCC-T1 and HIBEC cells with the RNeasy Total RNA System (Qiagen, Hilden, Germany) following the manufacturer's directions and was used for RT-PCR. For quantitative analysis, the real-time PCR assay was performed with by SYBER Green PCR Master Mix and an ABI PRISM 7700 Sequence Detection System (both from Applied Biosystems, Tokyo, Japan). The primers for fractalkine (forward, 5'-CTGCTGCCCTAACTCGAAAT-3'; reverse, 5'-AGGACCACAGACTCGTCCAT-3') and glyceraldehyde 3-phosphate dehydrogenase (forward, 5'-GGCCTCCAAGGAGTAAGACC-3'; reverse, 5'-AGGGGTCTACATGGCAACTG-3') were set according to the instruction. Data were assessed as the relative fold compared with unstimulated cells (phosphate-buffered saline [PBS]).

Detection of Soluble and Cell-Bound Fractalkine in HuCC-T1 Cells

HuCC-T1 was cultured for 3 days after stimulation with PBS or either LPS, IL-1β, IFN-γ, or TNF-α. Soluble fractalkine in the supernatant and cell-bound fractalkine in the cell lysate from HuCC-T1 were detected by enzyme-linked immunosorbent assay and Western blotting, respectively. Western blotting was performed with the same antibody used in the immunohistochemistry.

In Vitro Chemotaxis Assay of Fractalkine

The chemoattractant activity of fractalkine secreted by the HuCC-T1 cells stimulated with LPS was assessed in 24-well plates assembled with Chemotaxicell (8-μm pore size; Kurabou, Osaka, Japan)[26] and THP-1[27] (RCB1189; RIKEN, Tokyo, Japan), which are monocytes expressing CX3CR1 and showing efficient chemotaxis and adherence in a fractalkine-dependent manner.[28,29] Briefly, HuCC-T1 was cultured with LPS (1 μg/mL) for 3 days. As described in Results, soluble fractalkine was detectable by enzyme-linked immunosorbent assay in the supernatant of subconfluent HuCC-T1 after LPS treatment. After assembly of the Chemotaxicell, a THP-1 suspension (2×10^5 cells/100 μL) was seeded and incubated for 90 minutes. The migrated THP-1 cells were counted in 10 randomly chosen high-power fields (×400) under a microscope, according to Vitale et al.[30] To evaluate further the chemotactic processes, THP-1 cells were preincubated with human recombinant fractalkine (1 μg/mL; Chemicon, Temecula, CA) comprising only the chemokine domain,[31] which is expected to bind CX3CR1 expressed on THP-1 cells or sufficient anti-CX3CR1 antibody (10 μg/mL; IBL) in the culture for 15 minutes, which is expected to block CX3CR1 on THP-1. Normal rabbit IgG (10 μg/mL; Santa Cruz Biotechnology, Santa Cruz, CA) was used as a negative control.

Statistical Analysis

Data were analyzed using the Kruskal-Wallis test and Spearman's correlation coefficient. P values less than .05 were considered statistically significant.

Results

Fractalkine Expression in Liver Tissue

Immunohistochemistry. In normal livers, small bile ducts were negative or slightly positive for fractalkine (Fig. 1A). Some infiltrating mononuclear cells, endothelial cells of small vessels, smooth muscle of arteries, and periportal hepatocytes were negative or faintly positive. In EBO, CVH-C, and PSC livers, small bile ducts and proliferated bile ductules were negative or slightly positive for fractalkine. In PBC livers, expression of fractalkine in BECs was rather strong in approximately two thirds of small bile ducts, particularly injured bile ducts (Fig. 1B), whereas the remaining one third were negative or slightly positive. Endothelial cells around injured bile ducts also were positive.

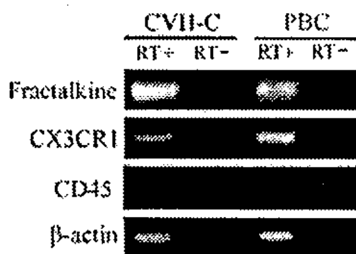

Fig 2. Fractalkine and CX3CR1 messenger RNA (mRNA) in microdissected small bile ducts by Laser Capture Microdissection System PixCell II. By reverse-transcriptase polymerase chain reaction, fractalkine and CX3CR1 mRNA were amplified at the predicted size (263 bp and 296 bp, respectively) from RNA samples of microdissected bile ducts in chronic viral hepatitis C (CVH-C) and primary biliary cirrhosis (PBC). β-actin (275 bp) was used as an internal control. CD45 (leukocyte common antigen, 220 bp) is not amplified. RT+, addition of reverse transcriptase for reverse transcription; RT−, addition of distilled water for reverse transcription.

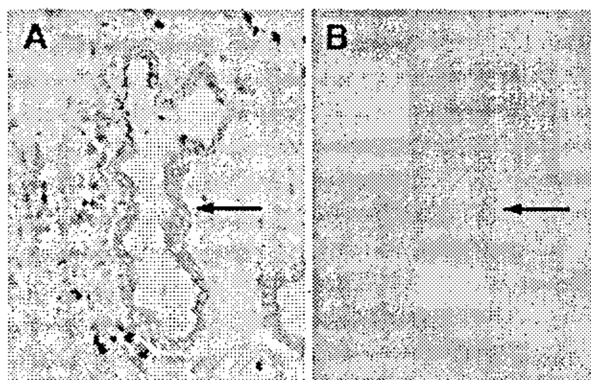

Fig 3. (A) *In situ* hybridization shows fractalkine messenger RNA in the cytoplasm of bile duct (**arrow**) of primary biliary cirrhosis. Positive signals also are detected around the bile duct, corresponding to infiltrating mononuclear cells and endothelium of small vessels. (B) These signals disappear in the bile duct (**arrow**) and also in the periductal nonbiliary cells when the slide is hybridized with a sense probe. Panels A and B are serial sections from the same area of primary biliary cirrhosis (original magnification, ×200).

RT-PCR for Fractalkine mRNA in Microdissected Samples. Fractalkine mRNA was detected by RT-PCR in microdissected samples of small bile ducts of CVH-C and PBC in which CD45 amplification was not evident (Fig. 2), suggesting that fractalkine mRNA was present in BECs.

