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Review

Recent progress in the etiopathogenesis of pediatric biliary disease, particularly Caroli's disease with congenital hepatic fibrosis and biliary atresia

Yasuni Nakanuma[1], Kenichi Harada[1], Yasunori Sato[1] and Hiroko Ikeda[2]

[1]Department of Human Pathology, Kanazawa University Graduate School of Medicine, and [2]Department of Diagnostic Pathology, Kanazawa University Hospital, Kanazawa, Japan

Summary. Recent progress in elucidating the etiopathogenesis of pediatric biliary diseases, particularly Caroli's disease with congenital hepatic fibrosis (CHF) and biliary atresia (BA), is reviewed. The former is characterized by multiple saccular dilatations of the intrahepatic bile ducts. An animal model of this disease, the PCK rat, is being extensively studied. PCK rats and Calori's disease with CHF belong to autosomal recessive polycystic kidney disease (ARPKD) with ductal plate malformation. Mutations of *PKHD1* have been identified in ARPKD, and fibrocystin, a product of *PKHD1* located in the cilia of bile ducts is lacking in the pathologic intrahepatic bile ducts of ARPKD. Disordered cell kinetics, including apoptosis of biliary epithelial cells (BECs), may be significantly related to ductal plate malformation, and laminin and type IV collagen were immunohistochemically reduced in the basement membrane of intrahepatic bile ducts of ARPKD, and such a reduction is an additional factor for the dilatation of bile ducts. Abundant connective tissue growth factor retained diffusely in heparan sulfate proteoglycan in the fibrous portal tracts are responsible for non-resolving hepatic fibrosis. In addition, pathologic BECs of ARPKD may acquire mesenchymal features and participate in progressive hepatic fibrosis by producing extracellular matrix molecules. In an animal model of BA, an initial virus-induced, T-cell mediated autoimmune-mediated cholangiopathy has been reported. In human BA, virus-induced apoptosis of BECs by a TNF-related apoptosis-inducing ligand followed by the progressive obliteration of bile ducts is also suggested, and epithelial mesenchymal transition of BECs induced by viral infection may be involved in the fibrotic process in sclerosing cholangitis. However, the role of viral infections in the affected tissues is controversial. Comprehensive and analytical studies of ARPKD and BA using human materials and animal models may lead to the clarification of their etiopathogenesis and open the way for new therapeutic strategies.

Key words: Intrahepatic bile duct, Cystogenesis, Hepatic fibrosis, Innate immunity, Epithelial mesenchymal transition

Offprint requests to: Yasuni Nakanuma, M.D., Department of Human Pathology, Kanazawa University Graduate School of Medicine, Kanazawa 920-8640, Japan. e-mail: pbcpsc@kenroku.kanazawa-u.ac.jp

Introduction

Recently, there has been considerable progress in elucidating the etiopathogenesis of Caroli's disease with congenital hepatic fibrosis (CHF) and biliary atresia (BA). In the former, the gene product responsible, fibrocystin, is being clarified (Masyuk et al., 2003; Zhang et al., 2004), and by using several animal models, particularly the polycystic kidney (PCK) rat, the pathogenesis of bile duct lesions is being explored (Sanzen et al., 2001; Sato et al., 2005). In the latter, viral infection and its related sequela, such as innate and acquired immunity, are now being clarified by using human cases and animal models (Mack et al., 2006; Harada et al., 2007). There are two types of BA, embryonic and perinatal. As for the pathogenesis of perinatal BA, the most common type, an initial virus-induced, progressive T cell-mediated inflammatory obliteration of bile ducts which has been demonstrated in an animal model of BA, is now being tested.

Herein, the pathogenesis of these biliary diseases, particularly their bile duct lesions, is reviewed. First, the anatomy and development of the intrahepatic biliary tree are briefly reviewed for a better understanding of these diseases.

Anatomy and development of the intrahepatic biliary tree

Anatomy

In humans, the ducts proximal to the right or left hepatic duct can be classified into two main categories: intrahepatic large and small bile ducts (Nakanuma et al., 1997). The former consists of the segmental ducts, the area ducts, and the first and second branches of the area ducts and are grossly visible. The septal and interlobular bile ducts, which are visible only microscopically, are finer branches of large intrahepatic bile ducts. Like the large intrahepatic ducts, the septal ducts are surrounded by a layer of collagenous wall. In contrast, the interlobular bile ducts are lined by cuboidal cells and are connected to the bile canalicular network by ductules or the canals of Hering.

The biliary tree of rats is divided into extrahepatic and intrahepatic bile ducts: the former refers to the biliary tree that derives from the duodenum, and the latter are the branches of the extrahepatic bile ducts located within the hepatic parenchyma. Smaller bile ducts, including bile ductules, are included in the intrahepatic bile duct in this study (Sanzen et al., 2001).

Development

Human intrahepatic bile ducts arise from the ductal plate, a double-layered cylindrical structure at the interface between the portal mesenchyme and primitive hepatocyts (Terada et al., 1997). The ductal plate first appears from primitive hepatocytes at around 8 gestational weeks and undergoes remodeling; some parts of the ductal plate disappear while other parts migrate into the portal mesencyme (remodeled bile ducts) (Desmet, 1992) During this remodeling, apoptosis and cell proliferation occur with the expression of apoptosis-related proteins. Fas and c-myc were constantly detected in the ductal plate, remodeling ductal plate and remodeled ducts. Bcl-2 which was not detected in the ductal plate and remodeling ductal plate, was apparently present in remodeled ducts. These findings suggest that c-myc, Fas, and Bcl-2 modulate the apoptosis of fetal intrahepatic biliary epithelial cells (BECs), probably via stimulative (c-myc and Fas) or inhibitory (Bcl-2) effects (Terada and Nakanuma, 1995).

In rats, immature biliary cells around the portal vein assumed bile ductule-like structures with a slit-like lumen, appearing as pearl-like structures at 17 days of gestation (Van Eyken et al., 1988). In neonates after birth, these immature biliary elements between portal tracts and hepatic lobules begin to bud into the portal mesenchyme. In neonates from 7 to 42 days after birth, pearl-like structures generally disappear and the bile ducts and bile ductules are present in the portal tracts.

An anatomical structure exactly corresponding to the ductal plate, which forms during the development of human intrahepatic bile ducts, is not seen in rodents. Instead, pearl-like structures equivalent to the ductal plate occur in rats. They undergo similar remodeling processes in the development of the intrahepatic bile ducts. Therefore, the changes to pearl-like structures and intrahepatic bile ducts in rats resembling ductal plate malformation in humans are called “ductal plate malformation” in this study.

Caroli’s disease and CHF

Autosomal recessive polycystic kidney disease (ARPKD), which is characterized by the fusiform dilatation of collecting renal tubules and by biliary dysgenesis and hepatic fibrosis with an incidence of 1 in 20,000 live births, is known to show variable clinical manifestations. CHF and Caroli’s disease with CHF are representative of ARPKD (Desmet, 1992; Ozaki et al., 2005).

