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TOPIC HIGHLIGHT

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Involvement of cholangiocyte proliferation in biliary fibrosis

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Abstract

Cholangiocytes are the epithelial cells that line the biliary tree. In the adult liver, they are a mitotically dormant cell population, unless ductular reaction is triggered by injury. The ability of cholangiocytes to proliferate is important in many different human pathological liver conditions that target this cell type, which are termed cholangiopathies (i.e. primary biliary cirrhosis, primary sclerosing cholangitis and biliary atresia). In our article, we provide background information on the morphological and functional heterogeneity of cholangiocytes, summarize what is currently known about their proliferative processes, and briefly describe the diseases that target these cells. In addition, we address recent findings that suggest cholangiocyte involvement in epithelial-to-mesenchymal transformation and liver fibrosis, and propose directions for future studies.

Key words: Biliary epithelium; Cholangiopathies; Cholestasis; Integrins; Liver fibrosis; Proliferation

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INTRODUCTION

Cholangiocytes are epithelial cells that line the biliary system and make up 3%-5% of the liver cell population[1,2]. The biliary system is a tree-like, three-dimensional network of ducts that range in size from small (< 15 μm) to large bile (> 15 μm) ducts in animal models[3,4]. These ducts are lined by cholangiocytes that also vary in size, dependent upon the size of the bile duct[5]. For example, large bile ducts are lined by large cholangiocytes and small bile duct by small cholangiocytes[3,4,6]. The major functions of the biliary system are to deliver bile from the liver to the gallbladder to the duodenum and the modification of

bile of canalicular origin[5,7,8]. Cholangiocytes modify bile[9-13] through a series of re-absorptive and secretory processes involving water, ions, and solutes, which are heavily regulated by gastrointestinal hormones, such as secretin[11,13]. Secretin receptors (SR) are present only on cholangiocytes in the liver[14]. Large but not small cholangiocytes express SR and are responsive to secretin in the normal rodent liver[3]. Small cholangiocytes *de novo* express secretin receptor during pathological conditions where large functionally active cholangiocytes are damaged[12,15]. In large cholangiocytes, secretin increases cyclic adenosine 3', 5'-monophosphate (cAMP) levels[4,16-19] and induces the opening of the Cl- channel (cystic fibrosis transmembrane conductance regulator, CFTR), which leads to the activation of the Cl-/HCO_3- anion exchanger 2 (AE2) and the secretion of bicarbonate in bile[3]. In addition to their normal biliary function, cholangiocytes have also been found to detoxify xenobiotics[9-12] and they also provide one of the first lines of defense against microbes in the biliary system[7,20]. Our knowledge of the factors that control cholangiocyte function has greatly increased in recent years, due to an increased interest in liver diseases, such as biliary cancer, biliary fibrosis and cholestatic liver disease[7,9-11].

Cholangiocytes are the target of many diseases, referred to as cholangiopathies, with a high impact in terms of morbidity and mortality in both children and adults[7,10,21,22]. These diseases have a diversity of etiologies, such as genetic predisposition, e.g. Alagille's syndrome, Cystic Fibrosis, fibropolysystic diseases; immune-mediated diseases like PSC, PBC[3,7,23,24], liver allograft rejection, and graft-versus-host disease[3,7,10,23,24]; a variety of infections - bacterial, fungal, parasitic and viral cholangitis; AIDS cholangiopathy; biliary atresia; idiopathic causes such as sarcoidosis; and malignant ones, such as cholangiocarcinoma[3,7,23,24]. Cholangiopathies are the leading cause of liver transplantations in pediatric patients (50%) and the third leading cause in adults (20%)[3,23,25]. Cholangiopathies are characterized by cholestasis, the loss of cholangiocytes through necrosis or apoptosis, with cholangiocyte proliferation resulting in the formation of new side branches to ducts in an effort to regain function[13,26,27], and portal/periportal inflammation[26]. Obstructive cholestasis contributes to hepatic cirrhosis and portal hypertension[28]. Portal fibroblasts and hepatic stellate cells (HSCs) are recruited to the area, and followed by parenchyma invasion and biliary fibrosis[26]. This remodeling process involves crosstalk between mesenchymal cells and cholangiocytes, the latter being able to secrete chemokines [interleukin 6 (IL-6), tumor necrosis factor-alpha (TNF-α), interlukin 8 (IL-8), Monocyte chemotactic protein-1 (MCP1) and profibrogenic factors (platelet derived growth factor-BB (PDGF-BB), endothelin-1 (ET1), connective tissue growth factor (CTGF), and transforming growth factor-beta 2 (TGFβ2)][26,29].