In Situ *Hybridization for Fractalkine mRNA.* Signals for fractalkine mRNA in small bile ducts were weak in normal liver and strong in PBC liver (Fig. 3A). Fractalkine mRNA also was detectable in smooth muscle, endothelial cells, and several mononuclear cells, including fibroblasts in portal tracts and fibrous septa. The distribution of cells positive for fractalkine mRNA was similar to that of expression of fractalkine protein. No signals were detected in these cells using sense probes as a negative control (Fig. 3B).

Serum Level of Soluble Fractalkine

As shown in Fig. 4A, the soluble fractalkine level was significantly elevated in the sera of PBC patients compared with healthy volunteers, CVH-C patients, or EBO

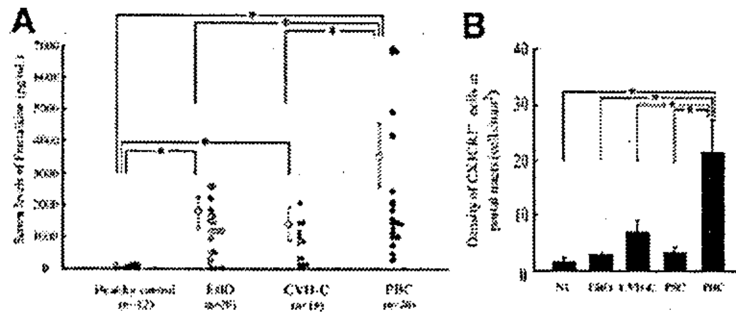

Fig 4. (A) Serum level of soluble fractalkine measured by enzyme-linked immunosorbent assay is higher in primary biliary cirrhosis (PBC; 3,736 ± 838 pg/mL, mean ± SE) than in healthy subjects (32 ± 6.8 pg/mL), chronic viral hepatitis C (CVH-C; 1,622 ± 417 pg/mL), and extrahepatic biliary obstruction (EBO; 1,910 ± 425 pg/mL; *$P < .05$, Kruskal-Wallis test). Serum value of soluble fractalkine is higher in EBO and CVH-C livers than in those of healthy volunteers (*$P < .05$, Kruskal-Wallis test). Numbers of patients with each disease examined are shown in parentheses. (B) Density of CX3CR1-positive mononuclear cells in an area of the portal tract excluding the portal vein, bile ducts, and arteries. The number of CX3CR1-expressing mononuclear cells in the portal tract is 1.6 ± 1.5/mm² (mean ± SE) in normal liver (NL), 2.8 ± 1.0/mm² in livers with extrahepatic biliary obstruction (EBO), 7.0 ± 2.5/mm² in livers with chronic viral hepatitis C (CVH-C), 3.3 ± 1.6/mm² in livers with primary sclerosing cholangitis (PSC), and 21.5 ± 4.2/mm² in livers with primary biliary cirrhosis (PBC). CX3CR1-positive cell numbers are increased in PBC compared with other diseases (*$P < .01$, Kruskal-Wallis test).

patients. The serum level was higher in CVH-C and EBO patients than in healthy volunteers.

CX3CR1 Expression in Liver Tissue

Immunohistochemistry. Although CX3CR1-positive mononuclear cells were very few in portal tracts of normal livers and infiltrated variably in portal tracts and fibrous septa in EBO, CVH-C, and PSC livers, they were significantly increased in PBC livers ($P < .05$; Fig. 4B). CX3CR1 also was expressed in BEC (Fig. 1C-D) and in arterial and venous branches of portal tracts. Small bile ducts expressing CX3CR1 were more frequent in PBC livers (77% of 85 bile ducts) than in the others, except for PSC livers (24% of 75 bile ducts in normal livers, 44% of 50 bile ducts in EBO livers, 31% of 45 bile ducts in CVH-C livers, and 51% of 45 bile ducts in PSC livers; $P < .05$, Kruskal-Wallis test).

Confocal laser microscopy showed that CX3CR1 was expressed in the membranous parts (basolateral and luminal) of bile ducts and also in their perinuclear cytoplasm (Fig. 5A). In addition, endothelial cells of small arteries and fibroblast-like spindle cells[32] in portal tracts were positive for CX3CR1 (Fig. 5B).

RT-PCR for CX3CR1 mRNA in Microdissected Samples. CX3CR1 mRNA was detected by RT-PCR in the microdissected samples of small bile ducts of CVH-C and PBC livers in which CD45 amplification was not evident (Fig. 2), suggesting that CX3CR1 mRNA was present in BECs.

Double Immunostaining of CX3CR1 and CD3, CD4, or CD8. CD3$^+$ T cells (Fig. 6A, red) mainly were present in portal tracts and fibrous septa. In PBC livers, CX3CR1 was shown evidently in the cytoplasm and occasionally in the membranous parts, whereas in other dis-

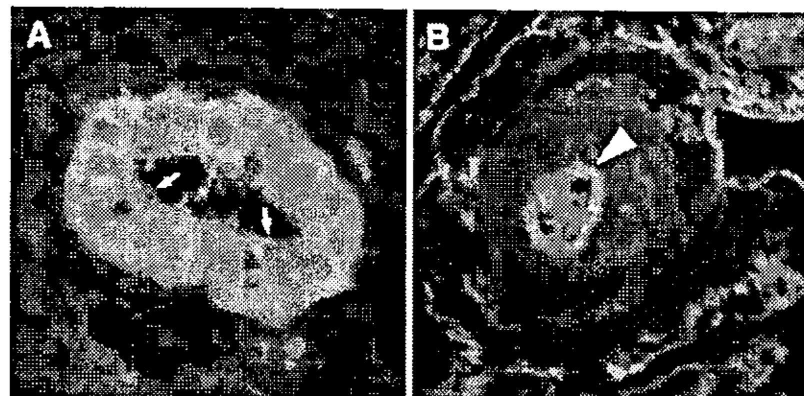

Fig 5. Immunofluorescent staining for CX3CR1 in the liver of primary biliary cirrhosis using the Envision-AP method with Fast Red (Envision+; DAKO, Tokyo, Japan) and viewed under a confocal laser microscope. (A) In one small bile duct, membranous staining of CX3CR1 (**arrows**) and perinuclear cytoplasmic staining are evident (original magnification, ×400). (B) Endothelium of the hepatic artery (**arrowhead**) and that of portal veins is strongly positive for CX3CR1. Such staining is not evident in the smooth muscles of the artery (original magnification, ×630).

