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ABERRANT EXPRESSION OF STEM CELL FACTOR ON BILIARY EPITHELIAL CELLS AND PERIBILIARY INFILTRATION OF c-kit-EXPRESSING MAST CELLS IN HEPATOLITHIASIS AND PRIMARY SCLEROSING CHOLANGITIS: A POSSIBLE CONTRIBUTION TO BILE DUCT FIBROSIS

KOICHI TSUNEYAMA, NAOKO KONO, MASASHI YAMASHIRO, WATARU KOUDA, AMURA SABIT, MOTOKO SASAKI AND YASUNI NAKANUMA

Second Department of Pathology, Kanazawa University School of Medicine, Kanazawa 920, Japan

SUMMARY

Hepatolithiasis and primary sclerosing cholangitis (PSC) are intractable chronic biliary diseases. In hepatolithiasis, bilirubin-calcium stones are packed in multiple irregularly dilated intrahepatic bile ducts. In PSC, small bilirubin-calcium stones develop terminally. The progressive periductal fibrosis with dilated and stenotic bile ducts in these two diseases may play a role in their incurability. This immunohistochemical study has investigated the expression of some factors that might be involved in fibrogenesis in hepatolithiasis and PSC. Many mast cells positive for c-kit were found in the periductal and ductal fibrosis around the intrahepatic large bile ducts and also around the proliferative peribiliary glands. These mast cells also expressed basic fibroblast growth factor and/or tumour necrosis factor-*α*, which are known as fibrogenetic factors. It was of interest that the aberrant expression of stem cell factor (SCF), a ligand of c-kit, was demonstrated on biliary epithelia of the dilated and stenotic bile ducts showing periductal fibrosis and inflammation and also of the proliferated peribiliary glands in hepatolithiasis and PSC, while no such expression was seen in non-affected bile ducts in hepatolithiasis or in the bile ducts in normal livers. Some of the infiltrating mononuclear cells around the SCF-expressing bile ducts were also positive for SCF. It seems likely that aberrantly expressed SCF on biliary epithelial cells accumulates and stimulates mast cells via the c-kit receptor and that these up-regulated mast cells induce progressive periductal and portal fibrosis by displaying fibrogenetic factors in hepatolithiasis and PSC. Copyright © 1999 John Wiley & Sons, Ltd.

KEY WORDS—**stem cell factor; c-kit; intrahepatic biliary tree; hepatolithiasis; primary sclerosing cholangitis**

INTRODUCTION

Hepatolithiasis is an intractable biliary disease associated with cholangitis and liver failure. Multiple calcium-bilirubinate stones and portal and bile duct fibrosis with a proliferation of peribiliary glands and inflammatory cell infiltration are characteristic features of this disease.[1] As for aetiology, bile stagnation, bacterial infection, alteration of bile composition, and bile duct damage have been suspected,[1–5] but the exact mechanism of this disease, particularly that underlying the bile duct damage, remains unclear. It is very difficult to control hepatolithiasis surgically or medically because of frequent recurrence. Progressive ductal fibrosis with alternating dilatations and stenoses of the bile duct lumen is regarded as an important factor in multiple stone formation and recurrence.[2] Primary sclerosing cholangitis (PSC) is also characterized by progressive bile duct and portal fibrosis, with luminal obliteration and dilatation.[6] This disease is progressive and finally leads to biliary cirrhosis; small calcium-bilirubinate stones or sludges are frequently found in the dilated ductal lumina.[6]

We have previously reported the marked infiltration of mast cells around the fibrotic bile ducts in hepatolithiasis and PSC.[7] It was recently noted that mast cells play an important role in fibrogenesis in several organs, including the liver.[8–14] However, it is not known why or how the mast cells accumulate around the damaged bile ducts in hepatolithiasis and PSC, and the role of these cells in bile duct damage and periductal fibrosis is still speculative. We therefore examined the expression of stem cell factor (SCF), a ligand of c-kit-expressing mast cells.[15–17] c-kit is essential for the development and up-regulation of mast cells.[18] In addition, we examined the expression of basic fibroblast growth factor (b-FGF) and tumour necrosis factor-*α* (TNF-*α*), well-known fibrogenetic factors.[13,17–22] The aberrant expression of SCF on biliary epithelial cells and a significant infiltration of c-kit-expressing mast cells may play important and cooperative roles in the portal and biliary fibrosis of hepatolithiasis and PSC.