CHF

CHF is a rare disease and mainly manifests during childhood and youth. The long-term prognosisis poor. CHF patients usually present with portal hypertension, which may lead to bleeding from gastroesophageal varices. CHF is characterized by an overgrowth of portal connective tissue and tortuous and abnormally shaped dilated bile ducts and ductules in enlarged portal tracts at the microscopic level (McDonald and Avener, 1991; Nakanuma et al., 1982). The hepatic parenchyma is subdivided by the overgrowth of portal fibrous tissue, while the regenerative activities of subdivided parenchyma are not evident, thus differing from cirrhotic regenerative nodules. In addition, portal vein branches are hypoplastic or unidentifiable in the fibrous portal tracts.

Caroli’s disease

Caroli´s disease is characterized by congenital, progressive multiple and segmental saccular or cystic dilatations CHF with Caroli’s disease (Summerfield et al., 1986; Desmet, 1992). CHF itself is frequently associated with grossly visible saccular or cystic dilatations of the intrahepatic bile ducts (Fig. 1A). Caroli’s disease without CHF is rarely reported. The age of onset of the initial symptoms of Caroli’s disease has been reported to range from a few weeks to 60 years. Saccular dilatations of the intrahepatic bile ducts in Caroli’s disease with CHF are a predisposing factor in the stagnation of bile and bacterial infection associated with chronic cholangitis, the formation of stones, and secondary biliary fibrosis, and this condition may give rise to cholangiocarcinoma (Ikeda et al., 2007; Yamato et al., 1998). Chronic cholangitis is frequently associated with goblet cell metaplasia and the aberrant expression of mucus core protein-2 (MUC2).

The PCK rat is an animal model of Caroli's disease and CHF

The polycystic kidney (PCK) rat recently reported by Katsuyama et al. (2000), is a spontaneous mutant derived from a colony of Crj:CD rats showing polycystic lesions in the liver and kidneys and an autosomal recessive inheritance. Interestingly, these hepatic polycysts were found to be multiple segmental and saccular dilatations of the intrahepatic bile ducts (Fig. 1B) (Sanzen et al., 2001). Histologically, irregular dilatations with pearl-like structures were observed around the portal veins in the fetal livers and these dilated biliary structures frequently surrounded the portal connective tissue. The dilatation spread throughout the liver and the degree of dilatation increased with aging. Overgrowth of portal connective tissue associated with biliary lesions also became evident after birth and increased thereafter. In addition, the hepatic parenchyma was subdivided by fibrously enlarged portal tracts with a preserved hepatic lobular pattern. These features are identical to those of Caroli's disease with CHF (Nakanuma et al., 1982; McDonald and Avner, 1991).

Ductal plate malformation in Caroli's disease with CHF and PCK rats

Excessive immature biliary elements, including ductal plate in the humans and pearl-like structures in rats, disappear during remodeling of the normal intrahepatic biliary tree (Van Eyken et al., 1988; Terada et al., 1995). However, some hepatic fibropolycystic diseases, particularly ARPKD consisting of CHF and Caroli's disease with CHF, show a failure of remodeling of the ductal plate, persistence of ductal plates and their dilatation and proliferation after birth (Desmet, 1992). These characteristic features are known collectively as "ductal plate malformation". The following findings are regarded as indications of ductal plate malformation: i) bulbar protrusions of the duct wall to the lumen, ii) bridge formation of the duct wall across the lumen, and iii) duct lumen surrounding part of the portal tract fibrous tissue (Nakanuma et al., 1982). These features are found, grossly as well as microscopically, in the hepatobiliary lesions in PCK rats, and also in cases of Calori's disease with CHF (Fig. 2A,B), suggesting that ductal plate malformation is involved in the pathogenesis of these hepatobiliary lesions, including saccular and segmental dilatations of the biliary tree. Taken together, the PCK rat is regarded as an animal model of slowly progressive ARPKD (Sanzen et al., 2001). In this review, Caroli's disease with CHF and PCK rats are collectively called as ARPKD

Genetic alterations in ARPKD

Fibrocystin and PKHD

Recently, mutations to *PKHD1* have been identified in cases of ARPKD, including Caroli's disease with CHF as well as the PCK rats. These genetic changes are now regarded as principle changes in both ARPKD patients and PCK rats. It seems likely that the liver and kidney lesions in ARPKD patients and in PCK rats are related to these mutations to *PKHD1* (Masyuk et al., 2003; Harris and Rosetti, 2004; Zhang et al., 2004). In

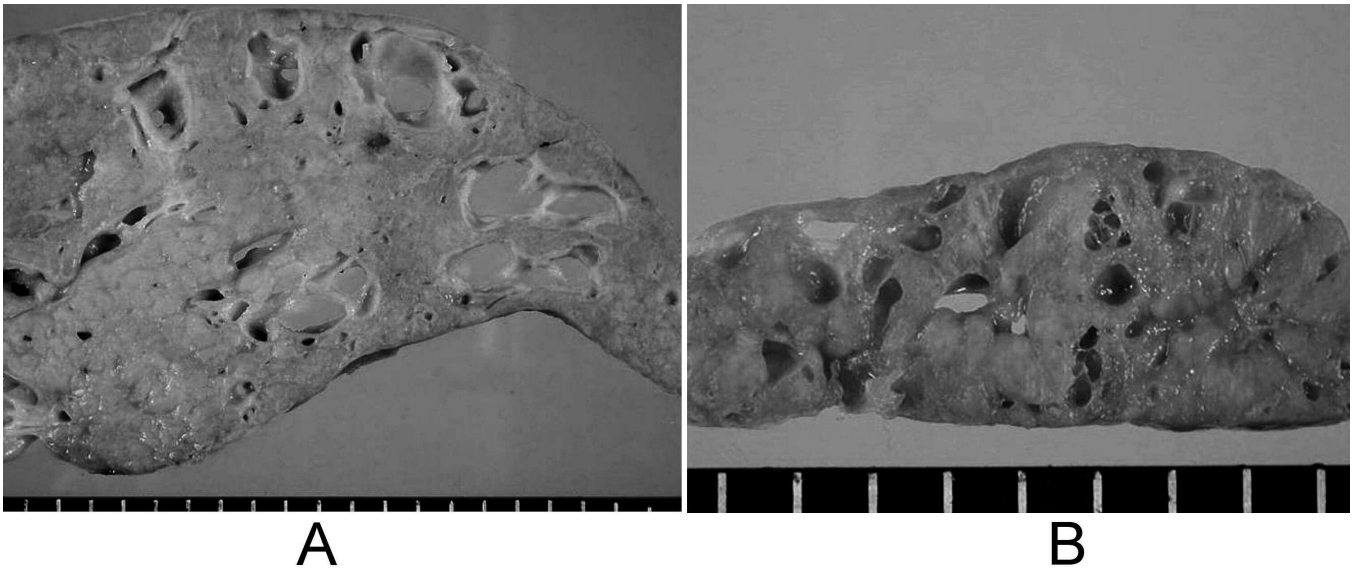

Fig. 1. Gross findings of Caroli's disease with congenital hepatic fibrosis **(A)** and the polycystic kidney rat **(B)**. Both livers show cystic dilatation of the intrahepatic bile ducts and fibrosis.

the PCK rat, a spontaneous splicing mutation of *Pkhd1* initiates the development of hepatic cysts. *PKHD1* is a large gene from which multiple transcripts may be generated by alternative splicing. Developing and mature intrahepatic bile ducts express the *PKHD1* protein, fibrocystin, whereas bile ducts of ARPKD patients do not. Mice with a targeted mutation of *PKHD1* also develop cystic biliary dysgenesis and portal fibrosis. However, the exact causal relations, including pathogenesis between mutations to *PKHD1* and the pathologic conditions including the hepatobiliary dysgenesis in ARPKD patients and the PCK rats, remains unclear.