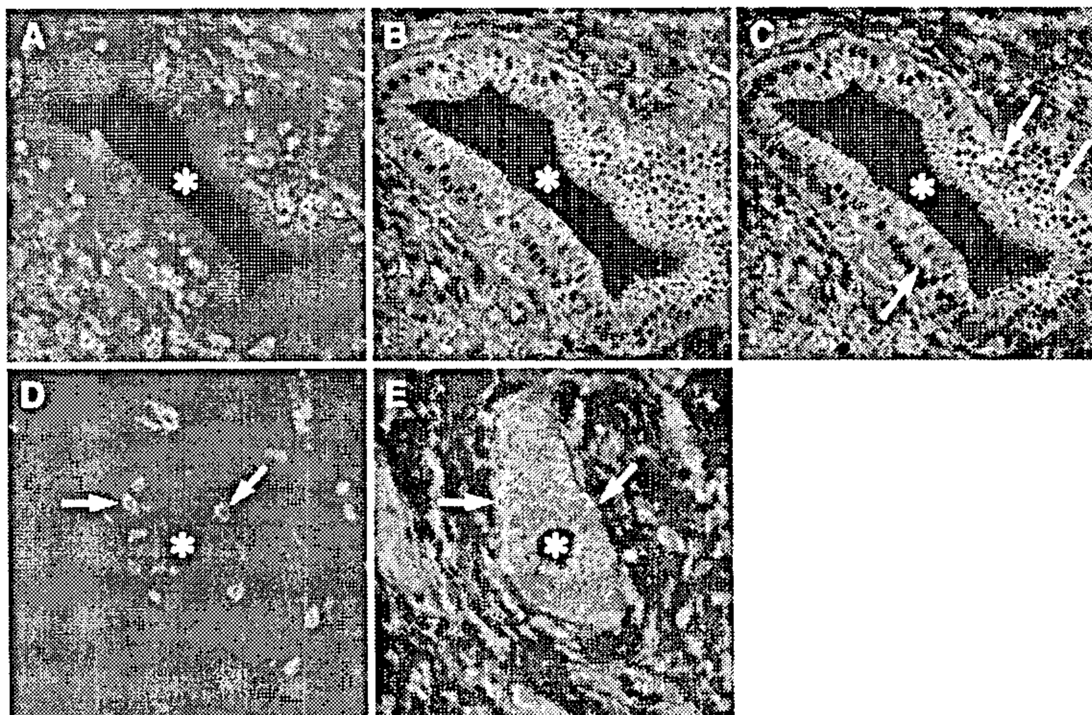

Fig 6. Double immunostaining of CX3CR1 and CD3, CD4, or CD8 in the liver of primary biliary cirrhosis. CX3CR1 was visualized by Alexa Fluor 488 (**green fluorescence**) and CD3, CD4, or CD8 was visualized by Alexa Fluor 594 (**red fluorescence**). Panels A, B, and C are from the same area of the same section, and panels D and E are also from the same area of the same section. All panels are viewed by fluorescence microscope. *Lumen of the bile duct. (A) Many $CD3^+$ T cells (**red**) are seen around the bile ducts and also within the biliary epithelia (original magnification, ×200). (B) $CX3CR1^+$ mononuclear cells (**green**) are seen around the bile duct. Biliary epithelial cells of this bile duct also show membranous and supranuclear cytoplasmic staining of CX3CR1. (original magnification, ×200). (C) Lymphocytes positive for $CD3^+$ and CX3CR1 (**yellow**) are seen within the biliary epithelia (**arrows**) and also around the bile ducts. In addition, red mononuclear cells expressing CD8 alone are also seen (original magnification, ×200). (D) $CD8^+$ T cells (**red**) are found within the biliary epithelia and also around the bile ducts (original magnification, ×400). (E) Lymphocytes positive for $CD8^+$ and CX3CR1 (**yellow**) are seen within the biliary epithelia (**arrows**) and also around the bile ducts (original magnification, ×400).

eases, CX3CR1 was slightly or faintly positive on the membranous parts or in the cytoplasm of bile ducts (Fig. 6B, green). Most biliary IEL of injured bile ducts and of lymphoid cells around these bile ducts in PBC livers were positive for both CX3CR1 and CD3 (Fig. 6C, yellow), although some mononuclear cells in portal tracts were positive for CD3 alone (Fig. 6C, red). In normal and other diseased livers, a few biliary IELs detectable in small bile ducts and lymphoid cells in portal tracts infrequently were positive for both CX3CR1 and CD3.

$CD4^+$ and $CD8^+$ T cells were seen within biliary epithelia (Fig. 6D, red), and most of them also expressed CX3CR1 (Fig. 6E, yellow). $CD4^+$ and $CD8^+$ T cells expressing CX3CR1 also were found around the injured bile ducts of PBC patients.

Expression and Secretion of Fractalkine and CX3CR1 in Cultured BECs

Expression of Fractalkine mRNA on Cytokine Treatment. Quantitative analysis using real-time PCR revealed that fractalkine mRNA was weakly expressed in both cultured HuCC-T1 cells and HIBECs without any stimulants (PBS), whereas treatment with LPS, IL-1β, IFN-γ, and TNF-α induced an upregulation of the expression in these cultured cells, especially LPS and IFN-γ in HuCC-T1 and IFN-γ in HIBECs ($P < .05$; Fig. 7). Stimulation with IL-4 or IL-6 did not result in such upregulation in these cultured cells, suggesting that Th1 cytokines and LPS upregulate fractalkine expression in cultured BECs.

