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*TOPIC HIGHLIGHT*

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# Heterogeneity of the intrahepatic biliary epithelium

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## Abstract

The objectives of this review are to outline the recent findings related to the morphological heterogeneity of the biliary epithelium and the heterogeneous pathophysiological responses of different sized bile ducts to liver gastrointestinal hormones and peptides and liver injury/toxins with changes in apoptotic, proliferative and secretory activities. The knowledge of biliary function is rapidly increasing because of the recognition that biliary epithelial cells (cholangiocytes) are the targets of human cholangiopathies, which are characterized by proliferation/damage of bile ducts within a small range of sizes. The unique anatomy, morphology, innervation and vascularization of the biliary epithelium are consistent with function of cholangiocytes within different regions of the biliary tree. The *in vivo* models [e.g., bile duct ligation (BDL), partial hepatectomy, feeding of bile acids, carbon tetrachloride ($CCl_4$) or α-naphthylisothiocyanate (ANIT)] and the *in vivo* experimental tools [e.g., freshly isolated small and large cholangiocytes or intrahepatic bile duct units (IBDU) and primary cultures of small and large murine cholangiocytes] have allowed us to demonstrate the morphological and functional heterogeneity of the intrahepatic biliary epithelium. These models demonstrated the differential secretory activities and the heterogeneous apoptotic and proliferative responses of different sized ducts. Similar to animal models of cholangiocyte proliferation/injury restricted to specific sized ducts, in human liver diseases bile duct damage predominates specific sized bile ducts. Future studies related to the functional heterogeneity of the intrahepatic biliary epithelium may disclose new pathophysiological treatments for patients with cholangiopathies.



**Key words:** cAMP; Gastrointestinal hormones; Growth factors; Mitosis; Nerves

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## ANATOMICAL AND MORPHOLOGICAL CHARACTERISTICS OF THE BILIARY EPITHELIUM

Two kinds of epithelial cells, hepatocytes and cholangiocytes, are present in the liver[1-3]. While hepatocytes initially secrete bile into the bile canaliculus[4], cholangiocytes modify bile of canalicular origin by a series of coordinated spontaneous and hormone/peptide regulated secretion/reabsorption of water and electrolytes before it reaches the small intestine[3,5-7]. For more information on the mechanisms of bile formation we refer to recent reviews[4,5]. The human biliary system is divided into extrahepatic bile ducts and intrahepatic bile

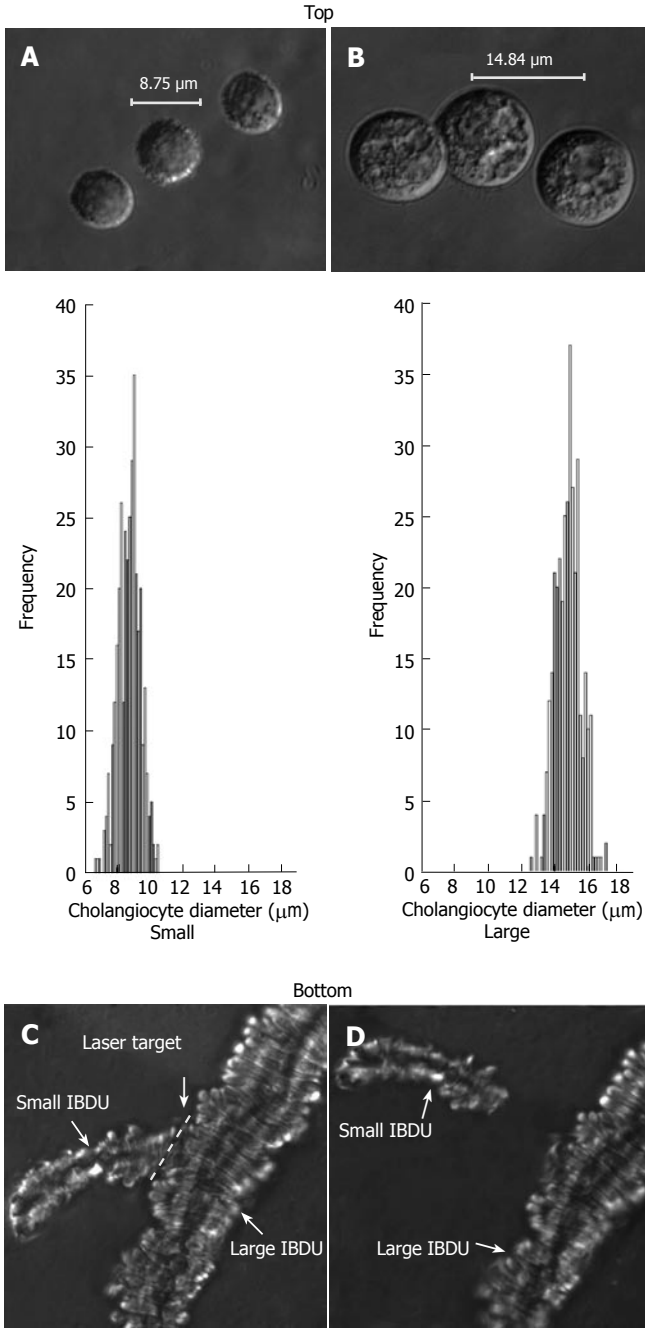


**Figure 1** [**Top**] Isolation of small (**A**), approximately 8 μm diameter] and large (**B**), approximately 14 μm diameter] cholangiocytes from small and large ducts, respectively, from normal rats. Small and large cholangiocytes were purified by counterflow elutriation followed by immunoaffinity purification. Original magn., × 625. Reproduced with permission from Ref[12]. [**Bottom**] Isolation of small (**C**) and large (**D**) IBDU from normal rat liver. Small (< 15 μm in diameter) and large (> 15 μm in diameter) IBDU were pruned off from large ducts by a nitrogen pulsed dye laser and subsequently separated (**D**) by picking up IBDU with a micromanipulator micropipet. Original magnification × 2000. Reproduced with permission from Ref 13.

