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Detection of Apoptosis and Expression of Apoptosis-Related Proteins during Human Intrahepatic Bile Duct Development

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We investigated apoptosis by nick end labeling and the expression of apoptosis-related proteins by immunohistochemistry in fetal development of human intrahepatic bile ducts and hepatocytes. During intrahepatic bile duct development, apoptosis was present at all stages, and its positive ratio was high in the remodeling ductal plate, moderate in the ductal plate, and relatively low in remodeled ducts. The cell proliferative activity as determined by proliferating cell nuclear antigen was also high in the remodeling ductal plate, and relatively low in the ductal plate and remodeled ducts. fas antigen and c-myc protein were constantly positive in the ductal plate, remodeling ductal plate and remodeled ducts. Bcl-2 protein was negative or faintly positive in the ductal plate and remodeling ductal plate, but was apparently positive in remodeled ducts. Lewis^y as detected by the BM-1 antibody was present in the ductal plate, remodeling ductal plate, and remodeled ducts. p53 protein was not found in any cell types in the liver development. During hepatocyte development, many apoptotic and proliferating cell nuclear antigen-positive hepatocytes were noted. The developing hepatocytes expressed c-myc protein and fas antigen. Bcl-2 protein and Lewis^y antigen were also weakly positive in the developing hepatocytes. These findings showed that balanced cell proliferation and apoptosis are involved in the normal development of intrahepatic bile ducts and hepatocytes, and suggest that c-myc protein, fas antigen, Bcl-2 protein, and Lewis^y antigen modulate apoptosis of fetal intrahepatic biliary cells and hepatocytes, probably by stimulative (c-myc protein and fas and Lewis^y antigens) or inhibitory (Bcl-2 protein) effects. (Am J Pathol 1995, 146:67–74)

Several studies have elucidated the developmental process of human intrahepatic bile ducts.[1–10] These studies have revealed that human intrahepatic bile ducts derive from primordial immature hepatocytes around the portal veins.[1–10] The primordial hepatocytes give rise to an excess structure consisting of a double-layered cylinder called the ductal plate, which is positive for biliary-type cytokeratins.[1,2,4,5] The ductal plate is an excessive structure of the biliary anlagen that gradually undergoes remodeling via the removal of some cells of the ductal plate as well as via the incorporation of some primitive biliary cells from the ductal plate into the mesenchyma around the portal veins.[3,4] The incorporated primitive bile duct cells become immature bile ducts during the fetal stage.[1–10] The immature bile ducts further transform to mature bile ducts after birth.[5] Impaired remodeling of the ductal plate may lead to so-called "ductal plate malformation," which is an excessive structure of the postnatal period that arises from the failure of the ductal plate to disappear.[4]

Apoptosis (programmed cell death) is a type of cell death. It differs from necrosis in morphology, process of cell death, and other aspects.[11] Apoptosis is characterized by DNA fragmentation caused by the activation of endonuclease, and this fragmentation is recognized as the DNA ladder by electrophoresis.[11] Recently, it has been suggested that apoptosis plays an important role in fetal organogenesis.[12] In addition, it has been suggested that apoptosis is mediated by several proteins both inhibitory and stimulatory.[13–16] *fas* antigen,[13] p53 protein,[14] and c-*myc* protein[15] are stimulators of apoptosis, whereas Bcl-2 protein[16] is an inhibitor. Lewis^y antigen, which is recognized by BM-1 monoclonal antibody, has been recently reported to be expressed in damaged and apoptotic cells, and its expression may be followed by apoptotic cell death.[17]

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However, presence and absence of apoptosis and the role of apoptosis in the remodeling process of the ductal plate in human intrahepatic bile duct development have not been evaluated. In addition, expression of the modulators of apoptosis in the remodeling process of the ductal plate is not known. In this study, we investigated apoptosis by *in situ* nick end labeling and examined the expression of apoptosis-related proteins by immunohistochemistry during fetal intrahepatic bile duct development in humans.

Materials and Methods

Tissue Specimens

We collected 22 human fetal livers at various gestational ages: three each at 9 and 10 weeks; two each at 11, 12, 13, 14, 15, and 17 weeks; and one each at 18, 24, 25, 32, 33, and 37 weeks. All fetal livers were obtained from spontaneous abortions at our affiliated hospitals. Informed consent was obtained from every mother. The fetal livers obtained were immediately sliced frontally at the hepatic hilum, and one to two liver tissue specimens including the hepatic hilum were obtained. The specimens thus obtained were immediately fixed in 4% neutral buffered formaldehyde and embedded in paraffin. Several 3-μ serial sections were obtained from each paraffin-embedded block; one was stained with hematoxylin and eosin, and the rest were subjected to *in situ* nick end labeling for apoptosis as well as to the immunohistochemical staining of apoptosis-related proteins and proliferating cell nuclear antigen (PCNA).