Fractalkine Protein in Culture Supernatants and in Cell Lysate of HuCC-T1 Detected by Western Blotting. Supernatants of HuCC-T1 cultured for 3 days contained soluble fractalkine, and its level was increased by treatment with IL-1β (126.9 ± 39.6 pg/mL), LPS (39.8 ± 19.9 pg/mL), IFN-γ (54.0 ± 10.8 pg/mL), or TNF-α (16.1 ± 2.5 pg/mL) when compared with no stimulant (PBS; 10.4 ± 7.6 pg/mL). The increase caused by IL-1β treatment was significant ($P < .05$). Fractalkine protein was detected in cell lysate of HuCC-T1 cultured

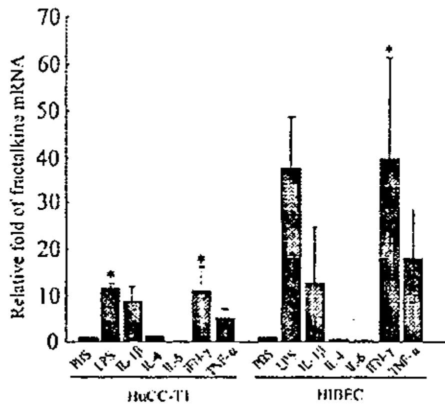

Fig 7. Expression of fractalkine messenger RNA (mRNA) induced by cytokines in cultured cells assessed by real-time polymerase chain reaction. Cultured HuCC-Tl cells and human intrahepatic biliary epithelial cells (HIBECs) were treated with either lipopolysaccharide (LPS) at 1 μg/mL, or interleukin (IL)-1β, IL-4, IL-6, interferon gamma (IFN-γ), or tumor necrosis factor α (TNF-α) at 1,000 U/mL for 3 hours. The increase in fractalkine levels on treatment with LPS, IL-1β, IFN-γ, or TNF-α are 11.7-fold, 8.9-fold, 11.1-fold, and 5.3-fold in HuCC-T1 cells, and 37.5-fold, 12.5-fold, 39.6-fold, and 17.9-fold in HIBECs, respectively, compared with each nonstimulant. Compared with control (phosphate-buffered saline [PBS]), the relative fold increase in the expression of fractalkine mRNA is higher for LPS and IFN-γ in HuCC-T1 cells and for INF-γ in HIBEC cells (*$P < .05$, Kruskal-Wallis test).

for 3 days with no stimulant and was increased after stimulation with LPS, IL-1β, IFN-γ, and TNF-α by Western blotting.

Fractalkine and CX3CR1 Expression in HuCC-T1 Cells Cultured Within Collagen Gel. HuCC-T1 cells cultured within collagen gels for 3 days showed cystic or branching growth.[26] Fractalkine and CX3CR1 were detected immunohistochemically diffusely in the cytoplasm of cultured cells and also in their lumen, suggesting that these cultured cells synthesized and secreted fractalkine and CX3CR1.

Chemotactic Effects of Fractalkine to THP-1 Cells

The number of THP-1 cells that transmigrated in the chemotaxicell chamber after LPS treatment (29.4 ± 1.5 cells/high-power field) was larger than that in the negative control (PBS; 13.9 ± 0.6 cells; $P < .01$; Fig. 8), suggesting that the fractalkine synthesized and secreted by HuCC-T1 cells treated with LPS chemoattracted THP-1. The number of LPS-induced transmigrated THP-1 cells was significantly reduced on preincubation with recombinant fractalkine (1 μg/mL; 20.8 ± 0.8 cells) or anti-CX3CR1 antibody (10 μg/mL; 12.6 ± 2.3 cells), confirming that increased migration of THP-1 was dependent on fractalkine derived from HuCC-T1 cells and also was dependent on CX3CR1 expressed on THP-1.

Discussion

In this study, fractalkine mRNA and protein were detectable in BECs of small bile ducts and also in endothelial cells of portal tracts of normal and diseased livers. Although the expression was weak in normal bile ducts, it was increased in injured bile ducts of PBC.

In normal intestine, fractalkine is expressed in epithelial and endothelial cells[8,33] and is involved in chemoattraction and adhesion of CX3CR1-expressing mononuclear cells into the propria,[8,33] and interestingly, in recruitment of lymphocytes, including $CD8^+$ T cells into the intestinal epithelium.[1,8,33,34] The fractalkine–CX3CR1 system plays an important role in the mucosal immune response in inflammatory bowel disease.[1,8,33,34] This study showed that CX3CR1-positive mononuclear cells infiltrated into portal tracts, and their number increased in PBC. Furthermore, most biliary IELs in injured bile ducts of PBC livers were positive for CX3CR1, suggesting that increased expression of fractalkine in BECs is responsible for chemoattraction of CX3CR1-positive lymphocytes into portal tracts and into biliary epithelia. Fractalkine expression was not strong in BECs of bile ducts in CVH-C and EBO livers, compatible with the finding that biliary IELs expressing CX3CR1 were few in these livers.

We investigated the regulation of fractalkine expression in two cultured BECs. The upregulation or induction of fractalkine expression in cultured endothelial or

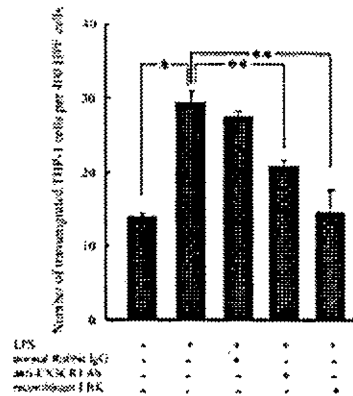

Fig 8. Chemotactic effects of fractalkine (secreted by HuCC-T1) on THP-1 cells. Number of THP-1 cells transmigrated in chemotaxicell chambers after lipopolysaccharide (LPS) treatment (1 μg/mL; 29.4 ± 1.5 cells/high-power field [HPF]) are larger than those without (13.9 ± 0.6 cells/HPF; *$P < .01$, Kruskal-Wallis test). Preincubation with anti-CX3CR1 antibody (10 μg/mL) or recombinant fractalkine (1 μg/mL) significantly reduced LPS-induced THP-1 transmigration (20.8 ± 0.8 cells/HPF and 12.6 ± 2.3 cells/HPF, respectively; **$P < .05$, Kruskal-Wallis test). However, normal rabbit immunoglobulin G (IgG; 10 μg/mL) did not affect LPS-induced THP-1 migration. Ab, antibody; FRK, fractalkine.