ducts, the latter further sub-divided into large and small bile ducts[2,3,8]. The intrahepatic bile ducts represent that part of the biliary tree proximal to the confluence of the hepatic ducts[9] extending from the canals of Hering to the large extrahepatic ducts[2,3,8]. In human liver, a study by Ludwig classified the intrahepatic bile duct system upon duct diameter[8], small bile ductules (< 15 μm), interlobular ducts (15-100 μm), septal ducts (100-300 μm), area ducts

**Table 1 Terminology and relationship between human and rat intrahepatic bile ducts**

| Terminology for human bile ducts (diameter in μm) | Terminology for rat bile ducts (diameter in μm) |
|---|---|
| (Large bile ducts) | |
| Hepatic ducts (> 800) | |
| Segmental ducts (400-800) | |
| Area ducts (300-400) | |
| (Small bile ducts) | |
| Septal bile ducts (100-300) | |
| Interlobular bile ducts (15-100) | Large bile ducts (> 15) |
| Bile ductules (cholangioles) (< 15) | Small bile ducts (< 15) |

These data have been obtained from studies[8,12,13] aimed to define the morphological characteristics of the biliary epithelium of rats, and humans. Reproduced with permission from Ref 2.

(300-400 μm), segmental ducts (400-800 μm) and hepatic ducts (> 800 μm)[8] (Table 1). Small ductules are lined by 4-5 cholangiocytes, have a basement membrane, tight junctions between cells and microvilli projecting into the bile duct lumen[10,11]. Cholangiocytes are progressively larger and more columnar in shape in larger bile ducts (lined by 10-12 cholangiocytes)[10,11].

In rats, morphological studies in liver sections and small and large intrahepatic bile duct units (IBDU) have shown[2,12-14] that the intrahepatic biliary tree is divided into: (1) small ducts (< 15 μm in external diameter) lined by small cholangiocytes (approximately 8 μm in diameter)[12,13]; and (2) and large ducts (> 15 μm in diameter) lined by large cholangiocytes (approximately 15 μm in diameter)[12,13] (Figure 1, Table 1). Specifically, we have shown[12] that the rat intrahepatic biliary epithelium is formed by ducts of different sizes (5 to 200 μm in external diameter) and cholangiocytes of different cell areas (3 to 80 $\mu m^2$). Furthermore, a direct relationship exists between cholangiocyte area and external duct diameter, a finding that demonstrates that small ducts are lined by small cholangiocytes, whereas larger ducts are lined by larger cholangiocytes[12-14]. The fact that small and large ducts are lined by small and large cholangiocytes, respectively, is important since it allows for the assignment of the secretory, apoptotic and proliferative functions (achieved in isolated small and large cholangiocytes) within the different portions of the intrahepatic biliary epithelium. Recently, Masyuk *et al*[15] have reconstructed the intrahepatic biliary epithelium that resembles a tree, with the common and hepatic ducts corresponding to the trunk, the intrahepatic bile ducts corresponding to the large branches and the small ducts corresponding to the smallest tree limbs of a tree.

Studies by Phillips *et al*[16] have shown that no major ultrastructural differences exist among cholangiocytes lining small and large bile ducts. However, in support of the concept that the intrahepatic biliary epithelium is morphologically heterogeneous, electron microscopic studies by Benedetti *et al*[14] in rat liver sections and IBDU have demonstrated that large bile ducts are lined by 8-15 cholangiocytes and small ducts by 4-5 cholangiocytes. The studies also showed that small and large cholangiocytes

have a multilobulated nucleus, numerous vesicles at the subapical region, tight junctions, high density of microvilli and lysosomes and a few mitochondria[14]. Other studies have shown the presence of microvilli and cilia in the apical plasma membrane of cholangiocytes[17,18], cilia that play an important role in the regulation of cholangiocyte functions[19,20]. While large cholangiocytes are columnar in shape, small cholangiocytes have a cuboidal shape[14]. Abundant Golgi apparatus was observed between the apical pole and the nucleus[14]. Rough endoplasmic reticulum was inconspicuous in the smallest ducts and increased only slightly in the largest[14]. While large cholangiocytes display a small nucleus and conspicuous cytoplasm, small cholangiocytes possess a high nucleus/cytoplasm ratio[14]. Cholangiocytes have distinct apical and basolateral membranes[14,17,18].Coated pits have also been observed on the apical and basolateral membranes of cholangiocytes, a finding suggesting receptor-mediated endocytosis at both domains of cholangiocytes[21]. Functional tight junctions are located between adjacent cholangiocytes in proximity to the apical domain[17].

## INNERVATION

There is growing information regarding the role of the nervous system in the regulation of the pathophysiology of the biliary epithelium[3,22-27]. In the liver, adrenergic and cholinergic nerves are located around the hepatic artery, portal vein, and the biliary epithelium[28,29]. The intrahepatic arteries, veins, bile ducts and hepatocytes are also innervated[28,29]. In the autonomic nervous system, there are a number of regulatory peptides including neuropeptide tyrosine (NPY)[30,31], calcitonin gene related peptide (CGRP), somatostatin, vasoactive intestinal polypeptide (VIP) (mostly associated with parasympathetic fibers), enkephalin and bombesin[31-35]. NPY-positive nerves are present in extrahepatic bile ducts[36] and have been suggested to regulate bile flow by autocrine/paracrine mechanisms[37]. We have shown that NPY inhibits cholangiocarcinoma growth by interaction with a G-protein coupled receptor by $Ca^{2+}$-dependent modulation of Src/ERK1/2 phosphorylation[38]. Nerve fibers containing CGRP and substance P are present around blood vessels and bile duct radicles within portal tracts[39,40]. VIP-positive nerve fibers are located in the walls of hepatic arteries, portal veins and bile ducts[41].