Pathogenesis of bile duct lesions in ARPKD

There are several hypotheses on the pathogenesis of bile duct lesions, including cystogenesis of Caroli's disease with CHF and PCK rats (ARPKD).

Defects in fibrocystin expression and ciliary structure

Recent studies have shown that the ARPKD protein fibrocystin is localized to the primary cilia of renal epithelial cells and is often absent in ARPKD tissue, while the mechanisms for the involvement of cilia in PKD-related biliary cystogenesis remain speculative (Masyuk et al., 2003; Zhang et al., 2004). In the PCK rat, BECs possess short and malformed cilia that do not express fibrocystin. In intrahepatic bile ducts of normal rats, each BEC has a single cilium that expresses fibrocystin, whereas the cilia of PCK rats are short and malformed and do not express fibrocystin. The cultured BECs of PCK rats grown on collagen formed a polarized monolayer with well-developed junctional complexes, and distinct apical and basolateral membranes. Compared to the BECs of normal rats, the cultured BECs of PCK rats had short and malformed cilia that did not express fibrocystin. A link between the development of cysts and ciliary dysfunction attributable to the lack of fibrocystin has been suggested, although the precise role of fibrocystin in cyst development remains unclear.

Disordered cell kinetics of BECs

The normal development of intrahepatic bile ducts requires a balance between the proliferation and apoptosis of BECs. Our recent study showed that the proliferative activity of BECs was greater in PCK rats than controls during development (Sanzen et al., 2001). In contrast, apoptosis of BECs was less extensive in PCK rats than the controls until 1 week after delivery, but greater after 3 weeks. Cultured BECs of PCK rats also exhibited increased proliferation, and interestingly, the cultured BECs of PCK rats seeded in collagen gel formed cystic structures which expanded progressively. However, the cysts formed by BECs of normal rats remained the same size (Sato et al., 2005).

Epidermal growth factor (EGF) is a major participant in the proliferation and differentiation of epithelial cells. By binding to EGF receptor (EGFR), EGF activats mitogen activated protein kinases (MAPKs). which relay signals from the cell membrane to the nucleus. The MAPK pathway consists of three proteinkinases that act sequentially within the pathway: a MAPK kinase kinase, a MAPK/ERK (extracellular signal-regulated protein kinase) kinase (MEK), and a MAPK (ERK) (Kato et al., 1998). In murine ARPKD

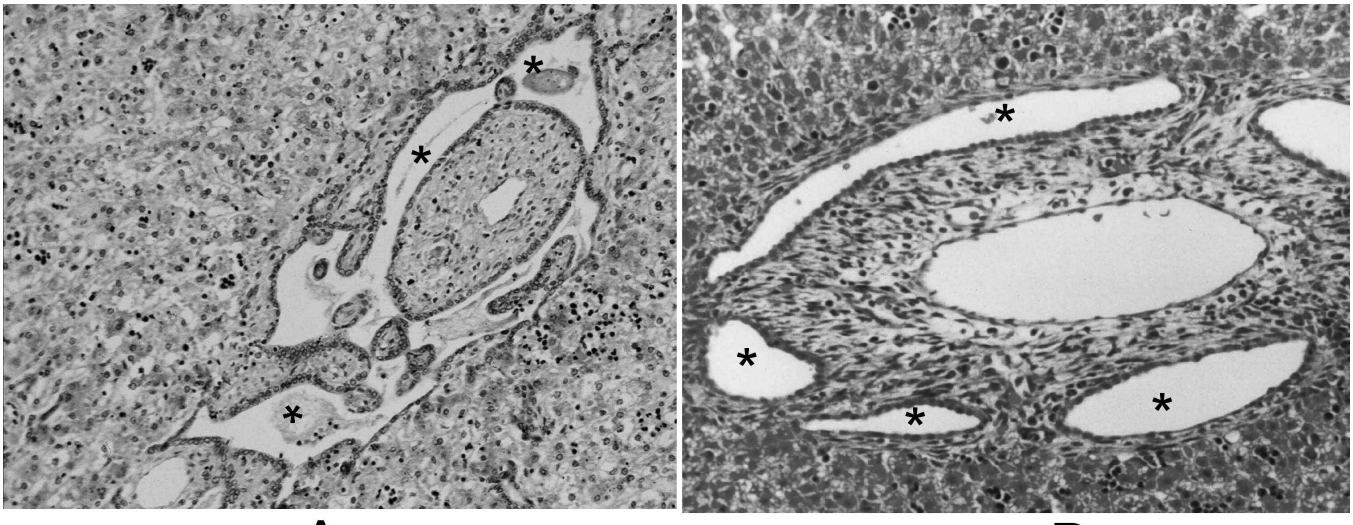

Fig. 2. Ductal plate malformation in Caroli's disease with congenital hepatic fibrosis **(A)** and the polycystic kidney rat **(B)**. Dilated and tortuous bile ducts (*) are found at the boundary of the portal tract.

models, abnormal expression of EGF and EGFR has been implicated in renal cystogenesis, and Nauta et al. (1995) demonstrated that BECs obtained from mice with ARPKD were hyperresponsive to EGF. Thse findings suggest that activation of the MAPK pathway may also be involved in the development of cystogenesis in the liver of PCK rats.

Interestingly, the increased proliferation of cultured BECs of PCK rats was accompanied by overexpression of MEK5, and subsequent phosphorylation of ERK5 (Sato et al., 2005). BECs of PCK rats diffusely expressed EGFR, and BECs isolated from the PCK rat wre hyperrreactive to EGF, which was accompanied by the activation of the MAPK pathway consisting of MEK5/ERK5. Thus, the involvemnt of the MEK5-ERK5 pathway seems essential in the abnormal growth of BECs of PCK rat. An EGFR tyrosine kinase inhibitor, gefitinib, also significantly inhibited the abnormal growth of cultured BECs of PCK rats *in vitro*, suggesting theat the MEK5/ERK5 pathway was was activated in the BECs of PCK rats. Activation of EGFR is known to trigger numerous downstream signaling pathways, such as ERK/MAPK and the phospho-inositide-3-kinase (PI13K)/Akt pathways, suggesting that the activation of the MEK5-ERK5 cascade plays a pivotal role in the biliary dysgenesis of PCK rats, and also the cystogenesis in cases of Caroli's disease with CHF.