smooth muscle cells in response to inflammatory stimuli has been reported.[8,31,35-38] In nonstimulated conditions, cultured HuCC-T1 and HIBECs expressed fractalkine mRNA negligibly. Interestingly, treatment with LPS, TNF-α (Th1 and Th2 types), IL-1β (Th1-type), or IFN-γ (Th1-type) upregulated fractalkine mRNA expression significantly in these cultured cells. In contrast, Th2 cytokines (IL-4 and IL-6) induced no such upregulation. Furthermore, HuCC-T1 cells cultured for 3 days after treatment with LPS, INF-γ, TNF-α, and especially IL-1β increased secretion of soluble fractalkine in culture supernatant. These findings suggest that the Th1 predominance around *in vivo* small bile ducts may lead to increased expression of fractalkine in BECs, followed by periductal chemoattraction of T cells and recruitment of biliary IELs in injured bile ducts of PBC livers. LPS in bile[39] to which BECs are facing also may contribute to this scenario. Th1-dominant cytokine milieu around bile ducts is known to be important for the development of cholangiopathy in PBC.[40,41]

Brand et al.[33] disclosed that fractalkine was able to induce expression of fractalkine via CX3CR1 on intestinal epithelial cells in an autocrine manner.[33] This study showed the expression of mRNA and protein of CX3CR1 and fractalkine in the bile ducts, and that the expression was upregulated in injured bile ducts of PBC. Furthermore, it was found that HuCC-T1 cells cultured within collagen gel produced and secreted both fractalkine and CX3CR1, raising the possibility that their coexpression leads to an autocrine/paracrine effect on fractalkine-induced fractalkine expression.[29,33]

Recent studies suggest potential pathophysiologic roles for fractalkine in glomerulonephritis and intestinal inflammatory diseases.[29,33,35-38,42-44] This study showed that expression of fractalkine and CX3CR1 was upregulated in injured bile ducts of PBC, CX3CR1-expressing mononuclear cells were dense around bile ducts and within the biliary epithelium, and cultured BEC expressed cell-bound fractalkine and secreted soluble fractalkine. So, we examined the chemotactic activity of mononuclear cells expressing CX3CR1, toward a fractalkine gradient, by using chemotaxicell microchemotaxis chambers and HuCC-T1 and THP-1 (monocytes expressing CX3CR1[28,31]). Transmigration of THP-1 toward cultured HuCC-T1 cells treated with LPS was increased, indicating that the fractalkine gradient difference might have chemoattracted THP-1. This fractalkine-dependent chemotaxis of THP-1 cells was confirmed by preincubation of THP-1 with either recombinant fractalkine or anti-CX3CR1 antibody, indicating that fractalkine secreted by HuCC-T1 is functional and that the binding of CX3CR1 by its ligand fractalkine inhibited the chemotactic transmigration activity of THP-1. These results suggest that fractalkine expressed on and secreted from BECs of small bile ducts, particularly injured ones in PBC livers, plays an important role in the migration and adhesion of CX3CR1-expressing mononuclear cells in portal tracts and into biliary epithelia.

T-cell–mediated cytotoxicity toward BECs of small bile ducts reportedly is important in PBC livers, and granzyme B- and Fas ligand-expressing T cells infiltrating around bile ducts in PBC are known to be effecter cells to induce biliary epithelial apoptosis.[45] This study showed that $CD3^+$, $CD4^+$, and $CD8^+$ T cells were present within epithelial layer as well as around injured bile ducts, and most of these T cells also were positive for CX3CR1. Interestingly, most of these injured bile ducts showed upregulation of fractalkine. It seems likely, therefore, that T-cell migration into biliary epithelium was mediated via the fractalkine–CX3CR1 system, and these T cells eventually could damage the bile ducts. PBC is characterized by a serological occurrence of antimitochondrial antibodies that recognize 2-oxo-acid dehydrogenase complex, particularly pyruvate dehydrogenase E2 component (PDC-E2).[46] Furthermore, autoreactive T cells against PDC-E2 are also detectable in the serum and liver in PBC.[47] $CD4^+$ T cells, especially $CD4^+$ and $CD28^-$ T cells recognizing PDC-E2 and $CD8^+$ T cells recognizing PDC-E2, were increased in number in the peripheral blood and liver tissue of PBC livers,[24,47] and these autoreactive T cells may be cytotoxic and responsible for immune-mediated BEC damage. This finding and the circumstantial evidences given above support that idea that fractalkine and CX3CR1 expression are relevant to duct injury in PBC livers via autoreactive and cytotoxic T-cell migration. Further study to demonstrate that $CD4^+$ and/or $CD8^+$ biliary IELs are reactive against PDC-E2 is needed.

Elevated serum soluble fractalkine levels of PBC may emphasize the systemic inflammatory component of PBC and seem to support the above-mentioned scenario regarding fractalkine and bile duct lesions. Eventually, secreted and cell-adherent fractalkine on injured bile ducts may be responsible for chemotaxis of mononuclear cells expressing CX3CR1 and in recruitment of biliary IELs. Similar data on the increased serum level of fractalkine and chemotactic migration of $CD4^+$ lymphoid cells into target tissues were reported in allergic asthma and rhinitis.[43] This study also raises the possibility that immunoneutralization of CX3CR1 attenuates development and persistence of chronic cholangitis of PBC, as is speculated to be the case in other organs.[31,48,49]

In conclusion, this study showed that fractalkine expression was upregulated in injured bile ducts in PBC.

Upregulation of fractalkine was induced by LPS and Th1-cytokines in cultured BECs, suggesting that in PBC livers, the Th1-cytokine predominance around the bile duct and possibly LPS in bile upregulate fractalkine expression in bile ducts. Secreted soluble fractalkine may be involved in the chemoattraction of mononuclear cells expressing CX3CR1 around these pathological bile ducts and in the recruitment of biliary IELs.