In Situ *Nick End Labeling Method for Apoptosis*

Apoptosis (DNA fragmentation) was detected by the modified terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP)-biotin nick end labeling (TUNEL) method according to Gavrieli et al.[18] In brief, after proteinase K digestion and removing of endogenous peroxidase, the sections were incubated at 37 C for 1 hour in a solution containing TdT and digoxigenin-labeled dUTP and deoxyadenosine triphosphate (dATP). The sections were then treated with the peroxidase-labeled anti-digoxigenin antibody solution for 30 minutes. The reaction products were developed with 3,3′-diaminobenzidine tetrahydrochloride and counterstained with methyl green. For negative control, phosphate-buffered saline was substituted by TdT containing digoxigenin-labeled dUTP and dATP, which resulted in no staining. All reagents were purchased from Sigma Chemical Co. (Saint Louis, MO) and Oncor Ltd. (Gaithersburg, MD).

Immunohistochemistry for Apoptosis-Related Proteins and PCNA

Six semiserial sections were immunohistochemically stained for apoptosis-related proteins (p53 protein, c-*myc* protein, Lewis^y antigen, *fas* antigen, and Bcl-2 protein) as well as for PCNA to evaluate cell proliferative activity,[19] using the standard avidin-biotin-peroxidase method of Hsu et al.[20] In brief, after deparaffinization and the elimination of endogenous peroxidase activity, the sections were heated by a microwave oven as described by Shi et al[21] to enhance antigen retrieval. The sections were incubated with normal serum for 20 minutes. Then, the sections were treated at 4 C overnight with the following monoclonal antibodies: anti-p53 protein (DO-7, mouse IgG2 class, dilution 1:20, Dako Corp., Glostrup, Denmark), anti-c-*myc* protein (9E10, AB-1, mouse IgG1 class, 20 μg/ml, Oncogene Science, Uniondale, NY), anti-Bcl-2 protein (mouse IgG1 class, dilution 1:40, Dako Corp.), anti-Lewis^y antigen (BM-1, mouse IgM class, dilution 1:50, JIMRO, Takasaki, Japan), anti-*fas* antigen (UB2, mouse IgG1 class, 10 μg/ml, MBL, Nagoya, Japan), and anti-PCNA (PC10, mouse IgG2 class, dilution 1:100, Novocastra Lab, Newcastle upon Tyne, UK). In selected specimens, immunostaining of c-*myc* protein was also performed, using two additional monoclonal antibodies to synthetic peptide of c-*myc* (mouse IgG1 class, dilution 1:10, Chemicon Inc, Tomecula, CA; mouse IgG1 class, 30 μg/ml, Cambridge Research Biochemicals, London, UK). The sections were then incubated with biotinylated anti-mouse IgG or IgM (Vector Laboratories, Burlingame, CA) for 1 hour. Next, the sections were treated with the avidin-biotin-peroxidase complex (Vectastain ABC Kit, Vector Lab) for 1 hour. Reaction products were developed by immersing the sections in 3,3′-diaminobenzidine tetrahydrochloride containing 0.1% H_2O_2. Nuclei were lightly stained with methyl green. A negative control study was performed by replacing the primary antibodies with nonimmune serum or phosphate-buffered saline, which resulted in negative staining.

Evaluation of Apoptosis and Proliferating Cell Nuclear Antigen

In each specimen, 200 cells of developing bile ducts or hepatocytes were randomly selected, and the

number of apoptotic or PCNA-positive cells was counted. The positive rate of apoptotic cells was expressed as a percentage, and that of PCNA as PCNA labeling index (PCNA-LI). The PCNA-LI is the percentage of PCNA-positive cells to all examined cells. Statistical analysis was conducted by Student's *t*-test.