Blockage of the EGFR signaling pathways is reported to result in the induction of apoptosis in EGFR-expressing cells. Our previous study showed that apoptosis was induced more frequently in the BECs of PCK rats by treatment with gefitinib (Sato et al., 2005). Because MEK5 is overexpressed in the BECsx of PCK rats, the cells may have a more sustainable apoptotic response, as well as show growth inhibition by gefitinib through the inhibition of the MEK5/ERK5 cascade.

Despite the strong inhibitory effects of gefitinib on the formation of biliary cystis *in vitro*, the administration of gefitinib to PCK rats resulted in incomplete inhibitory effects on the cystic dilatation of the intrahepatic bile ducts (Sato et al., 2006). The proliferation of BECs seems not to be simply mediated by the EGF/TGF/EGF-R pathway in the pathogenesis of PKD, indicating that the abnormal proliferation of BECs of PCK rats is not simply attributable to the activation of EGFR, and other factors may contribute to the pathogenesis *in vivo*.

EGFR mRNA and protein were similarly observed in the cultured BECs and in the BECs of liver tissue of both control and PCK rats. However, proliferative activities of the BECs in PCK rats are much stronger than those of the cells in control rats, suggesting that another factor is imporant in the proliferation of BECs of PCK rats. Immunohistochemically, mast cells located around the bile ducts were also found to be positive for EGF and have also been shown to express abundant EGF mRNA in the rat (Sato et al., 2005). These EGF-expressing mast cells were concentrated around small and large bile ducts of PCK rats, suggesting that they are involved in the proliferation of BECs and dilatation of bile ducts and mast cell-derived EGF and EGFR expressed on BECs is inolved in cystogenesis in the PCK rat.

Basement membranes and cystogenesis and biliary dysgenesis in ARPKD

For the development of intrahepatic bile ducts, the coordinated expression of basal laminar components such as laminin and type IV collagen and proteolytic enzymes is essential (Shiojiri and Sugiyama, 2004; Yasoshima et al., 2009). In normal livers, laminin and type IV collagen are invariably expressed in the basement membrane of intrahepatic bile ducts. Interestingly, the expression of laminin and type IV collagen around the dilated intrahepatic bile ducts was reduced or lost in Caroli's disease with CHF and the PCK rats, and the bile ducts with reduced expression of laminin and type IV collagen showed progressive dilatation with age after birth in PCK rats, while the expression of laminin and type IV collagen tended to be maintained around small bile ducts or non-dilated bile ducts.

It seems possible that the degradation of matrix proteins of the basement membrane, particularly laminin and type IV collagen, is related to the biliary dysgenesis in ARPKD (Yasoshima et al., 2009). Most of the proteolytic enzymes involved in these processes belong to the matrix metalloproteinases (MMPs) and the serine proteinases, in particular the plasminogen activator (PA)/plasmin system. Furthermore, plasmin contributes to activation of the zymogens of MMP-9 and MMP-13, which in turn degrade basement membrane components, including type IV collagen.

Interestingly, the amounts of laminin and type IV collagen in the gel used for the culture of BECs of PCK rats were significantly reduced. Furthermore, mRNA and protein of tissue-PA (tPA) and plasminogen were also overexpressed in cultured BECs of PCK rats. The addition of 2-antiplasmin, which inactivates tPA, to the culture medium inhibited the degradation of laminin and type IV collagen in the gel by the BECs of PCK rats. In vivo, immunostaining using frozen liver sections showed increased expression of tPA in the BECs of PCK rats at all ages, suggesting that biliary overexpression of plasminogen and tPA leads to the increased generation of plasmin, followed by plasmin-dependent lysis of laminin and type IV collagen, and then may contribute to the biliary dysgenesis in Caroli's disease with CHF and PCK rats (Yasoshima et al., 2009).

Other factors related to cystogenesis in PCK rats

The abnormal expression and location of ion transporters and water channels in BECs of PCK rats may also be related to hepatic cystogenesis. In the cultures using collagen gel, the BECs from PCK rats formed bigger cysts than cysts formed by normal BECs

under basal conditions and in response to secretin and hypotonicity. In normal BECs, the water channel aquaporin-1 (AQP1), the chloride channel cystic fibrosis transmembrane conductance regulator (CFTR), and the anion exchanger AE2 account for ion-driven water transport. In PCK rats, expressions of these three proteins are increased in BECs of PCK rats in comparison with BECs of normal rats. Hepatic cystogenesis in ARPKD may involve the increased accumulation of fluid because of the overexpression and abnormal location of AQP1, CFTR, and AE2 in cystic BECs (Banales et al., 2008).

The PCK rat is known to develop BEC-derived cysts associated with increased levels of intracellular adenosine 3',5'-cyclic adenosine monophosphate (cAMP). Recently, it was shown that the cAMP effectors Epac and PKA are also involved in the hepatic cystogenesis of PCK rats. In PCK rats, elevated cAMP levels stimulate the proliferation of BECs via two downstream effectors, the cAMP effectors Epac and PKA, and intracellular calcium is also involved in the hepatic cystogenesis of PCK rats (Banales et al., 2009).

Mechanism of portal fibrosis of the liver in ARPKD

Overgrowth of portal fibrous connective tissue is another feature of ARPKD. Abundant connective tissue growth factor (CTGF) reatained diffusly in heparan sulfate proteoglycan (HSPG) in the fibrous portal tracts may be responsible for non-resolving hepatic fibrosis in CHF (Fig. 3A,B) (Ozaki et al., 2005). Portal mononuclear cells and endothelial cells were positive for HSPG mRNA, and mononuclear cells were positive for CTGF mRNA, and such cells were accentuated around proliferated bile ducts in CHF. BECs of proliferating bile ducts also reportedly participate in the fibrotic process by reproducing CTGF. CTGF can promote fibrosis by triggering the proliferation of fibroblast and up-regulating ECM production.

In most types of chronic liver diseases with continuous hepatocellular damage, activated hepatic stellate cells (HSCs) play major roles in hepatic fibrosis. However, necroinflammatory changes and activated HSCs are lacking in hepatic parenchyma in ARPKD, suggesting that the hepatic fibrosis is mediated by other cell types, such as portal myofibroblasts. This effect was accompanied by the reduced expression of CTGF mRNA in the bile ducts, suggesting the involvement of pathological BECs in the progressive portal fibrosis in PCK rats (Sato et al., 2006).