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2．自然免疫の観点から

原田　憲一　　中沼　安二

I．はじめに

病原体(細菌，真菌，ウイルス)の侵入に対する防御を担う自然免疫は，昆虫から哺乳類まですべての生物が有する免疫機構であり，脊髄動物のみが有する獲得免疫と対向して用いられる．自然免疫は，免疫担当細胞や上皮細胞から産生される抗菌ペプチドなどの液性因子およびマクロファージなどの貪食作用による細胞性因子からなる．自然免疫の分子標的は，病原体の発生学的に変化に乏しい分子パターン(Pathogen-Associated Molecular Patterns, PAMPs)である．近年，PAMPsを認識する受容体(Pattern Recognition Receptors, PRRs)の一つとして，Toll-like receptor (TLR) familyがクローニングされ，自然免疫の病原体認識にかかわるシグナル伝達経路が明らかにされつつある．このTLRはマクロファージや樹状細胞などの免疫担当細胞のみならず，肺胞上皮や腸管上皮などの上皮細胞にも存在し，臓器特異的な生体防御機構を形成することがわかってきた．

胆汁は通常無菌であるが，リポポリサッカライド(LPS)などの菌体壁構成成分や細菌由来の遺伝子が胆汁中から検出され，さらに肝胆道系疾患では培養可能な細菌も胆汁中から検出される[1～7]．腸管由来の菌体成分が門脈を介して肝臓へ流入後，最終的に胆汁中に排泄されていることから，胆管上皮は断片化および修飾を受けた菌体成分に曝されていると推測される．さらに，解剖学的に胆道系は十二指腸へ開口していることから，十二指腸から上行性に細菌性胆管炎を来しやすく，このような病態が肝内結石症の結石形成や慢性増殖性胆管炎の成因に強く関与していると考えられている[8,9]．したがって，胆道は微生物からの防御に独自の自然免疫機構を有していると容易に推測される．本稿では，近年明らかになりつつある自然免疫の分子機構をもとに胆管細胞における知見について，我々の研究データを交えて紹介する．

II．液性因子

胆管上皮は，気管や腸管と同様，粘膜上皮としてとらえることができ，外界からの病原体侵入に対して，抗菌ペプチド，粘液，分泌型IgAなど粘膜免疫としての防御機構を有する．本項では，自然免疫の液性因子として，胆管上皮細胞で発現または産生を認める抗菌ペプチドについて列記する．

1) サーファクタント蛋白-D

サーファクタント蛋白-Dは，コラーゲン様構造を有するC型レクチン(コレクチン)に属し，胆管を含め涙腺，唾液腺，膵，尿細管など上皮系に広範に発現を認める[10]．その他，サーファクタント蛋白-Aやマンノース結合蛋白質(MBP)もコレクチンファミリーに属し，これらのコレクチンは細菌，原虫などの病原微生物に直接結合後，急性期反応物質として感染防御の最前線を担う生体防御レクチンである．サーファクタント蛋白-Aは，肺胞上皮のII型細胞やクララ細胞で産生されることから，肺特異的マーカーとしてよく知られている．しかし，サーファクタント蛋白-Dは，胆道系を含め比較的広範に分布する抗菌ペプチドであり，胆道系のみならずあらゆる上皮系自然免疫の一端を担っている抗菌ペプチドである．

2) ラクトフェリンおよびリゾチーム

ラクトフェリンは細菌が成長するために必要な第2鉄イオンと結合し，このイオン結合の欠乏により抗菌作用を示す．また，リゾチームは細菌溶解酵素ムラミダーゼであり，細菌の細胞壁に直接作用し分解させ，菌体の溶解により抗菌作用を示す．肝門部胆管の胆管付属腺には，ラクトフェリンおよびリゾチームの発現があり，特に肝内結石症ではこれらの発現亢進が見られる[11]．また，胆汁中にもラクトフェリンの存在が証明されており[12]，ラクトフェリン，リゾチーム共に胆道系感染防御に重要な抗菌ペプチドである．

3) デフェンシン

デフェンシンは細菌，真菌，ウイルスなど広範囲に抗微生物活性を有する塩基性抗菌ペプチドであり，特にヒトβデフェンシン(hBD，現在までhBD1からhBD6が同定)は気道や皮膚などの上皮細胞から産生され，粘膜における感染防御機構の重要な役割を担う．肝内胆管上皮では，hBD1のびまん性発現を認めるが，hBD2

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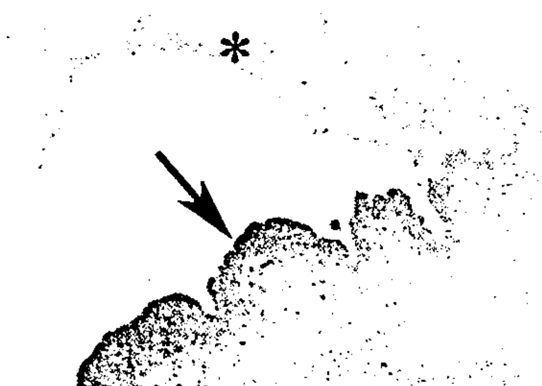

図1　β-defensin 2の免疫組織化学的染色(肝内結石症例)：胆管炎を伴った肝門部大型胆管にβ-defensin 2発現を認め(矢印)，また胆汁にも染色性を認める(*).

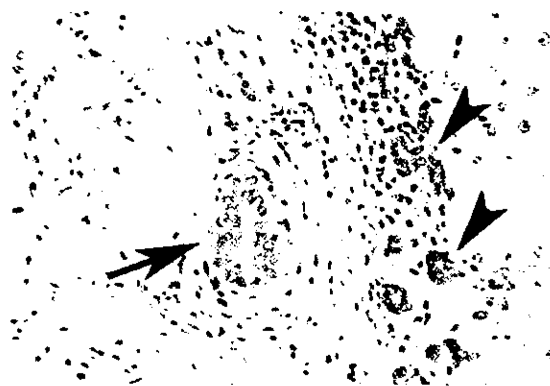

図2　TLR 4の免疫組織化学的染色(原発性胆汁性肝硬変症例)：小葉間胆管(矢印)および増生細胆管(矢頭)にTLR 4の発現を認める.