## VASCULARIZATION

The intrahepatic and extrahepatic bile ducts are nourished by a complex network of minute vessels [*i.e.*, peribiliary vascular plexus (PBP)], which originate from branches of the hepatic artery and flow principally into the hepatic sinusoids, either directly (lobular branch) or by portal vein branches (prelobular branches)[42,43]. Since the blood flows in the opposite direction (from the large towards the small ducts) to bile flow, the PBP presents a countercurrent stream of biliary reabsorbed substances to hepatocytes[44,45]. We have previously shown that the function of the intrahepatic biliary tree is linked to its vascular supply sustained by the PBP[44]. Changes in intrahepatic bile duct mass are associated with changes of the PBP architecture[44]. Following BDL, the PBP undergoes hyperplasia, thus supporting the increased nutritional and functional demands from the proliferating bile ducts[44]. In support of this concept, studies[46] have shown that following chronic feeding of ANIT (which induces increases in both cholangiocyte proliferation/apoptosis)[47], the hepatic artery and portal vein undergo marked proliferation, presumably to support the increased nutritional and functional demands of the proliferated bile ducts[44,46]. However, the proliferation of the PBP occurs only after the hyperplasia of bile ducts[44]. Recent studies have shown that small and large rat bile ducts have a different vascular supply[44]. The PBP is primarily present around large bile ducts and less visible around small bile ducts[44], a finding that may partly explain why large but not small cholangiocytes proliferate following BDL in rats[48] and why small and large ducts differentially proliferate or are damaged in other experimental models of cholangiocyte proliferation/loss including chronic feeding of certain bile acids (e.g., taurocholate and taurolithocholate)[49], ANIT[47] or acute gavage administration of $CCl_4$[50,51] (Figure 2) or partial hepatectomy[52].

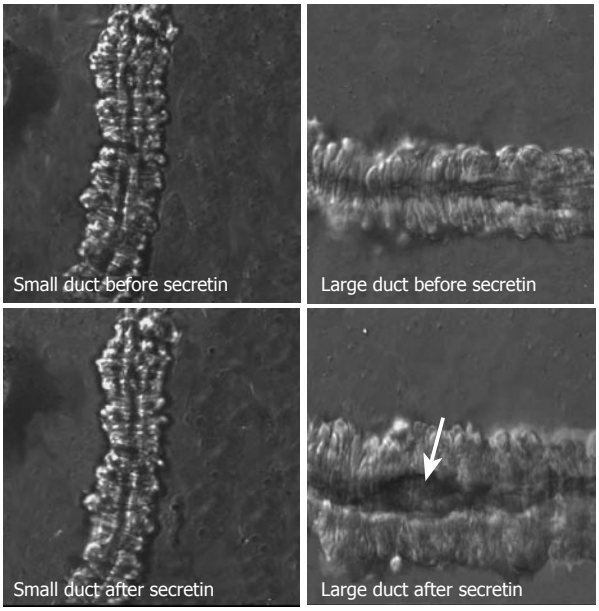


**Figure 2** Measurement of $H_3$ histone gene expression in small and large cholangiocytes from 1-wk BDL rats and 1-wk BDL rats treated with $CCl_4$ or mineral oil. $H_3$ histone gene expression in large cholangiocytes decreased on d 2 before returning to control values on d 7 after $CCl_4$ treatment. $H_3$ histone gene expression (which was absent in small cholangiocytes from BDL rats) was expressed by small cholangiocytes on d 1 and 2 before returning to control undetectable values on d 7 after $CCl_4$ treatment. Administration of mineral oil to 1-wk BDL rats did not alter $H_3$ histone gene expression in large cholangiocytes. The message for $H_3$ histone gene was absent in small cholangiocytes from oil-treated rats. Comparability of RNA used was assessed by hybridization for GAPDH (housekeeping gene). Autoradiograms were quantified by densitometry. Densitometric values are means of 2 experiments. Reproduced with permission from Ref 50.

## GENERAL BACKGROUND ON CHOLANGIOCYTE FUNCTIONS

The major function of cholangiocytes is to modify bile of canalicular origin[4] (by basal and hormone/peptide regulated secretion and reabsorption of

water and electrolytes) before reaching the small intestine[3,5,6]. Ductal secretion is coordinately modulated by gastrointestinal hormones (e.g., secretin, gastrin, insulin, somatostatin, bombesin and VIP)[3,5-7,12,53-58], gastrointestinal peptides (i.e., endothelin-1, ET-1)[59], enzymes (e.g., alkaline phosphatase)[60], bile acids (e.g., taurocholate, taurolithocholate, taurohyodeoxycholate, tauroursodeoxycholate, ursodeoxycholate and taurourso deoxycholate)[49,61-64] and cholinergic[23,26], adrenergic[65,66], serotoninergic[67] and dopaminergic[25] receptor agonists. Cholangiocytes, which have a low DNA turnover under normal physiological conditions[48,52,68], proliferate or are damaged in response to liver injury/toxins[2,3,6,26,47,48,50,52,68-72]. In rat liver, secretin is of particular importance since secretin receptors are only expressed by cholangiocytes[53], and its expression is upregulated under pathological conditions associated with enhanced cholangiocyte growth (e.g., after BDL)[48,71,72] and downregulated with cholangiocyte damage/loss (e.g., following acute $CCl_4$ administration)[50,51]. Thus, the secretin receptor is an important pathophysiological tool that allows us to evaluate the secretory, proliferative and apoptotic heterogeneity of the intrahepatic biliary epithelium in response to agonists and liver toxins/injury[2,3,12,13,47-51,54,61,71,73]. Interaction of secretin with its receptor is associated with increased intracellular cAMP levels[12,13,25,26,48,50-52,54,59,72]. Enhanced cAMP levels leads to phosphorylation of PKA[74], which induces opening of the cystic fibrosis transmembrane regulator (CFTR) channel[54] leading to the activation of the $Cl^-/HCO_3^-$ exchanger[12,23,52] resulting in biliary bicarbonate secretion[6,52].