MATERIALS AND METHODS

Classification of intrahepatic biliary tree

As in our previous report,[23] the intrahepatic biliary tree was classified into the right and left hepatic duct, the

*Correspondence to: Yasuni Nakanuma, MD, Second Department of Pathology, Kanazawa University School of Medicine, Kanazawa 920, Japan. E-mail: pbcpsc@kenroku.kanazawa-u.ac.jp

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Table I—Expression of stem cell factor (SCF) on the biliary epithelial cells (bile duct-lining cells and peribiliary glands) and periductal infiltrating mononuclear cells in hepatolithiasis (HL), primary sclerosing cholangitis (PSC), and normal livers

Disease	SCF on BE	SCF-positive mononuclear cell infiltration
HL (10 cases)	6/10 (60%)	4/5 (40%)
PSC (5 cases)	2/5 (40%)	2/5 (40%)
With stones	2/2 (100%)	2/2 (100%)
Without stones	0/3 (0%)	0/3 (0%)
Paraquat poisoning	0/4 (0%)	0/4 (0%)
Normal	0/5 (0%)	0/5 (0%)

BE=biliary epithelial cells; PT=portal tract.

segmental ducts (the first major branch of each hepatic duct), the area ducts (the first major branch of each segmental duct), and their finer branches. The hepatic, segmental and area ducts and their first branches are accompanied by peribiliary glands and were collectively termed the intrahepatic large bile ducts. Their finer branches were septal bile ducts with an external diameter greater than 100 μm; the finer branches of the septal bile ducts were divisible into interlobular bile ducts and bile ductules. This study examined mainly the intrahepatic large bile ducts.

Liver specimens and tissue preparation

Surgically resected liver specimens from ten patients with hepatolithiasis and five patients with PSC were studied. All of the specimens included the intrahepatic large bile ducts. All of the hepatolithiasis specimens showed multiple calcium-bilirubinate stones in the intrahepatic biliary tree, and the bile ducts showed ductal and periductal fibrosis, with variable inflammatory cell infiltration and proliferation of peribiliary glands (chronic proliferative cholangitis).[1] The intrahepatic large bile ducts of the PSC specimens showed chronic cholangitis with variable stenosis and dilatation and variable proliferation and inflammation of the peribiliary glands.[24] Two of the five PSC specimens also showed small secondary intrahepatic stones classifiable as calcium-bilirubinate stones (Table I). As a disease control in which there is bile duct injury but no fibrosis, we used four autopsied cases of acute paraquat poisoning which showed hydropic and necrotic biliary epithelial injuries from bile ductules to the intrahepatic large bile duct but no periductal fibrosis.[25,26] As a normal control, we used five histologically normal livers with an age and sex distribution comparable to those of the hepatolithiasis and PSC cases, all of which were surgically resected livers; the intrahepatic large bile ducts were available for examination in these cases. The histologically normal surgically resected livers were from ruptured livers due to trauma.

All of the liver tissues were fixed in 10 per cent neutral buffered formalin and embedded in paraffin. Approximately 20 sections, 4 μm in thickness, were cut from each paraffin block. Some of these sections were processed for routine staining including haematoxylin and eosin (H&E) and Mallory's azan, and the remainder were used for immunohistochemistry.

Primary antibodies and immunohistochemistry

Our preliminary study revealed that formalin-fixed, paraffin-embedded sections as well as frozen tissue sections could be used for the immunohistochemical detection of SCF, human mast cell tryptase (HMCT), b-FGF, TNF-α, and c-kit, by using a standard avidin–biotin detection system.[27,28] HMCT is a specific and sensitive marker protease of human mast cells.

As a primary antibody, we used rabbit polyclonal antibodies against human SCF [Medical Biological Laboratories (MBL), Nagoya, Japan] and c-kit [Immuno-biological Laboratory (IBL), Fujioka, Japan], mouse monoclonal antibodies against bovine b-FGF (Upstate Biotechnology, Lake Placid, NY, U.S.A.), HMCT (DAKO, Glostrup, Denmark), and goat polyclonal anti-human TNF-α (R&D Systems, Minneapolis, MN, U.S.A.).