Classification of Intrahepatic Bile Duct Development

The process of intrahepatic bile duct development was classified into three stages: the ductal plate, the remodeling ductal plate, and remodeled bile ducts. The ductal plate is an excess structure consisting of a double-layered cylinder in the periportal hepatocytes. The remodeling ductal plate is the stage characterized by the incorporation of ductal plate cells into the mesenchyma as well as by the disappearing ductal plate. The remodeled bile ducts are characterized by newly formed bile ducts as well as by the disappearance of the ductal plate. The development of intrahepatic bile ducts proceeded from the hilar portions to the peripheral portions, and occasionally two or more of the developing stages of intrahepatic bile ducts were present in the same liver specimen.

Results

Apoptosis

Apoptosis was identified as nuclear staining by the modified TUNEL method. The number of apoptotic cells in the development of intrahepatic bile ducts and hepatocytes is shown in Figure 1. It was high in the remodeling ductal plate (Figure 2B), intermediate in the ductal plate (Figure 2A), and rather low in remodeled bile ducts (Figure 2C). Apoptotic cells were scattered among the developing hepatocytes (Figure 2D) and were also present in the mesenchyma and in hematopoietic cells.

Apoptosis-Related Proteins

The expression of apoptosis-related proteins is summarized in Table 1. The immunostaining using the three antibodies to c-*myc* protein showed the same staining patterns. During intrahepatic bile duct development, c-*myc* protein was expressed in the ductal plate (Figure 3A), the remodeling ductal plate (Figure 3B), and the remodeled bile ducts (Figure 3C), mainly in the cytoplasm. Developing hepatocytes were diffusely positive for c-*myc* protein.

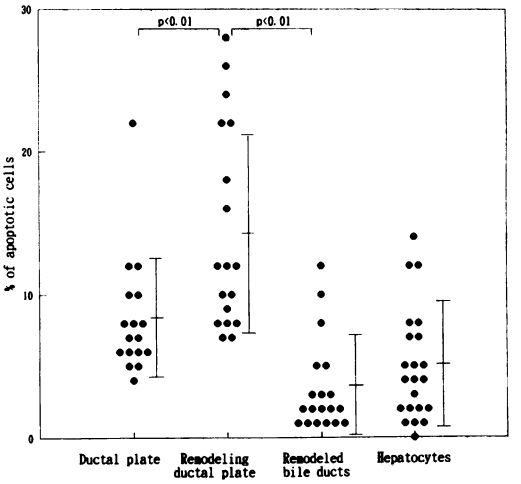

Figure 1. *Percentage of apoptotic cells during human liver development. The percentage is high in the remodeling ductal plate, intermediate in the ductal plate, and relatively low in remodeled bile ducts and hepatocytes. The percentage of apoptotic cells is significantly higher in the remodeling ductal plate than in the ductal plate and in remodeled bile ducts* ($P < 0.01$).

The expression of Bcl-2 protein was negative or faintly positive in the ductal plate and in the remodeling ductal plate, but was positive in the cytoplasm of the remodeled bile ducts. Developing hepatocytes were weakly and diffusely positive for Bcl-2 protein.

fas antigen was expressed in the cytoplasm of the ductal plate (Figure 4A), remodeling ductal plate (Figure 4B), and remodeled bile ducts (Figure 4C), although the expression in remodeled bile ducts was very weak (Figure 4C) and some cells were negative for *fas* antigen. Developing hepatocytes were diffusely positive for *fas* antigen.

Lewis^y expression was present in the cytoplasm of the ductal plate (Figure 5A), remodeling ductal plate (Figure 5B), and remodeled bile ducts (Figure 5C). The developing hepatocytes were weakly and focally positive for Lewis^y.

Nuclear p53 expression was not found in any cell types, but weak expression was noted in the cytoplasm of a few developing hepatocytes.

PCNA

As shown in Figure 6, the PCNA-LI was high in the remodeling ductal plate and developing hepatocytes, but relatively low in the ductal plate and remodeled bile ducts.

Discussion

The present study revealed that the frequency of apoptotic cells was high in the remodeling ductal plate,