CHOLANGIOCYTE PROLIFERATION

In animal models of cholestasis and biliary tract injury, cholangiocyte proliferation is coordinately regulated by a number of neuroendocrine factors, such as hormones, neuropeptides and neurotransmitters, which have been recently reviewed[1,3]. Proliferating cholangiocytes display neuroendocrine phenotypes, and as such, secrete and respond to a number of hormones, neuropeptides and neurotransmitters[9]. The capacity of cholangiocytes to proliferate is evident under specific experimental con ditions in animal models as well as in human pathological conditions[9,30,31]. Four types of "ductular reaction" have been described in animal models[9,30,31]. Cholangio cyte proliferation is described as "ductular reaction", a term coined by Popper to identify the expanded popula tion of epithelial cells at the interface of the biliary tree, which refers to proliferation of pre-existing ductules, progenitor cell activation, and the appearance of intermediate hepatocytes[5,9,30,31]. The ability of cholangiocytes to proliferate is important in many different human pathological conditions. Type I or "typical" cho langiocyte proliferation results in an increased number of intrahepatic bile ducts (hyperplasia), which re mains confined to portal spaces[31]. The proliferating cho langiocytes form a well-differentiated three-dimensional network of tubular structures with a well-defined lumen,which originates from pre-existing bile ducts located within portal areas[31]. In humans, this type is observed in acute obstructive cholestasis, extrahepatic biliary atresia[9,31,32], and in early phases of chronic cholestatic liver diseases (in association with "atypical" pro liferation)[9,32]. In the rat, "typical" cholangiocyte proliferation occurs after bile duct ligation (BDL), partial hepatectomy, chronic a-naphthylisothiocyanate (ANIT) fe eding, chronic L-proline treatment and prolonged oral administration of certain bile acids[31].

Type Ⅱ or "atypical" proliferation is characterized by irregular proliferation of intrahepatic bile ducts not only confined to portal areas, but also sprouting into periportal and parenchymal regions[9,31,32]. This implies that the newly formed bile ducts are functionally ineffective[31]. In humans, this type is observed in PSC, PBC, after massive hepatic necrosis, focal nodular hyperplasia, chronic cholestatic diseases, alcoholic liver diseases, and a long-standing hepatic biliary obstruction[9,31,32]. Type Ⅱ refers to ductular metaplasia of liver cell plates, predominantly observed in chronic cholestatic conditions like PBC[6,31]. It is thought that the "atypical" proliferation arises from both proliferation of pre-existing ductules and expansion of the hepatic progenitor cell compartment[31].

Type Ⅲ ductular proliferation, sometimes called "ductular hyperplasia" or "oval cell" in the past[32] consists in the massive proliferation of ductular hepatocytes (derived from oval cells) or progenitor cells in the liver, with submassive hepatic necrosis[9,31]. It involves activation and proliferation of hepatic progenitor cells,

appearing as periportal ductular structures in the case of submassive hepatocellular necrosis, and representing an alternative parenchymal regeneration when hepatocellular regeneratory capacity is insufficient, which is primarily the case in chronic liver disease[6,31].

Type IV ductular hyperplasia, now called "oval cell", occurs in early stages of carcinogenesis in rat liver and is caused by chemicals, like ethionine, 2-acetylaminofluorene, and furan[9,32,33]. This type of proliferation induces the formation of disorganized tubular structures with a poorly defined duct lumen, which randomly sprout into hepatic lobules, creating a distorted hepatic architecture[31]. "Oval cells" are cell types activated to proliferate in early stages of intoxication with carcinogens. The nature of these cells, that is, whether they are fibroblasts, endothelial cells, transformed hepatocytes, or biliary ductules, is a subject of debate[6].