表1　現在まで報告されているヒトTLRのリガンド

TLR1 & TLR2(ヘテロ2量体)	大腸菌のリポ蛋白
TLR2 & TLR6(ヘテロ2量体)	マイコプラズマ由来のリポ蛋白
TLR2	ペプチドグリカン，リポテイコ酸，酵母由来のザイモザン
TLR3	ウイルスの2本鎖RNA
TLR4 & MD-2(ヘテロ2量体)	LPS
TLR5	細胞のフラジェリン(鞭毛タンパク質)
TLR7	imidazoquinolines(抗ウイルス作用を示す合成化合物)
TLR8	imidazoquinolines，ウイルスの1本鎖RNA
TLR9	CpG DNA(細菌に特異的な非メチル化CpGモチーフDNA)
TLR10	(不明)

は肝内結石症や胆道感染症に見られる化膿性胆管炎の上皮細胞に発現があり(図1)，これらのhBDは胆汁中からも検出される[13]．さらに，我々は培養胆管細胞を用いてhBDの発現誘導機序について検討した結果，hBD1は恒常的に発現していたが，hBD2は大腸菌やLPS刺激で初めて発現誘導されることを明らかにした[13]．したがって，hBD1，hBD2共に抗菌ペプチドとして胆道系の感染防御にかかわっているが，hBD2は胆道感染発生時に新たに胆管上皮から産生され，局所的な感染防御に関与していると推測される．

III．細胞膜結合型受容体・TLRファミリー

近年TLRが同定されて以来，自然免疫の分子機構が明らかとなり，生体防御による生命維持のみならず，慢性炎症性腸疾患や自己免疫疾患への関与についても自然免疫の重要性が注目されている[14, 15]．TLRは，構造上，細胞外菌体認識ドメインであるleucin-rich repeat (LRR)および細胞内シグナル伝達ドメインであるToll-IL-1 receptor (TIR)から構成されている[16, 17]．現在まで，ヒトではTLRファミリーに属する10種の受容体(TLR1〜TLR10)が確認されており，これらのマウス相同体(マウスTLR1〜TLR10)も確認されている[16, 17]．我々は，マウス正常胆管由来およびヒト肝内胆管癌由来の培養胆管細胞を用いて検討し，胆管細胞は少なくともTLR2，TLR3，TLR4，TLR5およびTLR4に会合する分子MD-2を発現していることを明らかにした[3]．また，免疫組織化学的検討にて，ヒト肝内胆管はTLR2〜TLR5を発現し(図2)，また胆道系の解剖学的レベルによって発現パターンがやや異なることも確認しており，生体内でも胆管はTLRによる自然免疫を形成していると推測される．

表1に示す如く各TLRは特有の1種または複数種のリガンドを細胞外LRRドメインで認識後，細胞内ドメ

インのTIRを媒介してシグナル伝達アダプター分子であるMyD88（MyD88依存系）やTRIF（MyD88非依存系）に伝達する．引き続いて，NF-κBやMAP kinaseの活性化により種々の炎症性サイトカインや抗菌ペプチドの産生が誘導され，感染防御機構が作動する．我々は，最も解析が進んでいるTLR2とTLR4について検討したところ，胆管細胞はLPS，ペプチドグリカン，リポタイコ酸刺激にてNF-κBが活性化され，TNF-αの産生誘導が見られることを確認した[3]．以上の所見は，胆道系に少なくともTLR2とTLR4を介した機能的な自然免疫機構が存在することを示すものであり，今後，TLRによる胆道系自然免疫機構の全容を明らかにする必要がある．

IV．細胞質局在型受容体・NODファミリー

NOD1とNOD2は細菌認識ドメインLRRを有する細胞質内局在型の菌体認識受容体であり，構造上，中央にヌクレオチド結合性多量体領域（nucleotide-binding oligomerization domain, NOD）を有するNODファミリーに属する．NOD1はグラム陰性菌ペプチドグリカンのジアミノピメリン酸（DAP）構造を認識し，NOD2はグラム陽性菌および陰性菌のペプチドグリカンのムラミルジペプチド（MDP）構造を認識し，NOD1，NOD2ともにNF-κBの活性を誘導する[18]．近年，慢性炎症性腸疾患のクローン病ではNOD2の菌体認識部位LRRの変異があり，病原体に応答したNF-κBの活性化が誘導できないことが報告された[19]．その後の免疫組織化学的検討によりNOD2は腸管のパネート細胞に存在し，腸内細菌に対する自然免疫応答にかかわっていると考えられている[20, 21]．我々は，胆管細胞にもNOD2分子が存在していることを確認しているが，機構的解析については現在進行中である．TLRのみならず，NODファミリーも病原体に対する自然免疫応答に重要であると考えられ，今後，胆道系NOD分子の解析も必要である．

V．エンドトキシントレランス機構

PAMPs，特にエンドトキシンであるLPSはマクロファージを活性化させるが，同時にその活性を抑制する機構も誘導され，エンドトキシントレランスとして知られている．このエンドトキシントレランスが，腸管ホメオスタ－シスの維持に重要である．すなわち，腸管上皮は常に腸内細菌に曝されているにもかかわらず，炎症を引き起こすことはないが，その抑制機序の一つとしてエンドトキシントレランスが考えられている[22]．培養胆管細胞においても，連続するLPS刺激で容易にエンドトシキントレランスを誘導でき，胆道系においてもトレランスがホメオスターシスの維持にかかわっていると想定される．現在まで，エンドトキシントレランスの分子機構として，①TLRの発現減弱，②細胞内シグナル分子であるIRAK-1のdegradation，③シグナル伝達の抑制因子（IRAK-M，Tollip，SOCS-1，PPAR-γなど）の発現誘導が報告されているが[23]，現在のところ詳細は不明である．