Briefly, after incubation in 1:10 diluted normal horse serum (Sigma Chemical, St. Louis, MO, U.S.A.) for both the mouse monoclonal and the goat polyclonal antibodies or 1:10 diluted goat serum (Sigma) for the rabbit polyclonal antibodies, the deparaffinized sections were incubated with the primary antibodies anti-SCF (diluted 1:75), anti-HMCT (1:200), anti-b-FGF (1:100), anti-TNF-α (1:100), and anti-c-kit (1:100) for 1 h at room temperature. All slides were then washed several times in Tris-buffered saline (TBS), followed by a 30-min incubation with biotinylated horse anti-mouse IgG (heavy and light chain) (Vector Laboratories, Burlingame, CA, U.S.A.; 1:200) for mouse monoclonal antibodies, biotinylated horse anti-goat IgG (heavy and light chain) (Vector Laboratories; 1:200) for the goat polyclonal antibody or biotinylated goat anti-rabbit IgG (heavy and light chain) (Vector Laboratories; 1:200) for the rabbit polyclonal antibodies. For the subsequent staining, both the avidin–biotin–peroxidase complex (ABC-PO) staining system and the streptavidin–biotin–alkaline phosphatase (sABC-AP) staining system were used. For the substrate of the enzyme, 3,3′-diaminobenzidine (DAB) (Sigma) for ABC-PO and Fast Red (Vector Laboratories) with one drop of levamisol (Vector Laboratories) for sABC-AP were used. For the dilution and washing buffer, phosphate-buffered saline (PBS) (pH 7·6) for ABC-PO and TBS (pH 7·6) for sABC-AP were used.

All sections stained by Fast Red were also observed by a confocal laser microscope (LSM 410, Carl Zeiss, Gottingen, Germany), using a 514 nm argon laser selected for scanning, because Fast Red sheds red fluorescence as rhodamine. In each step of staining, a buffer solution (PBS or TBS) was used instead of the antibodies to clarify the specificity of the reaction; this always resulted in negative staining.

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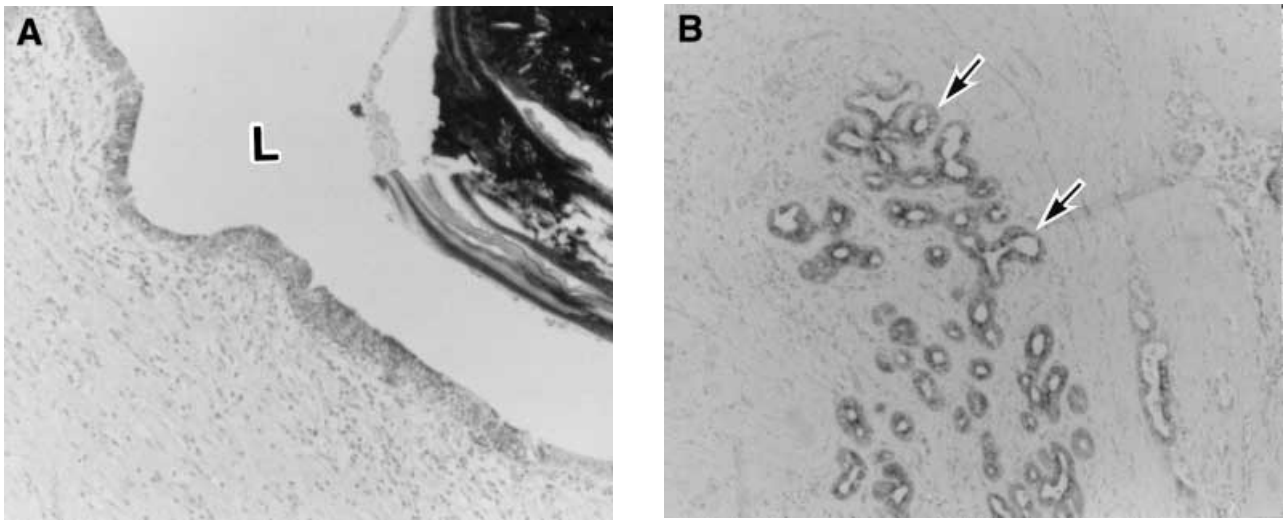

Fig. 1—Immunostaining for stem cell factor (SCF) in the liver of a hepatolithiasis patient. (A) Lining biliary epithelial cells of the stone-containing intrahepatic bile ducts show diffuse and intense cytoplasmic staining of SCF. L=bile duct lumen. (B) Epithelial cells of proliferated peribiliary glands (arrows) around the stone-containing bile duct also show diffuse and intense cytoplasmic staining of SCF. (A) AB complex/PO method; × 200, reduced to 95 per cent in printing. (B) AB complex/PO method; × 100, reduced to 95 per cent in printing

