American Journal of Pathology, Vol. 147, No. 5, November 1995

Short Communication

Expression of Matrix Proteinases during Human Intrahepatic Bile Duct Development

A Possible Role in Biliary Cell Migration

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Primitive biliary cells are known to migrate from the ductal plate into the mesenchyme during human intrahepatic bile duct development, and this migration process is essential for normal development of intrahepatic bile ducts. However, its molecular mechanism is unknown. Matrix proteinases play an important role in cell migration during cancer invasion and organ development. In this study, we therefore investigated* in situ *expression of matrix metalloproteinases (MMP) and tissue inhibitors of MMP (TIMP) during human intrahepatic bile duct development, using 32 human fetal livers. We also examined* in situ *expression of trypsinogen/trypsin, chymotrypsinogen/chymotrypsin, and cathepsin B, which are matrix proteinases and activators of MMP. MMP-1 expression was noted in the ductal plate and migrating primitive biliary cells. MMP-2, MMP-3, and MMP-9 were expressed in the ductal plate. TIMP-1 and TIMP-2 were expressed in the ductal plate and migrating primitive biliary cells. Trypsinogen/trypsin, chymotrypsinogen/chymotrypsin, and cathepsin B were also expressed in primitive biliary cells. These data suggest that MMP, trypsinogen/trypsin, chymotrypsinogen/chymotrypsin, and cathepsin B play a critical role in biliary cell migration during human intrahepatic bile duct development by degrading extracellular matrix proteins. The data also suggest that MMP inhibitors (TIMP-1 and TIMP-2) and MMP activators (trypsin, chymotrypsin, and cathepsin B) play an important role in biliary cell migration. The coordinated expression of MMP, MMP inhibitors, and MMP activators may be necessary for the normal development of human intrahepatic bile ducts. (Am J Pathol 1995, 147: 1207–1213)

The developmental process of human intrahepatic bile ducts has been well studied.[1–11] These studies have revealed that human intrahepatic bile ducts arise from primitive immature hepatocytes around the portal veins. The primitive hepatocytes give rise to a double-layered cylindrical structure known as the "ductal plate." Primitive bile duct cells migrate from the ductal plate into the mesenchyme around the portal veins. The migrating primitive bile duct cells become immature bile ducts, which further transform into mature bile ducts after birth.[1–11] This migration of primitive biliary cells into the mesenchyme is thought to be essential for the normal development of intrahepatic bile ducts.[1–11] However, the molecular mechanism of this migration is unknown, although a possible role of laminin and tenascin has been speculated.[3,8]

Proteolytic enzymes, collectively known as matrix proteinases, are known to play an important role in the cell migration of cancer cells[12,13] and during organ development[14,15] by degrading extracellular

Accepted for publication July 18, 1995.

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matrix (ECM) proteins. Matrix proteinases are classified into matrix metalloproteinases (MMPs), serine proteinases, cysteine proteinases, and aspartic proteinases.[12] Among these, MMPs are the most powerful proteinases.[12,13] Trypsinogen/trypsin and chymotrypsinogen/chymotrypsin, both serine proteinases, are known to play a role in cell migration by degrading ECM proteins.[16–18] Cathepsin B, a cysteine proteinase, is also involved in cell migration by degrading ECM proteins.[19,20] In cancer or developing tissues, there are inhibitors of these matrix proteinases, such as tissue inhibitors of MMP (TIMP) and pancreatic secretory trypsin inhibitor.[12–18] However, the *in situ* expression and role of matrix proteinases have not been examined during human intrahepatic bile duct development.

In this study, we therefore examined the *in situ* expression of MMP-1 (interstitial collagenase), MMP-2 (72-kd gelatinase/type IV collagenase (gelatinase A)), MMP-3 (stromelysin), MMP-9 (92-kd gelatinase/type IV collagenase (gelatinase B)), TIMP-1, TIMP-2, trypsinogen, chymotrypsinogen, and cathepsin B in primitive biliary cells of human fetuses in order to determine the possible role of these enzymes in the migration of primitive biliary cells.