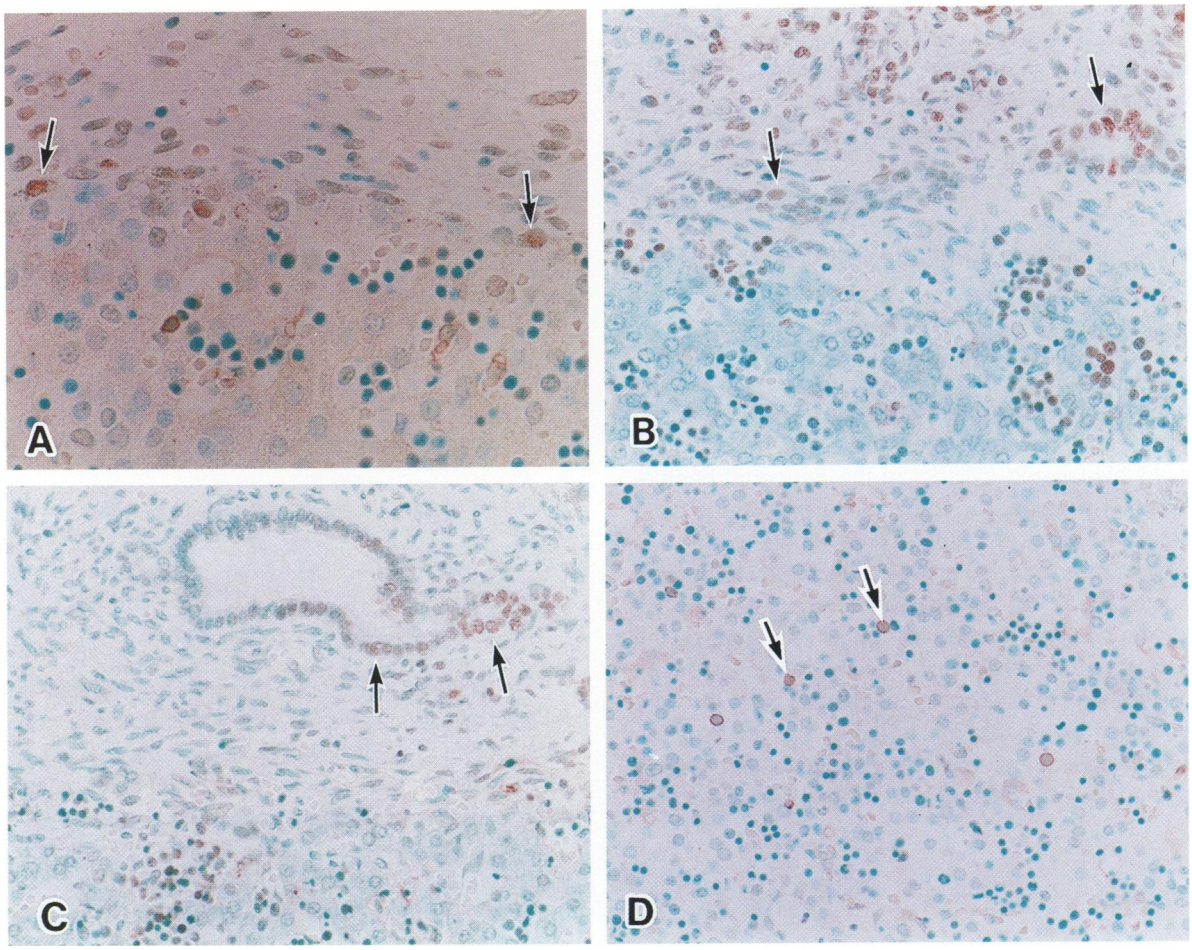

Figure 2. *Apoptotic cells during human fetal liver development are recognizable as nuclei stained by the TUNEL method. Cells of the ductal plate* (A), *remodeling ductal plate* (B), *and remodeled bile ducts* (C), *and immature hepatocytes* (D) *show apoptosis* (*arrows*). *A,* ×350; *B, C, D,* ×300.

Table 1. *Expression of Apoptosis-Related Proteins During Fetal Development of Intrahepatic Bile Ducts and Hepatocytes*

Apoptosis-related proteins	Expression site			
	Ductal plate	Remodeling ductal plate	Remodeled bile ducts	Hepatocytes
C-myc protein	++	++	++	++
Bcl-2 protein	±	±	++	+
Fas antigen	+++	++	+*	+++
Lewis^y	+	++	++	+
p53 protein	–	–	–	–

+++, strongly positive; ++, moderately positive; +, weakly positive; ±, negative or faintly positive; –, negative.
* very weak positivity in the luminal surface; some cells were negative.