Double immunostaining of SCF/HMCT, b-FGF/HMCT, c-kit/HMCT, and TNF-α/HMCT

To clarify the distribution of the antigen(s) in the same specimens, a double immunostaining method was performed in selected sections. In the case of the double immunostaining of SCF/HMCT and TNF-*α*/HMCT, two antibodies for individual antigens developed in different species were applied (see the section on primary antibodies and immunohistochemistry). After standard immunostaining using the ABC method by one antibody as described above, another antibody was applied with a different visualization system. That is, in the case of the SCF/HMCT, b-FGF/HMCT, c-kit/HMCT, and TNF-*α*/HMCT double immunostaining, SCF, b-FGF, c-kit, and TNF-*α* were firstly immunostained and coloured by the benzidine reaction (brown colour), and then HMCT was immunostained and coloured by Fast Blue (Vector Laboratories) (blue colour). Counterstaining by haematoxylin was omitted. The number of positive cells was counted in a visual field of middle magnification (× 200) in several different areas of each sample.

To avoid interference due to the double recognition of the primary antibody applied in the step of secondary antibody detection, the specimens were soaked in boiling water for 10 min[29] after visualization of the first immunostaining, for the purpose of denaturing the immunoglobulins. We preliminarily confirmed the negative staining after this hot-bath boiling method by applying peroxidase-conjugated secondary antibodies against mouse IgG.

RESULTS

Immunohistochemical expression of stem cell factor

Intrahepatic bile ducts and peribiliary glands—Six of the ten hepatolithiasis specimens (60 per cent) and two of the five PSC cases (40 per cent) showed diffuse intense cytoplasmic staining of SCF in the lining epithelial cells of intrahepatic large bile ducts and of proliferated peribiliary glands for SCF (Figs 1A and 1B). Two PSC specimens positive for SCF contained stones in the intrahepatic large ducts. The staining was intense in the dilated and stenotic bile ducts containing or adjacent to bililubin-calcium stones in the PSC and hepatolithiasis specimens. Even in specimens showing intense SCF expression, the biliary epithelial cells of the non-affected biliary tree were negative for SCF, while in the remaining four hepatolithiasis specimens and three PSC specimens, faint staining of SCF in the biliary and glandular epithelial cells was faintly seen. There were no significant histopathological differences between specimens showing intense SCF expression and those showing weak SCF expression.

In all paraquat toxicity and normal livers, there was no positive staining of SCF in the epithelial cells lining the intrahepatic bile ducts or in the peribiliary glandular epithelium (Table I).

Non-epithelial elements in portal tracts—SCF was also expressed in some of the infiltrating mononuclear cells scattered around the SCF-positive bile ducts. Such mononuclear cells were not encountered around the bile ducts without the epithelial expression of SCF. As shown by the double immunostaining of SCF/HMCT, almost all of the SCF-positive cells were negative for HMCT, suggesting that they were different from mast cells (Figs 2A and 2B). SCF-positive mononuclear cells and mast cells seemed to adhere together or were in close proximity here and there, showing a 'kissing' appearance.

Mast cells in portal tracts and around the biliary tree

HMCT-positive mast cells were observed to infiltrate variably in large-sized portal tracts and around the