Materials and Methods

Tissue Specimens

We collected 32 human fetal livers at the gestational ages of 9, 10 (n = 3), 11 (n = 3), 12 (n = 2), 13 (n = 2), 14 (n = 2), 15 (n = 2), 17 (n = 2), 18, 19, 22, 24 (n = 2), 25, 26, 28, 29, 30, 32, 33, 36, 37, and 40 weeks. The fetuses were either aborted or autopsied fetuses. All of the aborted fetuses were spontaneously aborted, and informed consent was obtained from the mother in each case. The liver was sliced frontally at the hepatic hilum, and one liver specimen including the hepatic hilum was obtained from each liver. The specimens thus obtained were fixed in 4% neutral formaldehyde solution and embedded in paraffin. Several 5-μm sections were obtained from each paraffin-embedded block; one of them was stained with hematoxylin and eosin, and the rest were subjected to immunohistochemical staining.

Antibodies and Specificity

For the development of mouse monoclonal antibodies immunoglobulin G (IgG class) against MMP-1, MMP-2, MMP-9, synthetic peptides of these antigens were prepared by the Fmoc-Bop method on a Bioresearch model 9600 peptide synthesizer (MilliGen/Bioresearch, Tokyo, Japan).[21] ProMMP-3 was purified from the culture medium from the rheumatoid synovial fibroblast.[22] TIMP-1 and TIMP-2 were also isolated from human placental tissues.[23] These peptides and proteins were used as antigens and used to immunize BALB/c mice. Monoclonal antibodies were first screened by enzyme-linked immunosorbent assay, and the positive clones were further characterized by immunoblotting. IgG antibodies specific to each MMP or TIMP were obtained by ammonium sulfate fractionation and Affi-Gel protein A MAPS-II kit from the ascites fluids of Pristane-treated BALB/c mice injected with each hybridoma. The specificity of each MMP or TIMP was confirmed by Western blot analysis.[21]

Mouse monoclonal antibodies to trypsinogen and chymotrypsinogen were commercially obtained from Chemicon Corporation (Tomecula, CA). The specificity of these antibodies was confirmed by Western blotting[24] and absorption testing.[11] Polyclonal antibody to cathepsin B was obtained from Binding Site Ltd. (Birmingham, UK), and the specificity was confirmed by the absorption test.[18]

Immunohistochemical Procedure

Sections from each paraffin block were immunohistochemically stained for MMP-1, MMP-2, MMP-3, MMP-9, TIMP-1, TIMP-2, trypsinogen, chymotrypsinogen, and cathepsin B, using the avidin-biotin-peroxidase complex method of Hsu et al.[25] In brief, after deparaffinization, endogenous peroxidase activity was abolished by immersing the sections for 20 minutes in methanol containing 0.3% H_2O_2. The sections were then treated with normal serum for 20 minutes, followed by treatment at 4°C overnight with the monoclonal antibodies (5 to 10 μg/ml) or polyclonal anti-cathepsin B (dilution, 1:50). The sections were then treated for 1 hour with biotinylated anti-mouse IgG (Vector Laboratories, Burlingame, CA) or biotinylated anti-rabbit IgG (Vector Laboratories), followed by treatment for 1 hour with avidin-biotin-peroxidase complex (Vectastain ABC Kit, Vector Laboratories). The reaction products were developed by immersing the sections in 3,3′-diaminobenzidine tetrahydrochloride solution containing 0.03% H_2O_2. The nuclei were lightly stained with hematoxylin. No staining was obtained when nonimmune serum or phosphate-buffered saline was used instead of the primary antibodies.

Staging of Intrahepatic Bile Duct Development

The developing intrahepatic bile ducts were classified into the following four stages: ductal plate; mi-

