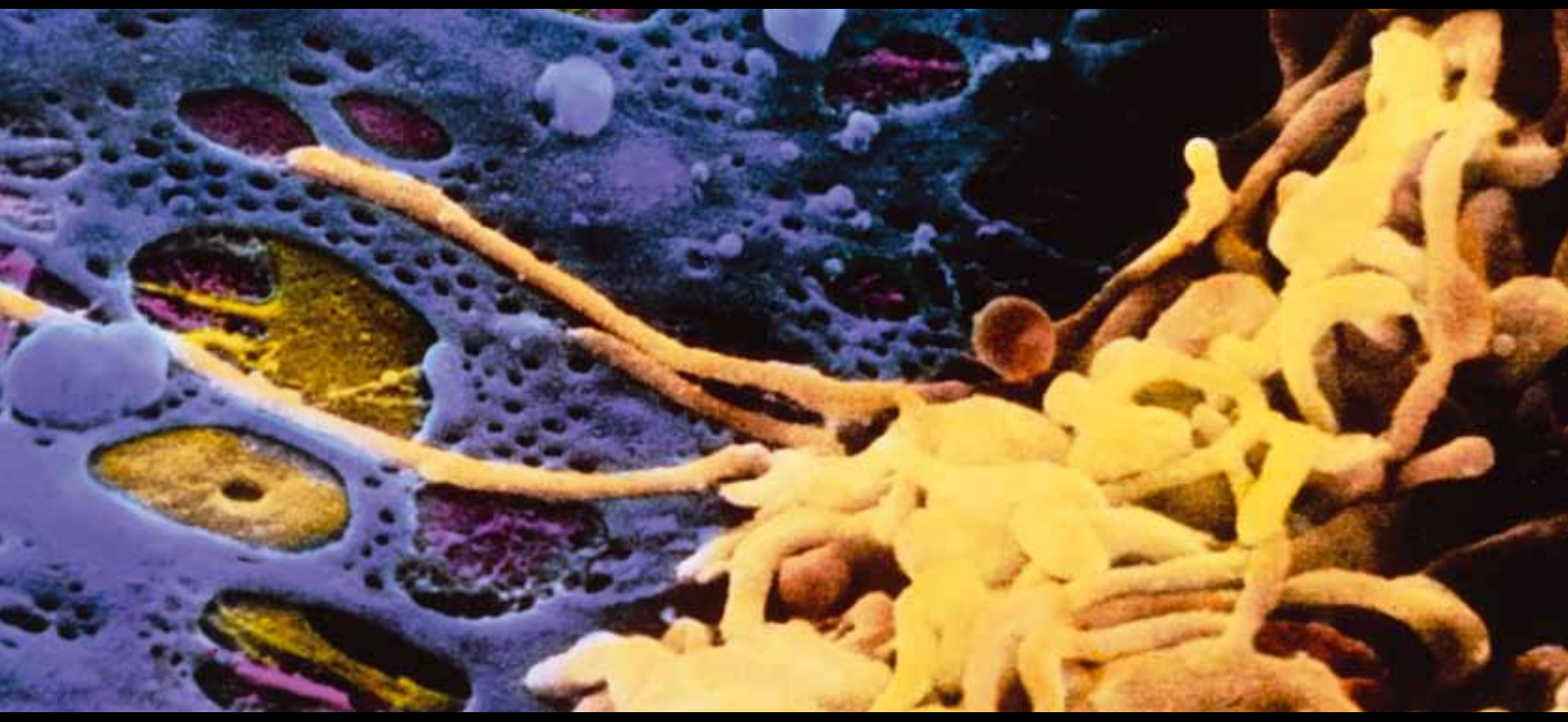
International Journal of Hepatology

Cholangiopathy: Genetics, Mechanism, and Pathology

Guest Editors: Yasuni Nakanuma, Anthony J. Demetris, Yoshiyuki Ueno, and Alberto Quaglia



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Editorial Board

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Editorial

Cholangiopathy: Genetics, Mechanism, and Pathology

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Cholangiopathy is pathologically and pathogenetically heterogeneous and presents a broad spectrum of clinical manifestations. A majority of them are known for many years, while some are newly emerging diseases. Recent advances in biology and medicine have introduced new technologies to study the cholangiocyte biologies and physiologies and the genetics, and the pathogenesis and pathology of cholangiopathy is now being evaluated from the aspects of experimental and clinical studies. Several animal models have been developed for autoimmune and genetic cholangiopathy such as primary biliary cirrhosis and polycystic disease of the liver and biliary tract. Knowledge and understanding of these conditions have led to the development of promising therapies and novel tools to characterize these clinical conditions.

In this special issue of cholangiopathy, several important aspects of cholangiophany are addressed with respect to recent progress. First, in the tutorial review, the anatomy and physiology of the biliary tree and physiologic functions of biliary epithelial cells are briefly introduced for better understanding of cholangiopathy. Several important and basic mechanisms and clinicopathological features of cholangiopathy are also described. Then, Drs. M. Harada and Y. Nokanuma describe the immunological aspects of biliary tree itself and also cholangiopathy with respect to innate immunity. Several biliary diseases, particularly biliary atresia, are now being discussed from the aspect of innate immunity. Then, Dr. Zen et al. introduce a newly emerging disease of IgG4-related disease with respect to biliary tract. Many clinicians and pathologists try to categorize this disease family showing a well response to steroid therapy and involving several organs. Dr. Shimoda et al. and Dr. Sasaki et al. focused on the pathogenesis of primary biliary cirrhosis from aspects of fractalkine and cellular senescence, respectively. Dr. Shimoda et al. stress the importance, of innate and acquired immunity and Drs. K. Sasaki and Y. Nakanuma notice the participation of autophagy in the induction of cellular senescence of biliary epithelial cells. Drs. K. Tsuneyama et al. discuss the animal models of primary biliary cirrhosis which are available. Dr. Ninomiya et al. introduced a new animal model of primary biliary cirrhosis. These models will contribute to the understanding of the pathogenesis of primary biliary cirrhosis in near future. Dr. Y. Sato et al. report their accumulating data on unique animal model of Caroli's disease and discuss its molecular mechanism. This animal model resembles the autosomal-recessive polycystic disease of the kidney and liver. Lastly, Drs. G. Fava and I. Lorenzini discuss molecular pathogenesis of cholangiocarcinoma, another topic of neoplastic cholangiopathy. New biomarker and molecular targeting therapies are now rapidly evolving in this field. We are sure that this special issue will contribute to the further progress of understanding and treatment of cholangiopathy.

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Review Article

Tutorial Review for Understanding of Cholangiopathy

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The biliary tree consists of intrahepatic and extrahepatic bile ducts and is lined by biliary epithelial cells (or cholangiocytes). There are also peribiliary glands around the intrahepatic large bile ducts and extrahepatic bile ducts. The biliary tree is a conduit of bile secreted by hepatocytes and biliary epithelial cells and also of the peribiliary glands and has several physiological roles. A number of diseases affect mainly the intrahepatic and extrahepatic biliary tree, and, in this special issue, these cholangiopathies are reviewed in detail with respect to genetics, pathogenesis, and pathology. In this paper, the anatomy and physiology of the biliary tree, basic injuries to biliary epithelial cells from stress and bile duct damage, and representative cholangiopathies are briefly reviewed.

1. Introduction

A number of diseases affect the biliary tree (cholangiopathies), though the pathological mechanisms involved and the anatomical level of the biliary tree affected vary [1]. For example, small interlobular bile ducts are mainly affected by a Th1-dominated microenvironment and cell-mediated immune response in PBC [2], while a Th2-dominated microenvironment and increased numbers of regulatory T cells are the major features of IgG4-related sclerosing cholangitis which affects mainly the extrahepatic bile ducts [3]. Ischemic damage to the biliary tree is a serious complication in liver transplantations [4].

In this special issue, cholangiopathy with respect to genetics, pathogenesis, and pathology will be discussed in detail. Herein, the anatomy and physiology of the biliary tree, basic injuries to biliary epithelial cells, basic forms of bile duct damage, and etiological classifications of cholangiopathy are reviewed. This tutorial review will be helpful for a better understanding of cholangiopathy.

2. Anatomy and Characteristics of the Biliary Tree

2.1. Anatomy. The biliary tree is composed of extrahepatic and intrahepatic bile ducts [5]. The former include the right and left hepatic ducts and their confluence and the common hepatic. and bile ducts, while the latter include the bile ducts proximal to the right or left hepatic duct. The intrahepatic branching of the bile ducts is best visualized on a cholangiograph or biliary injection cast (Figures 1 and 2). The extrahepatic bile duct is lined by high columnar epithelial cells, and its wall is composed of dense collagenous tissue harboring scattered smooth muscular elements.

The intrahepatic bile ducts can be classified as large and small, though there is no sharp delineation of the various segments [1, 5]. The biliary epithelial cells or cholangiocytes compose approximately 4-5% of liver mass. The large type consists of the right and left hepatic bile ducts and their first to third branches (segmental and area bile ducts). These ducts are grossly visible and belong to the perihilar bile ducts. They are lined by a tall columnar epithelium and surrounded by a dense hypocellular collagenous duct wall. In contrast, small intrahepatic bile ducts, the branches of the large intrahepatic bile duct, are classified into septal and interlobular bile ducts which are visible only under a microscope. While the septal ducts (>100 μm in diameter) are lined by tall columnar cells with basal nuclei, the interlobular bile ducts are lined by cuboidal cells. The fibrous ductal wall is evident in the former like large intrahepatic ducts, but not in the latter. The interlobular bile ducts are connected to the bile canalicular network by ductules (<20 μm diameter) lined by no more than a few minimally differentiated cuboidal cells and the canals of Hering, which are lined partly by biliary

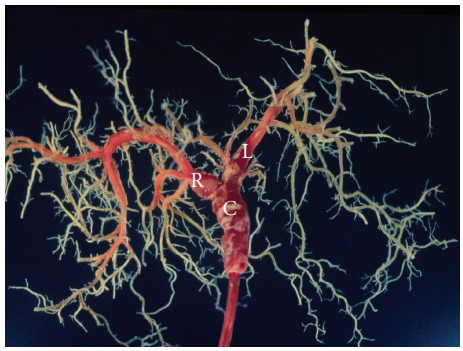

Figure 1: Biliary cast of normal liver. C: common hepatic duct, L: left hepatic duct, and R: right hepatic duct.

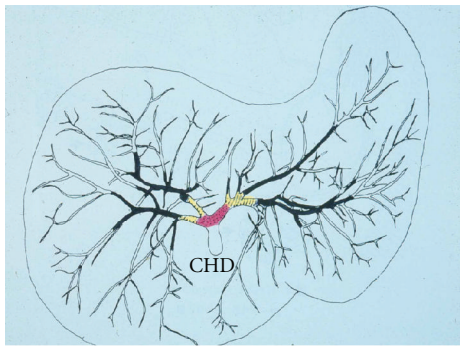

Figure 2: Diagram of the biliary tree. Red: right and left hepatic duct and their confluence, yellow: branches of the right or left hepatic ducts, and black: further branches. CHD: common hepatic duct.

epithelium and partly by hepatocytes. The intrahepatic stem cell niches are the canals of Hering in postnatal livers [6]. Bile ductules are very reactive anatomical elements in the liver, and proliferating bile ductules are reportedly involved in the fibrous progression of various chronic liver diseases and are easily identifiable by immunostaining of biliary cytokeratin (CK 7 and 19).

Peribiliary glands, the third biliary component, are present within the fibromuscular walls of extrahepatic bile ducts and also along the large intrahepatic bile ducts [1, 5, 7]. Glandular elements are also found at the neck of the gallbladder. Peribiliary glands around the large intrahepatic bile ducts (Figure 3) are subdivided into intramural glands, nonbranching tubular glands, and extramural ramified glands. The latter lie in the periductal connective tissue and, in a three-dimensional model, have a linear distribution along the opposite sides of the bile ducts and indirectly drain into the bile duct lumen via their own conduit. The extramural glands consist of serous and mucous acini. Pancreatic acini without Langerhans' islets are found intermingled with peribiliary glandular acini and are probably an intrinsic component of these glands. The glands are thought to have secretory activities. The extrahepatic stem cell niches are the peribiliary glands deep within the walls of the bile duct [6, 8].

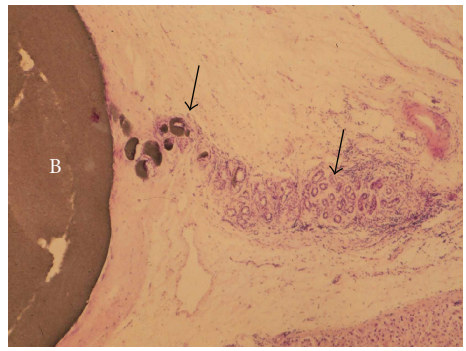

Figure 3: Peribiliary glands. B: bile duct; arrows: peribiliary glands and their conduits.

2.2. Distribution of Antigens along the Biliary Tree. The individual anatomical components of the biliary tree each have a rather characteristic antigen, probably reflecting a site-specific function [9, 10]. For example, the BECs lining large bile ducts are columnar and mucus is detectable in the supranuclear cytoplasm, but mucin is not detectable in the interlobular bile ducts and bile ductules. In contrast, in the adult liver, the BECs of intrahepatic large bile ducts constantly express MUC3, a membrane-binding type, whereas those of small bile ducts do not. MUC6 is constantly and focally expressed in BECs in the intrahepatic large bile ducts in normal liver. The expression of MUC1, MUC2, and MUC5 was infrequent in normal livers but increased in hepatolithiasis [9]. This study disclosed that the normal biliary tree has a specific expression of blood group antigens at different levels and that this expression is altered under pathologic conditions. In normal livers, large and septal bile ducts expressed A and B antigens in patients with comparable blood groups and also expressed H antigen frequently in patients with blood group O, A, or B and infrequently in patients with type AB. Lea and Leb are expressed in BECs at any level in secretors. As for cytokeratin, CK7 and CK19 are expressed in BECs of the biliary tree and also in peribiliary glands, while EpCAM is expressed in bile ductules. CK8 and 18 are expressed in hepatocytes and also BECs of the biliary tree.

2.3. Supply of Blood to the Biliary Tree. The intrahepatic and extrahepatic biliary tract is supplied by a network of fine vessels called the peribiliary vascular plexus (PBP) which exclusively derives from hepatic arterial branches [11–13]. The PVP can be histologically divided into the inner, intermediate, and outer layers, with respect to the bile duct walls [12]. These three layers are well and poorly developed in the large intrahepatic bile ducts and septal bile ducts, respectively, although the PBP around the interloblar bile ducts and bile ductules consists of scattered capillaries with no discernible layers [13]. This plexus has a fern-like appearance around the bile duct under the scanning electron microscope. The PBP drains into the sinusoids through "radicular portal veins" or communicates with portal venous

branches through "internal roots" or directly into the hepatic sinusoids in animals and probably in humans.

The inner layer, a layer of capillaries, is found just beneath the basement membrane of the epithelial layer and is regularly distributed like a chain. Ultrastructurally, the inner capillary layers are composed of fenestrated endothelial cells, and the number of fenestrae with a thin diaphragm is rather high on the capillary side facing the bile duct epithelium [13]. These observations suggest that the PBP, particularly the inner layer, may participate in the physiology of the bile ducts, particularly in the exchange of substances between blood in the peribiliary vascular plexus and bile in the bile ducts and in the supply and drainage of substances to and from the biliary epithelia [12].

2.4. Physiological Roles of the Biliary Tree. The biliary tree is lined by specialized epithelial cells called BECs or cholangiocytes [14] and is not only a conduit of bile secreted by hepatocytes and cholangiocytes but also a conduit of the peribiliary glands. The bile ducts and peribiliary glands play a number of physiological roles in the biliary system, contributing to about one-third of total bile secretion, participating in bile acid and water reabsorption, and secretion via transporters, and also mediating immune responses including innate immunity [15]. The primary hepatic bile secreted by hepatocytes is modified by BECs via a series of secretory and absorptive processes that provide additional bile water (BECs secrete ~40% of daily bile production in humans) or secrete HCO_3^- to induce an alkakine state [14]. BECs also interact with the immune system and microorganisms and are also involved in drug metabolism. To accomplish these functions, BECs display morphological and functional heterogeneity along the biliary tree.

2.5. Innate Immunity. The biliary tree is essentially sterile under normal conditions, but bile is potentially contaminated by bacterial components such as pathogen-associated molecular patterns (PAMPs) including lipopolysaccharide (LPS) and bacterial DNA originating from intestinal flora, which are actually detectable in bile of patients with chronic inflammatory biliary diseases [16]. In this context, the biliary tract is equipped with defence mechanisms, which are physical (bile flow and biliary mucus), chemical (bile salts), and immunological, such as secretory IgA. BECs also express Toll-like receptors (TLR) and intracellular adaptor molecules and secrete antibiotic peptides and (pro)inflammatory cytokines, thereby participating in the defense of the bile ducts [15].

Nonspecific bactericidal enzymes such as lactoferrin and lysozyme are also detected in the intrahepatic biliary tree, peribiliary glands, and bile [15]. Human β-defensins (hBDs) and cathelicidin, another antimicrobial peptide contributing to innate immunity at mucosal surfaces, are expressed in the biliary tree. hBD-1 is constitutively expressed in the biliary epithelium, while hBD-2 is expressed in large intrahepatic bile ducts in extrahepatic biliary obstruction, hepatolithiasis, and, to a lesser degree, PBC and PSC, suggesting a response to local infection or bacterial components, cytokines such as IL-1β and TNF-α, and/or active inflammation. Cathelicidin is expressed by normal biliary epithelial cells in addition to hepatocytes. Trefoil factor family (TFF) 1, 2, and 3 peptides expressed at the apical surface of the epithelium play a major role in mucosal repair.

IgA is known to be secreted into bile by binding with the secretory component (SC), and secretory IgA (SIgA) functions in a number of ways to protect the biliary tract. For example, it can directly bind and neutralize bacterial toxins. SIgA can bind to bacteria and prevent their adhesion to the mucosal membrane. Additionally, IgA has been demonstrated to neutralize intracellular microbes and their products. Biliary intraepithelial lymphocytes (bIELs), which are markedly increased in immune-mediated cholangitis, are occasionally encountered in normal intrahepatic bile ducts. Most of them are positive for CD8, some are positive for CD57, and these cells may participate in biliary innate immunity [17].

3. Basic Injuries of Biliary Epithelial Cells and Bile Duct Damage

3.1. Basic Injuries of BECs. Several pathologic agents and stress affect the intrahepatic and extrahepatic biliary tree including viral, bacterial, and even parasitic infections, oxidative stress, and immunological assaults, as well as biliary epithelial injuries from necrosis, apoptosis, and hyperplasia, and also bile duct damages.

3.1.1. Apoptosis and Necrosis. In some biliary diseases such as primary biliary cirrhosis (PBC) and chronic ductopenic allograft rejection, the ongoing apoptosis of BECs is important for progressive bile duct loss. In H&E stained sections, eosinophilic, shrunken slender cells with pyknotic nuclei in the biliary epithelial layer and fragmented and condensed nuclei in the bile duct lumen can be regarded as apoptotic bodies [2, 15]. Electron microscopically, shrunken BECs with a condensed cytoplasm and pyknotic nuclei are a marker of apoptosis. Apoptosis of BECs can be confirmed using in situ nick-end labelling and immunostaining of single stranded DNA, both of which detect DNA fragmentation. In contrast, the coagulative or lytic necrosis of the biliary epithelium is occasionally encountered in toxic cholangiopathy [18].

3.1.2. Cellular Senescence. Senescent BECs show characteristic features such as an eosinophilic cytoplasm, cellular and nuclear enlargement, multinucleation, and an irregular arrangement with uneven nuclear spacing [19]. Actually, these cells also express cellular senescent markers such as the cell cycle regulators, $p16^{INK4}$ and $p21^{WAF1/CIP}$, and increased activity of senescence-associated β-galactosidase (SA-β-gal). Recent studies showed that cellular senescence has at least two pathological effects in the development of biliary diseases: impaired regeneration and senescence-associated secretory phenotypes (SASPs).

Impaired Regeneration. Senescent cells no longer have the ability to proliferate and they are irreversibly arrested at

the G1 phase of the cell cycle [20]. The expression of senescence-related markers is increased in BECs during early chronic rejection in chronic liver allograft and PBC. Cellular senescence of BECs is involved in impaired regeneration and eventual and progressive bile duct loss in PBC and ductopenic chronic rejection [21, 22]. A relatively insufficient proliferative response of BECs due to cellular senescence (see below) is also responsible for the progressive loss of bile ducts due to apoptosis.

Senescence-Associated Secretory Phenotypes. Accumulating evidence suggests that senescent cells remain metabolically active and play an important role in modulating the microenvironment around them by secreting cytokines, chemokines, growth factors, and profibrogenic factors [19]. For example, senescent BECs of PBC expressing CCL2 and CX3CL1 may be involved in the recruitment of monocytes and possibly T lymphocytes into portal tracts, around injured and senescent BECs, and thereby responsible for the development of immune-mediated cholangitis such as PBC [19, 23].

3.1.3. Biliary Epithelial Cell Renewal. The homeostasis of physiological and pathological biliary epithelia operates through a balance between cell loss and cell renewal. Cell loss in the biliary epithelium is mainly due to apoptosis or senescence and mostly regulated by the *bcl-2* family of proteins or senescence-associated factors such as *p16* and *p21*. The biliary epithelial cells of bile ductules or small bile ducts may be replenished by bile ductular cells or hepatic progenitor cells in the canal of Hering, though such processes may be unlikely in the intrahepatic large bile ducts. As mentioned, the peribiliary glands themselves or progenitor cells located in these glands may be involved in renewal of the biliary epithelium of intrahepatic large bile ducts and extrahepatic bile ducts and also proliferation of the epithelia lining these bile ducts [7, 8].

3.1.4. Biliary Epithelial Hyperplasia. Inhibition of the apoptotic or senescent process in the biliary epithelia may cause hyperplasia with an increased risk of neoplastic transformation [24]. Hyperplasia of lining epithelia of the septal and large bile ducts manifests as micropapillary projections or as a stratification of the epithelium with or without dilatation of the duct lumen. Peribiliary glands, intramural or extramural, also show hyperplasia and proliferation and participate in the secretion of neutral, carboxylated, and sulphated mucins into the bile duct lumen. When prominent, in particular with *Clonorchis sinensis* infections or hepatolithiasis, the term adenomatous hyperplasia or chronic proliferative cholangitis has been used. As for the proliferation and hyperplasia of bile ductules and small interlobular bile ducts, they appear tourtous and increase in their number in the portal tracts. Some of these lesions are included in the so-called ductular reactions [25].

3.1.5. Metaplasia. Several kinds of metaplasia are reported in the biliary epithelium of the intra- and extrahepatic biliary tree, usually in cases of chronic biliary diseases such as hepatolithiasis, parasitic cholangitis, and primary sclerosing cholangitis (PSC). *Gastrointestinal metaplasia* resembling pyloric glands and goblet cells is not infrequently seen in chronically inflamed large bile ducts and peribiliary glands. This change is associated with the aberrant expression of gastric type mucus core protein (MUC) 5AC and MUC6 and also intestinal type MUC2. Such gastric and intestinal mucin is involved in lithogenesis in hepatolithiasis [9]. The so-called intramural glands with a gastric pyloric gland-like appearance are increased in long-standing biliary diseases and may reflect invagination of the biliary epithelium with gastrointestinal metaplasia. *Goblet cells* are occasionally encountered among bile duct-lining cells and also in peribiliary glands. *Intestinal metaplasia* with Paneth cells may also be encountered in the peribiliary glands. The expression of other molecules in intrahepatic large bile ducts, such as REG I and trefoil factors, appears to be related to intestinal or gastric metaplasia. While *pancreatic acinar metaplasia* is also reported infrequently in PSC, its differentiation from heterotopic pancreatic acini is controversial. *Hepatocytic metaplasia* occurs in interlobular bile ducts and bile ductules in various pathological situations but remains of unknown significance. *Squamous metaplasia* is rarely encountered in long-standing inflammation of large bile ducts such as PSC or in the lining of biliary cysts.

3.1.6. Biliary Intraepithelial Neoplasm (BilIN). Chronic biliary diseases such as hepatolithiasis and PSC are occasionally complicated by cholangiocarcinoma. In such cases, dysplastic or early neoplastic lesions are known to precede the invasive cholangiocarcinoma. Such biliary epithelial lesions are known as dysplasia or atypical hyperplasia of the biliary epithelium and characterized by atypical, enlarged, and hyperchromatic nuclei, an increased nucleocytoplasmic ratio, and a loss of polarity [5, 26]. Usually either micropapillary or flat lesions affect a portion or the circumference of the bile duct. These lesions were proposed to be called biliary intraepithelial neoplasm (BilIN), and this terminology was recently adopted by WHO [26]. They are divided into three grades according to cellular and structural atypia; BilIN-1, BilIN -2, and BilIN -3. In BilIn-1, cellular/nuclear atypia are mild or moderate but not enough for overt malignancy, and cellular polarity is minimally disturbed and corresponding to low-grade dysplasia. In BilIN-2, cellular/nuclear atypia are evident but not marked enough for overt malignancy, and the disturbance of cellular polarity is mild or focal, corresponding to high-grade dysplasia. BlIN-3 shows cellular/nuclear atypia corresponding to overt malignancy, and cellular polarity is diffusely disturbed, corresponding to a so-called carcinoma in situ of the biliary tract [26]. BilIN-1, BilIN -2, and BilIN -3 are seen in both large intrahepatic and extrahepatic bile ducts, peribiliary glands, and gallbladder and considered to reflect a multistep neoplastic transformation of the biliary epithelium.

3.2. Basic Pathology of Bile Duct Damage. In the biliary tree, there are several types of bile duct damage such as cholangiopathies and cholangitis. Representative pathological features of the biliary tree are as follows.

3.2.1. Cholangitis and Its Classification. Cholangitis is characterized by biliary epithelial damage with inflammatory cell infiltration. Some cholangitis is also associated with ductal and periductal fibrosis. It occurs along the biliary tree, and the term cholangitis is used for inflammatory damage to bile ductules.

Cholangitis can be histologically classified into suppurative and nonsuppurative forms. *Suppurative cholangitis* implies the presence of numerous polymorphonuclear cells around and within the wall as well as within the lumen of the ducts. This may involve ducts of any size and is occasionally associated with abscess formation—cholangitic abscess. A microbial infection is often responsible, but the change also occurs in the presence of sterile bile, particularly after bile extravasation. The release of chemokines or cytokines is the likely cause in some cases.

"*Nonsuppurative cholangitis*" includes a spectrum of bile duct inflammation which may be granulomatous cholangitis, lymphoid cholangitis, fibrous cholangitis, and pleomorphic cholangitis according to the predominant type of inflammatory reaction present [27]. *Granulomatous cholangitis* almost always seems to be destructive. This type involving the interlobular bile ducts constitutes the hallmark of PBC and is also found in drug-induced liver disease and sarcoidosis. The other types can be either destructive or nondestructive. *Lymphoid cholangitis* refers to a close association between duct branches, usually interlobular bile ducts, and lymphocytic aggregates, which may show a follicular arrangement. This is found in PBC and PSC with concomitant bile duct destruction or in nonbiliary disorders, in particular autoimmune and viral hepatitis C. *Pleomorphic cholangitis* is associated with inflammatory cell infiltration. All other types of cholangitis are found in CAH, PBC, PSC, and other liver diseases. *Fibrous cholangitis (also called sclerosing cholangitis)* with evident ductal fibrosis develops as a consequence of long-standing bile duct inflammatory, obstruction, or ischemic injury; it can be obliterative or nonobliterative. BECs show variable damage. The former is characteristic of PSC, though, in our experience, it may be seen in acquired forms of sclerosing cholangitis too. BECs of obliterative type are actually lost in fibrous lesions, appearing as a fibrous core. Sclerosing cholangitis with bile duct obliteration suggests a diagnosis of PSC in adults.

3.2.2. Bile Duct Sclerosis. In long-standing sclerosing cholangitis and also in other biliary diseases such as ischemic cholangitis, the bile duct wall shows a marked deposition of collagen fiber (bile duct sclerosis). The affected bile ducts in sclerosing cholangitis show a marked increase in the number of c-kit receptor-expressing mast cells which secrete fibrogenic factors such as histamine, basic fibroblast growth factor (bFGF), and/or tumour necrosis factor-alpha (TNF-α). The biliary epithelium itself produces and secretes fibrogenic substances such as bFGF, transforming growth factor-beta (TGF-β), and platelet-derived growth factor (PDGF), as well as basement membrane proteins and extracellular matrix proteins. In biliary atresia, BECs of the affected bile ducts variably express mesenchymal markers such as vimentin and might have acquired phenotypes of mesenchymal cells, though distinct morphological epithelial mesenchymal transition (EMT) of biliary epithelium is hardly recognizable [28, 29]. In all forms of bile duct sclerosis, a marked attenuation of the peribiliary vascular plexus is seen within the sclerotic duct wall, but it remains unknown whether these changes are secondary to, or responsible for, the bile duct fibrosis.