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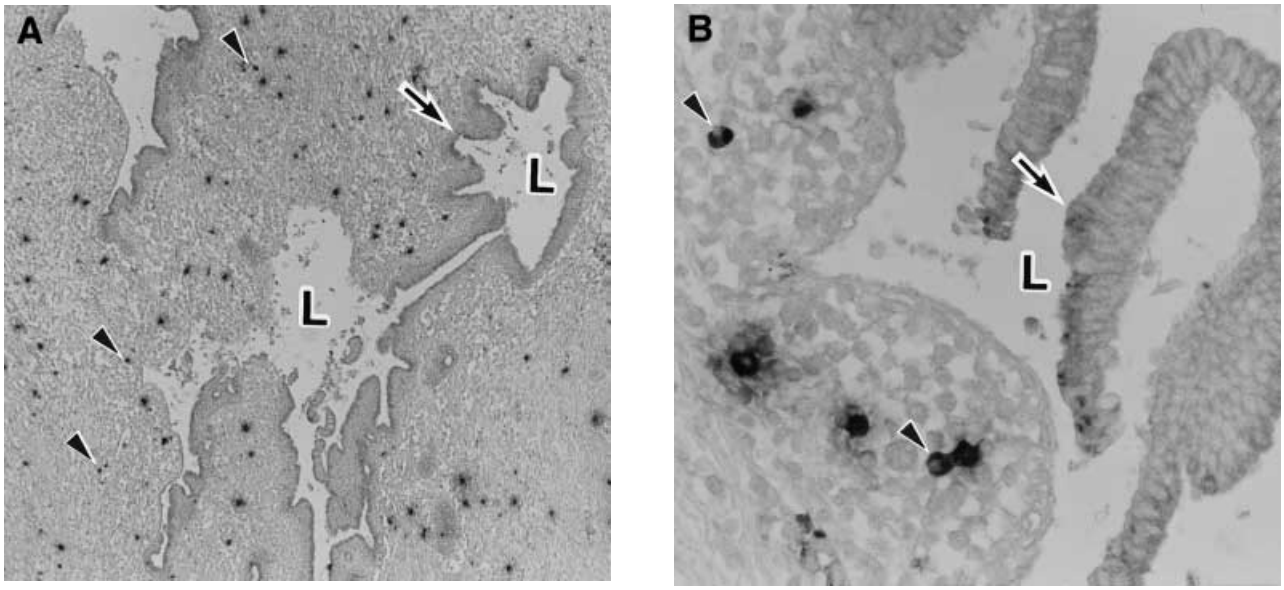

Fig. 2—Double immunostaining for stem cell factor (SCF) and human mast cell-specific tryptase in an intrahepatic large bile duct showing marked inflammatory changes of hepatolithiasis. (A, B) benzidine reaction method for SCF and AB complex/AP method for human mast cell tryptase. (A) × 200 and (B) × 400—both reduced to 95 per cent in printing. The bile duct lumen (L) is distorted. SCF is stained by the benzidine reaction (brown) and human mast cell tryptase by Fast Blue (blue). Intense staining of SCF was diffusely demonstrated in the cytoplasm of biliary duct epithelial cells (arrow) and also in the cytoplasm of some infiltrating mononuclear cells (arrow-heads) (A, B). Considerable mast cell infiltration was found around the bile duct (A). SCF-positive cells and mast cells adhere together or are found in close proximity here and there, sometimes showing a 'kissing' appearance

intrahepatic large bile ducts and peribiliary glands in all of the hepatolithiasis and PSC specimens (Figs 2A and 2B). A large number of HMCT-positive mast cells were identifiable around the dilated and stenotic bile ducts containing or adjacent to stones in the hepatolithiasis specimens. These ducts showed marked ductal and portal fibrosis. Rarely, mast cells were identifiable in the biliary epithelial layer. These HMCT-positive mast cells were also abundant around the intrahepatic large and septal bile ducts and peribiliary glands showing active fibroplasia and inflammation in the PSC specimens.

In the double immunostaining examination, almost all of the HMCT-positive mast cells co-expressed b-FGF (Fig. 3A), c-kit (Fig. 3B) and TNF-α (Fig. 3C) diffusely in their cytoplasm. In brief, there were 115 b-FGF-positive cells in a visual field of middle magnification (× 200) and 17 HMCT-positive cells, in the double staining of b-FGF/HMCT. The number of double positive cells was 12; that is, 70 per cent of HMCT-positive cells and 10 per cent of b-FGF-positive cells. In the double staining of c-kit/HMCT, there were 115 c-kit-positive cells and 14 HMCT-positive cells. The number of double positive cells was 11; that is, 79 per cent of HMCT-positive cells and 10 per cent of c-kit-positive cells. In the double staining of TNF-α/HMCT, there were 162 TNF-α-positive cells and 19 HMCT-positive cells. The number of double positive cells was 11; that is, 58 per cent of HMCT-positive cells and 7 per cent of TNF-α-positive cells. HMCT-negative cells showing b-FGF, c-kit, and TNF-α were also mainly identifiable around the dilated and stenotic bile ducts containing or adjacent to stones.