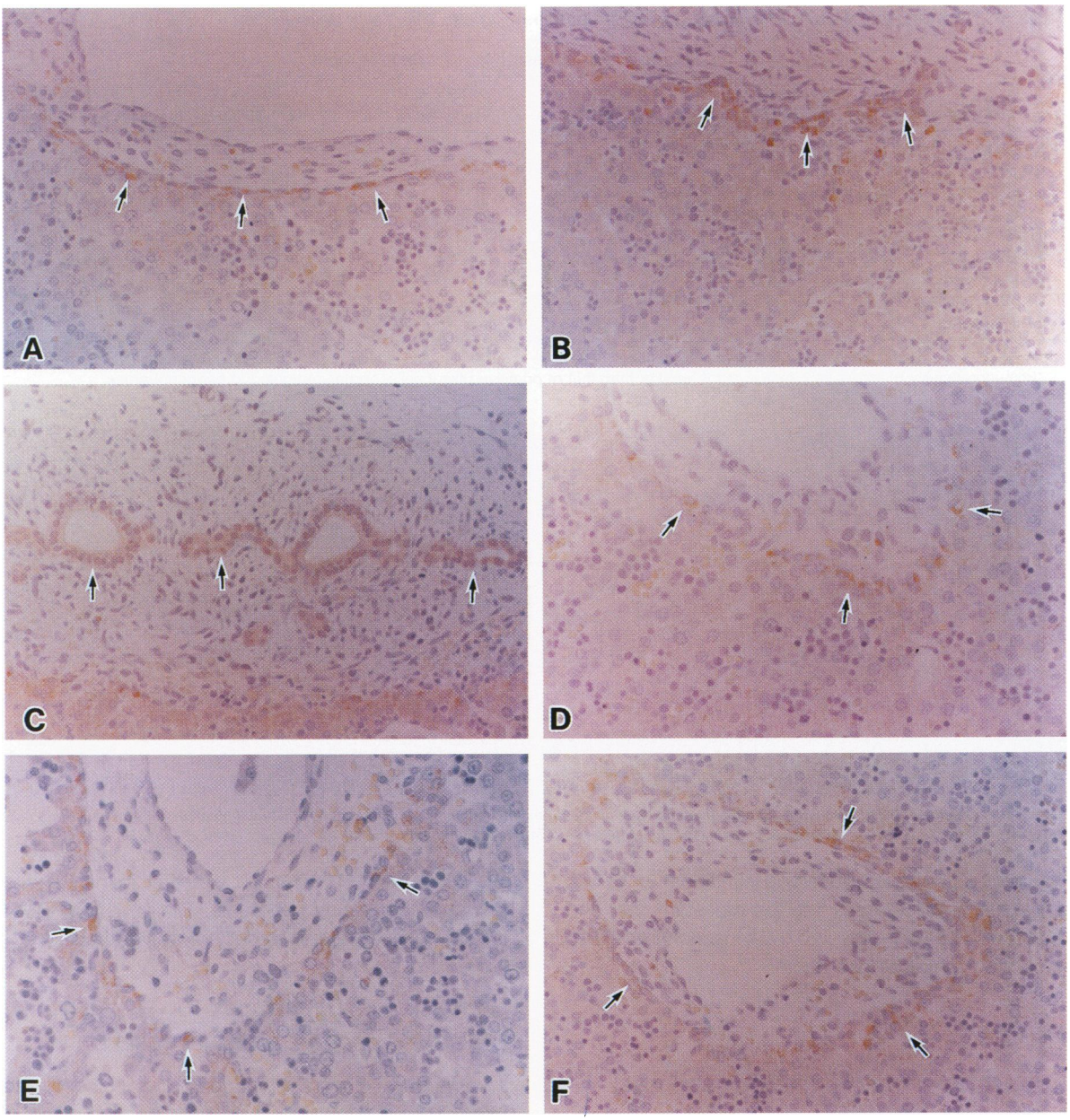

Figure 1. *Expression of MMPs during human intrahepatic bile duct development.* A: *MMP-1 is expressed in the ductal plate* (arrows). B: *MMP-1 is expressed in the biliary cells* (arrows) *migrating from the ductal plate into the mesenchyme.* C: *MMP-1 is expressed in immature bile ducts* (arrows). D: *MMP-2 is expressed in the ductal plate* (arrows). E: *MMP-3 is expressed in the ductal plate* (arrows). F: *MMP-9 is expressed in the ductal plate* (arrows). *Immunostaining for MMP, ×300.*

gration of biliary cells from the ductal plate into the mesenchyme (remodeling stage); immature bile ducts (remodeled stage); and maturing bile ducts. The maturing bile ducts were distinguished from the immature bile ducts as follows. The former were well-developed circular ductal structures without neighboring primitive biliary cells, whereas the latter were semicircular or slit-like ductal structures with neighboring primitive ductal elements. Intrahepatic bile duct development progressed from the hepatic hilum to the periphery.[1–11] The approximate period of each stage during the development of large bile ducts near the hepatic hilum was as follows: 9 to 12 gestational weeks in ductal plate, 13 to 17 gestational weeks in migration of biliary cells, 18 to 25 gestational weeks in immature bile ducts, and 26 to 40 gestational weeks in maturing bile ducts. Intrahepatic bile duct development differed according to the bile duct size. Therefore, two or more of these stages were frequently observed in the same liver;