moderate in the ductal plate, and relatively low in remodeled bile ducts. These findings suggest that the remodeling process of human intrahepatic bile duct development occurs by means of apoptosis, and that this process is marked in the remodeling stage of intrahepatic bile duct development. We also found that cell proliferative activity as documented by PCNA immunostaining was high during intrahepatic bile duct development, particularly in the remodeling ductal plate. This suggests that intrahepatic bile duct anlagens, particularly the remodeling ductal plate, have high cell proliferative activity. These findings indicate that the normal development of intrahepatic bile ducts involves marked cell proliferation and apoptosis. It is likely that some primitive biliary cells proliferate while others undergo apoptosis, and that their coordination results in normal intrahepatic bile duct development. The impaired regulation of apoptosis in the fetal development of intrahepatic bile ducts may lead to postnatal "ductal plate malformation."[4]

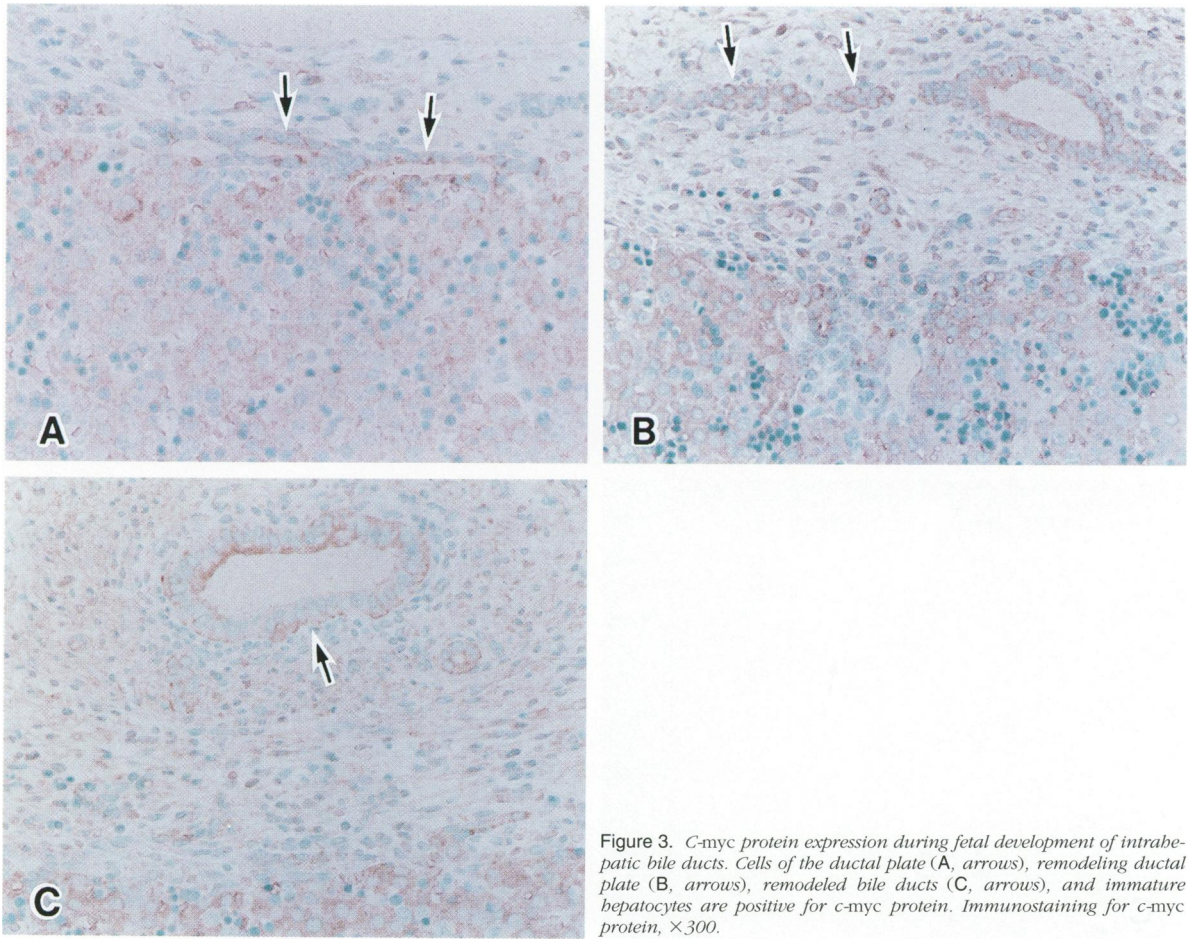

Figure 3. *C-myc protein expression during fetal development of intrahepatic bile ducts. Cells of the ductal plate (A, arrows), remodeling ductal plate (B, arrows), remodeled bile ducts (C, arrows), and immature hepatocytes are positive for c-myc protein. Immunostaining for c-myc protein, ×300.*