VI．終わりに－自然免疫から獲得免疫へ－

獲得免疫の主役はB細胞やT細胞であり，多彩な抗原受容体形成によって高い親和性と高い特異性で抗原を認識する．しかし，自然免疫は獲得免疫に比べ特異性や親和性は高くないが，外来微生物のあらゆるPAMPsに対する応答を示し，さらに，その後の獲得免疫の成立にも重要な役割を果たす．例えば，TLRの活性化によりIL-12が産生され，T細胞はIFN-γを産生するTh1細胞に機能分化し，細胞性獲得免疫へと効率良く橋渡しがなされる．本稿では，胆管上皮に発現する抗菌ペプチドとTLRを中心とした自然免疫の分子基盤をもとに胆管上皮細胞での新しい知見について述べたが，腸管系自然免疫に較べるとまだまだ不明な点が多い．我々は，胆道系炎症性疾患の病態形成に異常な自然免疫の応答が加担していると想定し，研究を進めている．

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細菌／ウイルス感染とPBC

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索引用語：大腸菌，AMA，分子相同性，自然免疫，胆管

1 はじめに

原発性胆汁性肝硬変(PBC)は，免疫機序による肝内小型胆管の進行性破壊と消失および自己抗体であるミトコンドリア抗体(AMA)の出現で特徴付けられる[1,2]．AMAの主要対応抗原は，ミトコンドリア内膜に粗に結合する 2-oxo-acid dehydrogenase complex(2-OADC)の構成成分であるピルビン酸脱水素酵素複合体E2成分(PDC-E2)である[3]．このPDC-E2，特にinner lipoyl domainは種を越えてよく保存されており，また，AMAが大腸菌などの微生物由来PDC-E2に対して交差反応性を示すことから，PBCの病態発生の病因として外来抗原，特に菌体成分との分子相同性が注目されている[4,5]．また，PBC患者から得られたPDC-E2に対する自己反応性T細胞クローンのエピトープは，B細胞エピトープとほぼ同じ領域であることが示されている[6,7]．さらに，そのエピトープ内にT細胞によるペプチド認識に必須のアミノ酸配列(ExDKモチーフ)の存在も明らかにされ，PDC-E2のみならず，その他の2-OADC構成成分，さらに大腸菌のPDC-E2にも類似のモチーフが存在することが分かり，分子相同性による病態発生の可能性は高いと推定される[8,9]．本稿では，PBCの病因としての細菌またはウイルス感染症に関する既報を表1に示す微生物について総覧し，最後に胆管周囲の微小環境および胆管免疫に

表1 PBCとの関連性が報告されている微生物

細菌
グラム陰性菌
Escherichia coli（変異株を含む）
Helicobacter pylori
Chlamydia
Novosphingobium aromaticivorans
グラム陽性菌
Mycobacterium gordonae
Probionibacterium acnes
ウイルス
Retrovirus
Human immunodeficiency virus-1
Mouse mammary tumor virus
Retrovirus derived from human breast cancer samples
Herpesvirus
Espstain-Barr virus
Reovirus

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ついて感染症の観点からわれわれの知見を中心に紹介する.

2 PBCの病因と細菌感染症

PBCの病因として感染症因子の関与が古くから提唱されてきたが, 肝組織中に存在するPBC特異的な細菌種を培養や生化学的な方法で検出・同定できたという報告はない. 胆管が走行する門脈域内において細菌が生菌として存在しているとは考えにくく, 通常の細菌培養では細菌の同定は困難と思われる. また, PBCにおける胆管特異性を考えた場合, 胆管および胆管周囲への局所的な外来抗原の存在が病態に関与しているものと推測され, 抗原量の点からPBC特異的な感染性因子の特定は困難と考えられる. しかし, 近年, 免疫組織化学的および分子生物学的手法により肝組織, 胆汁中, 肉芽腫, 肝所属リンパ節からいくつかの細菌種または菌体成分が検出・同定され, 分子相同性による病態解明を含めPBCと細菌との関連性が検討されつつある.

1. 尿路感染症 (大腸菌)

PBCと尿路感染症との関連性は古くから指摘されている. すなわち, Burrgoughsら[10]はPBC患者の合併症として高度の細菌尿がみられることを指摘し, また細菌尿の程度がPBCの予後因子となること[11]や大腸菌による敗血症発症時にAMAが検出された症例[12]も報告されている. また, Bogdanosら[13]は, PBC特異的な抗核抗体であるsp100, gp210, lamin B受容体に対する自己抗体と反復性尿路感染症との関連性を検討し, 尿路感染症を伴うPBC患者では高率(74 %)にsp100抗体が陽性であることから, PBC特異的な自己免疫現象の誘導に尿路感染症の関与が重要であると報告している. また, 近年の米国における最新の疫学コホート調査でもPBCと細菌感染(尿路感染および膣感染症)との関連性が確認されている[14]. しかし, 細菌尿の合併率, 培養による大腸菌の陽性率などを検討した結果, 対照疾患との間に有意な差がないとの報告もあり, 必ずしも統一した成績と見解は得られていない[15,16].

AMAと大腸菌との交差反応に関する血清学的研究は, 1976年にSayersら[17]により初めて報告され, その後, Flanneryら[18]およびFusseyら[5]は, ほ乳類のPDC-E2と大腸菌由来のPDC-E2が抗原決定基であるlipoyl domain領域で交差反応性を示すことを明らかにした. また, Bogdanosら[19]は, 蛋白データベース検索からヒトPDC-E2抗原決定基類似のモチーフを有する微生物由来非PDC-E2ペプチド配列(大腸菌由来の6種, *Helicobacter pylori* (*H.pylori*), *Pseudomonas aeruginosa, Haemophilus influenzae*, *Cytomegalovirus*からおのおの1種)を選び出し, これらの合成ペプチドとPBC患者血清との反応性を検討したところ, 大腸菌由来のペチプド配列との交差反応性が有意に高率であったと報告している. さらに, 変異株による反応性の違いも検討されており, 腸内細菌の中のR (rough)-form変異株(O抗原欠損株)大腸菌に対する抗血清がAMAの対応抗原と反応するが, 野生型に対する抗血清は反応しないと報告されている. しかし, 大腸菌との交差反応性はPBCに特異的でないとの報告もある. 例えば, 肝機能が正常でR-form変異株大腸菌による尿路感染症を繰り返す女性は, 低力価ではあるがAMAを有しており[23], また, 大腸菌のライセートを用いた大腸菌由来PDC-E2との交差反応はPBC以外の肝疾患や健常人においても低力価ながら高率に認められる[24]. これらの報告は, 尿路感染が