3.2.3. Bile Duct Loss or Ductopenia. The balance of cell death or dropout due to apoptosis or necrosis and the regeneration of lining biliary epithelia is important for the maintenance of bile ducts, and apoptotic activity that exceeds the proliferative response of bile duct cells results in progressive ductopenia. Ductopenia is defined as a loss of bile ducts from the portal tract in which hepatic arterial branches and bile ducts of similar size run parallel. Thus, portal tracts without evident bile ducts indicate a loss of bile ducts. Immunostaining of biliary cytokeratins such as CK7 and CK19 is helpful for the recognition of bile ducts. Ductopenia is usually defined as the absence of interlobular bile ducts in at least 50% of portal tracts. Extensive ductopenia is usually associated with chronic cholestasis and biliary fibrosis. Ductopenia is typically found during chronic liver allograft rejection with chronic cholestasis and also the advanced stages of PBC.

3.2.4. Mucobilia and Hemobilia. Mucin is impacted in the duct lumen and this is occasionally marked, leading to leakage and extravasation with the formation of mucus lakes. Drainage of mucin from Papilla of Vater is also a clinical manifestation of mucobilia, as seen in intraductal papillary mucinous neoplasms of the pancreas. Mucobilia is usually found in the neoplastic bile ducts and nonneoplastic bile ducts of "intraductal papillary neoplasms of the bile duct" (formerly known as "biliary papillomatosis") or mucin-producing bile duct tumors [26, 30]. When such changes are encountered in nonneoplastic biliary diseases such as PSC and hepatolithiasis, usually microscopic neoplastic biliary lesions are found in the affected bile ducts.

In cases of hemobilia, impacted erythrocytes are encountered in bile duct lumens. Recent endoscopic or surgical biliary manipulations in association with a primary or secondary malignancy may be underlining diseases for hemobilia.

3.2.5. Ductular Reaction. To date, many pathological terms such as oval cell proliferation, intermediate cells, and atypical bile ductular proliferation have been used to describe the "increased ductule-like cells or clusters of small epithelial cells different from mature hepatocytes" in the portal tract or the periportal area. This is a reaction of the ductular phenotype, possibly but not necessarily of ductular origin, commonly seen in many kinds of acute and chronic

Table 1: Etiologic classification of cholangiopathy.

(1) Immune-mediated cholangiopathy
(2) Infectious cholangiopathy
(3) Genetic cholangiopathy
(4) Ischemic cholangiopathy
(5) Drug- or toxin-induced cholangiopathy

hepatobiliary diseases. Recently, an international working group proposed the term "ductular reaction" for this lesion [25]. "Ductular reaction" implies a reaction of ductular phenotype, possibly but not necessarily of ductular origin. The epithelial component of a ductular reaction may actually derive from several sources: not only from the proximal branches of the biliary tree but also from the circulation (often if not always from bone marrow) and from biliary metaplasia of hepatocytes. "Reaction" encompasses the complex of stroma, inflammatory cells, and other structures of diverse systems, all of which participate in the reactive lesion. Bile ductular reaction is usually characterized by increased numbers in the periportal and portal areas and a common and frequent process in a number of hepatobiliary diseases.

A ductular reaction itself is heterogeneous in its development and has many meanings. There are several reports that bile ductules are very reactive anatomical elements in the liver, and proliferated bile ductules are involved in the progression of various chronic liver diseases. Our recent studies showed that bile ductular cells in PBC, PSC, and also NAFLD may undergo cellular senescence, and these cells could produce and secrete biologically active molecules and thereby be involved in hepatic fibrogenesis and other pathologic features of the liver.

3.2.6. Ductal Plate Malformation. Ductal plate malformations (DPMs), which are different from reactive changes of bile ducts or ductules, develop as a result of a remodeling failure of the ductal plate followed by the development of intrahepatic bile ducts. DPMs are characterized by increased numbers of abnormal bile duct-like structures and show a bridge-like structure in the dilated lumen and bulbar protrusion of biliary epithelia [31]. DPMs are observed in congenital hepatic fibrosis and Caroli's disease, biliary atresia, and other fibropolycystic liver diseases.

4. Cholangiopathies

Diseases that mainly target the biliary tree (cholangiopathies) can be divided into several categories according to the pathogenetic mechanism involved (Table 1). However, in many cholangiopathies, more than one pathogenetic mechanism is operative.

4.1. Immune-Mediated Cholangiopathies. The biliary tree could be affected by immunological assaults, and lymphoplasmacytic infiltration is evident around the damaged bile ducts. Primary biliary cirrhosis (PBC) and primary sclerosing cholangitis (PSC) are representative immune-mediated cholangiopathies [32]. Autoimmune pathogenesis is operative in PBC and PSC. There is a mixture of immunocompetent cells in the affected bile ducts, and CD3+, CD4+, and CD8+ T cells that bear the T-cell receptor α/β are predominant in PBC, supporting that Th1 immune response-predominant cytotoxicity and/or cytokine release are involved in the pathogenesis of the bile duct lesions of PBC. HLA-class II antigens are aberrantly expressed in the affected bile ducts of PBC, PSC, and chronic allograft rejection [33]. Biliary innate immunity is also involved in the pathogenesis of cholangiopathies in patients with PBC and biliary atresia (BA) [9]. BECs possess an innate immune system consisting of the Toll-like receptor (TLR) family and recognize pathogen-associated molecular patterns (PAMPs). In PBC, CD4-positive Th17 cells characterized by the secretion of IL-17 are implicated in the chronic inflammation of bile ducts, and the presence of Th17 cells around bile ducts is causally associated with the biliary innate immune responses to PAMPs. In BA characterized by a progressive, inflammatory, and sclerosing cholangiopathy, dsRNA viruses are speculated to be an etiological agent and to directly induce enhanced biliary apoptosis via the expression of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). Moreover, the epithelial-mesenchymal transition (EMT) of biliary epithelial cells is also evoked by the biliary innate immune response to dsRNA. In addition, intrahepatic small bile ducts and bile ductules are a main target in graft-versus-host disease and also hepatic allograft rejection. Recent studies showed that IgG4-related sclerosing cholangitis is also associated with altered immunity. Upregulation of regulatory T cells (Tregs) associated with Th2 predominance is reportedly important in the pathogenesis of IgG4-related sclerosing cholangitis [3]. The anatomical level of the biliary tree affected is different among these immune-mediated cholangiopathies. Interestingly, the peribiliary glands are also involved in PSC, graft-versus-host disease, and IgG4-related sclerosing cholangitis.

4.2. Infectious Cholangiopathies. The biliary tree is affected by several types of infectious diseases, such as bacterial, fungal, protozoan, parasitic, and viral cholangitis. Stagnation of bile due to biliary stenosis or obliteration is followed by bacterial cholangitis, frequently with sepsis or abscess formation. Parasitic infections are also reported in the biliary tract including the liver, and liver flukes such as *Clonorchis sinensis* and *Opisthorchis viverrini* are endemic in East Asia, particularly northern Thailand and some parts of Korea, and cholangiocarcinoma is a serious complication of parasitic cholangitis [26]. Hepatolithiasis is predominantly a disease of the Far East and is causally also related to infectious cholangitis, especially bacterial cholangitis [27, 34]. Mucin plays an important role in the development of hepatoliths, which are formed within the intrahepatic large bile ducts. Clinically, patients may present acutely with recurrent bacterial cholangitis and its possible complications, such as liver abscesses and septicemic shock, or with chronic complications, such as cholangiocarcinomas

and intraductal papillary neoplasms [35]. Pathologically, it is characterized by pigmented calcium bilirubinate stones within dilated intrahepatic bile ducts featuring chronic inflammation, mural fibrosis, and proliferation of peribiliary glands, without extrahepatic biliary obstruction. A transient viral infection such as type A rhesus rotavirus and type 3 reovirus is reported as an initiating mechanism of biliary atresia (BA), particularly perinatal type.

4.3. Genetic Cholangiopathies. Genetic alterations affecting the biliary tree manifest as biliary dilatation, bile duct paucity, obstruction, proliferation, stone formation, and so on. Caroli's disease with congenital hepatic fibrosis (CHF) is a representative genetic cholangiopathy. Some cases of biliary atresia (BA) also belong to this category. The former is characterized by multiple saccular dilatations of the intrahepatic bile ducts. Caroli's disease with CHF belongs to autosomal recessive polycystic kidney disease (ARPKD) with ductal plate malformation characterized by a disordered remodeling of the intrahepatic biliary tree [28]. Disordered cell kinetics, including the apoptosis of biliary epithelial cells (BECs), may be significantly related to ductal plate malformation, and laminin and type IV collagen levels were reduced in the basement membrane of intrahepatic bile ducts of ARPKD; such a reduction is an additional factor for the dilatation of bile ducts. Paucity of the intrahepatic bile ducts is a genetic cholangiopathy [36]. For example, Alagille syndrome with a mutation in a ligand for the Notch protein is characterized by paucity of intrahepatic bile ducts and other anomalies. Cystic fibrosis (CF) due to a mutation in the cystic fibrosis transmembrane conductance regulator (CFTR) is associated with focal biliary fibrosis, and the bile duct and ductules are filled with pink and amorphous secretions. Low phospholipid-associated cholelithiasis (LPAC) is characterized by a low biliary phospholipid concentration with symptomatic and recurring cholelithiasis, and LPAC syndrome is associated with mutations of the adenosine triphosphate-binding cassette, subfamily B, member 4 (ABCB4) gene encoding the hepatobiliary phospholipid translocator multidrug resistance protein 3 [37]. This causes recurrent cholelithiasis, continuous irritations of the biliary tract with cholangitis, chronic cholestasis, and even biliary cirrhosis.

4.4. Ischemic Cholangiopathies. Ischemic cholangiopathy is defined as focal or extensive damage to bile ducts due to an impaired blood supply [11]. Most causes of bile duct ischemia are iatrogenic, though some systemic vascular diseases also cause this type of cholangiopathy. This entity may be observed in various circumstances and is of clinical importance for practitioners involved in gastroenterology, oncology, abdominal surgery, and liver transplantation. Ischemic bile duct injury may occur when small hepatic arteries or the peribiliary vascular plexus are injured or when all possible sources of arterial blood supply are interrupted. Ischemic biliary injury may take the form of bile duct necrosis, bile leakage, biloma, bile duct fibrosis, or stenosis. Bile duct necrosis and bilomas develop predominantly where there is an abrupt and complete interruption of the arterial blood supply, for example, when HA thrombose in a liver transplant recipient. On the contrary, fibrous stenoses develop where there is progressive injury to the hepatic arterioles, for example, after several courses of intra-arterial chemotherapy. Cholangiographic findings include diffuse and multiple bile ducts lesions. Ischemic cholangiopathy is a serious complication during liver transplantation [4]. When biliary drainage or reconstruction is not possible or has failed, liver transplantation is the only potential cure.

4.5. Drug- or Toxin-Induced Cholangiopathies. Bile ducts, particularly interlobular bile ducts, are occasionally affected by drug-induced hepatobiliary damage, various bile duct injuries, various types of cholangitis, and bile duct loss (drug-induced cholangiopathy) [38]. This type of cholangitis is not infrequently associated with cholestasis. Some cases presenting with progressive ductopenia and cholangitis and prolonged cholestasis mimic PBC and also PSC. While the mechanism of drug-induced cholangitis remains speculative, immune-mediated processes including hypersensitivity may be operative. Some forms of drug-induced cholangiopathy develop after hepatic arterial infusion of floxuridine (FUDR) (floxuridine- (FUDR-) induced cholangiopathy). Ischemic changes to the peribiliary vascular plexus may be at least partly involved in this type of cholangiopathy. Although BECs have low metabolic activity compared with hepatocytes, cytotoxic or cytopathic bile duct injury has been produced experimentally or accidentally by toxic substances such as α-naphthylisothiocyanate, 4,4′-diaminodiphenylmethane, and paraquat (toxin-induced cholangiopathy) [18].

In conclusion, the anatomy and physiology of the biliary tree, basic injuries to biliary epithelial cells, basic forms of bile duct damage, and etiological classification of cholangiopathy were reviewed. This tutorial review will be helpful for better understanding cholangiopathies.

Conflict of Interests

The authors have no conflict of interests to declare.

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Review Article

Cholangiopathy with Respect to Biliary Innate Immunity

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Biliary innate immunity is involved in the pathogenesis of cholangiopathies in cases of biliary disease. Cholangiocytes possess Toll-like receptors (TLRs) which recognize pathogen-associated molecular patterns (PAMPs) and play a pivotal role in the innate immune response. Tolerance to bacterial PAMPs such as lipopolysaccharides is also important to maintain homeostasis in the biliary tree, but tolerance to double-stranded RNA (dsRNA) is not found. Moreover, in primary biliary cirrhosis (PBC) and biliary atresia, biliary innate immunity is closely associated with the dysregulation of the periductal cytokine milieu and the induction of biliary apoptosis and epithelial-mesenchymal transition (EMT), forming in disease-specific cholangiopathy. Biliary innate immunity is associated with the pathogenesis of various cholangiopathies in biliary diseases as well as biliary defense systems.

1. Introduction

Primary biliary cirrhosis (PBC), primary sclerosing cholangitis (PSC), and hepatolithiasis in adults and biliary atresia and choledochal cyst in infants are biliary diseases in which different anatomical levels of the biliary tree are specifically affected and characterized by cholangiopathy. The biliary tree, consisting of cholangiocytes, is a system of connecting ducts that drain the bile secreted by hepatocytes into the duodenum. Cholangiocytes provide the first line of defense in the biliary system against luminal microbes originating from the intestines via portal blood and duodenum [1]. In general, although human bile is normally sterile, it can contain bacterial components such as lipopolysaccharide (LPS), lipoteichoic acid, and bacterial DNA fragments, known as pathogen-associated molecular patterns (PAMPs) [2–5], and cultivable bacteria are detectable in bile of patients with biliary diseases [1, 6–8]. Enteric bacteria, in particular, may be responsible for the chronic proliferative cholangitis associated with hepatolithiasis [1, 6]. These findings indicate that cholangiocytes are exposed to bacterial PAMPs under physiological as well as pathological conditions.

Innate immunity was initially thought to be limited to immunocompetent cells such as dendritic cells and macrophages, but epithelial cells also possess TLRs and proper innate immune systems reflecting the specific micro-environment and function of each epithelial cell type. Recent studies concerning biliary innate immunity indicate that cholangiocytes express a variety of pathogen-recognition receptors such as Toll-like receptors (TLRs) [9, 10]. Infectious agents have been implicated in the etiology or progression of cholangiopathies including cholangitis, bile duct loss, and lithiasis as a trigger or aggravating factor. Notably, several enterobacteria and viruses are speculated to be primary or secondary factors for PBC, PSC, biliary atresia, hepatolithiasis, and chronic cholecystitis [2, 3, 11–15] (Table 1). Moreover, no microorganisms showing cholangiocyte-specific tropism have been identified, suggesting that an innate immune response specific to cholangiocytes rather than PAMPs is important in the pathogenesis of cho-lan-gi-op-a-thy. This review summarizes our current understanding of the biliary innate immune system against microbial infections including the various mechanisms employed by negative regulators and their associations with the pathogenesis of cholangiopathy in biliary diseases.

Table 1: Bacteria and viruses speculated to be etiologic factors in biliary diseases.

Primary biliary cirrhosis	
Lipopolysaccharide	
Lipoteichoic acid	
Helicobacter	
β-retrovirus	
Propionibacterium acnes	
Escherichia coli	
Mycobacterium	
Novosphingobium	
Lactobacillus	
Chlamydia	
Biliary atresia	
Reovirus	
Rotavirus	
Cytomegalovirus	
Adenovirus	
Enterovirus	
Ebstein-Barr virus	
Primary sclerosing cholangitis	
Helicobacter	
α-hemolytic streptococcus	
Hepatolithiasis	
Escherichia coli	
Klebsiella	
Streptococcus	
Pseudomonas	
Bacteroides	
Clostridium	
Campylobacter	

2. Molecular Mechanisms of Biliary Innate Immunity

2.1. Expression of PAMP-Recognizing Receptors and Intracellular Adaptor Molecules. The TLR family are the best characterized cell surface receptors recognizing PAMPs, and 10 members (TLR1-10) have been identified in humans [16, 17]. The response to LPS is mediated by interaction with TLR4 in conjunction with the TLR4 accessory proteins MD-2 and CD14, triggering the transduction of intracellular signals followed by the activation of TLR-associated adapter proteins, myeloid differentiation factor 88 (MyD88), and IL-1 receptor-associated kinase- (IRAK-)1, leading to the activation of nuclear factor-κB (NF-κB) and then to the synthesis of antibiotics and proinflammatory cytokines. In contrast to bacterial PAMPs, dsRNA including viruses are recognized by TLR3, IFN-inducible helicase retinoic acid-induced protein I (RIG-I), and melanoma differentiation-associated gene-5 (MDA-5). The stimulation of these receptors by dsRNA transduces signals to activate transcription factor interferon regulatory factor 3 (IRF3) as well as NF-κB.

Table 2: Expression of Toll-like receptors in cultured human biliary epithelial cells (BECs), cholangiocarcinoma, and murine BECs.

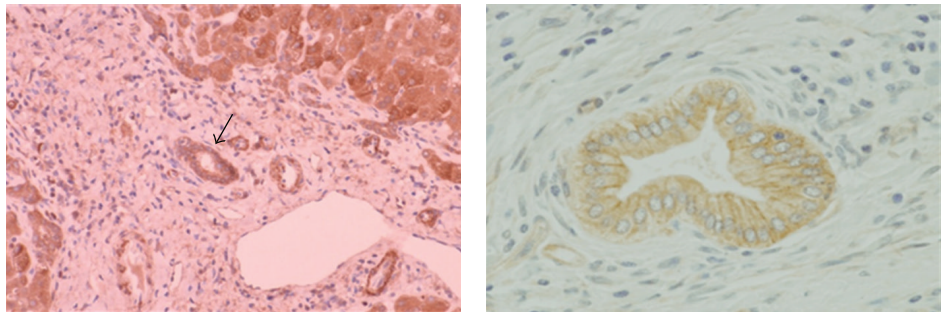
	Human		Murine
	BECs	Cholangiocarcinoma	BECs
TLR1	+ [19]		
TLR2	+ [19, 20]	+ [2]	+ [2]
TLR3	+ [19, 20]	+ [2]	+ [2]
TLR4	+ [19–21]	+ [2]	+ [2]
TLR5	+ [19, 20]	+ [2]	+ [2]
TLR6	+ [19, 20]		
TLR7	+ [19] / −*		
TLR8	+ [19] / −*		
TLR9	+ [19] / −*		
TLR10	+ [19]		

Blanks: no reports. *Our unpublished data. Parentheses denote reference numbers.

NODs (i.e., NOD1 and NOD2) are also involved in the intracellular recognition of microbes through specific interactions with derivatives of pathogen-specific peptidoglycans [18].

The expression of TLRs in human and murine cholangiocytes and several human cholangiocarcinoma cell lines has been confirmed by several groups (Table 2), implicating the possible activation of biliary mucosal immunity against microbial infections [2, 19–23]. Cultured human and murine biliary epithelial cells (BECs) possess at least TLR1-TLR5, related molecules (MD-2, MyD88, and IRAK-1), RIG-I, and MDA-5 [2, 20, 23, 24]. Moreover, SV40-transformed human cholangiocytes expressed mRNAs for all ten human TLRs [19]. Immunohistochemistry has confirmed that intracellular adaptor molecules (MyD88 and IRAK-1) as well as TLRs (TLR1-TLR5) are diffusely distributed in the intrahepatic biliary tree of normal and diseased human livers, irrespective of anatomical level (Figure 1) [2, 20–22, 24, 25]. As for NODs, cultured human BECs and cholangiocytes in intrahepatic bile ducts constantly express the mRNA and protein of NOD2, but cultured BECs do not respond to the NOD2 ligand (muramyl dipeptide, MDP), indicating a suspicious functional expression (our unpublished data).

2.2. Recognition of PAMPs. In addition to the expression of TLRs in cholangiocytes and the biliary epithelium, the activation of TLRs has also been demonstrated during bacterial, viral, and parasitic infections. Stimulation with PAMPs including Pam3CSK4 (TLR1/2 ligand), MALP-2 (TLR2/6 ligand), peptidoglycan (TLR2 ligand), and polyinosinic-polycytidylic acid (poly(I:C), a synthetic analog of viral dsRNA, TLR3 ligand) induced the activation of NF-κB, a major transcription factor downstream of TLRs, in cultured human BECs [2, 20, 23]. In addition to bacteria, *Cryptosporidium parvum* (*C. parvum*), a protozoan parasite causing intestinal and biliary diseases, also activates both TLR2 and TLR4 in cholangiocytes to initiate epithelial host responses, accompanying the recruitment of these TLRs and ganglioside GM1 to membrane rafts [26]. Membrane rafts have been implicated in TLR activation in several other cell



(a) (b)

Figure 1: Immunohistochemistry for TLR3 in chronic hepatitis C (a) and TLR4 in primary biliary cirrhosis (PBC). The expression of TLR3 existing in endosomes is found in interlobular bile ducts (arrow in (a)) and hepatocytes in a cytoplasmic pattern. In contrast, TLR4 expression is highlighted in a membranous pattern (b).

types, including epithelial cells, following microbial infection [27]. Moreover, viral PAMPs such as double-stranded RNA (dsRNA) are also recognized by cultured BECs; cultured human BECs expressed nuclear transcription factors including NF-κB and interferon regulatory factor-3 (IRF-3) on stimulation with poly(I:C), a synthetic analog of viral dsRNA [23]. These findings indicate that human BECs possess functional PAMP-recognizing receptors and an innate immune system against viruses as well as bacteria.

In addition to microorganism components, several families of proteins originating from and produced by autocells are involved in the recognition of pathogens and the products released from injured or dying cells. In particular, endogenous factors including HMGB1, S100A8/S100A9, and heat shock proteins are known as damage-associated molecular patterns (DAMPs) [28], but a detailed analysis has not been conducted in cholangiocytes.

2.3. Response to PAMPs. As part of the host's defenses against infections, cholangiocytes secrete polymeric immunoglobulin A and produce several antibiotics against bacteria (lactoferrin, lysozyme, and defensins) [29–31]. Defensins, in particular, are key elements in innate immunity. Basic peptides activate against a broad spectrum of microbes including bacteria and fungus, defensins are divided into two types, α- and β-defensins. Human beta-defensins (hBDs) consisting of hBD1-hBD6 are produced by several epithelial cells including cholangiocytes and play an important role in the defense against mucosal infection. hBD1 distributes throughout the intrahepatic biliary tree and is detected in bile. Moreover, studies using cultured human BECs and SV40-transformed human cholangiocytes confirmed the constant production of hBD1 and also hBD3 [19, 22]. In contrast, hBD2 is not physiologically expressed in nondiseased livers and *de novo* expression is detected in bile ducts showing suppurative inflammation in patients with biliary diseases such as hepatolithiasis and biliary infections and also in their bile [22]. Moreover, the expression of hBD2 via the activation of NF-κB occurred on stimulation by PAMPs including LPS, *E. coli,* and *C. parvum* in cultured human BECs [19, 22]. This finding suggests that hBD-1 is constantly detectable in bile samples while it plays a role in the constitutive antimicrobial defense of the hepatobiliary system and hBD2 plays a role in the localized biliary defense in cases of biliary infection. In addition to defending against bacteria, cholangiocytes possess an innate immune system to fight viral infections, because cholangiocytes have TLR3, RIG-I, and MDA-5 recognizing dsRNA viruses such as Reoviridae (reovirus and rotavirus). Stimulation with poly(I:C), a synthetic analog of viral dsRNA, induces the activation of NF-κB and IRF3 and the production of key components of antiviral immunity, IFN-β1 and MxA [23]. In normal human liver tissues, small numbers of Kupffer cells, but no hepatocytes and cholangiocytes, exhibited MxA expression. In contrast, strong expression of the MxA protein was identified in Kupffer cells and cholangiocytes in patients with chronic liver diseases and fulminant hepatic failure [19]. These findings suggest that cholangiocytes participate directly in innate immunity and show a prompt response to pathogens without any help from immunocompetent cells such as macrophages.

In addition to antibiotics, cholangiocytes produce several inflammatory cytokines and chemokines such as IL-6, TNF-α, IL-8, fractalkine, monocyte chemotactic protein-1 (MCP-1), and CXCL16 [2, 19, 20, 32–37]. IL-6 has been demonstrated to increase DNA synthesis in human cholangiocytes *in vitro*, indicating increased proliferative activity [38]. IL-8 is closely associated with neutrophilic infiltration and its expression is found in cholangitis lenta which is usually encountered in septic patients and characterized by bile ductular proliferation, ductular cholestasis, and ductular epithelial damage [33, 39]. Chemokines produced in cholangiocytes as part of the biliary innate immune response could result in the recruitment and activation of T cells, macrophages, neutrophils, hepatic stellate cells, and NK cells to protect against biliary infection and also play an important role in bile duct-specific acquired immunity by forming periductal cytokine networks and migrating immunocompetent cells, thereby contributing to biliary mucosal defense and subsequent acquired immunity.

Cholangiocytes may also function as professional antigen-presenting cells (APCs) and contribute to the control of

inflammatory reactions [40]. Cultured murine BECs constitutively expressed low levels of MHC Class I and MHC Class II molecules, and these levels were significantly enhanced by IFN-γ stimulation and murine cytomegalovirus (CMV) infection [41]. Moreover, murine BECs infected with murine CMV showed a progressive cytopathic effect. In contrast, in cultured human BECs, CMV-infection augmented the expression of MHC class I but not MHC class II molecules [42]. These findings suggest that CMV affects the immunogenic potential of cholangiocytes.

TLR signals influence from fuctions of tight junctions in cholangiocytes by activating various intracellular signaling pathways. LPS disrupted the tight junctions of a rat BEC monolayer via a TLR4-dependent mechanism and LPS and *C. parvum* increased paracellular permeability by activating c-Src in rat and human BECs [43, 44]. Therefore, biliary innate immune reactions are involved in the functional regulation of tight junctions in cholangiocytes.

3. Regulation of Biliary Innate Immunity

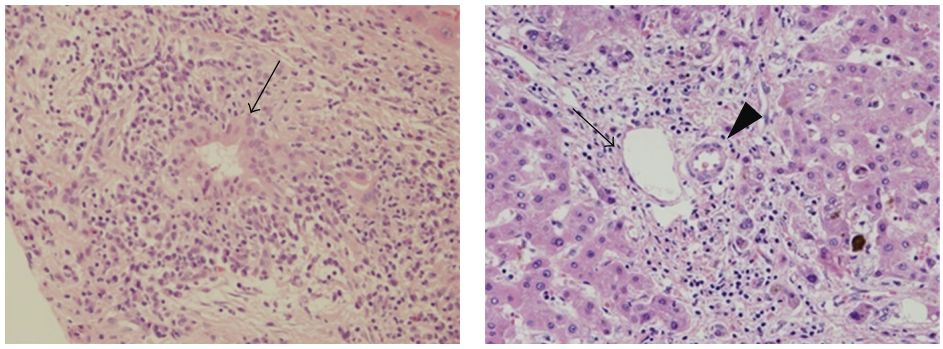
TLR signaling initiates adaptive immunity which then regulates the innate immune system to maintain mucosal homeostasis. The expression of TLRs in cholangiocytes is highly regulated, but its disruption has been associated with various hepatobiliary diseases. Infecting cultured human cholangiocytes with *C. parvum* induced a significant increase in TLR4 protein, a process that appears to be associated with the production of hBD2 [19]. T cell-derived inflammatory cytokines are known to participate in the regulation of TLR expression in several cells [45, 46]. The interactions of TLRs with Th1 cytokines, in particular, participate in the pathogenesis of inflammatory bowel diseases [47]. Cholangiocytes express receptors for cytokines such as IFN-γ, TNF-α, IL-4, IL-6, and IL17, and thus, are also the target of many periductal inflammatory mediators during biliary inflammatory diseases. A Th1-type cytokine, IFN-γ upregulates the mRNA expression of TLR2-TLR5 and accelerates the upregulation of PAMP-induced NF-κB activation in cholangiocytes, suggesting that a Th1-dominant peribiliary milieu leads to the increased susceptibility to PAMPs and the production of inflammatory cytokines and chemokines from BECs [20]. This impaired regulation of biliary innate immunity caused by the Th1-predominant milieu may be involved in the pathogenesis of cholangiopathy in biliary diseases including PBC [48]. In fact, upregulation of TLR4 and TLR9 in cholangiocytes has been reported in patients with PBC and PSC [25, 49].