The present findings suggest that several apoptosis-related proteins are involved in apoptosis during intrahepatic bile duct development. C-*myc* protein and *fas* antigen were mainly expressed in the ductal plate and remodeling ductal plate, in which apoptosis was most marked. Since these proteins are stimulators of apoptosis,[13,15] their expression in the biliary anlagens may modulate apoptosis probably via the stimulative effect. The finding that their expression was frequent in the ductal plate and remodeling ductal plate, where apoptosis was most pronounced, may support this notion. Expression of *fas* antigen was stronger in the ductal plate than in the remodeling ductal plate, but apoptotic cells were more frequent in the remodeling ductal plate than in the ductal plate, suggesting that although *fas* antigen promotes apoptosis, other factors are involved in regulation of apoptosis during intrahepatic bile duct development. Although c-*myc* protein is usually present in the nucleus, its expression was largely cytoplasmic in this study. The immunostaining using three antibodies to c-*myc* protein showed the same staining pattern, and negative control study showed no staining, suggesting that this cytoplasmic staining was due to the diffusion of nuclear c-*myc* protein into the cytoplasm. However, it is possible that the cytoplasmic staining of c-*myc* protein is a nonspecific reaction.

We found that Bcl-2 protein was positive in remodeled bile ducts where apoptosis was not pronounced, but it was negative or faintly positive in the ductal plate and remodeling ductal plate where apoptosis was marked. As this protein is an inhibitor of apoptosis,[17] its expression in remodeled bile ducts may inhibit apoptosis during intrahepatic bile duct development. The negative or faint expression of Bcl-2 protein in the ductal plate and remodeling ductal plate, where apoptosis was most pronounced, may indicate that apoptosis in these bile duct anlagens is stimulated by the negative or faint expression of Bcl-2 protein.

Hiraishi et al[17] have recently reported that Lewis^y-positive cells, which were identified by the BM-1 antibody, frequently showed cell damage and apoptosis, and suggested that Lewis^y expression may be followed by apoptotic cell death. In the present study, Lewis^y antigen as detected by the BM-1 antibody was expressed in the ductal plate, remodeling ductal