## EXPERIMENTAL MODELS

A number of *in vivo* models (e.g., BDL, acute administration of $CCl_4$, partial hepatectomy, chronic feeding of ANIT or bile salts)[47-52] demonstrated that the intrahepatic biliary epithelium is functionally heterogeneous, with specific sized bile ducts (i.e., small and large) differentially responding to liver injury/toxins with changes in proliferative, apoptotic and secretory activities[2,3,12,47-52,54,62,71-73]. A number of *in vitro* experimental models (i.e., small and large cholangiocytes and IBDU and small and large immortalized normal murine cholangiocytes) (Figure 1)[12,13,47,48,50,51,75] have allowed us to suggest that the intrahepatic biliary epithelium is morphologically and functionally heterogeneous[2, 3,12,47-52,54,62,71-73]. The very first approach that was employed and that significantly contributed to lay down the basis of this field of research was the purification of small and large cholangiocytes from rat liver by counterflow elutriation[12,54,76]. Coupling such a technique to immunoaffinity separation[12,18,54], it was possible to isolate two distinct subpopulations of small (approximately 8 μm in diameter, obtained at the centripetal flow rate of 25 ml/min) and large (approximately 14 μm in diameter, collected at the flow rate of 55 mL/min) cholangiocytes (Figure 1)[12,54]. The two subpopulations of small and large cholangiocytes are further purified by immunoaffinity separation[18] using an antibody against an unidentified antigen (expressed by all intrahepatic cholangiocytes)[18] and characterized morphologically (by computerized image analysis) (Figure 1)[12,54], phenotypically (expression of γ-glutamyltransferase and cytokeratin-19 genes)[12,54] and functionally (by measurement of gene expression of secretin receptor, CFTR and $Cl^-/HCO_3^-$ exchanger and basal and secretin-stimulated cAMP levels, $Cl^-$ efflux and $Cl^-/HCO_3^-$ exchanger activity)[12,54].

In addition, we have developed a technique for isolating small (diameter smaller than 15 μm) and large (diameter greater than 15 μm) IBDU from small and large bile ducts, respectively (Figure 1)[13]. This important tool allowed us to directly evaluate the differential secretory responses of different portions of the biliary epithelium to selected gastrointestinal hormones/peptides[13,25,65,77]. As shown in Figure 1, the small duct was pruned off from the large duct by a brief exposure of a laser focused on the junction between large and small ducts (arrow) leading to separation of small from large ducts[13]. Small and large IBDU were characterized by morphometric analysis, gene expression for secretin receptor, CFTR and $Cl^-/HCO_3^-$ exchanger, secretin-induced cAMP levels, and secretion by change in luminal size in response to agonists including secretin, insulin, the α1-adrenergic receptor agonist, the α2-adrenergic receptor agonist, UK14,304 and the D2 dopaminergic receptor agonist, quinelorane[13,25,65,66,77].

Most recently. we have immortalized, from normal mice (BALB/c), small and large cholangiocytes by the introduction of the SV40 large T antigen gene, that allowed, after cloning, to establish small and large cholangiocyte cell lines[75]. The characteristics of the two subpopulations were evaluated by electron microscopy (EM) and measurement of trans-epithelial electrical resistance (TER), and secretin-stimulated cAMP levels[75]. EM, TER and differential cAMP response to secretin are consistent with the concept that small and large immortalized cholangiocytes originate from small and large ducts, respectively[75]. Microarray successfully displayed characteristic differential cDNA expression between small and large cholangiocytes[75]. Using the above described methods individually or in tandem, has allowed us to clearly demonstrate heterogeneity of the intrahepatic biliary epithelium and to dissect the differential physiological responses of these distinct subpopulations of cholangiocytes to endogenous stimuli.

## HETEROGENEOUS EXPRESSION OF PROTEINS

The heterogeneous expression of some enzymes/proteins and membrane transporters/receptors in small and large ducts from mice, rats and humans is summarized in Table 2. In human liver, large septal bile ducts mainly express the sialylated $Lewis^a$ blood group antigen[78]. In normal and diseased human livers, hepatic, segmental, area, and septal bile ducts, and peribiliary glands express pancreatic enzymes such as pancreatic lipase, pancreatic α-amylase, and trypsin[79,80]. By microarray of RNA from small and large immortalized murine cholangiocytes, we have demonstrated the heterogeneous expression of approximately 80 proteins between small and large cholangiocytes[75]. The pathophysiological relevance of the