Micro-RNAs play important roles in a wide range of biological events through posttranscriptional suppression of target mRNAs. Recent studies indicate that micro-RNA-mediated posttranscriptional pathways may be critical to host-cell regulatory responses to microbial infections. Cultured human BECs express let-7 family members which posttranscriptionally downregulate TLR4 expression and infections of *C. parvum* decrease the expression of let-7 resulting in the upregulation of TLR4 [50]. Moreover, microRNA-98 and let-7 suppressing cytokine-inducible Src homolog 2-containing protein (CIS, a suppressor of cytokine signaling family) at the translational level are expressed in cholangiocytes and LPS and *C. parvum* infections downregulate these mirco-RNAs, suggesting the regulation of the TLR-mediated biliary innate immune response [51].

The luminal surface of the bile duct is continually exposed to PAMPs via bile, but cholangiocytes physiologically do not elicit an inflammatory response. This lack of response to PAMPs, especially LPS, could be due to "endotoxin tolerance" and this system is important in preventing endotoxin shock in infections as well as maintaining homeostasis in organs [52]. As for negative regulatory systems of innate immunity, mechanisms compete with TLR binding and suppress intracellular TLR signaling using several molecules including extracellular soluble TLRs (sTLRs), single immunoglobulin IL-1-related protein (SIGIRR), IRAK-M (homolog of IRAK-1), MyD88s (inactive splice variant of MyD88), SARM (negative regulator of TRIF), Toll-interacting protein (Tollip), A20, SHIP (a PI3K inhibitor), and suppressor of cytokine signaling-1 (SOCS1) [52–58].

Our previous study using cultured BECs and cholangiocarcinoma cell lines revealed that the activation of NF-κB and the increased expression of TNF-α caused by stimulation with PAMPs including LPS are gradually attenuated with time and that pretreatment with LPS significantly inhibits the response to subsequent stimulation, suggesting an induction of LPS (endotoxin) tolerance [59]. Moreover, pretreatment with Pam_3CSK_4 (TLR1/2 ligand) effectively induced tolerance to subsequent stimulation with LPS (TLR4 ligand) [52, 59]. Among several negative regulators, the expression of at least IRAK-M and Tollip has been demonstrated in human cholangiocytes and treatment with LPS and Pam_3CSK_4 upregulates the expression of IRAK-M, but not Tollip. IRAK-M negatively regulates TLR signaling by inhibiting the activation of IRAK-1 and MyD88 [55]. Furthermore, immunohistochemistry using human liver tissue sections confirmed that IRAK-M is diffusely expressed in intrahepatic biliary trees in both normal and diseased livers. This negatively regulated mechanism of innate immune response is important to escape hypercytokine milieu and tissue injury caused by excessive innate immune responses.

In contrast, treatment with poly(I:C), TLR3 ligand, significantly enhanced NF-κB activity in fresh cultured BECs and pretreatment did not lead to tolerance to poly(I:C). [60] Levels of production of MxA and IFN-beta1 were also preserved. Therefore, TLR tolerance to a viral PAMP (poly(I:C)) is not found in BECs. Although IRAK-M mRNA expression was upregulated by stimulation with dsRNA (TLR3 ligand), no tolerance to the dsRNA was found in cultured BECs. This is reasonable because the intracellular signaling of TLR3 is a MyD88-independent pathway, that is, the dsRNA-related response is not affected by IRAK-M [17]. These findings suggest that cholangiocytes lining biliary trees are resistant to nonpathogenic commensal bacterial PAMPs, but not virus-derived dsRNA, maintaining the homeostasis of biliary innate immunity in physiological conditions. Moreover, the upregulation of IRAK-M expression on treatment with poly(I:C) is speculated to cause dsRNA-stimulated BECs to become resistant to TLR2- and TLR4-related PAMPs including LPS. Therefore, once cholangiocytes are infected



(a) (b)

Figure 2: Primary biliary cirrhosis (PBC). (a) Chronic nonsuppurative destructive cholangitis (CNSDC). Damaged bile ducts (arrow) and infiltration of mixed chronic inflammatory cells surrounding bile ducts are found. (b) Bile ducts have disappeared in the portal tract. Arrowhead and arrow denote artery and portal vein, respectively.

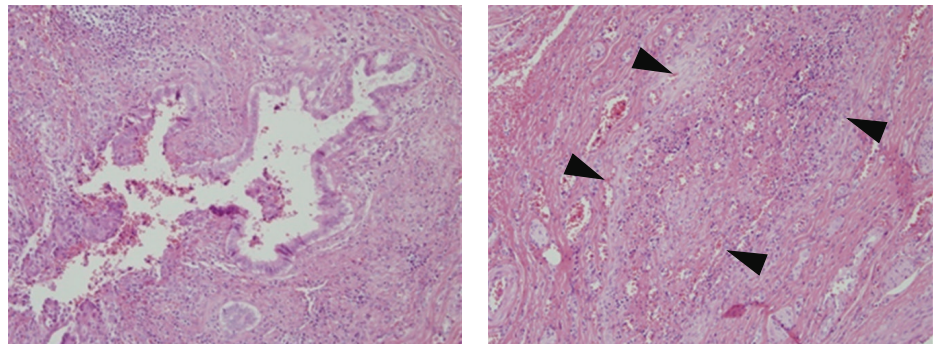
by a dsRNA virus, progressive destruction caused by the biliary innate response to dsRNA and resistance to bacterial infection continues until the virus is eliminated.

4. Disease-Specific Cholangiopathy Associated with Biliary Innate Immunity

4.1. PBC. PBC is characterized by the selective destruction and loss of interlobular bile ducts including chronic nonsuppurative destructive cholangitis (CNSDC) (Figure 2) [61]. The etiopathogenesis of PBC still remains speculative, but a high prevalence of vaginal and urinary tract infections and the presence of bacterial and viral components in bile and liver tissue and of the molecular mimicry of human and bacterial pyruvate dehydrogenase complex-E2 (PDC-E2, a major epitope of antimitochondrial antibody [AMA]) and xenobiotics are demonstrated (Table 1) [3, 5, 62–68]. Moreover, BECs translocate immunologically intact PDC-E2 to apoptotic bodies and create an apotope. The unique triad of BEC apotopes, macrophages from patients with PBC, and AMAs induces intense inflammatory cytokine production, providing a mechanism for the biliary specificity of PBC [69]. Innate immunity changes may be critical to the initiation and perpetuation of the autoimmune injury, as in the case of the enhanced response of immunocompetent cells (monocytes and memory B cells) as well as target BECs to infectious stimulation and environmental mimics [70, 71]. These findings suggest that the presence of microorganisms and the innate immune responses against them are involved in the pathogenesis, particularly cholangiopathy, of PBC.

In PBC, the expression of TNF-α and IL-6 was detected in cholangiocytes from the liver of patients with PBC, suggesting the result of some biliary response including a biliary innate immune response [72]. Several studies revealed that, compared with Th2, a Th1-dominant cytokine milieu is associated with the pathogenesis including bile duct injury in PBC [48]. Cholangiocytes possess the receptor for IFN-γ (Th1 cytokine) and IFN-γ upregulates the expression of TLRs and susceptibility to PAMPs in cholangiocytes, impairing the regulation of biliary innate immunity. Moreover, IL-4 (Th2 cytokine) and IFN-γ up- and downregulate the expression of peroxisome proliferator-activated receptor γ (PPARγ) showing anti-inflammatory activities in biliary innate immune response, respectively, in cultured human BECs [73, 74]. PPARγ is constitutively expressed in cholangiocytes of intrahepatic small bile ducts. PPARγ as well as IRAK-M, therefore, may also relate to the maintenance of biliary homeostasis as a tolerant regulator by attenuating inflammatory signals in cholangiocytes to commensal PAMPs in biles [73]. However, in PBC liver, PPARγ expression is significantly downregulated in the affected bile ducts as a Th1-dominant periductal cytokine milieu [73]. Moreover, the upregulation of TLR4 and TLR9 in cholangiocytes and of TLR3 and type I IFN signaling pathways in portal tracts and parenchyma are also found in PBC [24, 25, 49]. These finding indicate an increased susceptibility to PAMPs, suggesting an association with the pathogenesis of cholangiopathy in PBC.

In addition to Th1 and Th2 cells, a third pathogenic type, Th17 cells, are involved in the pathogenesis of chronic inflammatory diseases. Human Th17 cells are characterized by the production of IL-17 (IL-17A and IL-17F) and IL-6, IL-1β, and IL-23 (TGF-β instead of IL-1β in mice) are critical for driving the differentiation of naïve T cells into Th17 cells and maintaining or stabilizing the functions of Th17 cells [75, 76]. In inflammatory hepatobiliary diseases including PBC, IL-17-positive mononuclear cells are scattered at the interface areas, particularly showing interface hepatitis [32]. In PBC, moreover, the periductal accumulation, particularly around cholangitis including CNSDC accompanying the expression of IL-6, IL-1β, and IL-23 p19, of IL-17 positive cells is found, suggesting that the Th17-related peribiliary cytokine milieu is involved in the histogenesis of the sustained cholangiopathy of PBC [32, 77]. Moreover, an *in vitro* study using cultured human BECs revealed that bacterial PAMPs (LPS and Pam3CSK4) induced the production of Th17-inducing and -maintaining cytokines (IL-6, IL-1β, and IL-23 p19) [32]. These results indicate that biliary innate immunity plays a role in the induction and maintenance of Th17 cells in the periductal area in cases of PBC and the differentiation into Th17 cells in periductal dendritic



(a) (b)

Figure 3: Transverse section of extrahepatic biliary remnants in biliary atresia. (a) Distorted common bile duct showing luminal occlusion with surrounding fibroplasia and inflammatory cells. (b) The common bile duct has disappeared leaving a fibrous scar (arrowheads).

cells and macrophages. Th17 cells are part of the mucosal host defense system and also propagate and modulate the cholangiopathy in PBC.

Our recent study revealed that Langerin-positive Langerhans cells (LCs) are dominantly scattered around or within biliary epithelial layers of the damaged bile ducts in PBC. Moreover, experiments with cultured human BECs showed that an LC-attracting chemokine, macrophage inflammatory protein-3α, was produced by cholangiocytes in response to cytokines (IL-1β, TNF-α, and IL-17) and PAMPs [78]. Therefore, LCs existing around or within biliary epithelial layers are important as periductal antigen-presenting cells in PBC and the migration of LCs into bile ducts is closely associated with the periductal cytokine milieu and biliary innate immunity in PBC.

4.2. Biliary Atresia. Biliary atresia characterized by a progressive sclerosing obstruction of extrahepatic bile ducts (Figure 3), is a common infant biliary disease and subdivided to embryonic and perinatal types based on the clinicopathogenesis. Little is known about the etiology and pathogenesis of biliary atresia, but studies using human materials and a virus-infected rodent model suggest an association with Reoviridae (type 3 reovirus and type C rotavirus) having dsRNA, although conflicting results also have been reported [12, 79–81]. Imbalanced cell kinetics caused by enhanced apoptosis in cholangiocytes lining extrahepatic bile ducts is speculated as an important mechanism in obstructive cholangiopathy [23, 82, 83]. Human cholangiocytes are sensitive to tumor necrosis factor-related apoptosis-inducing ligand- (TRAIL-) and Fas- (CD95-)mediated apoptosis [20, 23, 84]. Moreover, because Reoviridae show epitheliotrophysm, the innate immune response against viruses is speculated to be directly associated with epithelial injury and death in biliary atresia. Our previous study demonstrated that stimulation with poly(I:C) induced the activation of NF-κB and IRF-3, followed by the production of antiviral IFN-β1 and also enhanced apoptosis via production of TRAIL [23]. Moreover, in biliary atresia, cholangiocytes lining the remnants of extrahepatic bile ducts diffusely and constantly expressed TLR3 and showed an enhancement of TRAIL and single-stranded DNA- (ssDNA-)positive apoptosis accompanying the activation of NF-κB and IRF-3 [20, 23]. A significant increase of TLR7 and antimicrobial peptide hepcidin and MxA at the mRNA and protein levels, was found in patients in the early stage of biliary atresia [85–87]. Therefore, cholangiocytes not only directly participate in the antiviral innate immune response, but also play a role in the generation of apoptotic responses to infected cells. Moreover, as described above, because the innate immune tolerance of dsRNA is lacking in cholangiocytes, the biliary damage caused by the biliary innate immune response continues until the virus disappears and directly forms the histogenesis of obstructive cholangiopathy in biliary atresia [60].

As the histogenesis of sclerosing lesion, the epithelial-mesenchymal transition (EMT) of cholangiocytes has been speculated to be associated with periductal fibrosis and portal fibrosis in biliary atresia [88–91]. Fundamental to EMT is a loss of normal epithelial features such as cell-to-cell adhesion molecules, the gain of mesenchymal phenotypes, and the acquisition of a fibroblast-like (spindle) morphology with cytoskeletal reorganization [92]. As mentioned above, although the biliary innate immune response to dsRNA reduces the viability of cultured human BECs via TRAIL-mediated apoptosis, the rate of cell death is approximately 70% [23]. The cells that evade apoptosis show a gradual loss of epithelial markers, CK19 (biliary-type cytokeratin in liver) and E-cadherin, and increased expression of a mesenchymal marker S100A4 (also known as fibroblast-specific protein 1) and an essential transcription factor for EMT, Snail, via increased susceptibility to transforming growth factor-β1 (TGF-β1) and the production of basic fibroblast growth factor (bFGF), demonstrating the occurrence of biliary EMT [23]. Because EMT confers resistance to apoptotic effects in fetal rat hepatocytes [93], biliary EMT is thought to be a survival mechanism and associated with an incomplete induction of apoptosis caused by the biliary innate immune response. In fact, *in vivo* studies reveal that mesenchymal markers (vimentin and S100A4) and Snail are expressed but CK19 and E-cadherin are not in cholangiocytes lining the remnants of extrahepatic bile ducts and peribiliary glands of biliary atresia [91, 94], suggesting that the occurrence

of EMT in cholangiocytes is associated with an incomplete induction of apoptosis caused by the biliary innate immune response and that these surviving cells play a role in the sclerosing cholangiopathy of biliary atresia without inducing tolerance until the clearance of the virus.

5. Conclusion and Perspectives

Biliary innate immunity consisting of an organ-specific system is important for the mucosal immunity in intrahepatic and extrahepatic bile ducts and also associated with the pathogenesis of several cholangiopathies in biliary diseases. We speculate that biliary innate immunity is solely associated with the etiology of biliary diseases as the initial event and that the presence of causative microorganisms is not necessary in the pathogenesis of cholangiopathy caused by a subsequent acquired immunity. It is mandatory to understand the molecular basis underlying the immunophysiology and immunopathology of cholangiopathy in terms of innate as well as acquired immunity.

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Review Article

IgG4 Cholangiopathy

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IgG4 cholangiopathy can involve any level of the biliary tree which exhibits sclerosing cholangitis or pseudotumorous hilar lesions. Most cases are associated with autoimmune pancreatitis, an important diagnostic clue. Without autoimmune pancreatitis, however, the diagnosis of IgG4-cholangiopathy is challenging. Indeed such cases have been treated surgically. IgG4-cholangiopathy should be diagnosed based on serological examinations including serum IgG4 concentrations, radiological features, and histological evidence of $IgG4^+$ plasma cell infiltration. Steroid therapy is very effective even at disease relapse. A Th2-dominant immune response or the activation of regulatory T cells seems to be involved in the underlying immune reaction. It is still unknown why IgG4 levels are specifically elevated in patients with this disease. IgG4 might be secondarily overexpressed by Th2 or regulatory cytokines given the lack of evidence that IgG4 is an autoantibody.

1. Introduction

IgG4-related disease is a unique systemic inflammatory condition characterized by tumorous swelling of affected organs and high-serum IgG4 concentrations [1–3]. Autoimmune pancreatitis is a prototype of IgG4 disease as Hamano et al. described in a landmark paper in 2001 [4]. Further studies have confirmed that IgG4-related disease can involve a variety of organs including the salivary glands (chronic sclerosing sialadenitis) [5, 6], lacrimal glands (Mikulicz's disease) [7, 8], and retroperitoneum (retroperitoneal fibrosis) [9, 10]. The bile duct lesion is called IgG4-related sclerosing cholangitis (IgG4-SC) [11] or IgG4-associated cholangitis [12, 13] (the former is used hereafter). Since we reported that IgG4-SC is a distinct entity which should be differentiated from primary sclerosing cholangitis (PSC) [11], clinical and pathological features have been clarified [12, 13]. In this paper, we describe the concept, pathology, differential diagnosis, and pathogenesis of IgG4-SC.

2. Spectrum of IgG4 Cholangiopathy

The relationship between IgG4-SC and autoimmune pancreatitis is summarized in Table 1. IgG4-SC can manifest as diffuse sclerosing cholangitis or a hilar pseudotumourous mass [11]. The former should be differentiated from PSC, whereas the latter radiologically resembles hilar cholangiocarcinoma [14]. Of note is that most case of IgG4-SC are associated with autoimmune pancreatitis. A study by the Mayo Clinic found that only 4 of 53 patients (7.5%) with IgG4 cholangiopathy had cholangitis without autoimmune pancreatitis [13]. Whether or not IgG4-SC can involve only peripheral small bile ducts like small-duct PSC is an interesting issue. Given that a recent study revealed that peripheral IgG4 cholangiopathy was always associated with large duct lesions [15], it seems safe to assume that IgG4-SC predominantly affects large bile ducts, which are detectable by cholangiographic or radiological examinations.

Recent papers have introduced IgG4-related autoimmune hepatitis [16], which accounts for 3% of cases of type 1 autoimmune hepatitis in the Japanese population [17]. The term IgG4 hepatitis should be only used for patients who do not have radiological biliary abnormalities and are found to have chronic hepatitis based on liver biopsies. Given that one cases of IgG4 hepatitis was complicated later by sclerosing cholangitis during followup [17], IgG4 hepatitis might also belong to a spectrum of IgG4 cholangiohepatitis.

3. Clinical Features and Autoantibodies

In our experience, patients with IgG4-SC usually present with obstructive jaundice due to a pancreatic head mass (autoimmune pancreatitis) or severe biliary stricture [12, 13]. Other patients are sometimes discovered to have IgG4-SC during a workup for other IgG4-related conditions such as sialadenitis, retroperitoneal fibrosis and kidney lesions. Weight loss or new-onset diabetes mellitus due to pancreatitis is another potential symptom.

Patients with IgG4 disease share serological abnormalities irrespective of the organ of origin. There is no doubt that elevated serum IgG4 levels are the most specific indicator. Other sensitive but not specific markers include hyper γ-globulinemia (observed in 50% of patients), hyper IgG (60–70%), antinuclear antibodies (40–50%), rheumatoid factor (20%), and eosinophilia (15–25%) [18, 19]. Autoantibody against SS-A (Ro) or SS-B (La), antimitochondria antibody, and antineutrophilic cytoplasmic antibody (ANCA) are all exceptional (<5%) [18, 19].

Studies on autoimmune pancreatitis have provided further data on autoantibodies which might participate in the pathogenesis. Antibodies against lactoferrin (LF) and carbonic anhydrase (CA) II are frequently detected in cases of autoimmune pancreatitis (73% and 54%, resp.) [20]. Interestingly, a strong positive correlation between increases in serum IgG4 levels and anti-CA-II antibody levels has been reported [21]. Anti-CA-IV, another autoantibody, was detected in 10 of 29 (34%) patients with autoimmune pancreatitis [22]. Given that LF and CAs are expressed in some exocrine organs, these autoantibodies may be related to systemic manifestations of IgG4-related disease. Of note is that autoantibodies of the IgG4 subclass have not been detected in patients with IgG4-related disease so far.

4. Diagnosis

4.1. Surgical Cases. It is not difficult to make a diagnosis of IgG4-SC if surgically resected specimens are available. The gold standard for the diagnosis of IgG4-SC is histology including characteristic features on H&E and extensive infiltration by $IgG4^+$ plasma cells on immunostaining. Pathological features can be summarized as follows: (1) diffuse lymphoplasmacytic infiltration, (2) storiform fibrosis, (3) obliterative phlebitis, (4) eosinophilic infiltration, and (5) numerous $IgG4^+$ plasma cells [11, 23]. Features unusual for IgG4-SC are neutrophilic infiltration with or without abscesses, xanthogranulomatous change, and mucosal erosive change. Obliterative phlebitis is a finding characteristic for IgG4-related disease irrespective of the organ affected. We speculate that endothelium may express chemotactic factors, but this has not been examined so far.

4.2. Patients with Autoimmune Pancreatitis. Serology, imaging, other organ involvement, and biopsy need to be considered for the diagnosis of nonsurgical cases. Given that most patients (>90%) with IgG4-SC have autoimmune pancreatitis, it seems most important to examine changes in the pancreas. Autoimmune pancreatitis is radiologically suspected in most cases, and the diagnosis can be confirmed by the serological examination of IgG4. Histological detection of $IgG4^+$ plasma cells is usually not necessary for cases showing typical radiological features (sausage-like diffuse swelling, peripancreatic capsule-like rim, and irregular narrowing of the pancreatic duct) [24] and high-serum IgG4 levels. But, if there are any unusual features on imaging or IgG4 levels are not elevated, biopsies should be considered to detect $IgG4^+$ plasma cells. Most institutions use 135 or 140 mg/dL as a cut-off point for serum IgG4 levels, with more than 300 mg/dL being highly specific for IgG4-related disease [4, 25].

4.3. Patients without Autoimmune Pancreatitis. It is challenging to diagnose IgG4-SC not associated with pancreatitis. In fact, most patients have been surgically treated for suspected biliary malignancy [13]. In our experience, detecting $IgG4^+$ plasma cell is recommended even if the patients have high-serum IgG4 concentrations. Three potential approaches have been proposed to detect infiltration by $IgG4^+$ plasma cells. Vater's ampulla biopsy is least invasive and technically easiest, and especially useful for patients discovered endoscopically to have ampullary swelling [26–29]. Another potential approach is a liver needle biopsy which can detect $IgG4^+$ plasma cells infiltrating into peripheral small portal tracts [15, 30, 31]. This is particularly useful for patients with intrahepatic biliary abnormalities on cholangiograms, but not useful for patients with only intrapancreatic bile duct stricture [15]. The last choice is a bile duct biopsy, the usefulness of which might depend on the ability and experience of the endoscopists [29]. The biggest advantage of this method is that not only $IgG4^+$ plasma cells but also other histological features such as storiform fibrosis and eosinophilic infiltration are detectable [29]. The specificity and sensitivity of the diagnosis by these three biopsies are summarized in Table 2. Normally, more than 10 $IgG4^+$ plasma cells are used as the diagnostic threshold for biopsy samples.

4.4. Steroid Trial. There is no international consensus regarding diagnostic steroid trials for IgG4-related disease. Some institutions use steroid trials for the diagnosis [32–34], whereas other groups, especially from Japan, are against doing so [35]. Given that IgG4-SC needs to be differentiated from malignant tumours (cholangiocarcinoma) different from other typical autoimmune diseases, much attention should be paid to diagnostic steroid trials.

5. Differential Diagnosis

5.1. PSC. IgG4-SC must be differentiated from PSC given that the steroid responsiveness is completely different. These two entities are histologically distinct. Large bile ducts affected by IgG4-SC show transmural inflammation with storiform fibrosis and obliterative phlebitis (Figure 1), whereas inflammation is more extensive on the luminal side with erosion or xanthogranulomatous changes in PSC [11]. Clinically, a young age (<40 years) and history of

Table 1: Disease spectrum of IgG4 pancreato-cholangiopathy and differential diagnosis.

IgG4-related pancreato-cholangiopathy	Differential diagnosis
Autoimmune pancreatitis without bile duct involvement	Pancreatic cancer
Autoimmune pancreatitis with IgG4 cholangitis	Pancreatic cancer and cholangiocarcinoma
IgG4-related sclerosing cholangitis	Primary sclerosing cholangitis
IgG4-related sclerosing cholangitis with hilar pseudotumor	Hilar cholangiocarcinoma

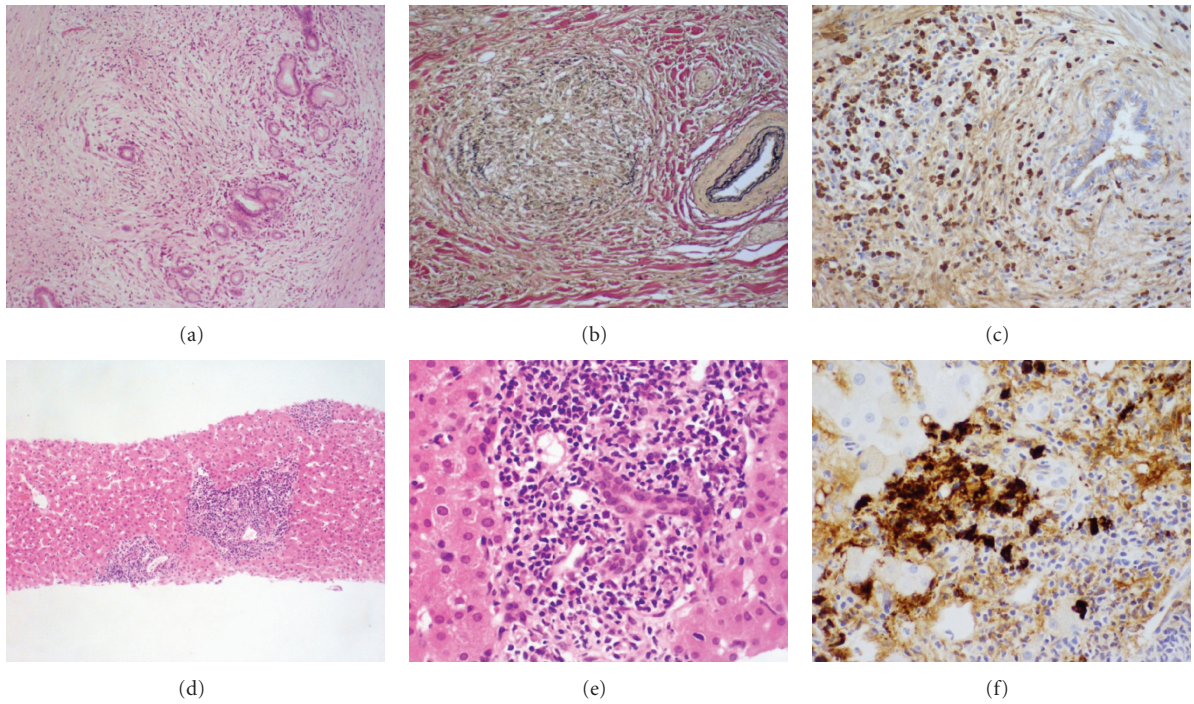

Figure 1: Histopathology of IgG4-related sclerosing cholangitis. The surgical specimens show diffuse inflammatory cell infiltration with fibrosis involving peribiliary glands (a), obliterative phlebitis (b), and infiltration of many IgG4$^+$ plasma cells (c). The liver needle biopsy reveals portal inflammation (d), bile duct damage (e), and IgG4$^+$ plasma cells (f).

Table 2: Sensitivity and specificity of detection of IgG4$^+$ plasma cells (≥10 cells/high power field) by ampullary, liver, and bile duct biopsies.

	Sensitivity	Specificity	Reference
Ampullary biopsy	80%	100%	[26]
	67%	100%	[27]
	53%	100%	[28]
	52%	91%	[29]
Liver needle biopsy	24%	100%	[30]
	60%	100%	[31]
	26%	100%	[15]
Bile duct biopsy	52%	97%	[29]

inflammatory bowel disease are features suggestive of PSC, whereas IgG4-SC is more likely in patients with other sclerosing lesions including autoimmune pancreatitis and retroperitoneal fibrosis. Serologically, IgG4 levels are most useful, but it should be noted that 9% of PSC patients show elevated IgG4 levels [36]. Positivity for ANCA is suggestive of PSC. Eosinophilia is similarly detectable in both diseases. Nakazawa et al. reported that PSC and IgG4-SC can be differentiated based on a detailed examination of cholangiograms [37]. Liver biopsy is also useful. Infiltration of IgG4$^+$ plasma cells or the presence of periportal "fibroinflammatory nodules [30]" in needle biopsy samples is suggestive of IgG4-SC (Figure 1), whereas ductopenia and periductal concentric fibrosis are more commonly seen in PSC [15, 31]. It is still unclear whether or not PSC can be differentiated from "burned-out" IgG4-SC.

5.2. PSC with Many IgG4$^+$ Plasma Cells. Zhang et al. [38] examined tissue infiltration of IgG4$^+$ plasma cells in explanted liver with PSC. Twenty-three of 98 livers (23%) showed more than 10 IgG4$^+$ cells per HPF which might be less specific given that most IgG4-related lesions show more than 100 IgG4$^+$ cells/HPF in surgical specimens. In addition, another group revealed that 2 out of 41 (5%) explanted livers with PSC showed more than 100 IgG4$^+$ cells/HPF [39]. These two studies suggested that explanted livers with PSC can sometimes show moderate degrees of IgG4$^+$ cell infiltration

(>10 cells/HPF) and rarely exhibit marked infiltration (>100 cells/HPF) around large bile duct lesions. Importantly, the other histological features of these cases were not typical of IgG4-SC but consistent with PSC [38, 39]. The histological diagnosis of IgG4-SC is not enough just based on the number of $IgG4^+$ plasma cells.