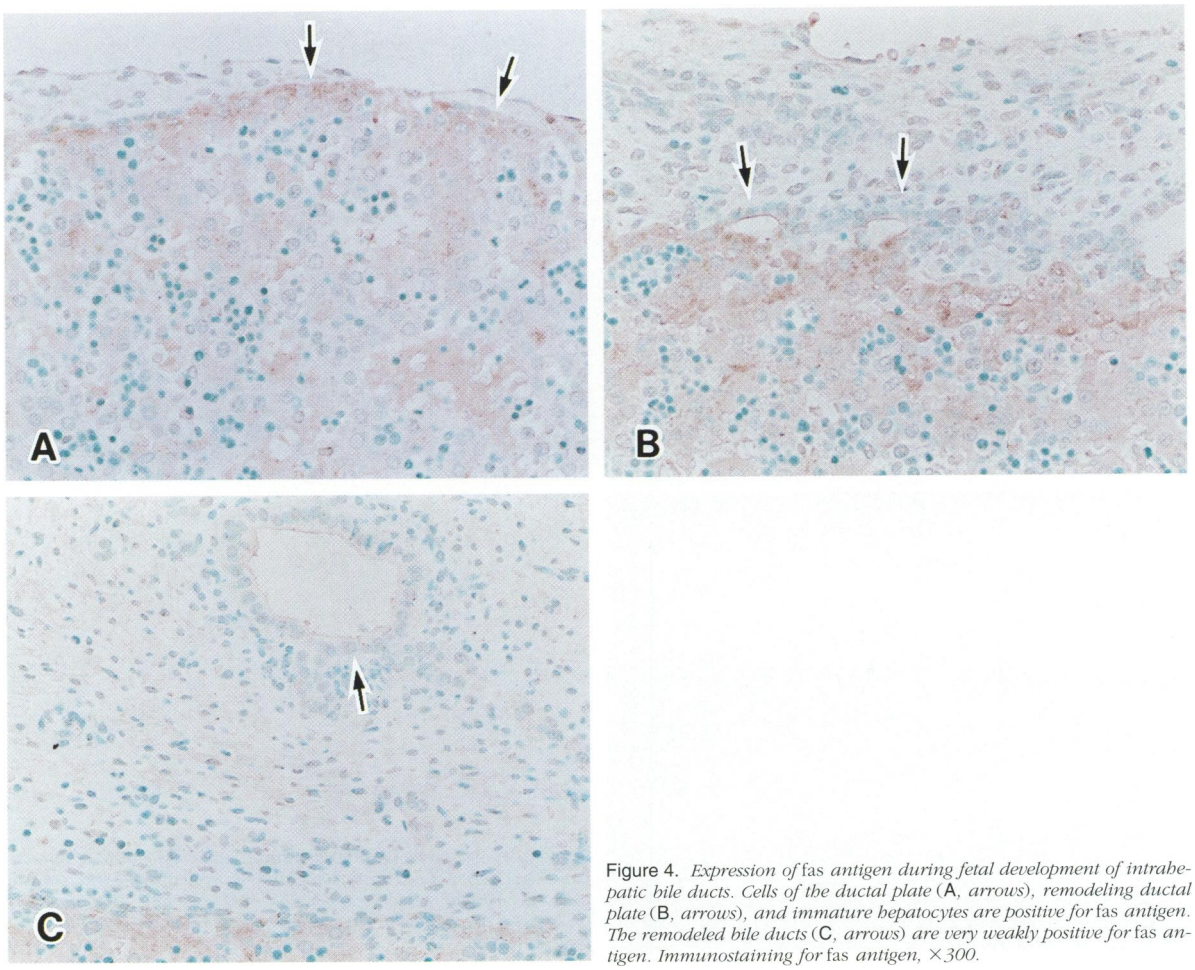

Figure 4. *Expression of* fas *antigen during fetal development of intrahepatic bile ducts. Cells of the ductal plate* (A, *arrows*), *remodeling ductal plate* (B, *arrows*), *and immature hepatocytes are positive for* fas *antigen. The remodeled bile ducts* (C, *arrows*) *are very weakly positive for* fas *antigen. Immunostaining for* fas *antigen,* ×*300.*

plate, and remodeled bile ducts. Its expression in the remodeling ductal plate where apoptosis is marked seems to support the hypothesis of Hiraishi et al.[17] However, Lewis^y antigen was also expressed in the ductal plate and remodeled bile ducts and weakly and focally in immature hepatocytes, where the frequency of apoptosis was relatively low, suggesting that other factors are involved in apoptosis during fetal development of human intrahepatic bile ducts and hepatocytes.

Nuclear expression of p53 protein, which is a potent stimulator of apoptosis under conditions such as cancer,[14] was not detected in any cell type, suggesting that p53 protein also has no effect on apoptosis during human intrahepatic bile duct development.

During hepatocyte development, many apoptotic and PCNA-positive cells were found, suggesting that hepatocyte development progresses via coordinated cell proliferation and apoptosis. Expression of c-*myc* protein, Bcl-2 protein, *fas* antigen, and Lewis^y antigen was found in developing hepatocytes, suggesting that these apoptosis-related proteins regulate apoptosis during the hepatocyte development.

In conclusion, the present study suggests that apoptosis and cell proliferation are involved during the development of human intrahepatic bile ducts and hepatocytes. In addition, c-*myc* protein, *fas* antigen, Bcl-2 protein, and Lewis^y antigen may modulate apoptosis of intrahepatic biliary cells and hepatocytes probably by stimulatory or inhibitory effects. An imbalanced apoptosis and cell proliferation during the fetal development of intrahepatic bile ducts, which may be caused by apoptosis-related proteins, may lead to "ductal plate malformation."