One of the functional characteristics of proliferating cholangiocytes is that they acquire a neuroendocrine phenotype, especially in "atypical" ductular reaction[9,32,34,35]. Proliferating cholangiocytes, from "atypical" proliferation, show phenotypical features of neuroendocrine cells like chromogranin A, S-100 protein glycolipid A2-B4, and a neural cell adhesion molecule[9]. During cholestatic liver diseases, the cholangiocytes express neuroendocrine phenotypes and respond to a number of hormones and neuropeptides[34]. For example, these proliferating cholangiocytes have increased expression and secretion of serotonin, endogenous opioid peptides and neurothrophins (and their corresponding receptors)[25]. They also show an enhanced response to hormones and neuropeptides such as secretin, gastrin, somatostatin, acetylcholine, and adrenergic and dopaminergic agonists[9,35]. Studies suggest that the formation of a neuroendocrine compartment is crucially instrumental in the progression of liver disease[9]. Thus, understanding how we can manage the proliferation of cholangiocytes is important for the development of treatments for liver diseases.

CHOLESTATIC LIVER DISEASES AND BILIARY FIBROSIS

In response to acute liver injury, cholangiocytes proliferate in an effort to regain proper liver function. Human chronic liver diseases are characterized by repetitive liver injury due to chronic infection by viral agents (hepatitis B and C viruses), toxin/drug exposure (alcohol consumption), and autoimmune attack (PBC/PSC)[26]. Chronic liver diseases cause a continuous activation of the wound-healing response that results in an accumulation of extracellular matrix, eventually leading to liver cirrhosis and hepatic failure[26]. Thus, cirrhosis can be defined as an advanced stage of fibrosis involving the formation of abnormal cell clusters surrounded by excessive extra- cellular matrix, which also results in significant changes in vascular framework[26,36-38]. As originally described, liver fibrosis during acute and chronic cholestasis involves the stepwise process that includes "ductular reaction", accompanied by polymorphonuclear leukocytes and an increase in matrix deposition, leading to periportal fibrosis and eventually biliary cirrhosis[39].

APOPTOSIS IN CHOLESTATIC LIVER DISEASES

Apoptosis is thought to play a major role in cholestatic liver diseases such as PBC, PSC and biliary atresia[40,41]. In immune-mediated liver diseases, such as PBC, PSC and autoimmune hepatitis, recent studies have indicated that programmed cell death ligands and circulating apoptotic markers might serve as diagnostic markers for these diseases[40,42]. Apoptosis of cholangiocytes has been observed in a number of animal models of cholestasis and biliary injury[12,15,43-45]. A recent study has shown that anti- death receptor 5 (DR5) monoclonal antibody induced cholangitis that exhibited the typical histological appearance of PSC and PBC[46]. These findings led them to believe the death signal mediated by TNF-related apoptosis-inducing ligand (TRAIL) receptor 2/DR5 may be a key regulator of cholestatic liver injury[46].

SECRETION OF PROFIBROGENIC FACTORS BY CHOLANGIOCYTES

Sedlaczek *et al*[47] demonstrated that during the progression of biliary fibrosis, proliferating bile duct epithelial cells are the predominant source of the profibrogenic factor CTGF. Newly formed bile ducts also express the message for alpha 1 (IV) procollagen, indicating that proliferating cholangiocytes are a potential source of hepatic collagen during fibrosis[48]. TGFb2 expression has been shown to be a specific property of proliferating bile duct epithelial cells and it has been postulated that its expression was related to the formation of specialized periductular connective tissue during bile duct proliferation[49]. In addition, platelet-derived growth factor is expressed in proliferating cholangiocytes during experimental biliary fibrosis in rats[50]. Administration of pentoxifylline exerts an antifibrogenic effect by reducing the PDGF-induced ERK-dependent signaling and proliferation of extracellular matrix- producing cells[51]. Other studies have shown that during biliary injury and fibrosis, the hedgehog pathway activation induces cholangiocyte production of chemokines that recruit natural killer T cells to portal tracts[52]. Hedgehog ligands regulate tissue-remodeling responses during embryogenesis and adult tissue repair[52,53]. Cholangiocytes produce and respond to hedgehog ligands[38,52]. Hedgehog pathway activation promotes proliferation and enhances viability of these cells, which unrestrained, could cause progressive fi

brosis and hepatic architectural distortion[52]. The targeting of the profibrogenic program that is activated in proliferating cholangiocytes and the profibrogenic factors they secrete, might provide an unique opportunity for the development of treatments for biliary fibrosis.