5.3. Follicular Cholangitis. This is a rare disease entity characterized by numerous lymphoid follicles around hilar or perihilar bile ducts [40, 41]. Most patients reported so far underwent surgical resection on suspicion of hilar cholangiocarcinoma. Follicular cholangitis is different from PSC in that the inflammatory cell infiltration is more extensive and biliary epithelial damage is not conspicuous. $IgG4^+$ plasma cell infiltration or obliterative phlebitis is usually not conspicuous, different from IgG4-SC [40].

5.4. Hilar Cholangiocarcinoma. In our experience, hilar cholangiocarcinoma is the most important and difficult differential diagnosis of IgG4-SC in the clinical field, particularly for patients without autoimmune pancreatitis. Radiological features of IgG4-SC sometimes resemble those of hilar cholangiocarcinoma [14]. Serum IgG4 levels can be mildly elevated in patients with cholangiocarcinoma, but titers of more than 300 mg/dL are highly suggestive of IgG4-SC. As described above, histological examination to detect $IgG4^+$ cell infiltration is needed for patients without autoimmune pancreatitis.

6. Treatment

IgG4-SC responds dramatically to steroid therapy the same as other IgG4-related lesions. This is one significant difference from PSC [12, 13]. At the moment, it is difficult to conclude the recommended dose and duration of steroid therapy for IgG4-SC because of a lack of published data. A Japanese study on autoimmune pancreatitis recommended an initial dose of 0.6 mg/kg/day, which was then reduced to a maintenance dose over a period of 3–6 months [42]. In that study, disease relapses appeared to be reduced but not eliminated by maintenance treatment with low-dose steroid [42]. Rituximab is a potential treatment for steroid-resistant autoimmune pancreatitis or IgG4-SC [43].

7. Pathogenesis

Recent papers have provided data suggesting that the Th2-type immune response is activated in IgG4-related disease including IgG4-SC [44–48]. Quantitative real-time PCR using RNA extracted from frozen tissue of affected organs including bile ducts revealed significantly higher ratios of IL-4/IFN-γ, IL-5/IFN-γ, and IL-13/IFN-γ in IgG4-related disease tissues than in tissues from patients with classical autoimmune diseases [49]. Lymphocytes expressing IL-4 were clearly demonstrated by *in situ* hybridization. Recent papers also showed that peripheral blood mononuclear cells collected from patients with IgG4-related disease produced predominantly Th2-type cytokines such as IL-4, IL-5, IL-10, and IL-13 after T-cell stimulation.

Interestingly, the number of regulatory T-cells (Tregs) is characteristically increased in both tissue and blood of patients with IgG4-related disease. Our investigation revealed that the mRNA expression of forkhead box P3 (Foxp3, a Tregs-specific transcriptional factor) was higher in IgG4-related disease than in classical autoimmune diseases. Two regulatory cytokines, IL-10 and transforming growth factor-β (TGF-β), are significantly overexpressed [49, 50]. Furthermore, $CD4^+CD25^+Foxp3^+$ Tregs could be detected within affected tissues by immunohistochemistry, in numbers significantly higher than in autoimmune and nonautoimmune disease controls. The number of $Foxp3^+$ cells was significantly correlated with the number of $IgG4^+$ plasma cells in IgG4-related cholangitis [51]. Miyoshi et al. examined the number of Tregs in the blood and reported that the number of $CD4^+CD25^{high}$ Tregs was significantly higher in patients with AIP than in patients with chronic pancreatitis and was correlated with the level of IgG4 in serum [52]. The number of naïve Tregs was significantly decreased. They speculated that hyporeaction of naïve Tregs might be involved in the development of IgG4-related disease, whereas hyperreaction of $CD4^+CD25^{high}$ Tregs could reflect IgG4-related disease progression [52].

The possible involvement of *H pylori* in the pathogenesis of AIP was reported in 2005 [53]. Gastric *H. pylori* infection triggers AIP in genetically predisposed subjects via molecular mimicry between human CA-II and alpha-carbonic anhydrase of *H. pylori* [54]. Frulloni et al. found that 94% of patients with AIP had antibodies against a plasminogen-binding protein of *H. pylori* [55]. The amino acid sequence of the plasminogen-binding protein exhibited homology with that of the ubiquitin-protein ligase E3 component n-recognin 2, an enzyme expressed in pancreatic acinar cells. However, the involvement of *H. pylori* in the pathogenesis of other IgG4-related lesions has not been reported so far.

8. Conclusion

IgG4-SC is a unique cholangiopathy which should be differentiated from classical PSC or biliary malignancy. An underlying immune response might be mediated by predominantly Th2 or regulatory cytokines.

Abbreviations

ANCA: Antineutrophilic cytoplasmic antibody
CA: Carbonic anhydrase
IgG4-SC: IgG4-related sclerosing cholangitis
PSC: Primary sclerosing cholangitis
TGF: Transforming growth factor
Tregs: Regulatory T cells
Foxp3: Forkhead box P3.

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Research Article

Fractalkine and Other Chemokines in Primary Biliary Cirrhosis

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Primary biliary cirrhosis (PBC) is characterized by the autoimmune injury of small intrahepatic bile duct. On this basis, it has been suggested that the targeted biliary epithelial cells (BEC) play an active role in the perpetuation of autoimmunity by attracting immune cells via chemokine secretion. To address this issue, we challenged BEC using multiple toll-like receptor (TLR) ligands as well as autologous liver infiltrating mononuclear cells (LMNC) with subsequent measurement of BEC phenotype and chemokine production and LMNC chemotaxis by quantifying specific chemokines, specially CX3CL1 (fractalkine). We submit the hypothesis that BEC are in fact the innocent victims of the autoimmune injury and that the adaptive immune response is critical in PBC.

1. Introduction

Primary biliary cirrhosis (PBC) is a chronic cholestatic liver disease recognized at histology as chronic nonsuppurative destructive cholangitis with an autoimmune pathogenesis supported by Th1 or Th17 cells producing IFN-γ or IL-17 [1, 2]. Several inflammatory cell populations, including T and B cells, are found around the affected intrahepatic bile ducts, and chemokines are believed to play a pivotal role for the infiltration of inflammatory cells [3].

A better understanding of the role of specific chemokines in liver injury is ancillary to understanding the molecular mechanisms regulating the autoimmunity process and is expected to unravel new strategies to treat PBC.

The observed patterns of chemokine expression in normal and PBC liver are illustrated in Table 1 [4]. In our recent experiments, we cultured EpCAM-positive cells (i.e., biliary epithelial cells and BEC) isolated by immunobeads from explanted liver tissue and examined the production of chemokines by protein array following the stimulation by inflammatory cytokines or Toll-like receptor (TLR) ligands [5]. Our data illustrated that BEC produce proinflammatory chemokines such as CXCL1, CXCL5, CXCL6, and CXCL8 without any specific stimulation as shown in Figure 1. On the other hand, BEC challenged with a TLR3 ligand (poly I : C) manifest a Th1 shift and the production of CCL3, CCL4, CCL5, and CXCL10. Such production of Th1 chemokines was further prompted by the interaction between CD40 on BEC and CD154 on liver infiltrating lymphocytes. Taken altogether, the evidence support the observation that BEC induces a proinflammatory environment in the absence of innate immunity stimulation and induces Th1-sifted environment when such stimulation is present.

2. Fractalkine

Fractalkine is characterized as a type-1 transmembrane molecule with the chemokine domain tethered by a 241-amino acid glycosylated stalk, a 19-amino acid transmembrane region, and 37-amino acid intracellular tail [6]. The surface-expressed transmembrane fractalkine induces the firm adhesion of leukocytes expressing its receptor CX3CR1. After shedding by the disintegrins and metalloproteinases (ADAM) 10 and 17, fractalkine also acts as a soluble leukocyte chemoattractant. Transmembrane fractalkine expressed on both endothelial and epithelial cells induces leukocyte

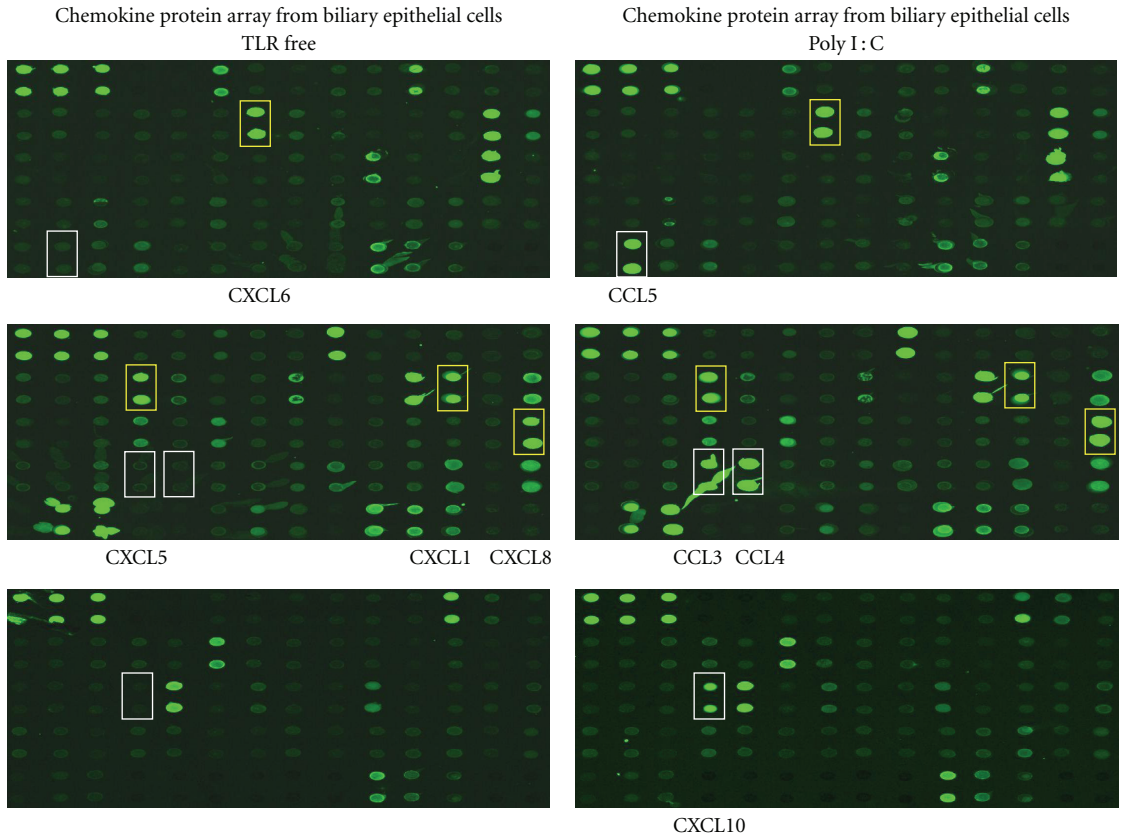

Figure 1: Chemokines produced by biliary epithelial cells under basal conditions or after stimulation with TLR3 ligand (poly I : C) for 48 hours. Cell-free culture supernatants were analyzed by a protein array kit to evaluate 174 different proteins simultaneously. Unstimulated cells produced detectable amounts of GRO-α/CXCL1, ENA-78/CXCL5, GCP-2/CXCL6, and IL-8/CXCL8, while poly I : C stimulation led to enhanced MIP-1α/CCL3, MIP-1β/CCL4, RANTES/CCL5, and IP-10/CXCL10.

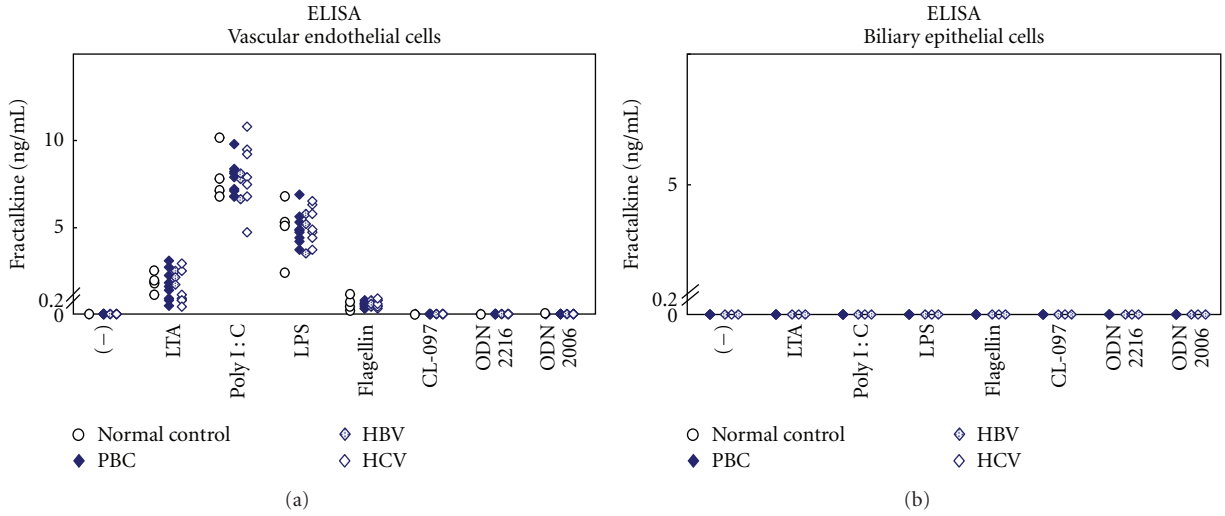

Figure 2: (a) Fractalkine production from endothelial cells from PBC and control (chronic hepatitis B and C) livers exposed to TLR ligands. Endothelial cells produced fractalkine with LTA, poly I : C, LPS, and flagellin with no significant differences observed between patients and control livers. (b) BEC did not produce fractalkine with any additional TLR ligand.

Table 1: Chemokine expression patterns in the portal tract, sinusoidal endothelium, and bile duct of normal and PBC liver.

Chemokine	Portal vein		Sinusoidal EC		Bile duct	
	Normal	PBC	Normal	PBC	Normal	PBC
CXCL9	±	+(?)	±	ND	−	+
CXCL10	±	+(?)	±	ND	−	+
CXCL11	±	ND	+	ND	ND	ND
CXCL12	−	−	−	−	+	++
CXCL16	+	+	+	+	+	++
CCL25	−	−	−	−	−	−
CCL28	−	+	−	−	−	++

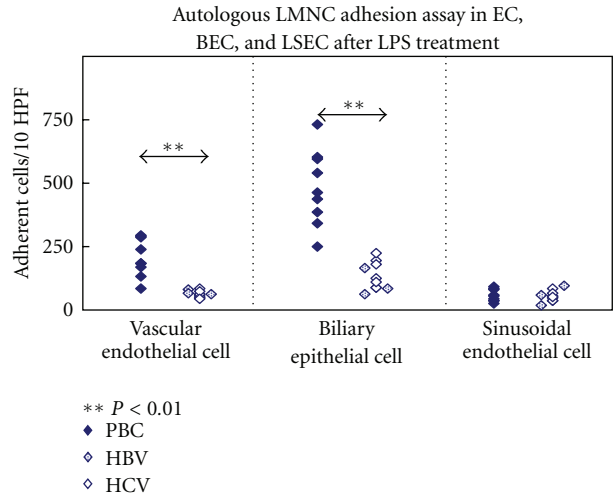

Figure 3: Autologous liver mononuclear cells adhesion assay using endothelial cells and BEC after stimulation with TLR4 ligand (LPS). Adherent liver mononuclear cells were stained and counted in ten random high-power microscopy fields. Liver mononuclear cells from PBC livers adhered in greater numbers than did liver mononuclear cells from controls using either endothelial cells or biliary epithelial cells, whereas liver mononuclear cells adhered only minimally to liver sinusoidal endothelial cells in all instances. Other TLR ligands did not accelerate liver mononuclear cells adhesion with neither endothelial cells nor biliary epithelial cells (data not shown).

transmigration [7]. Fractalkine is upregulated by inflammation cytokines such as TNF-α or IFN-γ, it has been proposed to contribute to inflammatory diseases by promoting the transmigration of CX3CR1-expressing cells to inflamed tissues in Crohn disease [8], rheumatoid arthritis, atherosclerosis [9], systemic lupus erythematosus [10], and most recently PBC [5]. CX3CR1 is expressed on natural killer cells, monocytes, macrophages, mucosal dendritic cells, $CD8^+$ T cells, and a subset of effector-memory $CD4^+$ T cells [11, 12]. Human Th1 cells express high levels of CX3CR1 mRNA, different from polarized Th2 cells [13, 14]. Fractalkine is expressed in limited amounts in the normal human liver, particularly near branches of the hepatic artery and in small bile ducts located at the interface between the portal tract and the hepatic lobule. In the case of acute or chronic viral hepatitis, fractalkine is detected in the areas of necrosis and inflammatory infiltration and also at the interface between the expanded portal tract and the regenerating nodule. Regenerating epithelial cells of the ductular reaction are also positive for fractalkine [15]. In kidney allograft transplantation, fractalkine is expressed in renal tubular epithelial cells, and the expression is upregulated by TNF-α, the recognized key proinflammatory cytokine in acute rejection [16]. The $CD4^+$ and $CD8^+$ T cells expressing CX3CR1 predominantly produce IFN-γ and TNF-α, and these T cells infiltrate the synovium in patients with rheumatoid arthritis [17]. In inflammatory bowel disease (IBD), intestinal microvascular endothelial cells produce high amounts of fractalkine, and IBD mucosa as well as periphery contained significantly more CX3CR1+ cells than control. Fractalkine is a major contributor to T- and monocytic-cell adhesion to endothelial cells [18]. In HCV infection, CX3CR1 is susceptible gene for hepatic fibrosis [19]. In mice models, it is unclear whether CX3CR1 positive cells are protective or trigger disease [20–25].

3. Fractalkine and PBC

Fractalkine is peripherally expressed dominantly in patients with PBC, and is upregulated in BEC of the PBC liver. CX3CR1 is expressed on infiltrating lymphocytes in the portal tracts and on intraepithelial T cells of injured bile ducts [26]. BEC manifesting senescent features in damaged

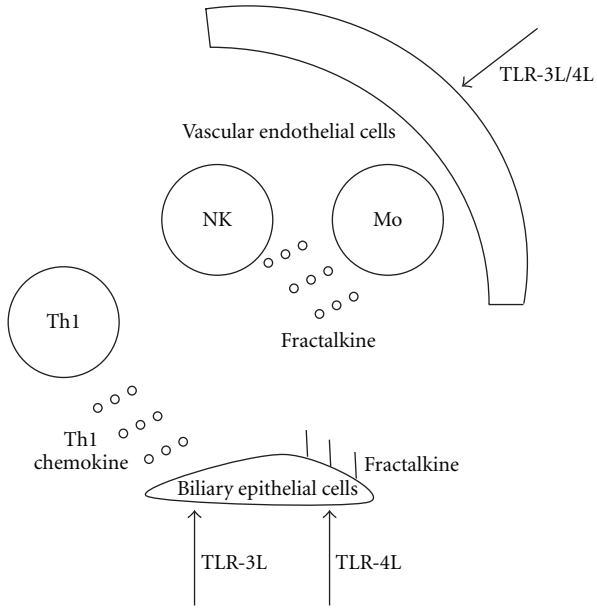

Figure 4: The proposed role of fractalkine is illustrated. TLR3 or TLR4 ligands stimulate vascular endothelial cells to produce fractalkine as chemokine, then fractalkine attracts CX3CR1 positive monocytes or NK cells. Subsequently, TLR4 ligand stimulated BEC produce fractalkine as cell adhesion molecule, then fractalkine recruit CX3CR1 positive cells around PBC target cells. This starts the chronic nonsuppurative destructive cholangitis and perpetuates the autoimmune pathogenesis of disease. Finally, TLR3 ligand stimulated biliary epithelial cells produce Th1 chemokines, and these chemokines are considered to contribute this autoimmune mechanism.

small bile ducts also overexpress fractalkine [27]. As previously introduced, in our recent work, we separated BEC as EpCAM positive and endothelial cells as CD31 positive by immunobeads and evaluated the production of fractalkine as chemokine by ELISA. Figure 2(a) illustrates the elevated production of fractalkine by endothelial cells challenged with TLR3 ligand (poly I : C) or TLR4 ligand (LPS). Conversely, BEC did not produce fractalkine with any other TLR ligand stimulation (Figure 2(b)), and this was not reversed with the addition of established inflammatory cytokines such as TNF-α or IFN-γ. Further, we investigated the production of fractalkine following the interaction between BEC or endothelial cells and liver infiltrating lymphocytes. As shown in Figure 3, mononuclear cells adhered with higher affinity to BEC compared to endothelial cells in the TLR4 ligand (LPS) stimulation, and this adherence was increased more in PBC than in other control diseases [5]. Fractalkine works to modulate inflammation in the BEC of PBC, thus suggesting that novel therapies to block fractalkine induced environment may prove beneficial. Based on our data, we propose a working model on the role of fractalkine as chemokine or cell adhesion molecule by vascular endothelial cells and BEC, summarized in Figure 4. First, fractalkine as chemokine from vascular endothelial cells stimulated via TLR3 or TLR4 induce CX3CR1 positive monocytes or NK cells. Second, fractalkine as cell adhesion molecule from TLR4-stimulated BEC recruit CX3CR1 positive cells around target cells. This mechanism may trigger the onset of chronic nonsuppurative destructive cholangitis and autoimmune mechanism perpetuating the cholangitis. We further submit that Th1 chemokines produced by BEC stimulated from TLR3 are important contributors to the autoimmune mechanism.

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Review Article

Novel Approach to Bile Duct Damage in Primary Biliary Cirrhosis: Participation of Cellular Senescence and Autophagy

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Primary biliary cirrhosis (PBC) is characterized by antimitochondrial autoantibodies (AMAs) in patients' sera and histologically by chronic nonsuppurative destructive cholangitis in small bile ducts, eventually followed by extensive bile duct loss and biliary cirrhosis. The autoimmune-mediated pathogenesis of bile duct lesions, including the significance of AMAs, triggers of the autoimmune process, and so on remain unclear. We have reported that cellular senescence in biliary epithelial cells (BECs) may be involved in bile duct lesions and that autophagy may precede the process of biliary epithelial senescence in PBC. Interestingly, BECs in damaged bile ducts show characteristicsof cellular senescence and autophagy in PBC. A suspected causative factor of biliary epithelial senescence is oxidative stress. Furthermore, senescent BECs may modulate the microenvironment around bile ducts by expressing various chemokines and cytokines called senescence-associated secretory phenotypes and contribute to the pathogenesis in PBC.

1. Introduction

Primary biliary cirrhosis (PBC) is a chronic, progressive cholestatic liver disease that affects usually middle-aged women and occasionally leads to liver failure and liver transplantation [1–5]. Autoimmune pathogenesis is suggested in PBC [1–4], because PBC is serologically characterized by a high titer of serum antimitochondrial autoantibodies (AMAs) and by an increased level of immunoglobulin M (IgM). PBC-specific antinuclear antibodies (ANAs), such as anti-gp210 are also detected in some patients [1, 2, 6–9]. AMAs are present in about 95% of patients with PBC, with disease specificity close to 100%. An inner lipoyl domain of the E2-component of pyruvate dehydrogenase (PDC-E2) and other 2-oxo-acid dehydrogenases is a major epitope for both B-cell and CD4 and CD8 T-cell response [9–12]. PBC is characterized histologically by the cholangitis of small bile ducts (chronic nonsuppurative destructive cholangitis; CNSDC), eventually followed by the extensive loss of small bile ducts and biliary cirrhosis [2, 3, 13]. Therefore, a major target of autoimmune-mediated injury has been thought to be biliary epithelial cells (BECs) in PBC.

There has been considerable progress in elucidating the immunopathological features [9–12], genetic factors [14–17], and environmental factors such as infectious agents and xenobiotics [5, 18–20] in the pathogenesis of PBC. The most accepted hypothesis states that PBC results from a combination of multiple genetic factors (susceptible genetic background) and superimposed environmental triggers. In this scenario, adaptive, both humoral and cellular (CD4 and CD8 T cells), and innate immunity have been proposed as coplayers in immune-mediated liver damage; however, the etiology and pathogenesis of PBC remain unclear. In particular, the significance of AMAs and autoantigen-specific T-cell response in the pathogenesis of bile duct lesions remains unknown. One hypothesis for a BEC-specific autoimmune reaction is a unique property of apoptosis in BECs, in which there is exposure of autoantigen to the effectors of the immune system [4, 21–23].

We have recently reported that cellular senescence and autophagy may be involved in bile duct lesions in PBC [24–28]. These two cellular processes may be related to autoimmune mechanism such as AMAs and the autoantigen-specific T cell and play a role to cause autoimmune-mediated

bile duct lesions in PBC. Recent studies have disclosed that autophagy plays an important role in innate immune responses and possibly autoimmunity [29–31]. Furthermore, it is plausible that senescent BECs modulate microenvironment around bile duct by expressing senescence-associated secretory phenotypes (SASPs) including various chemokines and contribute to the pathogenesis of bile duct lesions in PBC [32]. In this paper, we will focus on cellular senescence and autophagy in BECs in PBC and their possible involvement in the progression of diseases.

2. Cellular Senescence in the Damaged Small Bile Ducts in PBC

2.1. What Is Cellular Senescence? Cellular senescence is defined as a condition in which a cell no longer has the ability to proliferate. Senescent cells remain metabolically active, even though they are irreversibly arrested at the G1 phase of the cell cycle and do not respond to various external stimuli. Cellular senescence can be triggered by a number of cellular stresses including telomere dysfunction. Other causes include oxidative stress, nontelomeric DNA damage, epigenetic derepression of the INK4a/ARF locus, and oncogenic activation [33]. Several features, such as increased activity of senescence-associated β-galactosidase (SA-β-gal) (Figure 1), shortened telomeres, increased expression of $p16^{INK4}$ and $p21^{WAF1/CIP}$, and histological changes (Figure 1), are known to characterize cellular senescence [34–36]. Cellular senescence is a potent tumor suppression mechanism as well as apoptosis [33, 37]. Senescent cells are also seen in aged or damaged tissues, and they may decline tissue regeneration capacity with age [33]. Cellular senescence may play a role in limiting wound-healing responses following tissue damage [38]. Recent studies have disclosed that cellular senescence is involved in the pathophysiology of various chronic liver diseases, including chronic viral hepatitis and hepatocarcinogenesis [24, 26, 27, 38–44].

2.2. Bile Duct Lesion in PBC. "Chronic nonsuppurative destructive cholangitis (CNSDC)" is a characteristic bile duct lesion in PBC (Figure 1(a)) [2, 3, 13, 45]. Bile duct damage in early PBC mainly affects the septal and larger interlobular bile ducts, while the smaller interlobular ducts remain intact until later. The BECs in the affected bile ducts show irregular shape and arrangement with infiltration of mononuclear cells. The presence of epithelioid granuloma around the affected bile duct is also a feature of PBC. Bile duct loss eventually progresses and chronic cholestasis developes gradually. Hepatitis activity of varying degrees is frequently imposed on the liver at the same time. We proposed a new histological staging and grading system of PBC for comprehensive analysis of the histological progression of PBC (staging) toward extensive bile duct loss, chronic cholestasis and cirrhosis, and also the immune-mediated necroinflammatory activity of small bile ducts and hepatocytes [46].

2.3. Biliary Epithelial Senescence in Damaged Small Bile Ducts in PBC. BECs in damaged small bile ducts in PBC show senescent features, such as the expression of SA-β-gal and the increased expression of $p16^{INK4a}$ and $p21^{WAF1/Cip1}$ (Figure 1) [24–27]. Furthermore, a significant decrease in telomere length was observed in BECs in the damaged small bile ducts and bile ductules in PBC compared with normal-looking bile ducts and bile ductules in PBC, chronic viral hepatitis, and normal livers, when examined using quantitative fluorescence *in situ* hybridization [25]. γH2AX DNA damage foci were detected in BECs in damaged small bile ducts and bile ductules in PBC but were absent in BECs in control livers. The expression of $p16^{INK4a}$ and $p21^{WAF1/Cip1}$ increased corresponding to telomere shortening and γH2AX DNA damage foci in the damaged small bile ducts in PBC [25]. Taken together, telomere shortening and the accumulation of DNA damage coinciding with increased expressions of $p16^{INK4a}$ and $p21^{WAF1/Cip1}$ in the damaged bile ducts characterize biliary cellular senescence and may play a role in subsequent progressive bile duct loss in PBC [24–27]. Interestingly, chronic liver allograft rejection, which is characterized by bile duct loss similar to PBC, also shows similar biliary epithelial senescence [24, 40].