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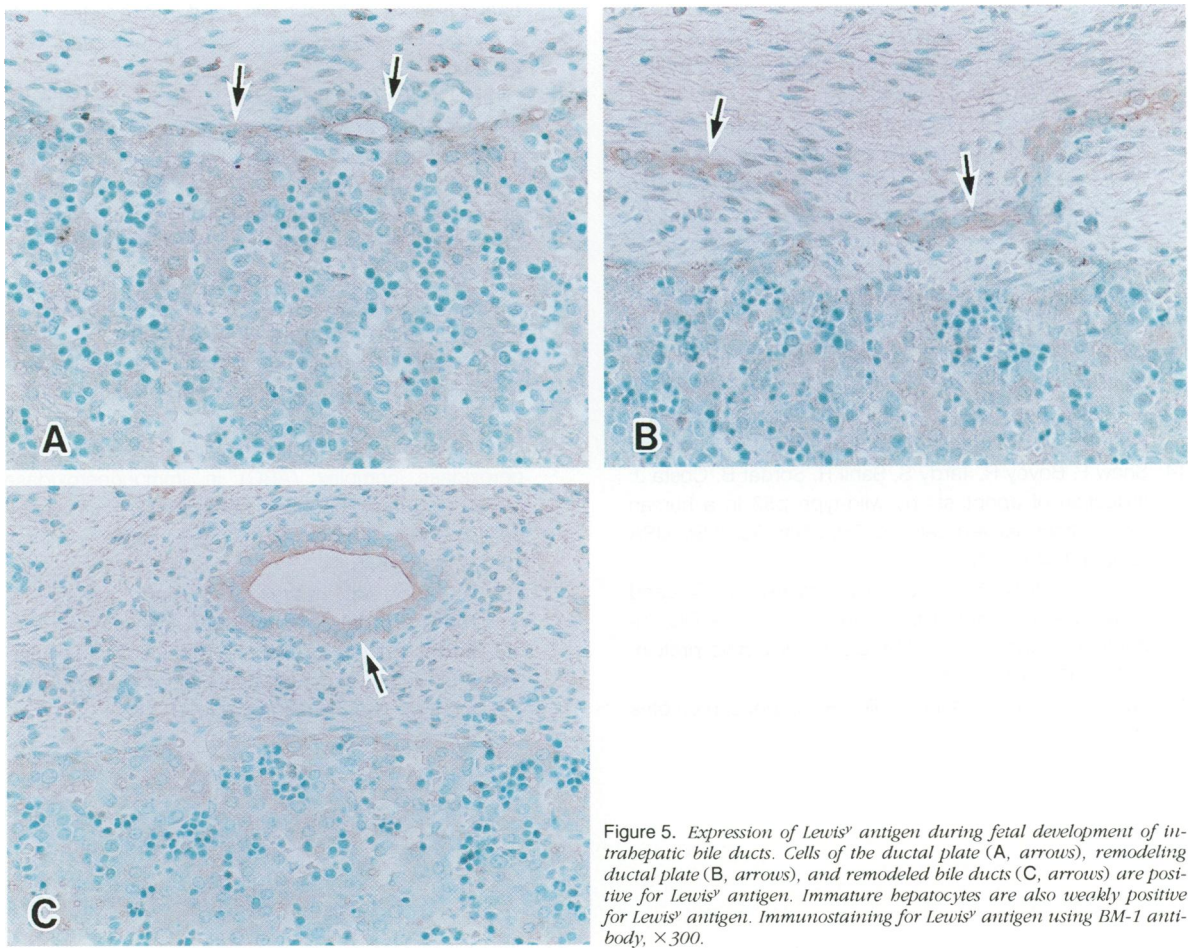

Figure 5. *Expression of Lewis^y antigen during fetal development of intrahepatic bile ducts. Cells of the ductal plate* (A, *arrows*), *remodeling ductal plate* (B, *arrows*), *and remodeled bile ducts* (C, *arrows*) *are positive for Lewis^y antigen. Immature hepatocytes are also weakly positive for Lewis^y antigen. Immunostaining for Lewis^y antigen using BM-1 antibody,* ×*300.*

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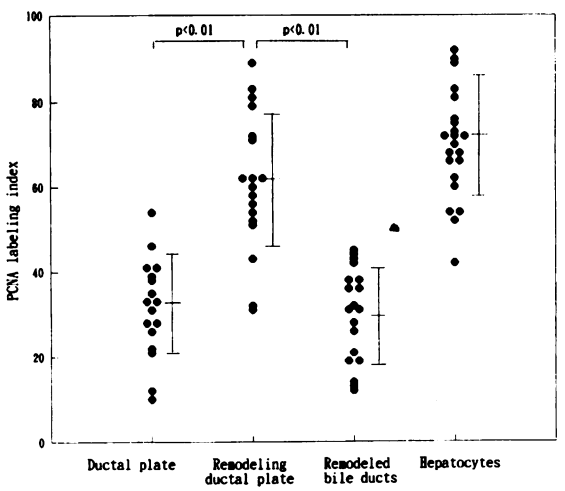

Figure 6. *The PCNA labeling index during human fetal liver development. The PCNA labeling index is high in the remodeling ductal plate and developing hepatocytes, whereas it is relatively low in ductal plate and remodeled bile ducts. The PCNA labeling index is significantly higher in the remodeling ductal plate than in the ductal plate or in remodeled bile ducts* ($P < 0.01$).

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