EPITHELIAL-TO-MESENCHYMAL TRANSITION (EMT)

Cholangiocytes normally exist in an highly differentiated state that allows them to modify bile of canalicular origins by a coordinated series of hormone- regulated secretory and absorptive processes[54]. Cholangiocytes proliferate in response cholestasis induced by bile duct ligation and during other pathological conditions such as, partial hepatectomy and CCl_4-induced liver damage[54]. Evidence suggests that proliferating cholangiocytes have a role in the induction of fibrosis, either directly through epithelial-mesenchymal transition[55], or indirectly through the activation of hepatic stellate cells[38]. EMT is a complex process that involves cross talk among several signaling pathways that collaborate to affect global, but gradual, changes in cell structure and function[56]. In this dynamic process cells eventually lose their typical epithelial characteristics (proteins that mediate cell-cell, cell-matrix contacts and cytoskeletal organization)[57,58]. These changes cause epithelial cells to disassociate from their neighbors, gradually acquire a motile phenotype, and eventually migrate out of epithelial sheets and into adjacent mesenchyme[21,56]. There are three biological subtypes of EMT, which have been previously reviewed[57,59]. Type 1 is present during implantation, embryogenesis and organ development. Type 1 also generates mesodermal and endodermal mesenchyme that can then undergo mesenchymal-to-epithelial transition (MET) to generate a secondary epithelia that can undergo additional rounds of EMT and MET to form various organs[57,59]. Although these concepts remain to be proven, it is possible that the balance between EMT and MET controls the outcome of chronic liver injury. Type 2 is associated with inflammation[57,59]. When there is injury with inflammation, this type generates fibroblastic cells that eventually cause organ destruction[57,59]. Type 3 is the result of genetic and epigenetic changes in cancer cells with invasion and spread of tumor cells that eventually form metastatic tumors apart from the primary tumor[57].

EMT is involved in tissues that are being developed or remodeled[57]. The presence of EMT in embryonic development[55,57] and cancer invasion and metastasis[60] is well established, and there is some evidence for EMT in the liver[55]. As a highly regenerative organ, the liver has the ability to restore its mass even in the face of extensive functional cell loss. However in situations of prolonged injury, this method of repair can lead to fibrosis[61]. Recent data suggest the classification at cellular, molecular and tissue level, multiple mechanisms for fibrosis as follows: (1) chronic activation of the wound-healing reaction; (2) oxidative stress and related reactive intermediates; and (3) profibrogenic derivatives of EMT[26]. Liver fibrosis develops from a heterogeneous population of profibrogenic hepatic myofibroblasts (MFs) that may originate from activated hepatic stellate cells and portal fibroblasts, bone marrow derived cells, or possibly cholangiocytes and hepatocytes that have undergone EMT[26]. These myofibroblasts are characterized by increased proliferation, migration, and contractility, and a relative resistance to apoptosis[37]. TGF(s) play a major role in the induction of EMT in development, carcinogenesis, and fibrosis, with different isoforms mediating various effects depending the cell type and setting[62]. EMT in response to TGFβ-1 and in fibrosis is mediated predominantly *via* Smad-dependent (mainly Smad3) pathways[63]. TGFβ-1 has previously been shown to play a critical role in the progression of liver fibrosis[64]. In fact, a recent study demonstrated that blockage of TGFβ-1 in proliferating biliary epithelia retards biliary fibrosis in an animal model of liver fibrosis[65]. Interestingly, a recent study demonstrated that EMT contributes to portal tract fibrogenesis during human chronic liver disease, which is characterized by chronic inflammation[65]. In fact, inflammation plays a key role as the convergence point between EMT and the progression of fibrosis in many organ systems, and has been previously reviewed[66].

EMT has been implicated as a key mechanism in the pathogenesis of liver fibrosis. In study of human samples from several types of liver diseases, Diaz and colleagues present convincing histological data revealing that EMT occurs in human liver fibrosis, particularly in disease associated with prominent bile ductular pro liferation, such as biliary atresia and PBC[55]. They observed significant colocalization between cytokeratin (CK-19, a cholangiocyte- specific epithelial marker) and other markers of EMT (i.e. vimentin, Snail, and fibroblast-specific protein 1) in biliary atresia and PBC. Robertson *et al*[67] have also demonstrated that biliary EMT occurs during post-transplantation recurrence of PBC. The study found that in livers affected by early recurrent PBC, there were indications that biliary EMT was occurring which was associated by cholangiocyte expression of S100A4 (a key marker of early fibroblast lineage), vimentin and pSMAD 2/3 with the data indicating that this process was driven by TGF-β[67]. S100A4 expression appears to occur prior to the onset of the appearance of other features of recurrent PBC, which suggests that EMT may be an initiating event, and may potentially explain the loss of bile duct epithelia during the course of the disease[67]. Rygiel and colleagues have also clearly demonstrated that EMT occurs during portal tract fibrosis[24]. Their work shows that cholangiocytes forming the small and medium sized bile ducts and responding with ductular reaction undergo