2.4. How Does Cellular Senescence Result in Bile Duct Loss?

The exact mechanism how cellular senescence of BECs cause bile duct loss in PBC is not clear. Cellular senescence is supposed to impair tissue integrity and cause persistent inflammation [47]. After cellular senescence occurs in injured BECs, these senescent cells are thought to remain *in situ* and not to be replaced by normal cells, although nonsenescent BECs proliferate in response to injury [48]. Therefore, it is plausible that the senescent BECs are prone to further injuries, accentuating inflammation by SASP, which is likely to be followed by bile duct loss in PBC. The fate of senescent BECs remains to be clarified: whether senescent BECs are removed by necrosis, apoptosis, or anoikis. Another possibility is that bile duct loss may be due to impaired function of hepatic stem/progenitor cells in PBC. Cellular senescence is also seen in bile ductular cells in a ductular reaction (DR), which is thought to harbor hepatic stem/progenitor cells in PBC [24, 25]. The impaired proliferation of hepatic stem/progenitor cells may fail to replace the damaged BECs in small bile ducts, subsequently cause bile duct loss.

2.5. Oxidative Stress Is a Potential Factor Inducing Cellular Senescence. Cellular senescence can be triggered by a number of cellular stresses, including telomere dysfunction, oxidative stress, nontelomeric DNA damage, epigenetic derepression of the INK4a/ARF locus, and oncogenic stress [33, 39]. The possible association of oxidative stress is suggested to be involved in the pathogenesis of cellular senescence in PBC [24, 26, 27]. For example, $p21^{WAF1/Cip1}$, activated/phosphorylated ATM, and an oxidative stress marker, 8-OHdG, were frequently and extensively coexpressed in the nuclei of CNSDC in PBC, and their expressions were

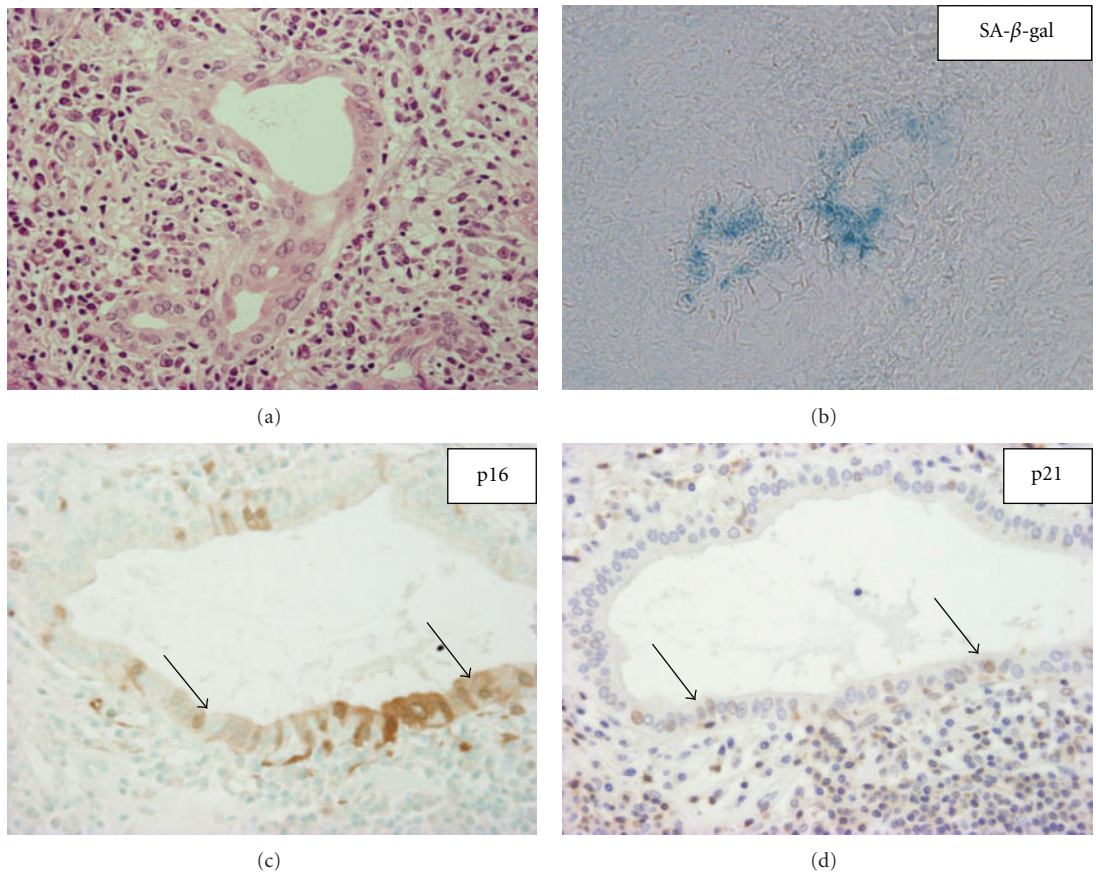

Figure 1: Biliary epithelial senescence in PBC. BECs in small bile ducts involved in chronic nonsuppurative destructive cholangitis (CNSDC) show histological features of senescence, such as cytoplasmic eosinophilia, cellular and nuclear enlargement, and uneven nuclear spacing (a). SA-β-gal activity is detected in BECs in PBC (b). Senescent markers, $p21^{WAF1/Cip1}$ and $p16^{INK4a}$, were expressed in BECs in damaged small bile ducts in PBC (c) and (d). Immunostaining for $p21^{WAF1/Cip1}$ and $p16^{INK4a}$. Original magnification: $\times 400$.

correlated [26]. Cell culture study suggests that oxidative stress and proinflammatory cytokines, such as IFN-β, IFN-γ, and TNF-α, which induce ROS generation, activate the ATM/p53/$p21^{WAF1/Cip1}$ pathway, followed by biliary epithelial senescence [49]. The expression of polycomb group protein Bmi1 is significantly decreased in damaged bile ducts in PBC, coordinating with the increased expression of $p16^{INK4a}$ [27]. The decreased expression of Bmi1 is induced by oxidative stress, followed by the increased expression of $p16^{INK4a}$ in cultured BECs [27]. Since an antioxidant, N-acetylcysteine can inhibit cellular senescence induced by oxidative stress and proinflammatory cytokines [49], antioxidants may have therapeutic implications in PBC.

3. Cellular Senescence in Ductular Reaction (DR) in PBC

DR is a reactive lesion at the portal tract interface composed of increased bile ductules with an accompanying complex of stromal and inflammatory cells [50]. DR is thought to harbor hepatic stem/progenitor cells [50]. We investigated the pathological significance of DR in chronic liver diseases, including PBC, with respect to cellular senescence [24, 25, 51]. The expression of senescence-associated markers ($p16^{INK4a}$ and $p21^{WAF1/Cip1}$) was frequently expressed in ductular cells in the advanced stage of chronic liver diseases, especially in PBC. Double immunostaining disclosed that neural cell adhesion molecules (NCAM) were frequently coexpressed in ductular cells showing senescence-associated markers ($p16^{INK4a}$ and $p21^{WAF1/Cip1}$) and cell cycle G1-phase marker (cyclin D) (Figure 2) [51]. These findings suggest that DR is heterogeneous in cell kinetics and the expression of NCAM and that some ductular cells in DR in chronic liver diseases were at G1 arrest and undergoing cellular senescence. Such senescent cells may be involved in the progression of fibrosis of these diseases, particularly in PBC [51]. This study raises the possibility that NCAM can be used as a cellular senescent marker developing in DRs. Furthermore, our recent study revealed that CCL2 expressed by senescent BECs can induce the cell migration of hepatic stellate cells (HSCs), which may play a role in the periportal fibrosis in chronic advanced liver diseases [52].

4. Autophagy in Damaged Small Bile Ducts in PBC

4.1. What Is Autophagy? Autophagy, or cellular selfdigestion, is a cellular pathway that results from various cellular

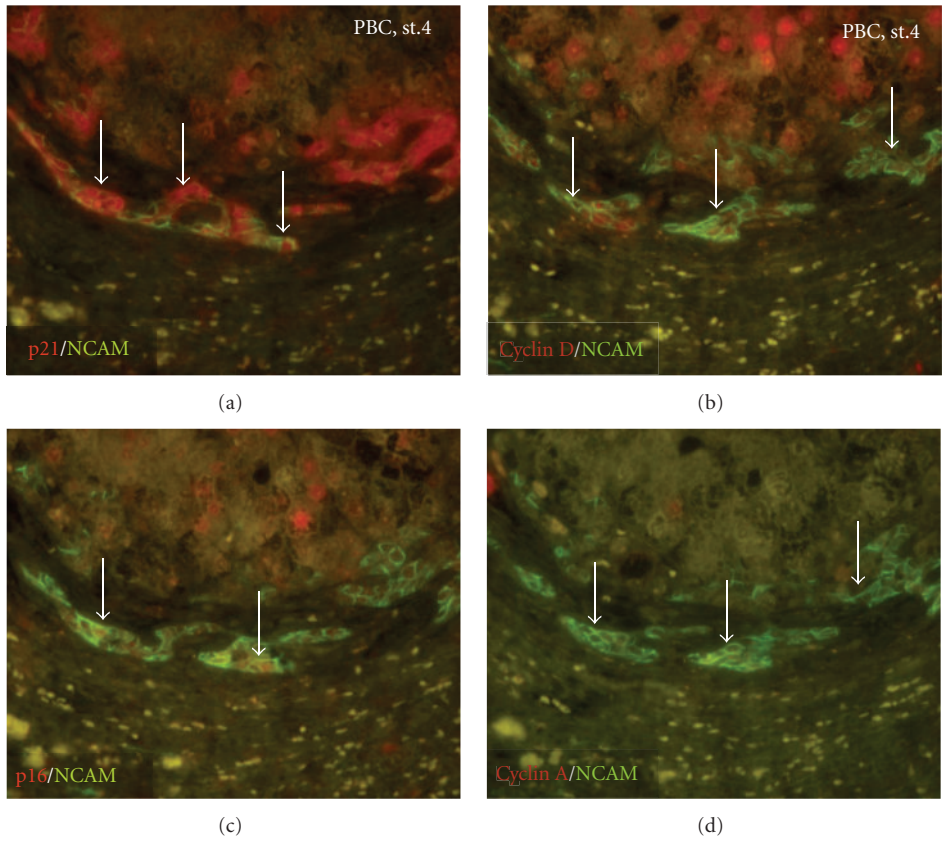

Figure 2: Double immunostaining for senescence markers ($p16^{INK4a}$ or $p21^{WAF1/Cip1}$) and cell cycle markers (G1-phase, cyclin D; S-phase, cyclin A) (red) and NCAM (green) in PBC, stage 4. (a), (c) The expression of senescent markers $p16^{INK4a}$ and $p21^{WAF1/Cip1}$ is seen in NCAM-positive ductular cells (arrows) in PBC, stage 4. (b), (d) Most NCAM-positive ductular cells (arrows) express cyclin D, whereas there is no cyclin A expression in DRs in PBC, stage 4. Original magnification ×400.

stresses, such as nutrient starvation, anoxia, and activation of the endoplasmic reticulum stress pathway [53, 54]. Three types of autophagy, macroautophagy, microautophagy, and chaperone-mediated autophagy, have been classified, and macroautophagy is the major type [53–55]. It is becoming evident that macroautophagy (hereafter referred to as autophagy) is important for development, differentiation, survival, homeostasis, and also many pathological processes. Autophagy occurs physiologically at low basal levels in cells to perform homeostatic functions such as protein and organelle turnover. It is rapidly upregulated through an inhibition of mammalian target of rapamycin (mTOR) when cells need to generate intracellular nutrients and energy, for example, in starvation [53–55]. Microtubule-associated protein-light chain 3β (LC3), a homologue of autophagy-related protein 8 (Apg8p), which is essential for autophagy and associated with autophagosome membranes after processing, is a widely used marker of autophagy [56, 57].

4.2. Cellular Senescence, Apoptosis, and Autophagy. An appropriate cellular stress response is critical for maintaining tissue integrity and function and for preventing diseases [58]. Cellular senescence, apoptosis, and autophagy are cellular responses to stress, correlating with each other [58]. Cellular stresses cause adaptation, repair, autophagy, apoptosis, or cellular senescence in cells [58]. These cell fate decisions are critical to dealing with the emergence of damaged and potentially dangerous cells that can cause cancer. Interestingly, a recent study disclosed that autophagy is induced during and facilitates the process of senescence [56]. Cellular senescence can be a failsafe program against a variety of cellular insults, as well as apoptosis. Cellular senescence is a typical delayed stress response involving multiple effector mechanism, in contrast, cytotoxic signals converge to a common mechanism in apoptosis. With the onset of cellular senescence cells can remain viable within tissues for long periods; resistance to apoptosis is a characteristics of senescent cells [41, 59].

4.3. Biliary Epithelial Autophagy in PBC. We have reported the upregulated autophagy in the damaged small bile ducts along with cellular senescence in PBC [28] (Figure 3). LC3, a commonly used marker of autophagy, was characteristically expressed in cytoplasmic vesicles in bile duct lesions in PBC [28]. Autophagic marker LC3 was coexpressed with senescent markers $p21^{WAF1/Cip1}$ and $p16^{INK4a}$ in damaged bile ducts in PBC [28]. The inhibition of autophagy reduced stress-induced cellular senescence in cultured cells with stress [28]. This finding is in consistent with a recent study in which the involvement of autophagy is reported in the process of

senescence [56]. Taken together, biliary epithelial autophagy may mediate the process of biliary epithelial senescence in bile duct lesions in PBC and it may be involved in the pathogenesis of bile duct lesions in PBC.

4.4. Autophagy and Autoimmune-Mediated Processes in PBC. An unsolved problem is how autophagy and cellular senescence are involved in the autoimmune-mediated processes such as AMA and other PBC-related autoantigens in PBC. Regarding apoptosis, it has been reported that BECs manifest unique features during apoptosis and that the combination of AMA and BECs apoptotic bodies (apotopes) could activate innate immune response with involvement of some inflammatory cytokines [21]. This study provides a mechanism for the biliary specificity of PBC and the involvement of AMA in autoimmune pathogenesis [21]. Recent studies reveal a crucial role for the autophagy pathway and proteins in immunity and inflammation [29–31]. The autophagy pathway and autophagy proteins may function as a central fulcrum that balances the beneficial and harmful effects of the host response to infection and other immunological stimuli [31]. Autophagy proteins function in adaptive immunity, including in the development and homeostasis of the immune system and in antigen presentation [31]. Furthermore, autophagy proteins play a role in both the activation and inactivation of innate immune signaling [30, 31]. On the contrary, it is demonstrated that autophagy is regulated by immune-signaling molecules, such as toll-like receptors (TLRs), IFN-γ, and NF-κB [30, 31].

The dysfunctional autophagy related to the regulation of immunity may contribute also to chronic inflammatory diseases and probably autoimmune diseases. A well-characterized link is between mutations in autophagy regulators and Crohn's disease, a chronic inflammatory bowel disease, in which autophagy proteins, ATG16L1, NOD2, and IRGM are reported as susceptibility genes [60]. Abnormal autophagy/autophagy protein may also result in inflammatory autoimmune disease, although not yet proven. Autophagy-related processing of self-proteins provides a source of immunostimulatory molecules and autoantigens, that is, by MHC-class II presentation of cytosolic antigens and control of T-cell homeostasis [61–63]. It is of interest that genomewide association studies (GWAS) have linked several single nucleotide polymorphisms (SNPs) in ATG5, an autophagy protein, to systemic lupus erythematosus (SLE) susceptibility [64, 65]. SLE is a representative multisystem autoimmune disease characterized by an enormous array of autoantibodies such as ANAs and autoimmune responses against self-antigens generated from dying cells. To date, it is unclear how such SNPs affect the expression level and function of ATG5. Interestingly, in mice, the lack of ATG5-dependent negative thymic selection generates autoimmunity and multiorgan inflammation [66]. The autoimmunity and inflammation associated with SLE may be caused by loss of other ATG5-dependent effects, such as regulation of IFN and proinflammatory cytokine secretion, clearance of dying cells [67], and dendritic cell antigen presentation [68]. Taken together, a link between SLE pathogenesis and ATG5 mutation or mutation of other autophagy genes is plausible, although not yet proven.

Similar to SLE, it is possible that a dysfunctional autophagic process of BECs may play a role in autoimmune pathogenesis, for example, the immune tolerance breakdown of autoantigens, in PBC, although this is only speculative at this moment. Recent genetic studies of PBC including GWAS identified, in a reproducible fashion, genetic associations between PBC and human leukocyte antigen as well as polymorphisms in the genes encoding IL-12 α-chain and IL-12 receptor β-chain [14, 15]. GWAS also identified interferon regulatory factor 5 (IRF5)-transportin 3 (TNPO3), 17q12-21, MMEL1, and SPIB as new PBC susceptibility loci [14, 15]. These immune-related genes may be associated with dysfunction autophagy in PBC, although there have been no identified autophagy proteins such as ATG5 and ATG16L1 as PBC susceptibility genes. In fact, IRF5 plays a key role in the innate immunity response as part of the TLR signaling pathway and mediates apoptosis induced by tumor necrosis factor-related apoptosis-inducing ligand [69, 70]. Interestingly, IRF5 loci have been reported as associated loci with several autoimmune diseases including SLE and Sjogren's syndrome [71, 72]. Therefore, IRF5 might be related to dysfunctional autophagy in PBC, although not yet reported.

5. Senescence-Associated Secretory Phenotypes (SASPs) in PBC

5.1. What Are SASPs? An increasing body of work described the change in the cellular secretosome in senescent cells. Senescent cells play an important role in modulating the microenvironment by secreting biological active molecules, senescence-associated secretory phenotypes (SASPs). SASPs include diverse proinflammatory factors such as cytokines (IL-6, IL-1 and so on) and chemokines (CXCL8/IL-8, CCL2/monocyte chemotactic protein-1 (MCP)-1) and so on), growth factors and profibrogenic factors [73–77]. Previous studies have shown that BECs express a number of profibrogenic proinflammatory and chemotactic factors (e.g., IL-1, IL-6, CXCL8/IL-8, and CCL2/MCP-1) [78–81]. These factors can attract and activate inflammatory cells and also stellate cell lineage in humans with biliary disorders and in animal models of biliary fibrosis. Taken together, these cytokines and chemokines previously reported in PBC may belong to SASPs [73–77].

5.2. SASPs May Play a Role in the Pathogenesis of PBC. The upregulation of several cytokines and chemokines in damaged bile ducts in PBC has been reported [79, 80, 82], and these factors may represent SASPs, as described above [73–77]. We have recently reported that the involvement of senescent BECs in modulation of the inflammatory microenvironment around affected small bile ducts in PBC (Figure 4) [32]. In this study, we have shown that the expression of CCL2 and CX3CL1 was significantly higher in BECs in inflamed and damaged small bile ducts in PBC, than in noninflamed bile ducts and control livers

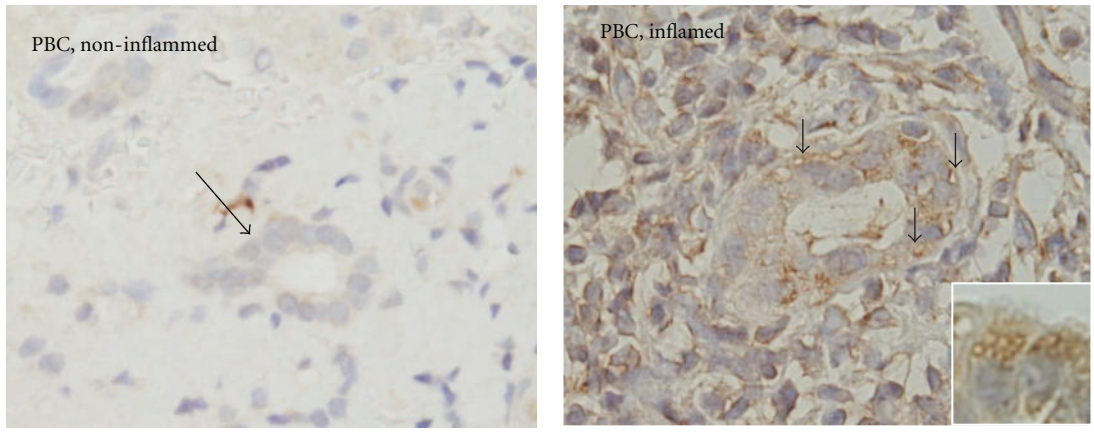

Figure 3: Biliary epithelial autophagy in PBC. (a) The expression of autophagy marker LC3 was not observed in BECs in noninflammed bile ducts (arrow) in PBC. (b) The expression of autophagy marker LC3 was detected in intracytoplasmic vesicles (arrows) in BECs involved in inflamed and damaged small bile ducts in PBC. Immunostaining for LC3. Original magnification, ×400 (inset, ×1000).

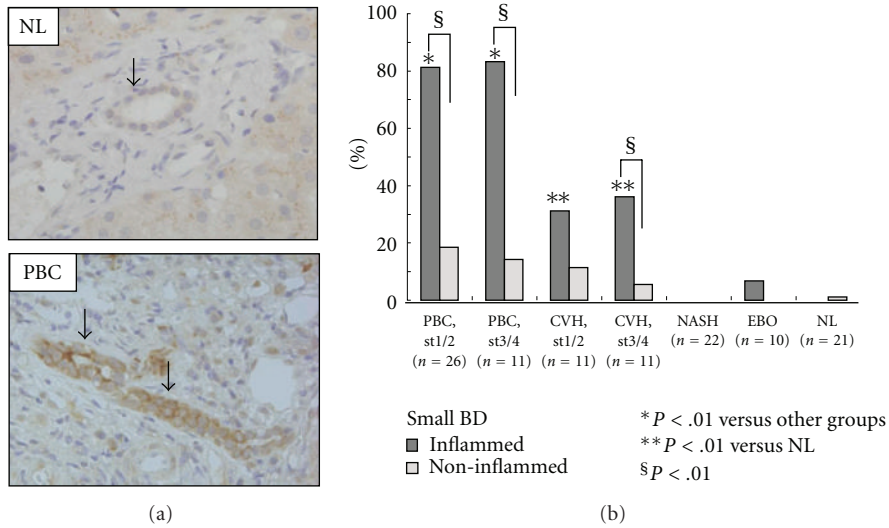

Figure 4: Increased expression of CCL2 in inflamed and damaged bile ducts in PBC. (a) The expression of CCL2 was absent or faint in biliary epithelial cells (BECs) in the small bile duct (arrow) in normal liver (top). CCL2 was extensively expressed in the membrane and cytoplasm of damaged and senescent BECs (arrows) in the early stage of PBC (bottom). Immunostaining for CCL2. Original magnification, ×400. (b) The expression of CCL2 was significantly more frequent and intense in inflamed small bile ducts in PBC, when compared with noninflamed small bile ducts in PBC and small bile ducts in control livers ($P < .01$). CVH: chronic viral hepatitis; NASH: nonalcoholic steatohepatitis; EBO: extrahepatic biliary obstruction; NL: normal liver.

(Figure 4). The expression of CCL2 and CX3CL1 was colocalized with the expression of senescent markers in damaged bile ducts in PBC [32]. In culture study, senescent BECs induced by cellular stresses expressed a significantly higher level of chemokines. Furthermore, senescent BECs significantly accelerated the migration of RAW264.7 cells, and neutralizing antibodies against CCL2 and CX3CL1 blocked in part the migration induced by senescent BECs [32]. These findings suggest that senescent BECs may play an important role in the pathogenesis of bile duct lesion in PBC by the accentuated inflammatory microenvironment through recruiting monocytes and other inflammatory cells via SASP (Figure 5). SASPs in senescent BECs in PBC may contribute to activation of the innate immune system around injured bile ducts. Furthermore, it raises the possibility that once biliary senescence develops, the change in the tissue microenvironment wrought by the SASP may induce senescence of surrounding BECs another types of cells in appositive feedback loop (Figure 5).

The mechanisms that initiate and maintain SASPs have not been clarified, so far [33, 73–75]. It is plausible that these stresses may induce SASPs via a common mechanism in the senescent state, because various cellular stresses, such as oxidative stress and serum deprivation, induce SASPs in senescent BECs [32].

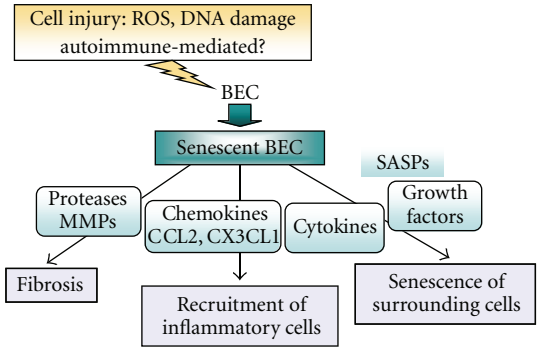

Figure 5: Possible regulation of microenvironment by senescent BECs expressing SASPs in PBC. Senescent BECs may function in modulation of the inflammatory microenvironment by recruiting monocytes and possibly other inflammatory cells by secreting chemokines and cytokines as SASPs. Senescent BECs may also participate in the induction of senescence in surrounding cells and progression of fibrosis via SASPs.

6. Summary

PBC is thought to result from a combination of multiple genetic factors and superimposed environmental triggers and apparently belongs to the "complex disease" category like most polygenic autoimmune diseases. Even though mitochondrial autoantigens and B-cell and T-cell autoepitopes have been well characterized in PBC, the pathogenesis of characteristic bile duct lesion and the exact role of AMA still remain to be elucidated. In this paper, we focused on a possible involvement of two novel cellular processes, autophagy and cellular senescence in BECs in bile duct lesions in PBC. Autophagy is expected to be a promising cellular mechanism involved in the autoimmune mechanism together with apoptosis. Cellular senescence may play a role in the immunopathology of BECs by expressing SASPs in PBC. Further studies are needed to disclose the autoimmune pathogenesis of PBC.

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Review Article

Pathological Features of New Animal Models for Primary Biliary Cirrhosis

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Primary biliary cirrhosis (PBC) is an autoimmune liver disease characterized by immune mediated biliary damage and frequent appearance of autoantibodies against mitochondrial enzymes. There is almost no useful animal model that is globally recognized and routinely used, however, several unique animal models manifested the characteristic clinical and pathological features of human PBC within the last 5 years. Herein, we compare the pathological features of previously reported and newly introduced novel animal models of PBC. Knowledge and understanding of the strengths and the limitations of each animal model have led to the development of promising therapies and novel tools to characterize these clinical conditions. Moreover, suitability of the model for the intended purpose should be confirmed by further research and analysis.

1. Introduction

Primary biliary cirrhosis (PBC) is an autoimmune disease of the liver that often develops in middle-aged women. Antimitochondrial antibodies (AMAs) appears in the serum of almost all cases of PBC while the occurrence of AMA is rare in other diseases. The major autoantigens recognized by AMA are identified as the E2 subunits of pyruvate dehydrogenase (PDC-E2), branched-chain 2-oxo acid dehydrogenase (BCOADC-E2), and 2-oxo-glutarate dehydrogenase (OGDC-E2) [1–3]. AMA or anti-PDC-E2 antibody is therefore an extremely useful diagnostic marker of PBC. Pathological destruction of interlobular bile ducts in the liver associated with lymphocytes and plasma cells is known as chronic nonsuppurative destructive cholangitis (CNSDC) and is considered the primary lesion of PBC, and eventually the interlobular bile ducts are destroyed, cholestasis occurs, bile ductules proliferate, and fibrosis develops as the disease advances. In some cases, an epithelioid granuloma is developed in the portal tract accompanied by varying degrees of eosinophilic infiltration [4–12]. PBC is considered a prototype of autoimmune diseases of the liver; it is responsible for both humoral (appearance of AMA/anti-PDC-E2 antibody) and cellular immunity (CNSDC, granuloma formation, etc.). An animal model of PBC that reflects both humoral and cellular immunological features is useful in elucidating the underlying pathophysiology of the disease or establishing an effective treatment. In addition, the characteristic pathophysiological findings of the disease such as the presence of related cytokines and chemokines, nature of inflammatory cells, extent of bile duct destruction, and granuloma formation are considered important aspects of the animal model of PBC. Moreover, ease of handling, the frequency with which relevant pathophysiology develops, and flexibility are important elements. The development of an animal model of PBC has been attempted at many research institutes over

a number of years; moreover, some animal models that show pathophysiological symptoms similar to those of PBC have been reported [12–22]. Currently, however, there is almost no useful animal model that is globally accepted and routinely used. Within the last 5 years, there have been also reports of several murine models that manifest the characteristic clinical features of human PBC. In this review, we compare the pathological features of previously reported animal models and a newly introduced novel animal model of PBC.