DISCUSSION

In the present study, we found that SCF was aberrantly expressed in the cytoplasm of the epithelial cells of dilated and stenotic ducts containing or adjacent to stones and of the proliferated peribiliary glands, in livers with hepatolithiasis; and of affected bile ducts containing stones and sludges, and their adjoining peribiliary glands, in livers with PSC. In contrast, almost all of the non-affected bile ducts in the hepatolithiasis specimens and the bile ducts without stones or sludges in the PSC specimens showed very faint or absent immunostaining, while the bile ducts in patients with paraquat poisoning and the normal bile ducts of control livers were negative for SCF. Paraquat-poisoned patients showed various degrees of epithelial injury in bile ducts of various sizes, but no fibrosis, as described by Matsumoto *et al.*[25] and Mullick *et al.*[26] These results suggested that the expression of SCF may not be a non-specific manifestation of bile duct injury, but may be related to bile duct injury associated with hepatolithiasis or PSC. The latter two were also associated with bile duct fibrosis. Using rodent bile duct ligation models, Omori *et al.*[8,9] reported that the expression of SCF occurred on the proliferated bile ductules and bile ducts 5 weeks after ligation and suggested that the expression of SCF on the bile ducts may be due to bile stasis. Our results are partly compatible with their hypothesis that the aberrant expression of SCF on the epithelial cells was more frequently and clearly seen in the damaged biliary tree with bile stagnation and stone formation in hepatolithiasis and PSC. Even in the same hepatolithiasis specimens, non-affected bile ducts did not express SCF clearly in their cytoplasm,

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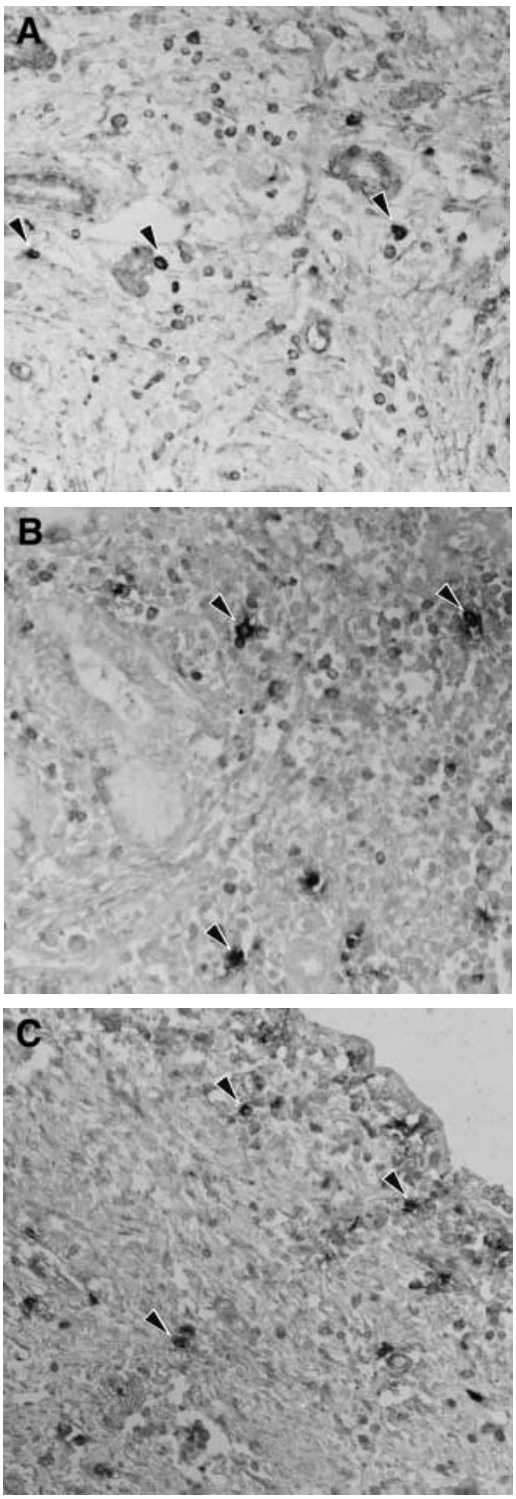