In addition, BECs of the PCK rat may acquire mesenchymal features in response to TGF-ß1 and participate in progressive hepatic fibrosis. In several chronic fibrotic disorders, epithelial cells acquire mesenchymal features, thereby contributing to the fibrogenic process, a phenomena known as epithelial-mesenchymal transition (EMT) (Liu, 2004). A recent study has demonstrated that BECs can undergo EMT, thereby contributing to hepatic fibrosis (Harada et al., 2009). Intrahepatic bile ducts of the PCK rat have two phenotypes: bile ducts lined by cuboidal-shaped (C-type) and flat-shaped BECs (F-type) (Sato et al., 2007). F-type BECs showed reduced expression of CK-19 and positive immunoreactivity for vimentin and fibronectin, and appeared to increase with age. The BECs of F-type contribute to progressive hepatic fibrosis of the PCK rat. However, E-cadherin expression was not reduced, and α-SMA expression was not observed in F-type bile ducts *in vivo*. TGF-ß initiates morphological transition of the cells from an epithelial to a fibroblastic appearance,

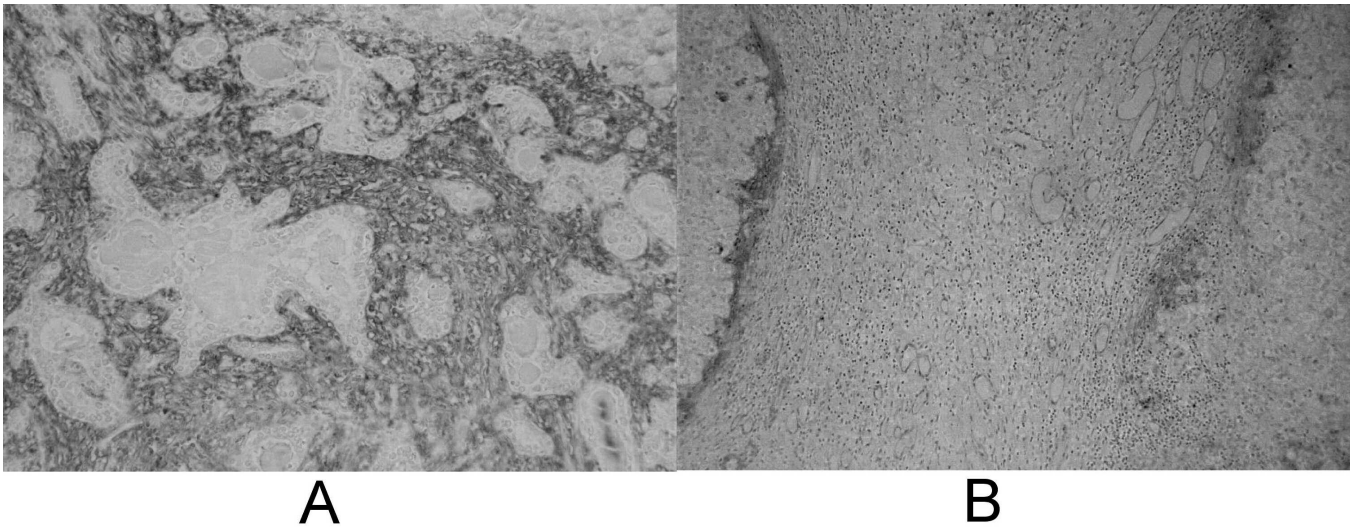

Fig. 3. Immunostaining of connective tissue growth factor (CTGF). **A.** Much deposition of CTGF in fibrous tissue in cases of of Caroli's disease with congenital hepatic fibrosis. **B.** CTGF is only found at the interface of chronic viral hepatitis C at the cirrhotic stage.

accompanied by a loss of epithelial cell markers and a gain of mesenchymal cell markers. Our recent study showed that TGF-ß1 reduced CK19 expression in the BECs of PCK rats, but the epithelial cell phenotype characterized by E-cadherin was not significantly affected by TGF-ß1. TGF-ß1 induced the expression of vimentin, collagen, and fibronectin in cultured BECs of PCK rats, although α-SMA expression was not induced in the BECs. These findings are consistent with the immunophenotype of F-type bile ducts *in vivo* (Sato et al., 2007). Thus, TGF-ß1 does not necessarily induce myofibroblast transdifferentiation in the BECs of PCK rats.

Immunohistochemically, TGF-ß receptor (TßR)-I and TßR-II are detected in both C- and F-type bile ducts *in vivo* (Sato et al., 2007). The cultured BECs of PCK rats expressed an increased amount of TßR-I mRNA. Although we have proposed the acquired nature of the development of F-type bile ducts in the PCK liver during aging, it remains unclear why a selected portion of intrahepatic bile ducts of the PCK rat underwent dedifferentiating events while acquiring mesenchymal phenotypes. Interestingly, many BECs of F-type bile ducts showed immunohistochemical expression of pSmad2, suggesting the transmission of TGF-ß signals from the cell surface into the nucleus.

Biliary atresia

BA is characterized by a progressive fibro-obliterative lesion of the extrahepatic biliary and subsequent cholestatic liver damage with a progressive loss of intrahepatic bile ducts (Fig. 4A,B). There are two main types in BA: embryonic (fetal or prenatal) and perinatal (acquired). The former affects 10–25% of patients and is characterized by a malformation of extrahepatic bile ducts and other concomitant congenital anomalies. The latter is the most common type and is characterized by aggressive fibro-obliterative and inflammatory cholangiopathy in the extrahepatic biliary tree. In both types an obstruction of bile flow causes cholestasis and ultimately cirrhosis and, clinically, a 1 to 2 month old child presents with clay colored stools and jaundice. Due to the progression of disease, untreated BA is fatal and the most common indication for liver transplantation in children. The incidence of BA is approximately 1 in 5000-12000 live births (Sokol et al., 2007; Wada et al., 2007). Familial cases including siblings including twins, and mother-to-daughter transmissin are also reported (Smith et al., 1991;