**Table 2 Expression and function of proteins and surface transporters in small and large ducts from rats and human**

| Markers | Small ducts | Large ducts | Function | References |
|---|---|---|---|---|
| γ-glutamyl transpeptidase | Not expressed | Interlobular large rat bile ducts | Glutathione metabolism | [81] |
| Alkaline phosphatase | Not expressed | Interlobular large rat bile ducts | Inhibition of secretin choleresis | [81] |
| Leucine amino peptidase | Not expressed | Interlobular large rat bile ducts | Undefined | [81] |
| Cytochrome P4502E1 | Not expressed | Expressed by large rat and human ducts | Dehalogenation of $CCl_4$ | [50,81,104,105] |
| Lipase, α-amylase and trypsin | Human septal ducts | Large human ducts, and peribiliary glands | Biliary tree development | [79,80] |
| Bcl-2 | Human small ductules | Not expressed | Anti-apoptotic protein | [109,143] |
| Secretin receptor | Not expressed | Expressed by large rat ducts | Stimulation of bicarbonate secretion | [12,13,48,54] |
| CFTR | Human but not rodent small ducts | Expressed by large rat ducts | Regulation of $Cl^-$-secretion | [54] |
| $Cl^-/HCO_3^-$ exchanger | Not expressed | Expressed by large rat and human ducts | Regulation of ductal bicarbonate secretion | [12,13,82] |
| Somatostatin receptor | Not expressed | Expressed by large rat ducts | Inhibition of secretin choleresis | [48] |
| D2 dopamine receptors | Unknown | Expressed by large rat ducts | Inhibition of secretin choleresis | [25] |
| α-1 adrenergic receptors | Expressed by small rat ducts | Expressed by large rat ducts | Stimulation of secretin choleresis | [65] |
| Endothelin receptors | Expressed by small rat ducts | Expressed by large rat ducts | Inhibition of secretin choleresis | [59] |
| $Na^+$-dependent ABAT | Not expressed | Expressed by large rat ducts | Regulation of ductal secretion | [88] |

Heterogeneous expression of proteins and membrane transporters that may play a role in the modulation of the heterogeneous properties of the intrahepatic biliary tree of rats and human. Modified with permission from Ref 73.

differential expression of these messages remains to be addressed.

### *Secretory activity*

Recent studies have demonstrated that large bile ducts are the major anatomical sites of cAMP-dependent ductal secretion by activation of cAMP/PKA/CFTR/$Cl^-$/$HCO_3^-$ exchanger (Figure 3)[3,12,13,48,54]. Specifically, studies in isolated small and large cholangiocytes and IBDU from normal and BDL rats have shown that large (but not small) cholangiocytes express the messages for secretin receptor, CFTR and $Cl^-/HCO_3^-$ exchanger and respond to secretin with increases in cAMP levels, $Cl^-$ efflux and $Cl^-/HCO_3^-$ exchanger activity and IBDU lumen expansion (Figure 3)[12,13,48,54]. In rat liver, large ducts express alkaline phosphatase and γ-glutamyltranspeptidase[81]. The expression of alkaline phosphatase in large ducts is consistent with our previous studies[81] showing that alkaline phosphatase inhibits secretin-stimulated choleresis by blockage of CFTR activity, which is expressed only in large ducts (Figure 3)[54]. Furthermore, large cholangiocytes (which is the only cholangiocyte subpopulation expressing the somatostatin receptor, $SSTR_2$)[48] are the major anatomical sites of somatostatin inhibition of secretin-stimulated ductal secretion (Figure 3)[48,55]. The inhibitory effects of somatostatin on secretin-stimulated secretion in large cholangiocytes are associated with reduced cAMP levels, $Cl^-$ efflux and $Cl^-/HCO_3^-$ exchanger activity[48,55,54]. The counter-regulatory effect of somatostatin on the choleretic effect of secretin is important in modulating ductal secretion in pathological conditions associated with cholangiocyte proliferation/loss[3]. Parallel with the findings observed in rat bile ducts[3,12,13,48, 4], in human liver secretin-stimulated duct secretory activity is heterogeneous, since only large bile interlobular ducts express the $Cl^-/HCO_3^-$ exchanger[82].

We have demonstrated the presence of insulin and CCK-B/gastrin receptors in large cholangiocytes from normal and BDL rats and have shown that these two hormones inhibit secretin-stimulated ductal secretion of BDL rats by $IP_3/Ca^{2+}$/PKCα-dependent decrease of cAMP levels[7,72,77]. Similarly, we found that $ET_A$ and $ET_B$ receptors are expressed by large cholangiocytes and that ET-1 inhibits secretin-stimulated cAMP levels and ductal bile secretion of BDL rats by interaction with $ET_A$ but not $ET_B$ receptors[59]. Furthermore, recent data have shown that: (1) the D2 dopaminergic receptors are expressed by large BDL cholangiocytes; and (2) the D2 dopaminergic receptor agonist, quinelorane, inhibits secretin-stimulated ductal secretion by activation of the $Ca^{2+}$-dependent PKCγ[25]. The α2-adrenergic receptor agonist, UK14, 304, inhibits secretin-stimulated cAMP-dependent $Cl^-$ efflux and $Cl^-/HCO_3^-$ in large cholangiocytes and secretin-stimulated lumen expansion in large IBDU of BDL rats[66]. The α1-adrenergic receptor agonist, phenylephrine, stimulates cAMP levels and secretin-stimulated secretion of large BDL cholangiocytes by $IP_3/Ca^{2+}$-dependent activation of PKCα and PKCβ II[65]. We have recently demonstrated[26] that acetylcholine, by interacting with M3 receptor subtypes, potentiates secretin-stimulated cAMP levels and $Cl^-/HCO_3^-$ exchanger activity in IBDU and purified cholangiocytes by a $Ca^{2+}$-calcineurin mediated but PKC independent modulation of adenylyl cyclase.