2. Previously Reported PBC Animal Models of PBC (up to 2005) (Table 2)

Attempts have been made to develop an animal model of PBC in many institutions. In 1989, Krams et al. immunized mice of various strains such as AKR/J, C3 H/J, and CBA/HeJ with recombinant polypeptides of dihydrolipoamide acetyltransferase, which is a constituent of the pyruvate dehydrogenase complex (PDC), and verified that an anti-PDC antibody appeared in each mouse strain although antibody titer levels varied among the strains. However, there were no signs of inflammation or bile duct lesions in the portal tract [13]. Krams et al. transferred lymphocytes from the peripheral blood of a PBC patient into severe combined immunodeficient (SCID) mice, which resulted in the appearance of AMA and anti-PDC-E2 antibodies, as well as marked lymphocytic infiltration around the interlobular bile ducts with slight morphological damage to the portal tract. This was a successful animal model from the perspective of humoral and cellular immunity [14]. However, this model was difficult to reproduce and was only moderately flexible; furthermore, it was not suitable for the analysis of pathological lesions. Masanaga et al. in 1998 used PDC to immunize A/J mice that were neonatally thymectomized, and they succeeded in inducing pathognomonic cholangitis/biliary damage. Although the bile duct lesion in this model was similar to that of human PBC, the appearance of AMA was not clearly described, and the removal of the thymus in the neonatal period posed as a technical difficulty [15, 16]. Tsuneyama et al. [17] and Ohba et al. [18] reported the presence of AMA in the serum of MRL/*lpr* mice, a model of autoimmune disease in which vasculitis, glomerular nephritis, arthritis, inflammation of the salivary glands, and interstitial pneumonia develop spontaneously in the same individual. Because inflammatory cell infiltrates and biliary damage in the portal tract similar to that seen in PBC also appeared in the liver of MRL/*lpr* mice, it was assumed that this mouse may serve as a model of PBC. However, the fact that only about 50% mice showed PBC-like features was a serious problem. The most widespread animal model of PBC is considered to be a graft versus host disease (GVHD) model [19–22]. Initiating the development of GVHD by transferring splenic immune cells from a donor mouse into a host mouse with major histocompatibility complex class-II antigens different from those of the donor leads to the appearance of an underlying autoimmune-like mechanism, such as hypergammaglobulinemia and the production of AMA. Furthermore, the initial pathological changes of PBC with similar associated findings appear in the liver. There have been several pathological analyses using the advantages of this animal model of PBC [19–22]. However, this model is now seldom used, due to its complexity and/or low flexibility.

3. Novel Animal Models of PBC (Since 2006) (Tables 3 and 4)

Several novel animal models of PBC have been reported since 2006. These can be roughly classified into spontaneous models, which employ genetic modifications seen in animals, and induced models immunized with xenobiotics whose structures are similar to that of PDC-E2.

Each of these new models shows autoantibodies characteristic of PBC, as well as the appearance of hepatic and bile duct lesions. Their profile also resembles that of PBC with respect to infiltrating inflammatory cells and the appearance of serum inflammatory cytokines. Furthermore, it is easy to establish experimental systems with these models, such as immune cell transfer and mating with other transgenic (Tg) and knockout (KO) mice. Therefore, they fulfill many of the requirements for a PBC animal model, as listed in Table 1, and are currently applied in various investigations of PBC worldwide. Although these animal models are currently considered among the most useful models of PBC, their pathophysiology needs further investigation because some models may show complications that are unusual in PBC, such as peritonitis or inflammatory bowel disease. The pathological features of each of these animal models are outlined below.

4. Spontaneous Models

4.1. The NOD.c3c4 Mouse. Nonobese diabetic (NOD) mice are a well-known model exhibiting susceptibility to the spontaneous development of autoimmune insulin-dependent diabetes mellitus (IDDM) [41]. Genetic loci associated with susceptibility to IDDM, as well as several insulin-dependent diabetes (Idd) loci and candidate genes, have been defined through the development of congenic mouse strains [42–44]. NOD mice are also prone to the development of other autoimmune syndromes in addition to IDDM [45]. In the NODc3c4 mouse model, the diabetes susceptibility genes on chromosomes 3 and 4 of the NOD mouse are replaced with the diabetes resistance genes of B6 and B10 mice, respectively. Although this helps in controlling the onset of diabetes in this mouse, autoimmune cholangitis and biliary dilatation similar to that seen in Caroli's disease appear. Serologically, AMA appears in 50–60% and antinuclear autoantibodies (ANA) in 80–90% of the animals. Immunohistochemical analysis demonstrated that the affected parts of the biliary epithelium are infiltrated with $CD3^+$, $CD4^+$, and $CD8^+$ T cells. Furthermore, treatment of NOD.c3c4 mice with monoclonal antibody to CD3 protects them from autoimmune biliary disease. NOD.c3c4-scid mice develop the disease after adoptive transfer of splenocytes or $CD4^+$ T cells, demonstrating a central role of T cells in pathogenesis of the disease in this model [23, 24]. Recently, aggregated lymphocytes surrounding the bile ducts resembling

Table 1: Requirements for the ideal animal model of PBC.

(i) Specific liver pathology (cellular immunity)
(1) Destruction of interlobular bile duct
(2) T-cell aggregation around the damaged bile ducts
(3) Epithelioid granuloma formation
(4) Fibrosis/cirrhosis
(ii) Specific autoantibodies (humoral immunity)
(1) Antimitochondrial autoantibodies (AMAs)
(2) Anti-PDC-E2 antibodies, anti-BCOADC-E2 antibodies, and anti-OGDC-E2 antibodies
(3) Antinuclear antibodies (ANAs)
(iii) Other immunological characters
(1) Increase in inflammatory cytokines
(2) Decrease in functional regulatory T cells
(3) Increase in natural killer T (NKT) cells
(iv) General versatility
(1) High reproducibility and disease frequency
(2) Simplicity of model production
(3) Long-term maintenance of disease
(4) Long lifespan without severe complicating disorders

Table 2: Representative PBC animal models reported up to 2005.

(1) PDC-immunized mice [13]
(2) Neonatally thymectomized mice with PDC immunization [15, 16]
(3) MRL/*lpr* mice [17, 18]
(4) GVHD model [19–22]

Table 3: Novel PBC animal models reported since 2006.

Spontaneous models
(1) NOD.c3c4 mice [23–25]
(2) Dominant negative TGF-β receptor II mice [26–31]
(3) IL-2 receptor $\alpha^{-/-}$ mice [32, 33]
(4) Scurfy mice [34]
(5) $Ae2_{a,b}{}^{-/-}$ mice [35]
Xenobiotic-immunized induced model
(1) 6-Bromohexanoate-immunized guinea pigs [36]
(2) 2-Octynoic acid-immunized mice [37–40]

the aggregations seen in Sjogren's syndrome, were observed in the salivary glands of this mouse [25]. Because Sjogren's syndrome is often seen as a complication in PBC patients, the pattern of inflammation seen in NOD.c3c4 mice has many similarities to those seen in PBC. However, the cyst-like dilatation of the affected bile duct that is characteristic of these mice is not seen in PBC patients at all. When the dilatation becomes marked, the biliary epithelium of NOD.c3c4 mice frequently exfoliates, and the exfoliated cells together with infiltrated histiocytes fill the lumen. If such dilatation becomes significant, neutrophil infiltration may be also observed resulting in a variable clinical picture such as cholangitis. Therefore, further pathological evaluation of this phenomenon is mandatory.

4.2. The Dominant Negative TGF-β Receptor II Mouse. Dominant negative TGF-β receptor II (dnTGF-βRII) mice overexpress the dominant negative form of TGF-β receptor type II under the control of the CD4 promoter [46]. Deficiency of TGF-β signaling results in various pleiotropic immunological abnormalities including colitis and relatively short lifespan [47–49]. dnTGF-βRII mice exhibit major serological and histological characteristics of human PBC, suggesting that the TGF-β signaling pathway is important in the pathogenesis of PBC. Serologically, AMA appears in 100% of these mice. The corresponding antigens include PDC-E2, BCOADC-E2, and OGDC-E2; these are the main autoantigens recognized by AMAs of PBC. Furthermore, hepatic lesions characteristic of PBC, such as lymphocytic infiltration, interlobular bile duct destruction, and granuloma formation in the portal tract, appear at high frequency. Various infiltrating cells are found in the portal tracts, including B cells, plasmacytoid dendritic cells, NK cells, and macrophages, in addition to $CD4^+$ and $CD8^+$ T cells. A particular characteristic is the increased ratio of $CD8^+$ T cells to $CD3^+$ T cells. This mouse strain presents mild inflammatory bowel disease and crypt abscesses similar to those of ulcerative colitis. Increased levels of inflammatory cytokines such as TNF-α, IFN-γ, IL-12p40, and IL-6 are also detected in the serum of these mice [26].

The dnTGF-βRII mouse is a spontaneous PBC model in which pathophysiological variations are minimum among individuals; furthermore, humoral and cellular immune responses appear to be reproducible and at high frequency. It has given rise to many models that are used for pathophysiological analysis. Yang et al. produced a model by transferring various fractions of splenocytes of dnTGF-βRII mice into Rag-$1^{-/-}$ mice. Their study revealed that PBC-like hepatic lesions were produced after the transfer of total splenic lymphocytes and that more severe hepatic lesions occurred after splenic $CD8^+$ T-cell transfer. On the other hand, PBC-like hepatic lesions did not appear, however the colitis worsened after splenic $CD4^+$ T-cell transfer. Currently, the $CD8^+$ T-cell transfer model shows maximum similarities to PBC, such as severe inflammatory cell infiltration, bile duct destruction, and granuloma formation in the portal tract [27]. A derived PBC model, produced by crossing dnTGF-βRII mice with a variety of genetically modified mice, is also used for pathological analysis. Moritoki et al. crossed dnTGF-βRII mice with mu-mutant mice ($Ig\mu^{-/-}$) to produce a B-cell-deficient model in order to study the role of B cells in the pathogenesis of PBC [28]. Chuang et al. studied NKT-cell commitment in a model produced by crossing dnTGF-βRII mice with $CD1d^{-/-}$ or $CD1d^{+/-}$ mice [29]. To investigate the roles of various cytokines, Yoshida et al. and Zhang et al. produced animal models by crossing dnTGF-βRII mice with IL12p40 KO and IFN-γ KO mice and by crossing dnTGF-βRII mice with IL-6 KO mice, respectively [30, 31]. Each of these derived models makes a considerable contribution to the pathological analysis of PBC. Interestingly, the grade of hepatic lesions in animal models produced by crossing

Table 4: Comparison of novel PBC animal models.

	Spontaneous models					Xenobiotic-immunized induced model	
	NOD.c3c4 mice	dnTGF-βRII mice	IL-2 R$\alpha^{-/-}$ mice	Scurfy mice	$Ae2_{a,b^{-/-}}$ mice	6-BH-immunized guinea pigs	2-OA-immunized mice
Advantages							
AMA	50–60%	100%	100%	100%	40–80%	100%	100%
Dominant AMA target protein	PDC-E2	PDC-E2	PDC-E2	PDC-E2	PDC-E2	PDC-E2	PDC-E2
Biliary damage	+	++	+−++	+−++	+−+++	+	+−++
Granuloma	+	+	− or +	−	?	++	++
Pro-inflammatory cytokines	+	+	+	+	+	+	+
Disadvantages	Biliary dilatation	moderate colitis	Severe colitis Severe hemolytic anemia	Short lifespan	Late onset	Late onset	peritonitis

does not vary greatly among individuals; moreover, it is easy to assign scores to different degrees of pathology for the purpose of evaluation. The dnTGF-βRII mouse model is now used in various analyses throughout the world, and it may be regarded as the most useful PBC animal model available at present. However, it is not totally satisfactory as a PBC model because the pathophysiological grade is not severe; furthermore, events that occur in the advanced stage of PBC, such as loss of the bile duct, cholestasis, and fibrosis, are rarely seen. Therefore, the pathophysiological model based on this mouse needs to be further developed.

4.3. The IL-2 Receptor $\alpha^{-/-}$ Mouse. IL-2 is critical for the development and peripheral expansion of $CD4^+$ $CD25^+$ Tregs that promote self-tolerance by *in vivo* suppression of T-cell responses [50, 51]. In IL-2 receptor $\alpha^{-/-}$ (IL-2R-$\alpha^{-/-}$) mice, the IL-2 signal, which is important in controlling the fate of mature T cells, is intercepted; these mice develop an inflammatory bowel disease and a lymphoproliferative autoimmune disease. Also, 25–50% mice develop severe hemolytic anemia at 8–20 weeks of age. It was reported that children with a genetic deficiency of IL-2R-α developed clinical manifestations similar to those of PBC [52]. Anti-PDC-E2 antibody is present in the serum of all IL-2R-$\alpha^{-/-}$ mice, and ANA is also present in the serum of 80% of these mice. There is profound lymphocytic infiltration in the portal tract and the interlobular bile duct is also damaged. $CD8^+$ T cells are predominant among the infiltrating lymphocytes, and $CD4^+$ T and B cells are also present in increased numbers. In addition, granulomas are formed, though in small numbers. Increased levels of inflammatory cytokines such as TNF-α, IFN-γ, IL-12p40, and IL-6 are present in the serum. The extent of inflammatory bowel disease is relatively severe and frequently associated with formation of crypt abscesses [32]. Using a derived model produced by crossing IL-2R-$\alpha^{-/-}$ mice with CD4 KO and CD8 KO mice, Hsu et al. showed that $CD8^+$ T cells participate in the pathogenesis of PBC [33]. The hepatic lesions of IL-2R-$\alpha^{-/-}$ mice are similar to those of PBC, though the complication of severe hemolytic anemia or colitis has not been evaluated. Moreover, the reduced lifespan of these mice makes it difficult to use them in various experiments, such as those involving mating; this is considered a limitation of this model.

4.4. The Scurfy Mouse. The Scurfy mouse is a mouse with loss of functional regulatory T cells caused by the *forkhead boxp3* (*Foxp3*) gene mutation. AMAs are present in all Scurfy mice at 3-4 weeks of age, and the portal tract shows moderate to marked lymphocytic infiltration and development of a severe interlobular bile duct lesion similar to PBC with high levels of cytokines such as TNF-α, IFN-γ, IL-6, IL-12, and IL-23 present in the serum and liver. However, Scurfy mice have an extremely short lifespan of about 4 weeks. This is a serious drawback with regard to its use in experiments [34].

4.5. $Ae2_{a,b^{-/-}}$ Mice. The anion exchanger (Ae)$2_{a,b}$-deficient mouse model was constructed by Salas et al. in Spain, based on a clinical investigation showing that *Ae2* gene expression was reduced in liver biopsy specimens and blood lymphocytes from patients with PBC [35]. $Ae2_{a,b^{-/-}}$ mice exhibit enhanced production of IL-12p70 and IFN-γ, an expanded $CD8^+$ T cell population, and a reduced number of Treg cells. Serum analysis by immunoblotting showed that 9 out of 11 $Ae2_{a,b^{-/-}}$ mice had AMAs. A histological study of liver sections from 11 $Ae2_{a,b^{-/-}}$ mice revealed mild to severe portal inflammation in 10 animals. Although the mechanism leading to the deficiency of *AE2* in the liver and blood mononuclear cells in human PBC is unclear, observations of $Ae2_{a,b^{-/-}}$ mice indicate a relationship between biliary epithelial dysfunction and the pathogenesis of PBC.

5. Immunity Induced by Xenobiotics in Mice

Not only genetic factors but also various environmental factors, such as bacterial infection and exposure to xenobiotics, are strongly implicated in the onset and development of PBC. Most importantly, prolonged exposure over an extended period of time to various xenobiotics with a structure similar to that of the inner lipoyl domain of PDC-E2 has attracted attention as a trigger for the development of PBC.

It has been revealed that two types of xenobiotics induce a pathophysiology that is very similar to that of PBC.

5.1. Guinea Pigs Immunized with 6-Bromohexanoate. 6-Bromohexanoate (6-BH) coupled with bovine serum albumin (BSA) has a structure similar to that of the inner lipoyl domain of PDC-E2 [53]. Increased levels of anti-PDC-E2 antibody, anti-BCOADC-E2 antibody, and anti-OGDC-E2 antibody appear in the serum of guinea pigs when they are immunized with BSA-coupled 6-BH. In addition, slight to moderate lymphocytic infiltration, interlobular bile duct irregularity, and granuloma formation in the portal tract are seen in the liver at an advanced age. Many vacuole-like lipid droplets are seen in the granulomas, and there are also aggregations of macrophages that phagocytoze lipids. Since these vacuolar changes are also seen in control animals to a slight extent, they may be related to immune reactions to foreign substances in the oil emulsion. The limitation of this animal model is the difficulty of use in some experiments because the extent of hepatic lesions is slight and lesions are slow in development, not appearing until 18 months after immunization [36].

5.2. Mice Immunized with 2-Octynoic Acid. 2-Octynoic acid (2-OA) is a xenobiotic widely used as a food additive and as a component of certain cosmetic products. 2-OA coupled to BSA has a structure similar to that of the inner lipoyl domain of PDC-E2 [54]. Wakabayashi et al. immunized C57BL/6 mice with BSA-coupled 2-OA and detected AMA and anti-PDC-E2 antibodies as well as increased serum levels of TNF-α and IFN-γ. Marked inflammatory cell infiltration and bile duct lesions in the portal tract frequently appeared, which were mainly associated with $CD8^+$ T cells [37]. Using the same methods, Wakabayashi et al. also succeeded in producing a PBC-like lesion in another mouse strain (nonobese diabetic (NOD) congenic strain 1101) [38]. This model is innovative because it induces PBC-like pathophysiology by administration of xenobiotics that may be related to the cause of PBC. As the reproducibility of this model is comparatively high and flexibility is also high, various pathophysiological analyses of this model are in progress in different countries' institutions [39, 40]. At present, however, the hepatic and bile duct lesions show many differences from those of PBC, and further evaluation of the model is needed. In the original immunization procedure used by Wakabayashi et al., BSA-coupled 2-OA was introduced into the abdominal cavity using complete Freund's adjuvant (*M. tuberculosis* in adjuvant oil), following which BSA-coupled 2-OA using incomplete Freund's adjuvant (adjuvant oil only) was administered every 2 weeks as a booster immunization. This model always developed peritonitis of various grades as an adverse effect. A preliminary experiment is necessary in order to check the extent to which peritonitis influences the development of pathological changes in the liver, particularly in that portion which is histologically evaluated. Moreover, a granuloma often appears in the portal tract or the hepatic parenchyma; this could be attributed to the complete Freund's adjuvant administered at the time of immunization. However, methods of immunization have improved; as a result, various modifications designed to induce more serious pathophysiology can be tested. Reports of studies using this model are eagerly awaited.

6. Conclusion

The pathophysiology of PBC involves both humoral and cell-mediated immunity, and it can be considered the prototype of autoimmune diseases of the liver. The production of a practical animal model of PBC has been a challenge for researchers interested in PBC and autoimmune diseases for many years. Although animal models based on a variety of mechanisms have been reported from many laboratories, the ideal animal model has still not been available. Recently, several PBC animal models based on different mechanisms were reported. While these newer animal models show the characteristic findings of PBC unlike earlier models, they still show different features from those of human PBC. Understanding the strengths and limitations of each animal model is required in order to match the model to the intended purpose; moreover, suitability of the model for the intended purpose should be confirmed by further research and analysis.

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Review Article

PBC: Animal Models of Cholangiopathies and Possible Endogenous Viral Infections

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Primary Biliary Cirrhosis (PBC) is considered an autoimmune disease characterized by immune-mediated destruction of the intrahepatic bile ducts and its characteristic serologic marker, the anti-mitochondrial antibody (AMA). Several factors were proposed to clarify the pathological and immunological mechanisms of PBC. Immunological reaction with a bacterial or a viral association was identified in the previous report, and it seems probable that PBC was thought to have such an etiology. The majority of patients with PBC was reported to have both RT-PCR and immunohistochemistry evidence of human betaretrovirus infection in lymph nodes or in 2008, the patient who developed PBC with high HIV viral load had an antiviral therapy and recovered. To understand the etiology of PBC associated with infection, several factors should be considered and especially animal models may be useful. In this paper, we introduce three typical animal models of PBC: the dominant-negative form of transforming growth factor-β receptor type II (dnTGFβRII) mouse, IL-2R$\alpha^{-/-}$ mouse and NOD.c3c4 mouse, are enumerated and described, and we discuss previous reports of viral infection associated with PBC and consider the etiology of PBC from our analysis of results in NOD.c3c4 mouse.

1. Introduction

Primary Biliary Cirrhosis (PBC) is considered an autoimmune disease characterized by immune-mediated destruction of the intrahepatic bile ducts and its characteristic serologic marker, the anti-mitochondrial antibody (AMA). AMA is a highly specific autoantibody found in about 90% of patients with PBC that reacts with an epitope on the E2 subunit of the pyruvate dehydrogenase enzyme complex (PDC-E2) [1–3]. The epitopes discerned by anti-PDC-E2 and CD4 and CD8 autoreactive T cells are present in the inner lipoyl domain of PDC-E2. A 100-fold increase in CD4 and a 10-fold increase in CD8 autoreactive T cells infiltrate into the portal tracts [4, 5]. Moreover, several factors were proposed to clarify the pathological and immunological mechanisms of PBC. Some biological features of the bile duct cells have been reported, suggesting a basis for their distinctive destruction [6–8]. Optionally, soon after autoimmune diseases were first recognized more than a century ago, immunological reaction with a bacterial or a viral association was identified and PBC was thought to have such an etiology (Table 1). [9–11]. The majority of patients with PBC were reported to have both RT-PCR and immunohistochemistry evidence of human betaretrovirus infection in lymph nodes [12], or in 2008, the patient who developed PBC with high HIV viral load had an antiviral therapy and recovered [13]. To determine whether PBC can be induced by infections, first autoimmunity needs to be defined. Autoimmune diseases occur when a response to a self-antigen involving T cells, B cells, or autoantibodies induces injury systemically or against a specific organ [14]. Although an autoimmune response occurs in most persons, it is only in a few persons that disease actually appears. In PBC, how can infection induce autoimmunity? The mechanism to explain the association of infection is molecular mimicry of autoepitops by peptides of microorganisms. This results in cryptic T-cell epitopes, the degeneracy of T-cell receptors, and the disruption of immune tolerance [15, 16]. This is of great significance for

Table 1: Viral infections in humans associated with autoimmune diseases.

Relevance or suspicion of autoimmune human diseases	Representative viruses
PBC	HIV-1 p24
	MMTV
Multiple sclerosis	Epstein-Barr virus (EBV) Measles virus
Type1 diabetes	Coxsackie virus B4
	Rubella virus
	Cytomegalovirus (CMV) Mumps virus
Rheumatoid arthritis	EBV
	Hepatitis C virus (HCV)
Systemic lupus erythematosus	EBV
Myocarditis	Coxsackievirus B3
	CMV
Myasthenia gravis	Herpes simplex virus
	HCV
Guillain-Barre syndrome	CMV
	EBV

PBC because of the tendency of several viruses to target particularly the liver. There are several mechanisms by which viruses are thought to induce an autoimmune response. These include the expression of some autoantigens, the expression of major histocompatibility complex molecules, and changes in cytokine production [16]. To understand the etiology of PBC associated with infection, several factors should be considered and especially animal models may be useful [14, 17]. The association of betaretroviral protein production and aberrant PDC-E2-like protein expression in the IL-2R$\alpha^{-/-}$ mouse and Nonobese diabetic (NOD).c3c4 mouse was reported recently [18].

In this paper, we introduce three typical animal models of PBC: the dominant-negative form of transforming growth factor-β receptor type II (dnTGFβRII) mouse, IL-2R$\alpha^{-/-}$ mouse, and NOD.c3c4 mouse are enumerated and described [19–21]. Additionally, we discuss previous reports of viral infection associated with PBC and consider the etiology of PBC from our analysis of results in NOD.c3c4 mouse.

2. Murine Model of PBC

2.1. DnTGFβRII Mouse. TGF-β is the most widely distributed cytokine with pleiotropic effects on cell growth and immunological controls, specifically having a promoting effect on the development of the regulatory T-cell compartment [22]. dnTGFβRII mice were originally developed by Gorelik and Flavell for the purpose of analyzing the role of this receptor, which regulates the activation of the T cell function [23]. To disrupt the intracellular domain of the normal receptor in this mouse, the receptor is incompetent of transduction after TGF-β ligation. The expression of dnTGFβRII is limited by the CD4 promotor which lacks CD8 silencer, and this transgenic mouse spontaneously develops features characteristic of PBC [23]. These features include the expression of AMA with specificity against PDC-E2, BCOADC-E2, and OGDC-E2, as in human PBC. Pathologically, the infiltration of lymphoid cells, especially $CD4^+$ and $CD8^+$ lymphocytes, in the portal tracts causes biliary duct destruction [19] and the accumulation of natural killer T cells (NKT) in the intrahepatic bile duct lesions, resembling the condition found in human PBC [24]. Although the granuloma formations around the portal tracts seen in human PBC are not present, some lymphocytic aggregations like immature granuloma formation could be observed [25]. Furthermore, the serum levels of cytokines such as IFN-γ,TNF-α,IL-12p40, and IL-6 are significantly increased, as seen in human PBC [26, 27].

2.2. IL-2R$\alpha^{-/-}$ Mouse. In 2006, Aoki et al. reported a male child with a genetic deficiency of IL-2 receptor αIL-2Rα,)CD25) expression who had liver dysfunction with serological expression of PBC. Histologically, there was lymphoid infiltration in the portal tracts and serum antibody to PDC-E2. The deficiency of $CD4^+$ $CD25^+$ subset of regulatory T cells was considered a key to elucidating of this clinical condition [20]. Based on these findings, Wakabayashi et al. established IL-2R$\alpha^{-/-}$ mice and evaluated their hepatic immunopathology [28]. These mice also show AMA positivity against PDC-E2 that localizes to the inner lipoyl domain of the autoantigen. Lymphoid cells, composed of $CD4^+$ and $CD8^+$ lymphocytes, infiltrate into portal tracts without a significant increase in NKT. Although mild interface hepatitis and biliary duct destruction are seen in the liver, granuloma formations around the portal tracts are not observed [28]. The circulating cytokine profiles are similar to those of dnTGFβRII mice, showing elevations of IFN-γ,TNF-α,IL-12p40, and IL-6, as identified in the serum of patients with PBC [26, 27, 29].

2.3. NOD.c3c4 Mouse. NOD.c3c4 mice were generated by the introgression of large genetic intervals on chromosome 3 and 4 into a NOD background [21, 30]. NOD and genetically modified NOD mice have been reported to progress to not only spontaneous autoimmune diabetes but also rheumatoid arthritis, Sjogren's syndrome, and thyroiditis [31–34]. NOD.c3c4 mice derived from NOD strains are considered to be an animal model of PBC with autoimmune biliary destruction [21, 30]. Most importantly, these mice show antibodies to PDC-E2. They express AMA positivity, unlike the dnTGFβRII mice and IL-2R$\alpha^{-/-}$ mice, and the rate of positivity has reached 50–60% [35]. Portal tract infiltration with $CD3^+$, $CD4^+$, and $CD8^+$ lymphocytes results in chronic nonsuppurative destructive cholangitis and epithelioid granuloma formations [21, 30]. However, the morphological features of the bile ducts lesions differ from those in human PBC, in which characteristic biliary cyst formations as well as apparent hepatomegaly are described [36].

Table 2: Antiviral trials for PBC.

Trial	Method	Subject	Design	Primary outcome	Year	Reference
Pilot studies of single and combination antiretroviral therapy	Lamivudine + Zidovudine versus Lamivudine	Human	Randomized controlled trial (RCT)	Serological improvements of alkaline phos-phatase, AST and ALT. Histological improvement in necroinflammatory score and a reduction in bile duct injury.	2004	[43]
Clinical trial: randomized controlled trial of lamivudine and zidovudine (Combivir)	Lamivudine + Zidovudine + UDCA versus UDCA	Human	RCT	Serological improvements in serial alkaline phosphatase, ALT and AST.	2008	[44]
Randomized controlled trial of lamivudine	Lamivudine versus UDCA	Human	RCT	One case showed a decrease of AMA titers.	2010	[45]
Combination antiretroviral therapy with Combivir	Lamivudine + Zidovudine versus Placebo	NOD.c3c4 mouse		Histological improvement in necroinflammatory score and a reduction in bile duct injury. No improvement on bile duct cyst. Decrease in viral burden.	2007	[46]
Highly active antiretroviral therapy with reverse transcriptase inhibitors and protease inhibitor	Combination of reverse transcriptase and protease inhibitor	NOD.c3c4 mouse		Serological improvements in alkaline phos-phatase and AST. Complete disappearance of cholangitis.	2008	[47]

3. Possibility of Viral Infection Associated with PBC

It has been thought that some viruses may associate with human diseases of oncogenesis or autoimmunity because of their genome integration or specific viral-encoding proteins. Especially, in 1998, Munoz et al. described that there was an antibody for human immunodeficiency virus-1 (HIV-1) in the serum of PBC patients [37]. To investigate for a possible immune response to the p24 gag protein of HIV-1, moderate-to-strong reactivity was found in about 30% of the patients with Sjogren's syndrome, as compared with less than 1% of healthy controls [38], and the 36% of systemic lupus erythematosus (SLE) patients produced antibodies to the p24 gag protein [39]. Mason et al. discovered HIV-1 p24 gag protein seroreactivity in 35% of patients with PBC, 29% of patients with SLE, and 39% of patients with either primary sclerosing cholangitis or biliary atresia, compared with only 4% of patients with alcohol-related liver disease or alpha1-antitrypsin-deficiency liver disease, and only 4% of healthy volunteers. Moreover, Western blot reactivity to the human intracisternal A-type particle (HIAP) proteins related to HIV-1 was found in 51% of patients with PBC, in 58% patients with SLE, and in 17% of those with other biliary diseases. None of the 23 patients with either alcohol-related liver disease or alpha1-antitrypsin deficiency and only one of the healthy controls showed the same reactivity to HIAP proteins [40]. Therefore, these antibody reactivities found in patients with PBC may be attributable to an immune response to uncharacterized viral proteins that share antigenic determinants with HIV-1-related retroviruses.