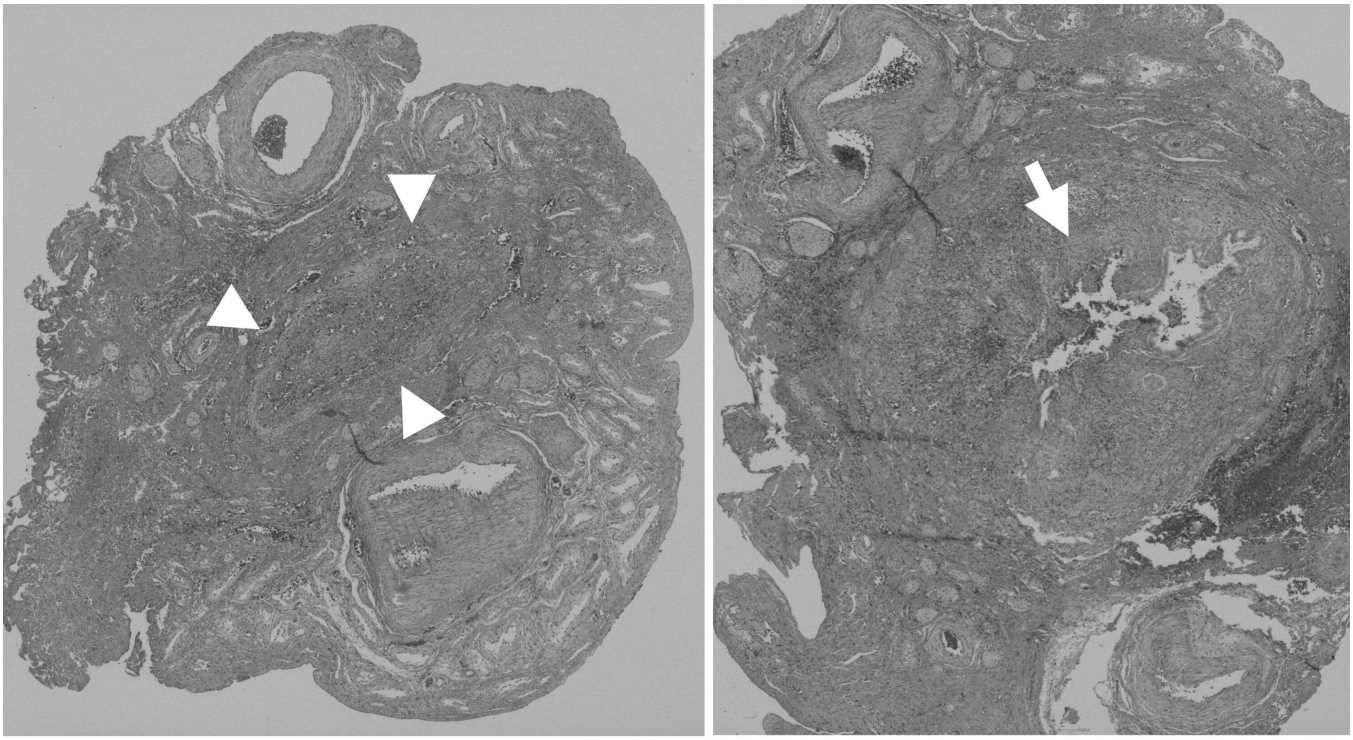

Fig. 4. Transverse sections of extrahepatic bile ducts in biliary atresia. **A.** Atretic bile duct showing luminal occlusion replaced by fibrous tissue (arrowheads). **B.** Distorted bile ducts (arrow) are found in the segments adjacent to **A**.

Kobayashi et al., 2008).

Histology

The morphologic features of extrahepatic bile ducts consist of epithelial damage, inflammation and fibrosis with obliteration of the lumen (Figs. 4, 5). In the early stage, at less than 3 months of age, obstructive changes such as ductular proliferation, variable portal edema, lobular cholestasis, and Mallory bodies are obvious within and around fibrously expanded portal tracts, and ductal plate malformation and periportal fibrosis with portal-to-portal bridging are also found. In parenchyma, nonspecific changes, such as focal multinucleated giant hepatocytes, extramedullary hematopoiesis, and hemosiderin in Kupffer cells, are seen. After 3 months of age, the portal fibrosis progresses surrounding and subdividing the hepatic lobule, thus resulting in biliary cirrhosis. The late stage of BA typically show less ductular proliferation and ductopenia develops (Raweily et al., 1990).

Morphological alterations of the liver in BA are very similar to and often indistinguishable from those of neonatal cholestasis. It is difficult to distinguish preoperatively between BA and other pediatric liver diseases. Liver biopsy specimens usually show only non-specific intrahepatic lesions related to the obstruction of the extrahepatic biliary tract, such as fibrous expansion of portal tracts and marked bile ductular proliferation, but these findings are the most useful distinguishable feature. Torbenson et al. (2003), reported that it is necessary to prove BA by immunostaining for CD56 as a helpful aid in differentiating various causes of neonatal cholestasis.

Etiopathogenesis of cholangiopathy

In the fetal type of BA, genetic abnormalities seem to play a role. Abnormal remodeling of the ductal plate leads to malformation (Nakanuma et al., 1997). This ductal plate malformation is a characteristic feature of CHF, but also found in the liver of BA. Polymorphisms in *HNF6*, *HNF1-B*, *JAGGED1*, and *PKDH1* genes regulating ductal plate remodeling are speculated to be susceptibility factors for the development of BA. Because mutations of *JAGGED1* lead to the malformation or dysfunction of intrahepatic bile ducts, *JAGGED1* gene abnormality is speculated to be an aggravating factor in severe cases of BA (Kohsaka et al., 2002). Complete deletion of the inversin (*inv*) gene in mice shows laterality defects in abdominal organ placement and also anomalous development of the hepatobiliary system (Mazziotti et al., 1999). Therefore, the inversin gene is also suggested to play an essential role in the morphogenesis of the hepatobiliary system, which raises the possibility that polymorphisms or

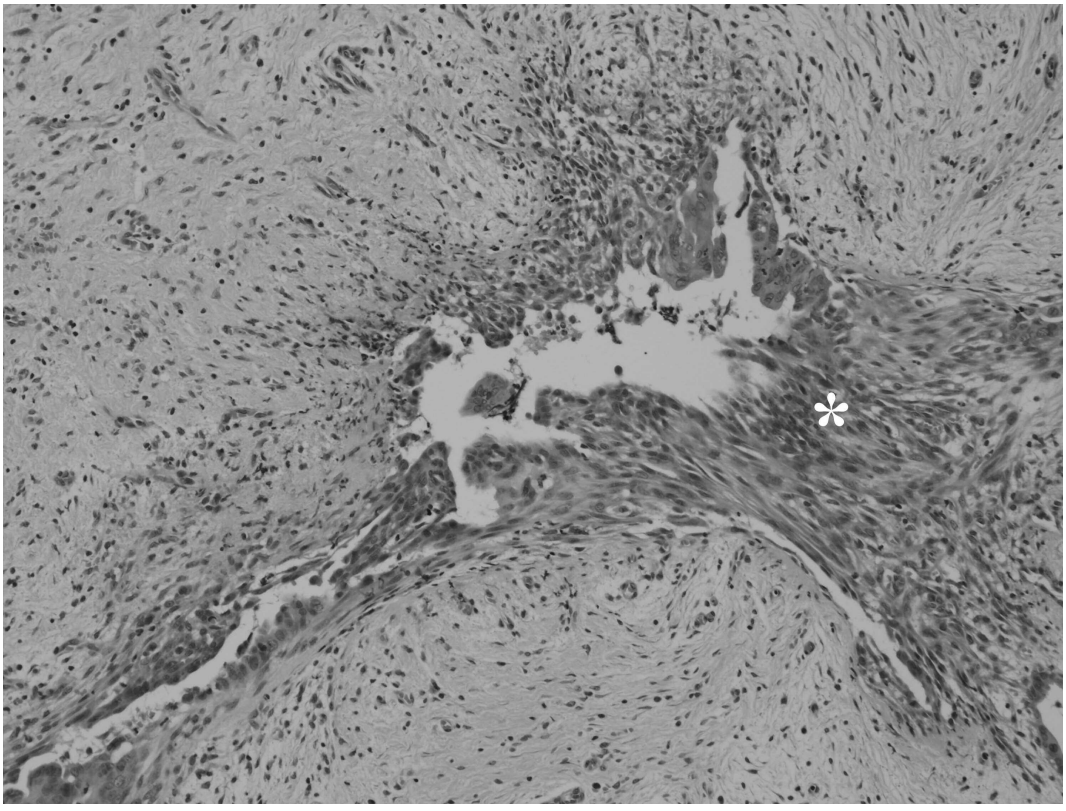

Fig. 5. Transverse section of extrahepatic bile ducts in biliary atresia. Bile ducts show a slit-like lumen lacking lining epithelia and accompanying periductal fibroplasia (*) and moderate inflammation.

mutations of inversin account for some cases of BA. However, human inversin is not frequently involved in the pathogenesis of BA and various congenital laterality defects, suggesting that the inversion gene is unlikely to be responsible for the majority of fetal cases of BA (Schon et al., 2002).