Following hepatocyte secretion[83], bile acids are reabsorbed by the biliary epithelium[84], then they return via the PBP to the hepatocytes for secretion into bile (cholehepatic shunting)[85]. As a mechanism for bile acids entry into cholangiocytes, the apical $Na^+$-dependent bile transporter, ASBT (structurally identical to the ileal bile acid transporter) is expressed on the apical membranes of large cholangiocytes[86]. Consistent with functional activity for ASBT in cholangiocytes, studies have shown $Na^+$-dependent and saturable uptake of taurocholate in normal

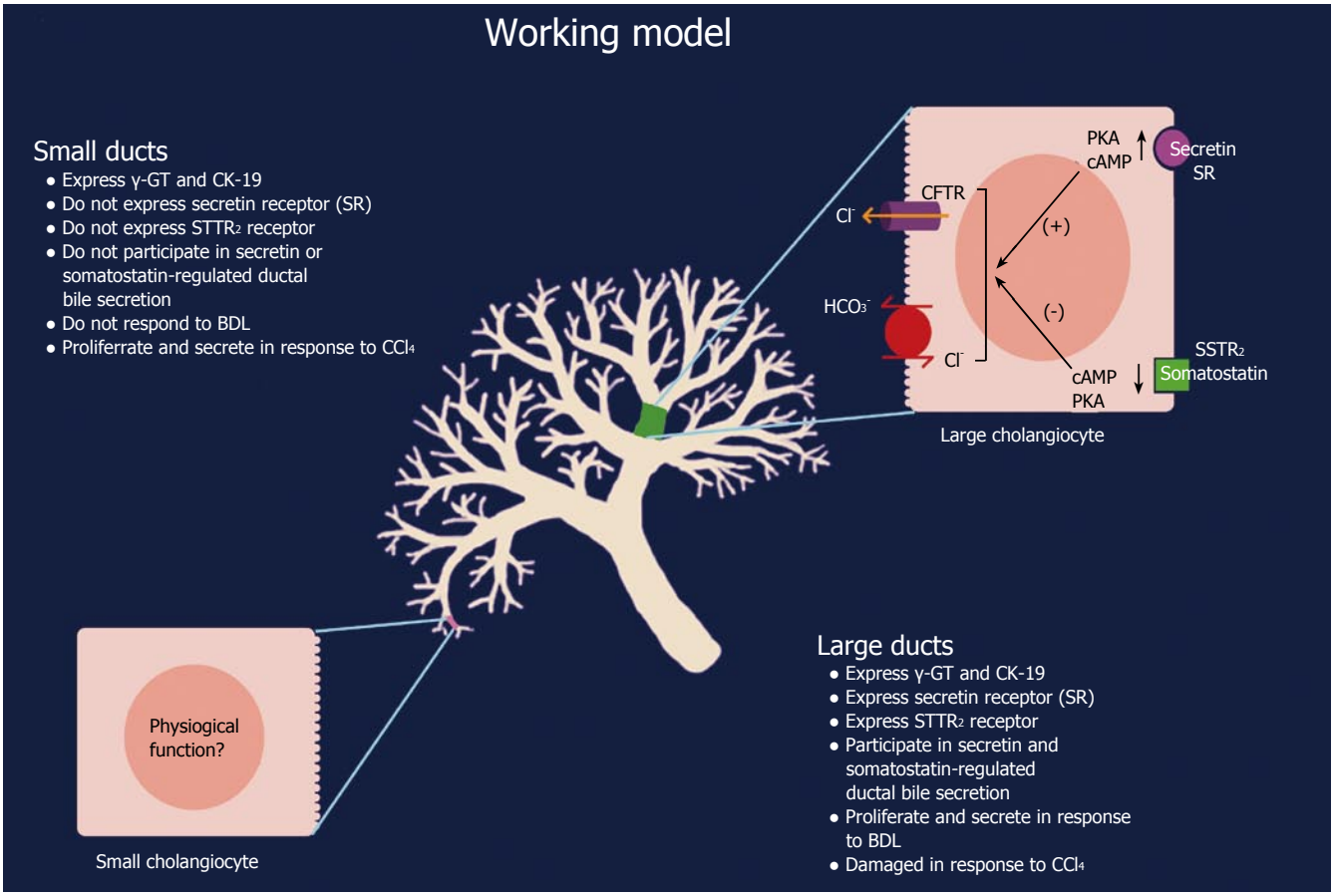


**Figure 3** Working model for the heterogeneity of the intrahepatic biliary epithelium. The model proposes that: (1) bile ducts are morphologically heterogeneous with small ducts lined by small cholangiocytes and large ducts lined by large cholangiocytes; (2) small and large ducts similarly express both γ-GT and cytokeratin-19; and (3) large (but not small) ducts express the secretin and somatostatin receptor, CFTR and $Cl^-/HCO_3^-$ and respond physiologically to these two hormones. The model also shows that following BDL, only large cholangiocytes proliferate and that a single dose of $CCl_4$ induces damage and loss of large duct function, whereas small cholangiocytes (resistant to $CCl_4$) *de novo* proliferate and secrete to compensate for the loss of large duct function. Reproduced with permission from Ref. 73.

cholangiocyte cultures[87] and large cholangiocytes[88]. These data suggests that after taurocholate and taurolithocholate enter into large cholangiocytes by ABAT, they stimulate secretin-stimulated ductal bile flow in these cholangiocyte subpopulations[88,89]. Other studies have shown that both taurocholate and taurolithocholate increase secretin-stimulated cAMP levels in large but not small cholangiocytes[89]. Chronic feeding of ursodeoxycholate and tauroursodeoxycholate to BDL rats inhibits secretin-stimulated ductal secretion in large cholangiocytes[62].