In 2003, a human betaretrovirus clone sequence was originally detected from the biliary epithelium cDNA library of a patient with PBC. When searching viral data registered in BLASTN, the initial partial pol gene fragment was found to exhibit 95% to 97% identities with mouse mammary tumor virus (MMTV) and with retrovirus sequences derived from human breast cancer samples within the overlapping sequence [12, 41]. Using a specific MMTV antibody, viral proteins were shown in the perihepatic lymph nodes but not in liver tissue samples from patients with PBC [41]. However, Selmi et al. expressed an opposing view concerning this result [42].

Some pilot studies were conducted to determine whether antiviral therapy impacted the disease progression (Table 2). First, Mason et al. performed a trial with reverse-transcriptase inhibitors (lamivudine group versus lamivudine/zidovudine group) for patients with PBC. The lamivudine/zidovudine group showed significant serological improvement in the activities of alkaline phosphatase, AST and ALT, and histological assessment revealed an improvement in the necroinflammatory score and a reduction in bile duct injury compared to the lamivudine group [43]. A further clinical trial was performed with a combination of lamivudine and zidovudine versus ursodeoxycholic acid (UDCA). Significant differences were observed in the antiviral therapy versus UDCA with serological improvements in serial alkaline phosphatase, ALT and AST as well as the clinical score [44]. Thus, reverse-transcriptase inhibitors are

expected to suppress retroviral proliferation and contribute to the improvement of PBC. We once investigated the efficacies of 90 day's administration of lamivudine to 20 PBC patients with unsatisfactory biochemical responses to UDCA in a randomized double blind control trial. As a result, no significant biochemical difference was seen between both groups. However, of interest, one case showed a decrease of AMA titers and biochemical response [45]. These results were similar to those of studies conducted by Mason et al. [43]; yet the true efficacy should be evaluated in large-scale control trials.

4. Are NOD.c3c4 Mice Infected with a Retrovirus?

NOD.c3c4 mice have been described as a mouse model with several features similar to PBC [30]. However, these mice develop marked biliary cyst formation that is not shown in human PBC. In human PBC, the destruction of cholangiocytes leads to ductpenia [48]. When we analyzed the gene expression of the cholangiocytes of such mice using microarray analysis, there was consistent liver-specific downregulation in the expression of Fas antigen (CD95) [36]. Fas (CD95) antigen is a member of the tumor necrosis factor family that binds to Fas ligand (FasL). This gene is situated at chromosome 19 in mouse [49]. Fas/FasL interactions play an important role in apoptosis [49–51]. Fas is detected in hepatocytes and plays an important role in inflammation and cell death in hepatitis virus-infected liver [52]. The Fas system on cholangiocytes has been also reported in human and rats, and enhanced expression of FasL on cholangiocytes has been implicated in progressive bile duct loss in PBC through apoptosis [53]. Furthermore, FasL expressed by cholangiocarcinomas was reported to induce lymphocyte cell death and escape immune surveillance [54]. It has been reported that the Fas/FasL system is strongly associated with biliary pathological conditions. It was thought that, because of the downregulation of Fas antigen, the apoptosis of cholangiocytes cannot easily occur and therefore biliary cyst formation was found in NOD.c3c4 mice [36]. However, it was considered that there are also other factors because the degree of the cyst formation differed according to the individual though the genetic expression was the same.

In 2007, Chen et al. reported that MMTV gag and pol gene expression was 4- to 25-fold higher in all three autoimmune biliary disease models, NOD.c3c4, dnTGF-βRII, and IL-2R$\alpha^{-/-}$, as compared to control mice when using real-time RT-PCR to quantify MMTV using gag and pol primers [55]. A randomized study was conducted using NOD.c3c4 mice treated with a combination therapy of lamivudine and zidovudine or with placebo (Table 2). Serial hepatic biochemistry studies showed diminished alkaline phosphatase in the mice treated with combination therapy. Histological evaluation showed a significant decrease in the necroinflammatory score and the grade of the bile duct injury in the combination therapy group. However, therapy had little improvement on bile duct cyst formation. When compared to mice receiving placebo group, combination therapy group reduced viral burden measured by the pol and gag gene RT-PCR [46]. Moreover, Graham et al. treated NOD.c3c4 mice with combination of reverse transcriptase inhibitors and protease inhibitor. Hepatic biochemistry showed significant improvements in alkaline phosphatase and AST, and the cholangitis completely disappeared [47]. Thus, the detection of MMTV in the NOD.c3c4 mice and resolution of biliary disease with antiviral therapy supports the retroviral hypothesis for PBC.

However, in order to prove that viral factors are involved in the pathogenesis of PBC, it is necessary to understand and elucidate the mechanism of viral replication, reproduction, the transcription of the virus genomes, the pathogenic roles of viral tropics, the integrated state, and the interaction with the host's immune system, especially the mechanism by which autoimmune tolerance is broken. Therefore, it is premature to relate the cause of the clinical condition of PBC to viral infection based only on the present report. However, given the unprecedented progress of biotechnology, a more detailed understanding of these issues can be expected in the near future.

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Review Article

Caroli's Disease: Current Knowledge of Its Biliary Pathogenesis Obtained from an Orthologous Rat Model

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Caroli's disease belongs to a group of hepatic fibropolycystic diseases and is a hepatic manifestation of autosomal recessive polycystic kidney disease (ARPKD). It is a congenital disorder characterized by segmental saccular dilatations of the large intrahepatic bile duct and is frequently associated with congenital hepatic fibrosis (CHF). The most viable theory explaining its pathogenesis suggests that it is related to ductal plate malformation. The development of the polycystic kidney (PCK) rat, an orthologous rodent model of Caroli's disease with CHF as well as ARPKD, has allowed the molecular pathogenesis of the disease and the therapeutic options for its treatment to be examined. The relevance of the findings of studies using PCK rats and/or the cholangiocyte cell line derived from them to the pathogenesis of human Caroli's disease is currently being analyzed. Fibrocystin/polyductin, the gene product responsible for ARPKD, is normally localized to primary cilia, and defects in the fibrocystin from primary cilia are observed in PCK cholangiocytes. Ciliopathies involving PCK cholangiocytes (cholangiociliopathies) appear to be associated with decreased intracellular calcium levels and increased cAMP concentrations, causing cholangiocyte hyperproliferation, abnormal cell matrix interactions, and altered fluid secretion, which ultimately result in bile duct dilatation. This article reviews the current knowledge about the pathogenesis of Caroli's disease with CHF, particularly focusing on studies of the mechanism responsible for the biliary dysgenesis observed in PCK rats.

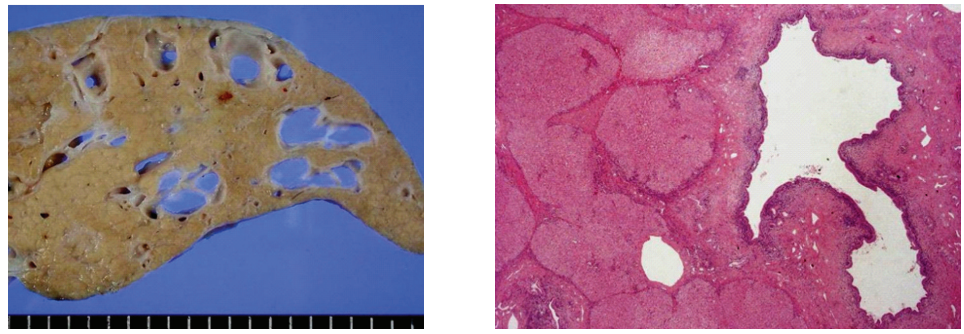
1. Introduction

Caroli's disease belongs to a group of hepatic fibropolycystic diseases [1, 2]. It is a congenital disorder characterized by a biliary abnormality consisting of segmental saccular dilatations of the large intrahepatic bile duct. It is frequently associated with varying degrees of portal fibrosis, corresponding to congenital hepatic fibrosis (CHF). Caroli initially described two variants of the biliary abnormality with and without CHF (Caroli's syndrome and Caroli's disease), and the form without CHF is quite rare.

A significant proportion of Caroli's disease cases involving CHF are transmitted in an autosomal recessive manner and are associated with autosomal recessive polycystic kidney disease (ARPKD). The incidence of ARPKD is 1 in 20,000 live births [3]. Renal failure may be present at birth, and the disease presentation is not limited to the neonatal period; it can be diagnosed in childhood or even adolescence or adulthood [4]. These late-presenting cases typically display less severe kidney disease, but more commonly involve liver disease complications.

Caroli's disease is a developmental anomaly, and the most viable theory explaining its pathogenesis is that it is related to ductal plate malformation at different levels of the intrahepatic biliary tree [5]. Intrahepatic bile ducts develop from bipotential liver progenitor cells that are in contact with the mesenchyme of the portal vein, which form from the ductal plates [6]. The ductal plates are then remodeled into mature tubular ducts. The ductal plate remodeling process begins from the larger ducts to the smaller peripheral ducts. The heredity factors causing Caroli's disease can exert their influence not only during the early embryological period in which large intrahepatic duct formation occurs, but also during the later development of the more proximal interlobular ducts involved in CHF.

The molecular pathogenesis of Caroli's disease is incompletely understood. Human and experimental data have suggested several potential mechanisms that could lead to cyst



(a) (b)

Figure 1: The liver of a Caroli's disease patient with CHF. Multiple cystic dilatations of the intrahepatic bile ducts are grossly (a) and histologically (b) visible. Hematoxylin-eosin staining (b).

formation in fibropolycystic liver diseases including those of Caroli's disease patients: (i) increased cell proliferation and apoptosis; (ii) enhanced fluid secretion; (iii) abnormal cell-matrix interactions; (iv) alterations in cell polarity; and (v) abnormal ciliary structure or function [7]. To study the cyst pathogenesis of ARPKD, different experimental animal models including cpk, bpk, and orpk mice and several types of knockout mice have been used [8–12]. Among them, the polycystic kidney (PCK) rat is an orthologous model of ARPKD that represents the phenotype of the slowly progressive form of ARPKD and is also a novel animal model of Caroli's disease with CHF [13].

This article reviews our current knowledge of the pathogenesis of Caroli's disease with CHF, particularly focusing on studies about the mechanism responsible for the biliary dysgenesis observed in the PCK rat. First, the clinicopathological and genetic aspects of Caroli's disease with CHF are described. In the following section, Caroli's disease refers to the form of the disease associated with CHF, since Caroli's disease without CHF is rare.

2. Caroli's Disease

2.1. Clinical Features. Renal involvement is encountered in up to 60% of patients with Caroli's disease [14]. Hepatic manifestations of ARPKD are present in 15–45% of patients and include an enlarged liver, portal hypertension, or abnormal findings on hepatic imaging [15]. A few rare cases of Caroli's disease have occurred in the setting of autosomal dominant polycystic kidney disease (ADPKD) [16].

The clinical manifestations of Caroli's diseases are related to both biliary abnormalities and portal hypertension due to CHF [14, 17, 18]. Its clinical progression and presentation are highly variable, and symptoms may appear late in life.

Bile ducts dilatation induces a predisposition to bile stagnation, leading to the formation of lithiases. Bacterial cholangitis occurs frequently and may be complicated by hepatic abscess formation and sepsis. Recurrent cholangitis dominates the clinical course and is the principal cause of morbidity and mortality. After cholangitis occurs, a large number of patients die within 5–10 years [14]. Secondary biliary cirrhosis can occur due to biliary obstruction.

Portal hypertension due to CHF may lead to ascites and esophageal variceal hemorrhaging. Splenomegaly and hepatomegaly are common. Children with Caroli's disease usually display earlier symptom onset and a more rapidly progressive disease because of the combined effects of cholangitis and portal hypertension.

Caroli's disease may progress to cholangiocarcinoma. The occurrence of cholangiocarcinoma has been reported in 7–14% of patients [19]. A rare case of cholangiocarcinoma arising in CHF has also been reported [20].

The laboratory findings of Caroli's disease are nonspecific. Transaminase levels may be slightly elevated. A complete blood count might reveal thrombocytopenia and leukopenia if portal hypertension and hypersplenism are present. An elevated white blood cell count and increased serum alkaline phosphatase or direct bilirubin levels could indicate cholangitis. BUN and creatinine values should also be measured to detect any associated renal disease.

2.2. Pathology. The biliary abnormalities of Caroli's disease are characterized by progressive and segmental saccular or cystic dilatation of the intrahepatic bile duct (Figure 1). The disease might be limited to one lobe of the liver, most commonly the left lobe. Histologically, the dilated ducts are lined by the biliary epithelium, which may be hyperplastic and ulcerated. In patients with cholangitis, an acute and chronic inflammatory cell infiltrate is seen around the dilated bile ducts. In the presence of CHF, dense portal fibrosis is observed, and the fibrotic region often contains variable numbers of abnormally shaped bile ducts and hypoplastic portal vein branches. The hepatic parenchyma is subdivided by the overgrowth of portal fibrous tissue, while no parenchymal regenerative activity is evident, which allows the condition to be differentiated from cirrhotic regenerative nodules.

The mechanism of the development of cholangiocarcinoma in Caroli's disease remains unclear. In chronic biliary diseases, it has become evident that cholangiocarcinoma

arising in the large bile ducts undergoes a multistep carcinogenic process, and biliary intraepithelial neoplasia (BilIN) is considered to be the precursor lesion [21]. BilIN is frequently seen in patients with hepatolithiasis, and it can be encountered in the livers of Caroli's disease patients. Biliary papillomatosis has also been observed in Caroli's disease [22]. Thus, cholangiocarcinoma in Caroli's disease probably arises despite a multistep carcinogenic process that is closely related to chronic epithelial damage.

2.3. Pathological Studies. There is limited data available from pathological molecular studies using human liver tissues from patients with Caroli's disease and/or CHF. Cholangiocytes from the livers of patients with Caroli's liver have been shown to overexpress vascular endothelial growth factor (VEGF), its receptors (VEGFR-1 and VEGFR-2), and angiopoietin-2 [23]. VEGF expression on cholangiocytes positively correlates with microvascular density around the bile ducts, suggesting that it has a proliferative effect on cholangiocyte growth by inducing the production of an abundant vascular supply. VEGF may also stimulate bile duct dilatation through the induction of cholangiocyte proliferation via an autocrine effect [24]. In addition, the activation of the mammalian target of rapamycin (mTOR) pathway has been implicated in the overgrowth of cholangiocytes in Caroli's disease [25].

Cholangiocyte overgrowth is linked to abnormalities in cell cycle progression and also to microRNA expression. The progression of cells through the cell cycle is controlled by a family of dual specificity phosphatases, Cdc25, that activate cyclin-dependent kinases. The biliary epithelium of CHF overexpresses Cdc25A protein (an isoform of Cdc25), which is accompanied by the downregulation of a microRNA (miR15a) [26].

Around intrahepatic bile ducts, basement membrane components such as laminin and type IV collagen, the major basal laminar components are degraded in Caroli's disease [27]. These findings indicate that the reduction of laminin and type IV collagen expression in the basement membrane, a supportive structure of intrahepatic bile ducts exacerbates the observed bile duct dilatation. The degradation of laminin and type IV collagen around bile ducts is also observed in foci of cholangiocarcinoma in situ arising in Caroli's disease, indicating that once cholangiocarcinoma in situ develops in the biliary epithelia of Caroli's disease patients, it tends to transform into invasive carcinoma [27].

In most types of chronic liver disease, activated hepatic stellate cells play major roles in hepatic fibrosis. However, necroinflammatory changes and the activation of hepatic stellate cells are not as marked in CHF as those seen in ordinary chronic liver diseases such as chronic viral hepatitis. The fact that abundant connective tissue growth factor (CTGF) is retained by heparin sulfate proteoglycans (HSPG) in the fibrous portal tracts could be responsible for the unresolved hepatic fibrosis observed in CHF [28]. Portal mononuclear cells and endothelial cells expressing CTGF and/or HSPG tend to collect around proliferated bile ducts in CHF, providing a possible explanation for the mechanism of the fibrosis that characterizes CHF.

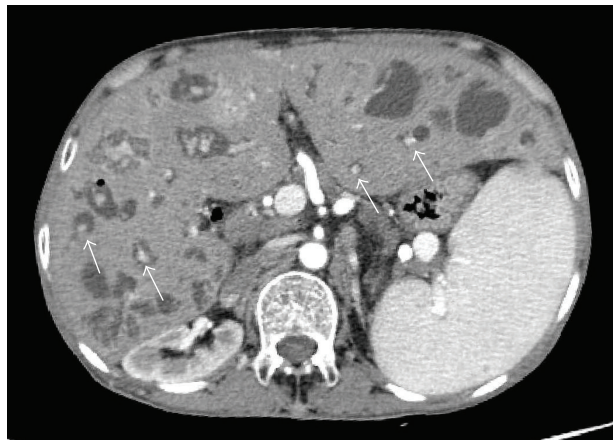


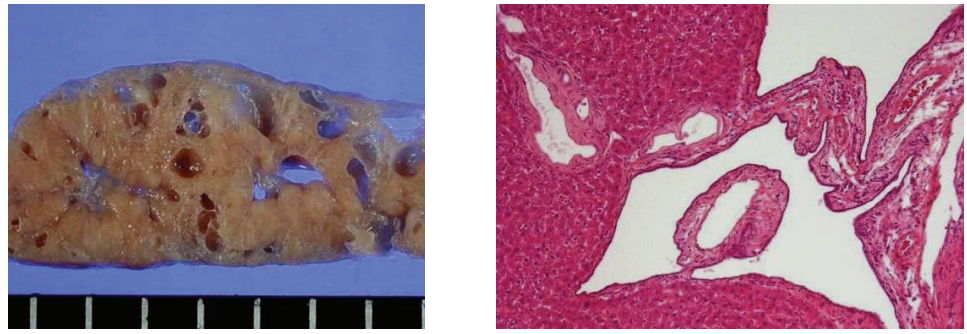
Figure 2: Dynamic CT reveals multiple cystic dilatations of the intrahepatic bile ducts in a patient with Caroli's disease with CHF. The arrows indicate the central dot sign.

2.4. Diagnosis. Caroli's disease is diagnosed by imaging studies showing nonobstructive, saccular, or fusiform dilatations of the intrahepatic bile ducts [29–31]. Ultrasonography and endoscopic retrograde cholangiopancreatography are the traditional methods of diagnosis. However, magnetic resonance cholangiopancreatography is emerging as the most useful diagnostic modality. On sonography, Caroli's disease presents as intrahepatic cystic anechoic areas in which fibrovascular bundles and linear bridges or septa may be present. The fibrovascular bundles are composed of the portal veins and hepatic arteries, which can be demonstrated by Doppler ultrasonography and are recognized as the central dot sign on CT with contrast enhancement (Figure 2). Overlapping imaging findings are often detected, which reflect its underlying pathology and associated complications, including fibrosis, ductal dilatation, cholangitis, lithiasis, and malignancy [32]. A liver biopsy is rarely required to make a diagnosis of Caroli's disease.

2.5. Treatment. Treatment for Caroli's disease is largely supportive and is directed toward treating the biliary infection and complications associated with portal hypertension [15]. Cholangitis, hepatic abscesses, and sepsis should be treated aggressively with appropriate antibiotics. Infections are particularly difficult to eradicate in the presence of bile stasis and intrahepatic lithiasis. Recurrent bouts of cholangitis can lead to end-stage liver disease.

Common bile duct stones may require endoscopic sphincterotomy and stone extraction, while the extraction of intrahepatic stones is difficult. Partial hepatectomy may be curative when the disease is confined to a single lobe of the liver [33]. Ursodeoxycholic acid has been used to treat intrahepatic lithiasis, which probably acts by increasing bile flow and decreasing bile stasis [34].

Variceal bleeding can be treated endoscopically with sclerotherapy or band ligation. A selective shunting procedure can provide relief from the complications associated with portal hypertension.



(a) (b)

Figure 3: The liver of a PCK rat. The gross (a) and histological (b) appearance of the adult rat liver closely resembles those of patients with Caroli's disease with CHF (Figure 1). Hematoxylin-eosin staining (b).

Liver transplantation is regarded as the ultimate treatment for patients who suffer recurrent bouts of biliary infection and those who also have complications related to portal hypertension [35]. Patient survival is reported to be excellent, and graft survival is comparable to or better than that of patients who have received transplants for other diseases [36]. Since the inheritance of the disease seems to occur in an autosomal recessive manner, it is important to provide genetic counseling to the patient's family.

2.6. Genetics. ARPKD is caused by mutations in a single gene, *PKHD1*, which has been localized to chromosome 6p21.1-p12. The gene consists of 86 exons and has a number of alternatively spliced transcripts. Its longest open reading frame contains 67 exons, which encode a 4074 amino acid protein called fibrocystin or polyductin [37, 38].

PKHD1 exhibits a high level of allelic heterogeneity, and more than 300 mutations have been described throughout *PKHD1*. A clear genotype/phenotype correlation has been described in ARPKD, with two truncating mutations associated with the most severe phenotype, while one or two missense changes are associated with milder disease [39]. The genetic basis for the differences between ARPKD with and without CHF has not been fully elucidated [40]. Mutations in *PKHD1* have also been identified in patients with Caroli's disease. *PKHDL1*, a homologous gene that is not involved in renal cystic disease has also been described [41].

2.7. Fibrocystin/Polyductin. Fibrocystin is a receptor-like membrane-associated protein. Structural predictions indicate that it has a large extracellular region with multiple copies of the TIG domain (an immunoglobulin-like fold), a single transmembrane region, and a short cytoplasmic tail. Based on its similarity with other TIG-containing proteins such as the hepatocyte growth factor receptor MET, fibrocystin is suggested to function as a receptor or ligand, since secreted forms can be generated from alternatively spliced transcripts [42]. In addition, the promoter might be directly regulated by hepatocyte nuclear factor-1β [43].

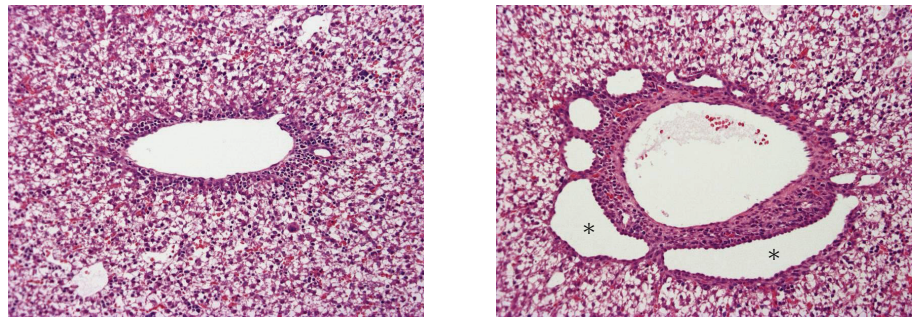
PKHD1 is preferably expressed in the kidneys with lower levels observed in the liver, pancreas, and lungs [37]. Similarly, fibrocystin is expressed in the cortical and medullary collecting ducts of the kidney as well as the biliary and pancreatic ducts [44], in which its distribution is consistent with the disease's phenotype. Fibrocystin is localized to primary cilia as well as to the basal body of epithelial cells and colocalizes with polycystin-2, the gene product responsible for ADPKD [42, 45]. Fibrocystin is also expressed in the normal ductal plate as well as in ductal plate malformations including CHF and colocalizes with stem cell markers in some ductal plate cells [46].

Fibrocystin can undergo notch-like processing, resulting in the release of the ectodomain from primary cilia [47]. Other studies have demonstrated cleavage of the fibrocystin ectodomain as well as the generation of a cytoplasmic fragment that translocates to the nucleus [48]. Such proteolytic cleavage can be elicited by the stimulation of intracellular calcium release or protein kinase C activation. Fibrocystin and polycystin-2 may act in a common molecular pathway to regulate calcium responses in the epithelia [49]. The structure and homologies of fibrocystin suggest that it plays a role in the regulation of cellular adhesion, repulsion, and proliferation and/or the regulation and maintenance of renal collecting tubules and bile ducts, but its exact role in normal and cystic epithelia remains unknown.

3. The PCK Rat

The PCK rat is derived from a Crj:CD (Sprague-Dawley) rat strain, originating in Japan [50]. The polycystic disease it suffers from is inherited in an autosomal recessive manner, and this model has a spontaneous mutation in its *Pkhd1* gene, an ortholog of human *PKHD1* [37]. The model has been rederived and is commercially available from Charles River Laboratories (Wilmington, MA).

In the livers of PCK rats, multiple segmental and saccular dilatations of the intrahepatic bile duct are observed (Figure 3). In addition, ductal plate malformations are evident in the livers of PCK rat fetuses (Figure 4), and the ductal dilatation spreads throughout the liver and increases in degree with age. The overgrowth of portal connective tissue progresses after delivery. All of the gross and histological



(a) (b)

Figure 4: Ductal plate malformation in the fetal liver of a PCK rat. Compared with a normal fetal rat (a), dilatation of the ductal plate is evident in the PCK liver (b, asterisks). Hematoxylin-eosin staining (a, b).

features of the PCK liver correspond to Caroli's disease with CHF.

To explore the mechanism of biliary dysgenesis suffered by PCK rats, a cholangiocyte cell line has been developed from the intrahepatic bile ducts of the PCK rat [51, 52]. The cholangiocytes of the PCK rat exhibit a higher rate of proliferation, with a doubling time of approximately half that of normal cholangiocytes, and maintain their biliary features during passaging.

Hereafter, based on the results of studies using PCK rats and/or the cholangiocyte cell line derived from them, the recent developments in our understanding of the cellular and molecular pathogeneses of the biliary dysgenesis observed in PCK rats are reviewed.

3.1. Proliferation and Apoptosis. Two key signaling pathways, 3′,5′-cyclic adenosine monophosphate (cAMP) activated B-Raf/MEK/ERK and AKT/mTOR/S6K/S6, have been implicated in the increased proliferation of PCK cholangiocytes.

Epidermal growth factor (EGF) and its receptor (EGFR) play important roles in promoting cholangiocyte proliferation. PCK cholangiocytes are hyperresponsive to EGF, and the increase in their proliferation is accompanied by activation of the MEK5/ERK5 pathway [51]. The phosphorylation of ERK1/2 is also increased in PCK cholangiocytes [53]. In contrast to other mouse models of ARPKD, EGFR is not overexpressed or mislocalized to the apical membrane in the PCK cholangiocytes [51, 54].

The cAMP levels of the PCK cholangiocytes are also increased [55]. These elevated cAMP levels stimulate cholangiocyte proliferation via downstream effectors and exchange proteins activated by cAMP (Epac1 and Epac2 isoforms) and protein kinase A (PKA) [56]. Hyperproliferation of the PCK cholangiocytes in response to PKA stimulation is associated with decreased intracellular calcium levels, and the restoration of calcium levels blocks PKA-dependent proliferation via the PI3K/AKT pathway. In addition, PCK cholangiocyte hyperproliferation is accompanied by the overexpression of Cdc25A protein and the downregulation of miR15a [26, 57]. miR15a overexpression in PCK cholangiocytes decreases Cdc25A levels, inhibits cell proliferation, and reduces cyst growth, indicating a potential therapeutic strategy for the disease.

The signaling pathway composed of AKT/mTOR/S6K/S6 is activated in PCK cholangiocytes (Ren XS et al., unpublished data). In the PCK rat liver, apoptosis of the biliary epithelium is less frequent than in normal rats until 1 week after delivery but is more common than in normal rats at 3 weeks after delivery [13]. Thus, dysregulated cell kinetics may be involved in the biliary abnormalities associated with PCK rats.

3.2. Fluid Secretion. Activated cAMP pathways can also lead to increased fluid secretion. Indeed, bile secretion is increased in the PCK rats compared with that in age-matched normal rats [58]. In normal cholangiocytes, the water channel aquaporin-1, the chloride channel cystic fibrosis transmembrane conductance regulator (CFTR), and the anion exchanger AE2 regulate ion-driven water transport. In PCK cholangiocytes, aquaporin-1, CFTR, and AE2 are overexpressed and show abnormal cellular localization, which could account for their altered fluid secretion [59].

3.3. Cell-Matrix Interactions. As is true in human Caroli's disease, the matrix proteins of the basement membranes of the intrahepatic bile ducts are degraded in PCK rats, and the biliary epithelium sits on the basement membrane and displays abnormal decreases in laminin and type IV collagen expression [27]. Since PCK cholangiocytes overexpress plasminogen and the tissue-type plasminogen activator, the generation of excessive amounts of plasmin and the subsequent plasmin-dependent lysis of extracellular matrix molecules may contribute to the progressive bile duct dilatation observed.