Table 1. *Expression of Matrix Proteinases during Human Intrahepatic Bile Duct Development*

Antigens	Stages of intrahepatic bile duct development				
	Ductal plate	Biliary cell migration	Immature bile ducts	Maturing bile ducts	Immature hepatocytes
MMP-1	+	+	+	−	−
MMP-2	+	−	−	−	−
MMP-3	+	−	−	−	−
MMP-9	+	−	−	−	−
TIMP-1	+	+	−	−	−
TIMP-2	+	+	−	−	−
Trypsinogen	±	+	+	+	+*
Chymotrypsinogen	+	+	+	+	+*
Cathepsin B	+	+	+	+	+*

+, positive; −, negative; ±, little or no immunoreactivity.
*Immunoreactivity of immature hepatocytes is weaker than that of primitive biliary cells, and accentuated in periportal areas.

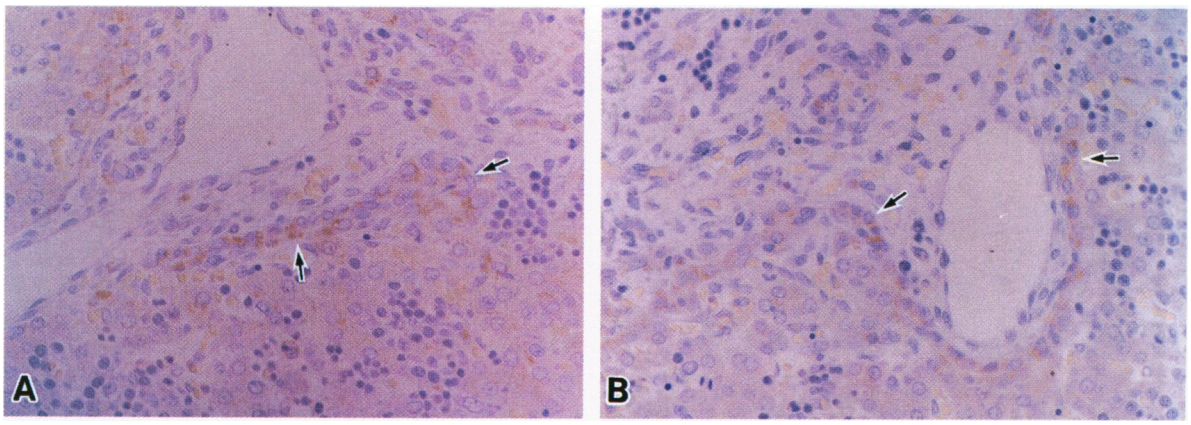

Figure 2. *Expression of TIMP during human intrahepatic bile duct development.* **A:** *TIMP-1 is expressed in the ductal plate and migrating biliary cells* (arrows). **B:** *TIMP-2 immunoreactivity is noted in the ductal plate and migrating primitive biliary cells. Immunostaining for TIMP, ×350.*

the ductal plate was observed simultaneously with migration of biliary cells into the mesenchyma, and migration of biliary cells into the mesenchyme coexisted with immature bile ducts.

Results

Expression of Matrix Metalloproteinases

MMP-1 was expressed in the ductal plate (Figure 1A), migrating primitive biliary cells (Figure 1B) and immature bile ducts (Figure 1C), but was not expressed in maturing bile ducts (Table 1). MMP-2, MMP-3, and MMP-9 were expressed in the ductal plate (Figure 1, D–F), but were not expressed either in migrating primitive biliary cells or in immature and maturing bile ducts. The pattern of MMP immunoreactivity in the cytoplasm was granular.

Expression of Tissue Inhibitor of Matrix Metalloproteinases

TIMP-1 and TIMP-2 were expressed in the ductal plate and migrating primitive biliary cells (Figures 2, A and B), but were not expressed either in immature bile ducts or in maturing bile ducts. The pattern of TIMP immunoreactivity in the cytoplasm was granular.

Expression of Trypsinogen, Chymotrypsinogen, and Cathepsin B

Trypsinogen, chymotrypsinogen, and cathepsin B were expressed in the ductal plate, migrating primitive biliary cells, immature bile ducts and maturing bile ducts (Figure 3, A–C), although little or no trypsinogen immunoreactivity was observed in the ductal plate. The immunoreactivity of trypsinogen and chymotrypsinogen showed diffuse cytoplasmatic pattern in the ductal plate, migrating biliary cells, and immature bile ducts, but supranuclear granular pattern was seen in maturing bile ducts. Cathepsin B immunoreactivity showed a diffuse cytoplasmic pattern.