As evidence against the notion that small cholangiocytes may be primitive, undifferentiated cells that do not display secretory activity, recent studies have shown that in pathological conditions associated with damage of large cAMP-responsive ducts (e.g., after acute $CCl_4$ administration) (Figure 3)[50,51], small cholangiocytes transiently compensate for large cholangiocyte damage by *de novo* activation of secretory (including expression of secretin receptor and secretin-stimulated cAMP response)[50,51] and proliferative[50,51] (see below) activities. Following ANIT feeding and partial hepatectomy, small cholangiocytes proliferate and secrete by the *de novo* expression of secretin receptor and activation of cAMP response[47,52]. Since preliminary data and unpublished observations (Alpini, 2005) show that small rat and mouse cholangiocytes express receptors ($ET_A$, CCK-B/gastrin, α1-adrenergic, D2 dopaminergic, insulin, H1 histamine) signaling by activation of $IP_3/Ca^{2+}$/PKC[59,90], we propose that there is a secretory gradient in the intrahepatic biliary tree with small cholangiocytes secreting water and electrolytes by activation of the $IP_3/Ca^{2+}$/PKC pathway, whereas large cholangiocytes secrete bile by activation of the cAMP/PKA/CFTR/$Cl^-/HCO_3^-$ exchanger[2,5,2,13,48,54].

## PROLIFERATION AND APOPTOSIS

Cholangiocyte proliferation is coordinately regulated by a number of factors including gastrointestinal hormones/peptides, growth factors, cAMP and $IP_3/Ca^{2+}$/PKC pathways, nerves and bile acids[2,3,24,49,61,62,68,70-72,91-93]. Recent studies have shown that different sized cholangiocytes differentially proliferate or are damaged by apoptosis in response to injury, toxins, nerve resection and selected diets[2,26,48-52]. Following BDL, large but not small cholangiocytes proliferate with increases in basal and secretin-stimulated choleresis (Figure 3)[2,3,48]. We propose that large cholangiocytes selectively proliferate in response to BDL due to: (1) the predominant expression of VEGF in large compared to small cholangiocytes (Alpini *et al*, 2005, unpublished observation); and (2) the presence of the PBP mainly around large bile ducts, and less discernable around small bile ducts[44]. In support of this

concept, in rats with BDL proliferation of the peribiliary plexus occurs only around large ducts[44]. Furthermore, we have recently demonstrated[93] that neutralization of VEGF levels of large cholangiocytes (by administration of a neutralizing anti-VEGF antibodies) reduces cholangiocyte growth typical of BDL rats[6]. In support of the concept that PBP and VEGF play a role in the regulation of large cholangiocyte function, hepatic artery ligation in BDL rats is associated with: (1) the disappearance of the PBP; (2) increased apoptosis and impaired proliferation of large cholangiocytes; and (3) decreased cholangiocyte VEGF secretion[92]. The effects of hepatic artery ligation on PBP and large cholangiocyte function were prevented by chronic administration of r-VEGF-A that, by maintaining the integrity of the PBP and large cholangiocyte proliferation, prevents bile duct damage following ischemic injury[92].

A number of gastrointestinal hormones/peptides have been shown to regulate the differential proliferative response of small and large cholangiocytes. We have shown that cholangiocytes express α1, α2, β1 and β2 thyroid hormone receptors and that the chronic administration of the thyroid hormone agonist, 3, 3′, 5 L-tri-iodothyronine to BDL rats reduces *in vivo* the proliferation of large cholangiocytes[94], the only cholangiocyte subpopulation proliferating in this model[48]. In addition, in BDL rats we have shown that somatostatin inhibits the growth of large cholangiocytes by a decrease in cAMP levels[48]. Furthermore, gastrin inhibits large cholangiocyte proliferation in BDL rats by $Ca^{2+}$/PKC-dependent inhibition of cAMP levels[72].

We have demonstrated that ovariectomy in BDL female rats reduces the proliferation of large cholangiocytes and induces a decrease in the expression of α and β estrogen receptors[69]. We propose that estrogens play a role in the management of chronic cholestatic liver diseases.

Recent studies have shown that nerves regulate the differential proliferative response of intrahepatic ducts. We have shown that the activation of serotonin 1 A and 1 B receptors in cholangiocytes leads to the inhibition of large cholangiocyte proliferation in BDL rats[67]. Serotonin inhibition of large cholangiocyte proliferation was associated with activation of the $IP_3/Ca^{2+}$/PKC signaling pathway and the consequent inhibition of the cAMP/PKA/Src/ERK 1/2 pathway[67]. Since cholangiocytes secrete serotonin, we propose that serotonin limits the growth of intrahepatic bile ducts in the course of chronic cholestasis by an autocrine mechanism. Similarly, we have shown that cholangiocytes secrete NGF and that NGF secretion increases in proliferating BDL cholangiocytes compared to normal cholangiocytes[24]. *In vivo*, immunoneutralization of NGF (with an anti-NGF antibody) decreased large cholangiocyte proliferation[24]. The data suggest that NGF regulates cholangiocyte proliferation by an autocrine mechanism.

We have demonstrated that sensory innervation via α-calcitonin gene related peptide (α-CGRP) plays a role in adaptive proliferative responses of large cholangiocytes during cholestasis following BDL[95]. Specifically, we have shown that small and large murine cholangiocytes express the CGRP receptor components (calcitonin like receptor or CLR, receptor component protein or RCP and receptor activity modifying protein or RAMP1)[95]. Large, but not small, cholangiocytes proliferate in response to α-CGRP, proliferation that was blocked by CGRP[8-37], α-CGRP receptor antagonist[96]. α-CGRP stimulation of large cholangiocyte proliferation was associated with increased cAMP levels and phosphorylation of PKA and p38[96]. We observed a decrease in the number of proliferating large cholangiocytes in BDL knock-out mice (lacking α-CGRP) compared to BDL wild-type mice[95].