Fig. 3—Double immunostaining for b-FGF (A), c-kit (B), and TNF-α (C) and human mast cell-specific tryptase in an intrahepatic large bile duct showing marked inflammatory changes of hepatolithiasis. There were many HMCT-positive mast cells in the fibrous area around the affected and enlarged bile ducts. Almost all of these HMCT-positive mast cells co-expressed b-FGF (A), c-kit (B) and TNF-α (C), respectively. The proportion of HMCT-positive mast cells among all b-FGF-positive mononuclear cells was nearly 10 per cent (A). The proportions of HMCT-positive mast cells among all c-kit (B) and TNF-α (C)-positive mononuclear cells were also nearly 10 per cent in each case. (A, B, C) Benzidine reaction method for b-FGF, c-kit and TNF-α and AB complex/AP method stained by Fast Blue (blue) for human mast cell tryptase. (A–C) $\times$ 400, reduced to 80 per cent in printing

and PSC cases without stones did not express cytoplasmic SCF in the biliary tree, supporting this suggestion. It is possible that bile stagnation and inflammation due to stone formation induce aberrant expression of SCF on the biliary epithelial cells in hepatolithiasis and PSC.

Our present findings confirmed our previous observation[1] that a considerable number of HMCT-positive mast cells infiltrated around the damaged intrahepatic large bile ducts and peribiliary glands. It is of interest that almost all of the mast cells around the large-sized portal tracts expressed c-kit, which is well known as the receptor of SCF.[8,9,15,16] It is possible that SCF expressed on the biliary and glandular epithelial cells may play an important role in the accumulation and possibly the proliferation of c-kit-positive mast cells.[30–34] In other words, there may be mutual paracrine signal transduction and activation via the SCF/c-kit system in the specimens with biliary SCF expression. In addition to the biliary epithelial expression of SCF, some of the infiltrating mononuclear cells around the SCF-positive bile ducts also expressed SCF strongly in their cytoplasm. To the best of our knowledge, there have been no previous reports of SCF expression in infiltrating mononuclear cells. The double staining with mast cell tryptase showed that these SCF-positive cells are different from mast cells. They did not co-express L26 (marker for B lymphocytes), UCHL-1 (marker for T cells), CD68 (marker for macrophages), b-FGF or TNF-α (data not shown). We could not clarify the nature of these SCF-positive cells, but other immune-associated cells such as natural killer (NK) cells may be candidates. The adhesion or approximation of SCF-positive mononuclear cells and HMCT-positive periductal mast cells was observed here and there in the specimens showing the biliary epithelial expression of SCF. Their 'kissing' appearance suggested a functional relationship between these cells, possibly relating to contact or paracrine pathways. For example, the SCF-positive cells may be involved in the local up-regulation or activation of mast cells.

Macrophages are well known as a source of b-FGF and TNF-α which are well-known fibrogenetic factors.[13,17–22] In our double staining analysis, mast cells accounted for nearly 10 per cent of b-FGF- and TNF-α-positive cells. It is becoming evident that mast cells play important roles in fibrogenesis in several organs.[8–11,13–22] After interacting with SCF, activated mast cells may co-express b-FGF and TNF-α. This suggests that the mast cell may be an important candidate regulator of fibrogenesis around the damaged bile ducts in hepatolithiasis and PSC.

Our data suggest the possibility of the following fibrogenetic mechanism in hepatolithiasis and PSC. Long-term bile stagnation or inflammation due to infection may induce aberrant expression of SCF in the biliary epithelial cells. SCF-expressing mononuclear cells also infiltrate around such bile ducts. The locally released SCF from the intrahepatic bile ducts and peribiliary glands may interact with c-kit-positive mast cells as a step in the paracrine pathway. After interacting with SCF, mast cells may be activated and begin themselves to synthesize and express fibrogenetic bioactive factors,

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such as b-FGF and TNF-*a*. These activated mast cells may thus induce the progressive portal and peribiliary fibrosis in hepatolithiasis and PSC.

Of course, this is not enough to explain completely the mechanism of biliary fibrosis. In the present study, several bile ducts without SCF expression also showed fibrosis in various degrees. We have no data on the time sequence of SCF expression in the bile ducts; SCF-negative ducts may have previously expressed SCF. It is difficult to distinguish between old and new fibrosis in H&E staining. Extracellular matrix components may change during the time course of fibrosis and further studies should examine not only SCF expression in the bile ducts, but also the surrounding connective tissue.

In conclusion, aberrant biliary expression of SCF and the interaction of c-kit-positive mast cells and SCF-positive epithelial cells might be involved in the progressive portal and periductal fibrosis in hepatolithiasis and PSC. Time sequential observations of the expression of SCF and c-kit, and of the fibrous tissue components around the affected bile ducts may clarify the causal relationship between periductal fibrosis and mast cell infiltration in these conditions.

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