Recently, the pathogenesis of BA has been clarified mainly using animal models. It is well-known that the infection of newborn Balb/c-mice with Reoviridae, including type A rhesus rotavirus (RRV) and type 3 reovirus (Abney), leads to cholestasis and biliary obstruction resembling human BA (Riepenhoff-Talty et al., 1993; Szavay et al., 2002). Moreover, adoptive transfer of hepatic T cells leads to a bile duct-specific inflammatory reaction in recipient severe combined immunodeficiency disease mice, implying that infections of the biliary tree and subsequent cellular autoimmunity are important for progressive cholangiopathy and loss in BA (Mack et al., 2006; Mack, 2007). In humans, the presence of several viruses, including Reoviridae (type 3 reovirus and type C rotavirus), cytomegalovirus (CMV), and Ebstein-Barr virus (EBV) in liver tissue or affected bile duct specimens of patients with BA has been demonstrated, though conflicting results have also been reported (Riepenhoff-Talty et al., 1996; Bobo et al., 1997; Saito et al., 2004; Rauschenfels et al. 2009). Immunostaining for Mx proteins, which are very sensitive markers for type I IFN activity to viral infections, reveals that hepatocytes and intrahepatic bile ducts in BA are positive for Mx, suggesting the presence and/or current history of infection in hepatocytes and BECs of BA (Al-Masri et al., 2006). Moreover, the presence of EBV-encoded RNA (EBER) transcripts has been demonstrated in hepatocytes and BECs of BA by *in situ* hybridization (Mahjoub et al., 2008a).

Acquired immunity

Aberrant expression of MHC class II (HLA-DR) and adhesion molecule (ICAM-1) in damaged bile ducts and depositin of immunoglobulin along the basement membranes of bile ducts are demonstrated in BA (Hadchouel et al., 1981; Broome et al., 1997). Moreover, the increased levels of immunoglobulin kappa light chain in sera of patients with late-stage BA suggest adverse immune modulation (Lee et al., 2007). Predominant infiltration of CCR4+ Th1 cells and CXCR3+ lymphocytes (Th1, Tc1) and enhanced expression of Th1-type cytokine profile with the expression of interleukin (IL)-2, interferon (IFN)-γ, tumor necrosis factor (TNF)-α, and IL-12, were demonstrated in portal tracts of BA (Shinkai et al., 2006; Baba et al., 2009). These findings suggest that CD4+ Th1 cell-mediated immunity and a specific immune response are involved in the pathogenesis of cholangiopathy in BA. Moreover, Mack et al.(2007) reported that BA is associated with oligoclonal expansion of CD4+ and CD8+ T cells within liver and extrahepatic bile duct remnant tissues, indicating the presence of activated T cells reacting to specific antigenic stimulation, though the definite human target organ is still unknown.

In RRV-infected mice, the involvement of a Th1-dominant cytokine milieu in the pathogenesis of BA was also confirmed. Shivakumar et al. (2004), found that the IFN-γ-driven obstruction of bile ducts is a key pathogenic mechanism of diseasease, using IFN-γ-deficinet mice infected with RRV. The presence of autoreactive T cells and autoantibodies specific to BECs and significant increase in IFN-γ-producing T cells in response to biliary epithelial autoantigen wre demonstrated in this animal model. Periductal immunoglobulin deposits and serum antibodies reactive to protein developed from BECs were also detected in RRV-infected mice, suggesting that both cellular and humoral autoimmunity exist in this murine model and that the progressive bile duct injury is due in part to a BEC-specific T cell-mediated immune response (Mack et al., 2006). In addition to IFN-γ (type II IFN), the importance of IFN-α and -ß (type I IFN) were also demonstrated by a study using mice with inactivated IFN receptors. Inactivation of the type I IFN-receptor significantly increases the incidence of BA following postpartum RRV infection, suggesting a type I IFN-linked deregulation of the innate immune system to be crucial for the induction of BA (Kuebler et al., 2006).

Apoptosis

Apoptosis of BECs is speculated to play an important role in the obstructive cholangiopathy of BA. Increased and disorganized cell turnover of BECs are related to malformation of the ductal plate or abnormal bile duct development (Funaki et al., 1998) and are caused by impaired expression of E-cadherin in bile ducts (Sasaki et al., 2001). TNF-receptor 1 (TNF-R1), Fas (CD95), and TNF-related apoptosis-inducing ligand (TRAIL) receptors belonging to the TNF receptor superfamily contain a death domain and induce apoptosis by cross-linking their ligands. Human BECs express Fas and TRAIL receptors, but lack TNF-R1, suggesting that BECs are sensitive to Fas ligand- and TRAIL-mediated apoptosis (Harada et al., 2000, 2006a). In BA, Fas ligand expression on BECs is speculated to be induced to counterattack the infiltrating lymphocytes (Liu et al., 2000). Moreover, we reported that enhanced TRAIL expression and single stranded DNA (ssDNA)-positive apoptosis were found in extrahepatic bile ducts of BA (Harada et al., 2007). Erickson et al. (2008) found that BECs undergo early activation of apoptosis and the synergistic role of IFN-γ and TNF-α in activating caspase-3 in BECs using RRV-infected mice, supporting a prominent role for apoptosis and Th1-predominant imbalanced cytokine milieu in the pathogenesis of BA.

Innate immunity

Reoviridae (rotavirus and reovirus) show epitheliotropism and induce apoptosis in intestinal epithelial cells (Sato et al., 2006) and reovirus-induced

apoptosis is mediated by TRAIL and requires activation of the transcription factor nuclear factor-κB (NF-κB) (Clarke et al., 2000). These findings suggest that the innate immune response to a virus could directly affect epithelial injury and epithelial cell death. We reported that human BECs possess a dsRNA-recognizing receptor, Toll-like receptor 3 (TLR3), IFN-inducible helicase retinoic acid-induced protein I (RIG-I), and melanoma differentiation-associated gene-5 (MDA-5) and treatment with poly(I:C) (a synthetic analog of viral dsRNA) activates the transcription factors NF-κB and interferon regulatory factor 3 (IRF3), and produces antiviral factors such as type I interferons (IFN-ß) and MxA, a characteristic virus-related immune phenomena in BA (Harada et al., 2007). Because the mechanism of 'endotoxin tolerance' which prevents excess responses to maintain innate immune homeostasis in organs, exists in BECs, LPS physiologically does not elicit an inflammatory response in the biliary tree (Harada et al., 2006b). However, BECs fail to show tolerance to poly(I:C) and once innate immunity is activated, it is sustained in poly(I:C)-free conditions (Harada et al., 2008). These findings suggest that the initiation of the immune response to dsRNA in BECs after the clearance of the virus, as well as at the start of infection, is closely associated with the progression of BA and support that Reoviridae infections directly relate to the pathogenesis of cholangiopathies in BA (Morecki et al., 1982; Tyler et al., 1998).