3.4. Primary Cilia and Ciliopathies. Cholangiocytes are ciliated cells, and cholangiocyte cilia extend from the apical plasma membrane into the bile duct lumen [60]. Cholangiocyte primary cilia are mechanosensory, osmosensory, and chemosensory organelles that can detect changes in bile flow and osmolarity and transduce them into intracellular signals. Changes in flow are communicated to other cellular response

elements via changes in intracellular calcium and cAMP concentrations. Increased flow causes a cilium-dependent rise in intracellular calcium followed by a decrease in the cAMP concentration via a calcium-inhibitable adenyl cyclase.

In PCK rats, a splicing mutation in *Pkhd1* results in structural and functional ciliary abnormalities [61]. Fibrocystin is normally localized to primary cilia, whereas defects in fibrocystin from primary cilia are observed in PCK cholangiocytes [62]. Defects in ciliary structure and their integrated sensory/transducing functions appear to result in decreased intracellular calcium and increased cAMP concentrations, causing cholangiocyte hyperproliferation, abnormal cell-matrix interactions, and altered fluid secretion. These modifications can lead to abnormalities in biliary tree differentiation, ultimately resulting in bile duct dilatation.

Other calcium channels such as Trpv4 are present in cholangiocyte cilia, and the activation of Trpv4 leads to increased intracellular calcium levels and reduces the hyperproliferative phenotype of PCK cholangiocytes [63].

In the liver, mutations in genes encoding ciliary-associated proteins cause a broad spectrum of genetically heterogeneous disorders, which are referred to as ciliopathies [64]. Since cholangiocytes are the only epithelial cells in the liver that possesses primary cilia, conditions affecting the liver are more appropriately called cholangiociliopathies [65].

3.5. Cholangitis. As PCK rats age, chronic suppurative cholangitis becomes a frequent histologic finding [66]. Although the clinical significance of cholangitis due to biliary infection is well recognized in Caroli's disease, the impact of biliary infection on its pathogenesis and progression is poorly understood.

Lipopolysaccharides (LPS) induce VEGF expression in PCK cholangiocytes via toll-like receptor 4 expressed on the cells, which is accompanied by the activation of NF-κB (Ren XS et al., unpublished data). Both LPS and VEGF increase the proliferation of PCK cholangiocytes, suggesting that LPS-induced overexpression of VEGF in the biliary epithelium leads to hypervascularity around the bile ducts, and concurrently, LPS and VEGF act as cell proliferative factors for cholangiocytes. Thus, biliary infection may exacerbate biliary cystogenesis through the induction of VEGF in the biliary epithelia of PCK rats.

Cholangitis is frequently associated with goblet cell metaplasia of the biliary epithelium in PCK rats. LPS induces upregulated CDX2 expression followed by aberrant mucus core protein-2 expression via the activation of NF-κB in PCK cholangiocytes, which accounts for the development of intestinal metaplasia in the setting of biliary infection [66]. Although recurrent cholangitis probably leads to the development of cholangiocarcinoma in some patients with Caroli's disease, we have not encountered the occurrence of cholangiocarcinoma in PCK rats.

3.6. Hepatic Fibrosis. A mechanism similar to the epithelial-mesenchymal transition (EMT) has been implicated in the hepatic fibrosis observed in PCK rats [67]. In PCK rat liver sections, the intrahepatic bile ducts display two different phenotypes, bile ducts lined by cuboidal-shaped (C-type), and flat-shaped (F-type) cholangiocytes. The flat-shaped cholangiocytes (F-type) show reduced immunohistochemical expression of the biliary epithelial marker cytokeratin19 and positive immunoreactivity for the mesenchymal markers vimentin and fibronectin. Treating PCK cholangiocytes with transforming growth factor-β1 (TGF-β1), a potent inducer of EMT induces the expression of vimentin and fibronectin in vitro, indicating that PCK cholangiocytes acquire mesenchymal features in response to TGF-β1 and participate in progressive hepatic fibrosis by producing extracellular matrix molecules. EMT has been also implicated in the pathogenesis of interstitial fibrosis in the kidneys of PCK rats [68].

In elderly PCK rats, suppurative cholangitis is a frequent histological finding in C-type cholangiocytes, while F-type cholangiocytes are not associated with suppurative cholangitis accompanied by polymorphonuclear leukocyte accumulation in their lumen [67]. In addition, F-type cholangiocytes occasionally show a fibrous scar-like appearance. Recent studies have shown that the majority of dilated intrahepatic bile ducts in PCK rats are initially connected to the biliary tree but over time become separated from it, resulting in true cyst formation [61]. It is speculated that F-type cholangiocytes are derived from the bile ducts that have been disconnected from the biliary tree, which may account for the observation of true biliary cysts in PCK rats.

The renin-angiotensin system is upregulated in the livers of PCK rats [69]. Angiotensin-converting enzyme (ACE) and angiotensin II, as well as their downstream target, the profibrotic mediator TGF-β, are overexpressed in the PCK liver, suggesting that the renin-angiotensin system activation is another important mediator of hepatic fibrosis.

3.7. Therapeutic Approaches. Understanding of the molecular mechanisms of cyst formation and growth has led to the discovery of novel potential therapeutic approaches for fibropolycystic diseases. However, in PCK rats, relatively few therapeutic reagents are effective for both liver and kidney cystogenesis.

Octreotide, a somatostatin analogue known to inhibit cAMP, decreases hepatic cyst volume, the hepatic fibrosis score, and mitotic indices in the PCK liver, and similar effects are observed in the kidneys [55]. Pioglitazone, a peroxisome proliferator activator receptor gamma agonist, inhibits bile duct dilatation and hepatic fibrosis as well as renal cyst growth, which is associated with decreased CFTR expression and reduced cell proliferation [53, 70].

As another example of therapies that are effective for both liver and kidney lesions in PCK rats, the inhibition of Src activity with SKI-606 ameliorates biliary ductal abnormalities and renal cyst formation [71]. The effects of Src inhibition suggest that the Erb2 and B-Raf/MEK/ERK pathways are involved in Src mediated signaling in ARPKD and that this occurs without any reduction in cAMP levels.

The inhibition of renal cAMP production by treatment with a vasopressin V2 receptor antagonist or by increasing water intake to reduce plasma vasopressin decreases cell proliferation and ameliorates kidney cystogenesis with an associated reduction in B-Raf/MEK/ERK activity, leading to

improved renal function in PCK rats [72–74]. However, consistent with the absence of the vasopressin V2 receptor in the liver, it does not have a significant effect on fibropolycystic liver disease. Similarly, Trpv4 activation induces a significant decrease in renal cystic area but causes a nonsignificant decrease in liver cyst formation [63].

ACE inhibition by chronic treatment with lisinopril decreases proliferative and apoptotic pathways in the kidneys of PCK rats, resulting in improved kidney function [75]. Chronic blockade of 20-hydroxyeicosatetraenoic acid (HETE) with a specific inhibitor of the CYP4A and CYP4F enzyme family prevents the formation of 20-HETE, resulting in a significant decrease in renal cyst formation in PCK rats [76]. The activation of calcium-sensing receptors with R-568 reduces the interstitial fibrosis, but not the cystogenesis, of the PCK kidney [77]. However, it is unclear whether these treatments, that is, the inhibition of ACE and 20-HETE synthesis, and the activation of calcium-sensing receptors, are effective treatments for biliary dysgenesis in PCK rats.

Gefitinib, an EGFR tyrosine kinase inhibitor, significantly improves biliary cystogenesis and hepatic fibrosis in PCK rats but has no beneficial effects on renal cyst pathogenesis [78]. In addition, EGFR tyrosine kinase inhibition with EKI-785 and EKB-569 has no effect on biliary dysgenesis in PCK rats, and the kidney lesions are unaffected or rather worsened by the treatment [54]. The inhibition of mTOR with sirolimus does not attenuate the progression of liver or kidney disease in PCK rats, which may be due to intrinsic or acquired sirolimus resistance [79].

4. Conclusions

The development of PCK rats has allowed us to explore the molecular pathogenesis of the disease and potential therapeutic strategies for Caroli's disease and ARPKD. The relevance of findings obtained from studies using PCK rats and/or the cholangiocyte cell line derived from them to the pathogenesis of human diseases is currently being analyzed, and several key signaling pathways have been elucidated. It seems likely that future treatments for Caroli's disease will involve combination therapies affecting several cystogenesis pathways.

Abbreviations

ACE: Angiotensin-converting enzyme
ADPKD: Autosomal dominant polycystic kidney disease
ARPKD: Autosomal recessive polycystic kidney disease
BilIN: Biliary intraepithelial neoplasia
cAMP: 3′,5′-Cyclic adenosine monophosphate
CHF: Congenital hepatic fibrosis
CFTR: Cystic fibrosis transmembrane conductance regulator
CTGF: Connective tissue growth factor
EGF: Epidermal growth factor
EGFR: EGF receptor
EMT: Epithelial-mesenchymal transition
HETE: Hydroxyeicosatetraenoic acid
HSPG: Heparin sulfate proteoglycan
LPS: Lipopolysaccharide
mTOR: Mammalian target of rapamycin
PKA: Protein kinase A
PCK: Polycystic kidney
TGF-β: Transforming growth factor-β
VEGF: Vascular endothelial growth factor
VEGFR: VEGF receptor.

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Review Article

Molecular Pathogenesis of Cholangiocarcinoma

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Epidemiological data from the last years show an increasing trend of incidence and mortality of cholangiocarcinoma (CC) worldwide. Many pathophysiologic aspects of this neoplasia are still unknown and need to be fully discovered. However, several progresses were recently made in order to establish the molecular mechanisms involved in the transformation and growth of malignant cholangiocytes. The principal concept that at least seems to be established is that cholangiocarcinogenesis is a multistep cellular process evolving from a normal condition of the epithelial biliary cells through a chronic inflammation status ending with malignant transformation. The bad prognosis related to CC justifies why a better identification of the molecular mechanisms involved in the growth and progression of this cancer is required for the development of effective preventive measures and valid treatment regimens. This Paper describes the scientific progresses made in the last years in defining the molecular pathways implicated in the generation of this devastating disease.

1. Introduction

Cholangiocarcinoma is originated by a malignant transformation of cholangiocytes, the epithelial cells lining the biliary ducts [1]. Since biliary cancers may arise from every portion of the biliary tree, they are anatomically classified as intrahepatic or extrahepatic [1]. Epidemiological data show that intrahepatic cholangiocarcinoma is increasing in incidence, prevalence, and mortality worldwide [2, 3]. In particular, in the past three decades, a progressive increase of mortality for intrahepatic CC has been reported, while mortality for extrahepatic CC is stable or slightly decreasing [3].

The poor prognosis of this cancer is also explained from the fact that no useful tools for early diagnosis for this neoplasia are still available. Because of, the lack of specific symptoms coupled with high invasiveness and frequent involvement of critical anatomical organs [1, 4], the patient typically presents with an unresectable disease at the diagnostic approach. This aspect justifies why a surgical curative treatment is often impossible. Besides surgery, the other types of treatments for CC are chemotherapy and radiotherapy [1]. However, CC cells do not respond or weakly respond to these approaches, which thus have often a palliative role. Recent therapeutic options include brachytherapy and photodynamic therapy (PDT), with promising results [1, 4].

CC develops from the accumulation of genetic and epigenetic alterations in regulatory genes in cholangiocytes that lead to the activation of oncogenes and the dysregulation of tumor suppressor genes (TSGs) [5–8]. The principal characteristics of malignant cholangiocytes can be summarized in (i) uncontrolled growth, (ii) high capacity of tissue invasiveness, and (iii) capacity to metastasize [5, 8]. In this paper, we have described in detail the principal molecular mechanisms involved in every passage of the multistep process of cholangiocarcinogenesis.

2. Molecular Mechanisms of Cholangiocarcinogenesis

The molecular mechanisms involved in the development of CC are incompletely defined in detail, although in the last years, several studies have contributed to codify them, at least in part [5]. With the term "cholangiocarcinogenesis" are named all the complex mechanisms that lead to the

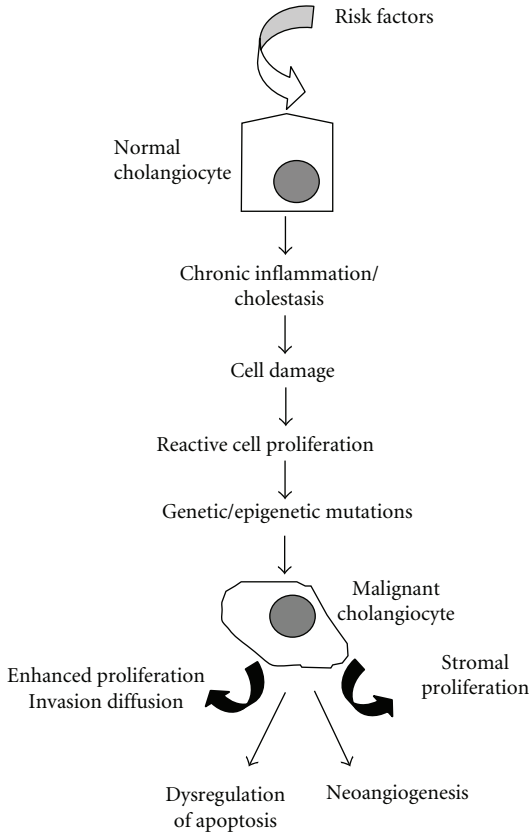

Figure 1: Proposed mechanisms leading to transformation of normal biliary cells into malignant cholangiocytes. Cholangiocarcinoma cells express altered molecular mechanisms, which enhance cell proliferation, decrease apoptosis, and increase the capacity of tissue invasion, stromal proliferation, and angiogenesis.

malignant transformation of cholangiocytes. These mechanisms can be simply described as a multistep process (Figure 1). CC usually develops in an environment of chronic inflammation of bile ducts with consequent cholangiocyte damage associated with the obstruction of bile flow [1, 5]. This tumor, especially when originating in the perihilar bile ducts, can develop in normal liver [5, 9].

Primary sclerosing cholangitis (PSC) is the most recognized risk factor for CC development [9]. Other risk factors for this cancer are specific parasites of endemic regions of Asia such as *Opisthorchis viverrini*, *Clonorchis sinensis*, and *Schistosoma Japonica* and bacteria such as *Salmonella typhi* [9, 10]. Hepatolithiasis, Caroli's disease, congenital choledochal cysts, bilioenteric surgical drainage and anomalous pancreaticobiliary junction, age greater than 65 years, bile duct adenoma, papillomatosis, liver cirrhosis, smoking, diabetes mellitus, thorotrast, dioxin and vinyl chloride intoxication, and HIV and HCV infections [9, 10] are also risk conditions for CC. However, the role of most of these conditions as predisposing factors for biliary cancer is still debated. Independently from the presence of one of the mentioned factors, the malignant transformation of cholangiocyte arises in a background of chronic inflammation. The high amount of cytokines and factors secreted during chronic inflammatory processes triggers and maintains the process of cholangiocarcinogenesis [5, 8] (Figure 1). Molecules participating in chronic inflammation promote neoplastic process by damaging protooncogenes, DNA mismatch repair genes/proteins, and tumor suppressor genes involved in cell growth, apoptosis, invasiveness, and neoangiogenesis. The final result is the uncontrolled cell proliferation and invasion (Figure 1).

Such as in many other cancer types, K-ras, p53, p14ARF, p16INK4a, and β-catenin genes can be mutated during the development of CC [6].

Two other genes recently described as implicated in the development of CC are *NKG2D* and *AID*. The natural killer group 2, member D cell receptor, also known as NKG2D, is expressed by NK cells and T-lymphocytes and plays a critical role in tumor surveillance by cell-mediated cytotoxicity [11]. Melum et al. recently showed that two single nucleotide polymorphisms (SNPs) of the NKG2D gene were associated with an increased risk of CC in PSC-affected patients [12]. Contrarily, homozygous condition for the no-risk alleles is related with a low risk of CCs [12].

Activation-induced cytidine deaminase (AID) is a member of the DNA/RNA-editing enzyme family. Recently, it was shown that AID production was significantly increased in human biopsies of PSC and CC-affected patients compared with normal liver parenchyma [13]. Aberrant expression of AID in biliary cells resulted in the generation of somatic mutations in tumor-related genes such as *p53*, *c-myc* and the promoter region of the *INK4A/p16* sequences [13]. The aberrant expression of AID gene induced by proinflammatory cytokines strengthens the link between chronic inflammation of the biliary tract and CC development [13].

3. Molecular Pathways Implicated in Cholangiocarcinogenesis

3.1. IL-6. IL-6 has an important role in the pathogenesis and growth of CC [14]. The mitogenic effect of IL-6 is suggested from the fact that the concentration of this molecule is increased during chronic inflammation of the biliary tract, a condition predisposing CC development [15]. IL-6 acts by both an autocrine and a paracrine manner stimulating several intracellular pathways involved in survival and growth of malignant cholangiocytes [16]. Among them, p44/p42 and p38 MAPKs have been largely studied [17]. Tadlok et al. showed that activation of p38 MAPK by IL-6 decreases expression of p21(WAF1/CIP1), a cell cycle controller protein, and mediates growth independent of anchorage signals, whereas activation of p44/p42 MAPK mediates an anchorage signal-dependent growth pathway [17]. IL-6 also influences the apoptotic process of malignant cholangiocytes. Several studies showed that IL-6 upregulates myeloid cell leukemia-1 or Mcl-1, a potent key antiapoptotic Bcl-2 family member protein. It has been recently shown that this effect of IL-6 is mediated by increased activation of STAT-3 (that is constitutively activated in malignant cholangiocytes), which regulates Mcl-1 transcription thus

increasing resistance to apoptosis [18]. In addition, Mcl-1 increases cancer cell resistance to tumor-necrosis-factor-related apoptosis-inducing ligand (TRAIL) [19] and, therefore, this molecule appears to have a fundamental role in CC development [20]. Conversely, inhibition of IL-6-induced iperexpression of Mcl-1 restores sensitivity to TRAIL [21].

3.2. JAK/STAT. The Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway is one of the key signaling mechanisms in CC cells, mediating their resistance to apoptosis [20]. In a recent study, Blechacz et al. showed that the multikinase inhibitor sorafenib may also block JAK/STAT signaling with consequent inhibition of CC growth [22]. The authors demonstrated that sorafenib induces STAT3 dephosphorylation by stimulating phosphatase SHP2 activity, sensitizes CC cells to TRAIL-mediated apoptosis, and is therapeutic in a syngeneic rat, orthotopic CC model that mimics human disease [22].

3.3. TGFβ. TGFβ is a cytokine implicated in several cell functions such as growth, differentiation, migration, apoptosis, adhesion, survival, and immunity. Several cell types of the liver secrete this cytokine [21]. Cholangiocytes, for example, express TGFβ in course of cholestasis but not in normal conditions [23]. It has been shown that TGFβ inhibits proliferation of human CC cells through modulation of the p21 cyclin dependent kinase inhibitor [24]. However, the mutations of TGFβ receptor and the alterations of intracellular signaling mediators (e.g., Smad4) together with the intracellular overexpression of cyclin D1 [25, 26] in CC cells induce a resistance to the inhibitory effect of TGFβ cells [27]. In addition, the lack of TGFβ signaling also stimulates the deposition of fibrotic tissue, abundantly expressed by biliary malignancies [27].

3.4. Smad4. DCP4/Smad4 is a tumor suppressor gene and also a downstream of TGFβ signaling [28]. Recently, it has been shown that Smad4 interacts with PTEN, another tumor suppressor gene, in order to regulate cellular cycle and escape the process of cholangiocarcinogenesis [29]. To strength this data, the blockage of these tumor suppressor genes favors the development of CC [29, 30]. It was also demonstrated that pTNM stage of intrahepatic CC appears to be correlated with the degree of Smad4 loss [30].

3.5. ErbB-2. The protooncogene-encoded receptor tyrosine kinase ErbB-2 is overexpressed in malignant cholangiocytes and plays an important role in the development and progression of biliary malignancies [31–33]. ErbB-2 acts in two different manners: first of all stimulates the proliferation of CC cells, then ErbB-2 stimulates the production of COX-2, which interacts with a subunit of the IL-6 receptor forming a complex [34]. This effect suggests a close link between IL-6 and ErbB-2 signaling [1, 34]. The mitogenic effect of ErbB-2 is also suggested from the fact that when normal cholangiocytes are transfected with the neu (the rat homologue of ErbB-2) oncogene, they undergo a malignant transformation that resembles the molecular aspects of human CC [35].

3.6. COX-2. The enzyme cyclooxygenase (COX) is responsible of the generation of prostaglandins, expressed in the course of the process of inflammation. COX exists in two specific isoforms: COX-1, normally expressed in many cell types and regulating the homeostatic functions of prostaglandins, and COX-2, the inducible isoform, which can be stimulated by a variety of molecules, such as cytokines and lipopolysaccharides [36]. The expression of COX-2 is major during inflammation, condition predisposing the development of CC [36]. Moreover, in rat CC cells, overexpression of COX-2 stimulates cell growth [37] while antisense depletion of COX-2 inhibits cell proliferation [37]. Recent studies showed that COX-2 is activated in human CC cells *in vitro* [38] by oxysterols, derivatives from cholesterol, which are present in bile of patients in course of cholestasis and inflammatory processes of the biliary tract [39]. The mitogenic effect of COX-2 towards CC cells justifies why the inhibition of COX-2-mediated pathway could represent a strategy to prevent CC development and growth. At this regard, recent data demonstrated that selective COX-2 inhibitors (e.g., celecoxib) reduce proliferation of CC cells by stimulating apoptosis [31, 37, 40, 41]. These studies also showed that the inhibitory effect towards CC cell growth by celecoxib was accompanied by an inhibition of PDK1 and PTEN, with a consequent reduction of Akt phosphorylation [42]. Moreover, celecoxib inhibits CC cells proliferation through activation of cyclin-dependent kinase inhibitors $p21^{waf1/cip1}$ and $p27^{kip1}$, with consequent cell cycle arrest at G1/S phase [43]. Sirica et al. recently demonstrated a link between the increase of COX-2 expression and CC development since the amount of this cytokine is high in cholangiocytes obtained from livers affected by PSC [31], a well known risk factor for CC [9]. These promising *in vitro* data, however, do not correspond to a good outcome in clinical practice since not all COX-2 inhibitors were shown to have a benefit in reducing CC cell growth [37] and also because the use of high doses of COX-2 inhibitors could cause serious side effects [37].

3.7. NO. Inducible nitric oxide (NO) synthase (iNOS) is an enzyme highly expressed during inflammatory and malignant processes of the biliary tract [44]. The activation of iNOS in course of inflammation determines an increase of intracellular NO, which triggers the process of cholangiocarcinogenesis by different ways: (1) inhibits DNA repair system thus allowing an accumulation of DNA damage and mutations [8, 45]; (2) stimulates COX-2 expression [44].

The carcinogenic effect of NO is at least in part due to Notch-1 signaling [46]. The role played by NO and Notch-1 in the development of biliary malignancies, as well as pancreatic cancer, is suggested by the evidence that Notch-1 is hyperexpressed both in cholangiocytes of PSC-affects patients and in CC cells where it colocalizes with iNOS [46]. A recent study by Ishimura et al. showed that iNOS is able to stimulate COX-2 expression through NO generation.

In particular, iNOS enhances COX-2 expression through activation of p38 MAPK and JNK1/2 [44]. The link between these two proteins explains their important role in the development and growth of CC [44].

3.8. Apoptosis. The process of apoptosis is fundamental to maintain the homeostasis of the biliary epithelium because it permits to remove the cells deeply damaged and with no reversible genomic mutations [47, 48]. After this premise, it is clear that a defect of apoptotic process favorites the survival of mutated cholangiocytes, which could go through a series of other mutations ending with the malignant transformation of the cell [47, 48].

Bcl-2 is a superfamily of antiapoptotic proteins. Bcl-2, which represents the prototype of this family [49] and is expressed in CC cells in a high amount. In these malignant cells, which possess a higher apoptotic threshold with respect to normal cholangiocytes [49], Bcl-2 exerts its antiapoptotic activity by reducing caspase 3 activation by preventing cytochrome-c release from the mitochondria [49].

Several other factors are implicated in the dysregulation of cholangiocyte apoptosis [8]. Among them, NO inhibits apoptosis of biliary epithelial cells. At this regard, the transfection of CC cells with nitric-oxide-synthase- (NOS-) cDNA induces a resistance to etoposide-induced apoptosis, an event that happens by caspase 9 nitrosylation [50].

Furthermore, Notch-1 and COX-2 reduce TRAIL-mediated apoptosis [21] and high levels of COX-2 inhibit Fas-induced apoptosis in CC cells [21]. To strengthened these data, a recent study demonstrated that celecoxib, a selective COX-2 inhibitor, induces cell death by apoptosis by the inhibition of the PI3-kinase signaling [37, 41].

The cytokine tumor-necrosis-factor-related apoptosis-inducing ligand (TRAIL) selectively stimulates apoptosis only in malignant cells without having any toxicity in normal tissues [51, 52]. CC cells resist TRAIL-induced apoptosis because they express high levels of myeloid cell leukemia protein-1 (Mcl-1) [19]. Thus, when specific small-interfering mRNA or stable transfection with Mcl-1 small hairpin RNA block Mcl-1 expression, CC cells become sensitive to TRAIL-induced apoptosis [19, 53]. The expression of Mcl-1 is also stimulated by bile acids, abundant in the course of cholestasis. Among them, deoxycholic acid, for example, increases Mcl-1 expression by blocking protein degradation through activation of an EGFR/Raf-1 pathway [54]. Indeed, Raf-1 inhibitors block the increase of Mcl-1, rendering the cells much more sensitive to Fas-induced apoptosis [55]. All these data suggest that TRAIL could be a target for novel drugs for the management of biliary tumors [56].

3.9. VEGF. Biliary tumors proliferate surrounded by a rich vascular network, which provides an adequate support of oxygen and metabolites to malignant cholangiocytes in order to enhance tumor development and growth [1, 57]. The proliferation of blood vessels is favored by high levels of vascular endothelial growth factor (VEGF) [57, 58]. This protein is stimulated by TGF-β and β-catenin [59] and is expressed by the surrounding mesenchymal cells and, even if in a lesser extent, by the malignant cells themselves. This evidence suggests the existence of an autocrine/paracrine mechanism for the VEGF production by malignant cholangiocytes [60].

3.10. Estrogens. It is well known the mitogenic effect of estrogens for CC cells. At this regard, 17-β estradiol stimulates human CC cells growth [61] while tamoxifen, an estrogen antagonist, decreases the proliferation of these cells *in vitro* and *in vivo* [61] by stimulating apoptosis through the Fas/APO-1 (CD95) signaling pathway via a calmodulin-dependent mechanism [62]. Alvaro et al. demonstrated that human intrahepatic CC cells express receptors for both estrogens and insulin-like growth factor 1 (IGF-1), which cooperate in the modulation of enhancing cell growth and reducing apoptosis [63]. Furthermore, HuH-28 human intrahepatic CC cell line expresses VEGF-A, VEGF-C, and related receptors, which are enhanced by stimulation with estrogens [64], and the stimulatory effect of 17beta-estradiol is blocked by estrogen receptor or insulin-like growth factor-1 receptor antagonists [64]. These data demonstrate that estrogens stimulate the proliferation of human CC by inducing the expression and secretion of vascular endothelial growth factor [64]. The result of these studies could be applied to the management of biliary malignancies in clinical practice. In fact, measuring IGF-1 levels in bile could help distinguish extrahepatic CC from pancreatic cancer or benign biliary stenosis and blocking estrogen, IGF-1, and VEGF receptors could be crucial to arrest CC cell proliferation [65].

3.11. Neuropeptides and Hormones. In the last years, many studies described as a multitude of hormones and neuropeptides are able to interact and regulate CC cells growth and invasion. Among them, gastrin, endothelin, serotonin, secretin, histamine, the α-2-adrenoreceptor UK14, 304, NPY, GABA, leptin, and opioid receptor modulators regulate the proliferation and apoptosis of CC cells [66–75]. All these novel data could contribute to clarify the complex mechanisms governing the process of cholangiocarcinogenesis.

4. Conclusion

The increasing worldwide incidence of CC together with the lack of its effective therapeutic tools explains the growing general interest for the study of this cancer type. The bad prognosis of people affected by CC is given by the fact that this cancer is often diagnosed when already at an advanced stage. Unfortunately, at this point, only palliative approaches are possible. With these premises, in the recent years, many researchers have focused their studies on the investigation of the molecular mechanisms involved in the development and growth of CC. Several works demonstrated that the conditions of cholestasis and chronic inflammation induce a local release of a network of mitogenic factors that induce genomic damages thus triggering the malignant transformation of cholangiocytes. The complete codification of molecular pathways involved in the pathogenesis of CC is

mandatory to discover novel tools for an early diagnosis and an efficacious specific therapy.

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