Discussion

During human intrahepatic bile duct development, the migration of primitive biliary cells into the mes-

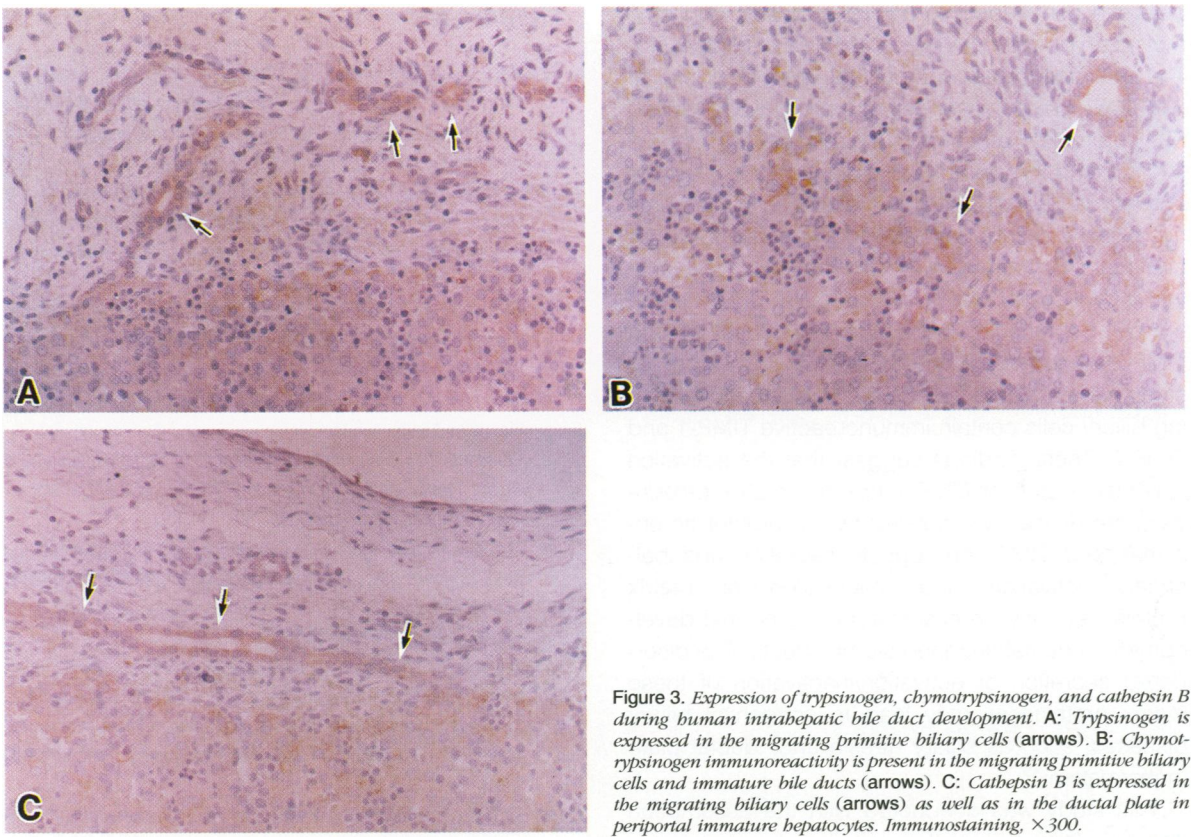

Figure 3. *Expression of trypsinogen, chymotrypsinogen, and cathepsin B during human intrahepatic bile duct development.* A: *Trypsinogen is expressed in the migrating primitive biliary cells* (arrows). B: *Chymotrypsinogen immunoreactivity is present in the migrating primitive biliary cells and immature bile ducts* (arrows). C: *Cathepsin B is expressed in the migrating biliary cells* (arrows) *as well as in the ductal plate in periportal immature hepatocytes. Immunostaining,* ×*300.*

enchyme is the critical event.[1–11] However, the molecular mechanism of this migration phenomenon is unknown. Proteolytic digestion of ECM is essential for cell migration.[12,13] MMPs are powerful proteolytic enzymes capable of degrading various ECM proteins, thus assisting in the migration of cancer cells[12,13] and during organ development.[14,15] Trypsinogen/trypsin, chymotrypsinogen/chymotrypsin, and cathepsin B are also proteolytic enzymes, but they are less powerful than MMP.[12,13,17–20] The demonstration in the present study of the presence of immunoreactive MMP, trypsinogen, chymotrypsinogen and cathepsin B in the primitive biliary cells of the human fetal liver suggests that primitive biliary cells contain these matrix proteinases and secrete these proteinases. It is strongly suggested that the secreted matrix proteinases are activated and that the activated proteinases play a critical role in biliary cell migration from the ductal plate into the mesenchyme by degrading the ECM proteins.