Fibrotic processes in sclerosing cholangiopathy

An imbalance between fibrogenesis and fibrolysis is important to sclerosing cholangitis in BA. Inceased gene expression of integrin αvß6 was demonstrated in BECs and may be involved in sclerosing cholangitis (Nadler et al., 2008). Integrin αvß6 is transmembrane receptor that is largely restricted to epithelial cells of the skin and mucosal surface and is known to regulate. TGF-ß function. Imbalance of MMP-3 and TIMP-1 expression (Baba et al., 2009) and of ECM degradation factors (MMP-7 and MMP-9) and their inhibitors (tissue inhibitors of metalloproteinases (TIMP)-1, plasminogen activator inhibitor (PAI-1), and TIMP-4) (Nadler et al., 2008) are also closely associated with sclerosing cholangiopathy.

Recently, EMT of BECs has been speculated to be associated with periductal fibrosis and portal fibrosis in several hepatobiliary diseases, including BA (Nakanuma and Kono, 1992; Sato et al., 2007; Diaz et al., 2008). TGF-ß1 and basic fibroblast growth factor (bFGF) are well-known inducers of EMT (Zavadil and Bottinger, 2005). In biliary remnants of affected bile ducts in BA, vimentin-positive and epithelial marker-negative biliary epithelial cells suggesting EMT are found in damaged and almost atretic areas (Fig. 6). We examined the

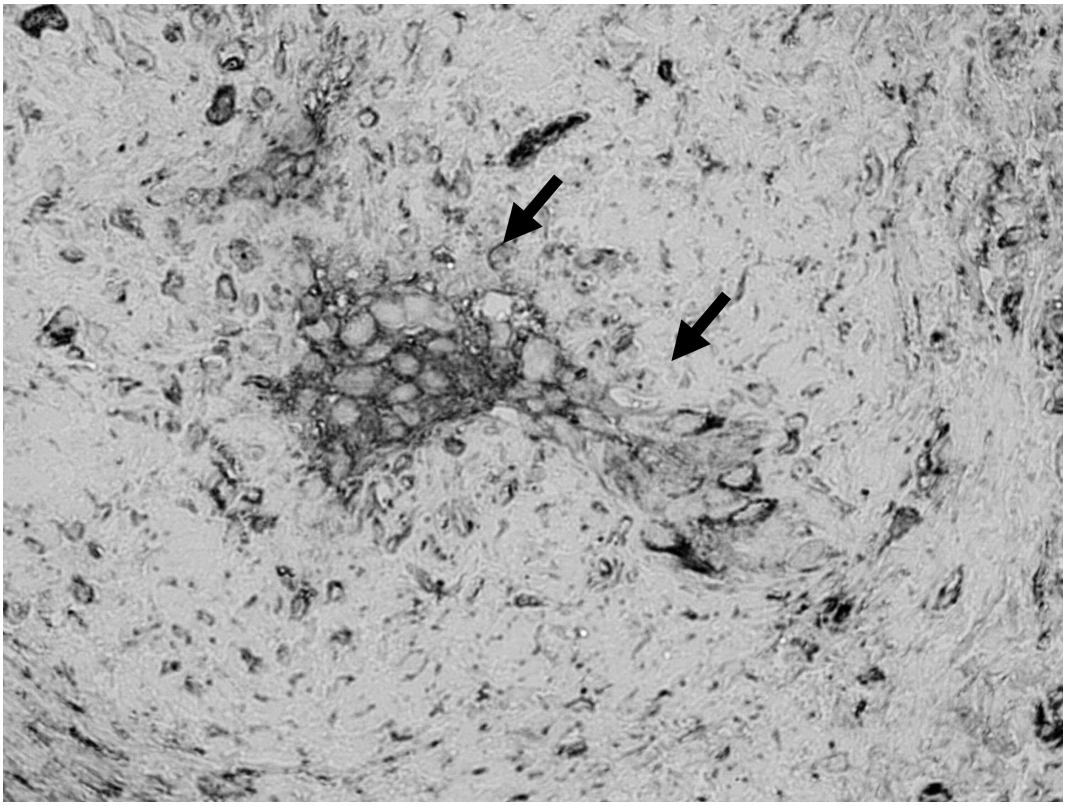

Fig. 6. Immunohistochemistry for the mesenchymal marker vimentin. Almost obstructed ducts express vimentin (arrows).

induction of biliary EMT and its association with viral infections to clarify the pathophysiological function of Reoviridae in BA. Consequently, we demonstrated that the biliary innate immune response to dsRNA viruses induces BECs to undergo EMT via the production of bFGF and increased susceptibility to TGF-ß1, suggesting tht the EMT contributes to the histogenesis of sclerosing cholangiopathy in cases of BA (Harada et al., 2009).

Progressive hepatic fibrosis

BA-related liver fibrosis is more rapid and aggressive than any other adult liver disease. In adult liver fibrosis, activated HSCs and fibrogenic molecules such as TGF-ß, platelet-derived growth factor (PDGF), and CTGF are important. TGF-ß regulates the synthesis of the ECM and PDGF is a key mitogen for the proliferation of HSCs. TGF-ß and PDGF were abundant in the early stage of BA as compared to its late stage (Faiz Kabir Uddin Ahmed et al., 2000). In BA livers, CTGF expression is high in BECs and correlates with the severity of fibrosis (Narkewicz et al., 2005). Moreover, MMPs and their endogenous tissue inhibitors (TIMPs) participate in ECM remodeling and degradation of hepatic fibrsois of BA, as seen in many organs. In contrast, Murata et al. (2008) demonstrated that the immunoreactivity of MMPs increased along with the degree of liver fibrosis in BA, whereas TGF-ß PDGF, and TIMP showed no difference in their expression with regard to the extent of fibrosis, and suggested that the liver in cases of BA is predominantly in a state of fibrolysis. Moreover, Ramm et al. (2009) suggested a key role for hepatocyte-derived monocyte chemotaxis protein-1 (MCP-1), potentially induced to express by elevated biliary and serum taurocholate levels under cholestatic conditions, in the recruitment of HSCs as reported for several other cholestatic liver injuries.

Conclusions

Recent progress in elucidating the etiopathogenesis of Caroli's disease with CHF and BA was reviewed. By using human tissue specimens and animal models, the mutations responsible for and the pathogenesis of biliary dysgenesis and fibrosis are now being clarified. An intial viral infection followed by innate and acquired immune responses are proposed for the progressive T cell-mediated inflammatory obliteration of bile ducts. Further studies are needed to explore new strategic approaches to these diseases.

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