EMT during chronic liver disease, which results in the formation of invasive fibroblasts[24]. Similar findings have been demonstrated in liver cells from rodents, and humans can undergo EMT[68]. This study found that both hepatic stellate cells and hepatic epithelial progenitor cells coexpress epithelial and mesenchymal markers indicating that EMT occurs in adult livers[68]. This recent evidence indicates that EMT probably plays a critical role in the process of portal fibrosis during chronic liver diseases.

INTEGRINS AND BILIARY FIBROSIS

Integrins are a family of heterodimeric transmembrane glycoproteins composed of α and β chain protein subunits that act as cell surface receptors[69-71]. Integrins are a large family of 24 heterodimers formed from eight β subunits and α subunits that have been identified. Integrins play a role in communicating messages between the cell and the environment *via* extracellular matrix interactions[69-71]. The binding to extracellular matrix to integrins results cytoplasmic signals in the integrin-expressing cell contributing to cell growth, differentiation, invasion, metastasis, and survival[65,72-74]. Integrins also play a key role in how cells sense to mechanical stimuli in the environment[65,72,73]. Integrins have been shown to interact with cell surface ligands, growth factors, pathogens, soluble proteases and transmembrane proteins[75,76]. The loss of integrin-mediated contacts, usually leads to apoptosis, a process called *anoikis*[74,77].

Two recent studies have demonstrated that targeting αvβ6 integrin expressed by proliferating biliary epithelia might provide a novel antifibrotic therapy[65,78]. Patsenker *et al* demonstrated that αvβ6 integrin is strongly upregulated in proliferating biliary epithelium in rodent (BDL, thioacetamide, Mdr2 (Abcb4)$^{-/-}$ mice) and human models (chronic hepatitis C[65,78]) and that it drives fibrogenesis *via* adhesion to fibronectin and stimulates auto/paracrine TGF-β1 activation[65]. Most importantly, they demonstrated in vivo that a single dose of a small molecule αvβ6 integrin inhibitor induced antifibrogenic and profibrinolytic genes, reduced activated cholangiocyte proliferation, and adhesion to fibronectin[65,78]. In addition, increased vascularization has a key role in the development of biliary fibrosis, as supported by fibrosis that has been limited in animal models where angiogenesis has been inhibited[14].

REVERSAL OF BILIARY FIBROSIS

Although we have made impressive progress in our understanding of the pathogenesis of liver fibrosis in the past two decades, translation of this knowledge into antifibrotic therapies has ground to a halt short of clinical trials[37]. The reduction of fibrosis within cirrhotic liver tissue would lead to a reduction of portal hypertension and consequent clinical complications, thus improving overall liver function, which would extend the complication-free patient survival time and reduce the need for liver transplants[26]. Studies have suggested the reversibility of liver fibrosis, but the mechanisms for such a reversal are poorly understood[79]. In BDL rats, Popov and colleagues introduced macrophages to damaged biliary epithelium *via* Roux-en-Y bilio-jejunal anastomosis. After engulfing apoptotic cholangiocytes, macrophages upregulate matrix metalloproteinases and become fibrolytic effector cells[79]. This suggests a link between apoptotic epithelial cells, macrophages, and the reversal of fibrosis.

FUTURE DIRECTIONS

In order to develop clinical treatments, we need to learn more about how cholangiocytes interact with other cell types, and the role that EMT contributes to biliary fibrosis. The studies presented in this review raise the important question of the relationship between cholangiocytes and myofibroblasts as to whether cholangiocytes may be an additional source of fibroblasts during chronic liver injury. Also, important will be to determine how cholangiocytes contribute to soluble factors and to the activation of myofibroblasts and to the deposition of extracellular matrix. Understanding the interactions and contributions of these cell types to the process of biliary fibrosis will be essential for determining whether different mechanisms of fibrosis occur in the various cholangiopathies, which, in turn, will aid in designing disease-specific therapies.

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