There was heterogeneity of the expression of MMP, trypsinogen, chymotrypsinogen, and cathepsin B. MMP-2, MMP-3, and MMP-9 were expressed in the ductal plate, whereas MMP-1 was expressed in the ductal plate as well as in migrating biliary cells and immature bile ducts. It appears that MMP-2, MMP-3, and MMP-9 play an important role in biliary cell migration from the ductal plate into the mesenchyme by degrading type IV collagen, laminin, and other ECM proteins. In fact, type IV collagen and laminin are present in the ductal plate.[8] This finding also suggests that MMP-1 plays a more important role than do MMP-2, MMP-3, and MMP-9. MMP-1 apparently assists in biliary cell migration during the stages of ductal plate, biliary cell migration, and immature bile ducts. MMP-1 was not present in maturing bile ducts, suggesting that ECM degradation by MMP-1 does not occur at this stage. Trypsinogen, chymotrypsinogen, and cathepsin B were expressed in the ductal plate, biliary cell migration, and immature bile ducts, suggesting that these proteinases play a role during these stages although these proteolytic enzymes seem less effective than MMP.

The activation mechanism of these matrix proteinases is complex. MMP is released from cells into the ECM in inactive forms.[13] Trypsinogen and chymotrypsinogen are also inactive forms. It is well known that cathepsin B activates proenzymes, such as trypsinogen and chymotrypsinogen, converting them into active forms, such as trypsin and chymotrypsin.[26,27] Therefore, it seems likely that trypsinogen and chymotrypsinogen in the primitive biliary

cells are converted into activated trypsin and chymotrypsin by cathepsin B in the primitive biliary cells. The activated trypsin and chymotrypsin may play an important role in biliary cell migration by degrading ECM proteins. Cathepsin B is also known to activate MMP,[28] and trypsin and chymotrypsin are known to activate MMP,[29] suggesting that MMPs in the primitive biliary cells are transformed into active forms by cathepsin B, trypsin, and chymotrypsin. The activated MMPs may play a critical role in primitive biliary cell migration. On the other hand, TIMP-1 and TIMP-2 inactivate the activated MMP.[12] The present study demonstrated that the ductal plate and migrating biliary cells contain immunoreactive TIMP-1 and TIMP-2. These findings suggest that the activation and inactivation of MMP and other matrix proteinases are delicately regulated by the proteolytic enzymes and TIMP. Appropriate secretion and balanced activation and inactivation of matrix proteinases may be essential for the normal development of human intrahepatic bile ducts. The disordered secretion or activation/inactivation of these matrix proteinases may lead to congenital biliary diseases, such as biliary atresia and "ductal plate malformation."[5]

This study was conducted with the assumption that transformation of the ductal plate into mature bile ducts occurs through active migration of biliary epithelial cells. However, there is an alternative possibility on this issue. That is, a synthesis of ECM components may take place, resulting in an engulfment of parts of the ductal plates by connective tissue. In the postnatal liver, ECM components are synthesized mainly by fat-storing cells (Ito cells) and mesenchymal cells.[30] Hepatocytes and bile duct cells do not contribute to the ECM synthesis.[30] In the fetal liver, however, cellular sources of ECM components are apparently unknown. Although this alternative hypothesis may be possible, we believe that primitive biliary cells actively migrate from the ductal plate into the mesenchyme. The demonstration of mRNA of ECM components by *in situ* hybridization is needed in fetal livers to test this alternative hypothesis.

Finally, the present study is only an immunohistochemical study, and the hypothesis that the activities of the matrix proteinases and TIMP are directly involved in the human intrahepatic bile duct development is not directly tested in the present study. To test this hypothesis directly, development of experimental models, such as ductal plate cell culture, organ culture, and animal, may be needed. However, this is very difficult at the present time, and this issue remains to be resolved in the future.

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