The role of the second messenger, cAMP, in the regulation of hepatic cell proliferation has been demonstrated in a number of animal models that stimulate hepatocyte and cholangiocyte proliferation via cAMP dependent mechanisms[26,50-52,54,97,98]. Following partial hepatectomy, there is an increase in intracellular cAMP levels in regenerating hepatocytes[99] and cholangiocytes[52]. Activation of $G\alpha_s$ coupled receptors leads to activation of adenylyl cyclase and increased cAMP levels, whereas activation of $G\alpha_i$ coupled receptors results in inhibition of AC activity and lowered intracellular cAMP levels[100]. cAMP response elements mediating transcriptional activation in response to increased intracellular cAMP levels have been identified[101]. In support of these findings, we have shown that chronic administration of forskolin to normal rats increased cAMP levels and the proliferation of large but not small cholangiocytes compared to rats receiving saline[70]. In purified cholangiocytes, forskolin increased large (but not small) cholangiocyte proliferation[70], which was blocked by Rp-cAMPs (a PKA inhibitor)[74], PP2 (a Src inhibitor)[102] and PD98059 (a MEK inhibitor)[103]. The effects of forskolin on large cholangiocyte proliferation were associated with increased phosphorylation of PKA, Src Tyr 139 and ERK1/2[70]. Maintenance of cAMP levels by forskolin administration prevents the effects of vagotomy on large cholangiocyte apoptosis (activation) and proliferation (inhibition)[26].

The acute administration of $CCl_4$ to normal and BDL rats induces decreased cAMP levels and loss of function of large cholangiocytes at d 2 and transient elevation of cAMP levels in small cholangiocytes[50,51]. In these models, small cholangiocytes *de novo* express secretin receptors, a key component of the biliary proliferative and secretory mechanisms, suggesting that intracellular cAMP plays a key role in the: (1) *de novo* expression of large cholangiocyte phenotypes by small cholangiocytes (to compensate for loss of large cholangiocyte function); and (2) perhaps the differentiation of small cholangiocytes towards a cholangiocyte subpopulation that has the capacity to secrete and proliferate by cAMP-dependent pathway[50,51].

Following partial hepatectomy, both small and large cholangiocytes proliferate and participate in the regeneration of the intrahepatic biliary epithelium[52]. A single gavage dose of $CCl_4$ to normal and BDL rats induces damage of large, cAMP-responsive cholangiocytes, whereas small cholangiocytes (resistant to $CCl_4$) *de novo* proliferate and secrete (by the activation of the secretin receptor and secretin-stimulated cAMP levels) to compensate for the damage and loss of functional activity of large cholangiocytes[50,51]. The differential resistance of small and large cholangiocytes to $CCl_4$ is presumably due to the presence

of cytochrome P4502E1 (the enzyme that converts $CCl_4$ to its radicals)[104] in large but not small cholangiocytes[50,51]. Chronic administration of the toxin, ANIT, induces proliferation of both small and large cholangiocytes, proliferation that (in contrast to other models including BDL)[26] was associated with enhanced apoptosis[47]. We propose that following ANIT or $CCl_4$ feeding, the proliferation of small cholangiocytes may be due to the presence of cholangiocyte apoptosis in these models[47,50,51]. We also propose that the lack of small cholangiocyte proliferation in BDL rats may be due to the absence of cholangiocyte apoptosis in this model[26]. Similar to what is observed following acute $CCl_4$ administration[50], the differential responses of small and large cholangiocytes to liver injury/toxins may be due to differential expression of other enzymes/proteins in small and large cholangiocytes. In support of this concept, phase I or mixed-function oxygenase enzymes (e.g., microsomal cytochrome P-450, aminopyrine-N-demethylases, G-6-$PO_4$, and NADPH cytochrome C reductase) and phase II or glutathione redox cycle enzymes (e.g., GSH-peroxidase, UDP-glucuronosyltransferase, and glutathione-S-transferase) drug-metabolizing enzymes are heterogeneously expressed by cholangiocytes[50,81,105]. Similarly, since small murine cholangiocytes express annexin-V[106] (that regulates cell apoptosis)[107], this finding may explain partly why small ducts are more resistant than large ducts to some hepatic injury/toxins[50, 51]. In support of this concept, recent studies have shown that bcl-2 (an anti-apoptotic protein)[108] is expressed by small bile ducts in normal human liver and human liver with cirrhosis and focal nodular hyperplasia[109], a finding that may also explain partly the greater resistance of small cholangiocytes to damage[3,50,51].

*In vitro* treatment of normal cholangiocytes with taurocholate and taurolithocholate increases the proliferation of large but not small cholangiocytes[89]. Chronic feeding of taurocholate and taurolithocholate to normal rats induces the *de novo* expression of ASBT and activation of proliferation of small cholangiocytes, which do not constitutively express ASBT and are mitotically quiescent, and increases the proliferation of large cholangiocytes[49]. Prolonged feeding of ursodeoxycholate and tauroursodeoxycholate to BDL rats reduces the growth of large cholangiocytes[62] that selectively proliferate in this hyperplastic model[48]. Furthermore, depletion of endogenous bile acids reduced large cholangiocyte proliferation compared with BDL rats[91]. Re-infusion of taurocholate to bile acid-depleted rats prevented the decrease in cholangiocyte proliferation that was maintained at levels similar to those of BDL rats[91].

Histamine, an aminergic neurotransmitter, regulates many pathophysiological functions. Four G-protein coupled histamine receptors ($H_1$, $H_2$, $H_3$ and $H_4$) exist[110]. While $H_1$ histamine receptors act via $G\alpha_q$ mobilizing $[Ca^{2+}]_i$[111], activation of $H_2$ histamine receptors is modulated by $G\alpha_s$ proteins, coupled to adenylyl cyclase[112]. $H_3$ and $H_4$ histamine receptors couple to $G\alpha_{i/o}$ proteins that inhibit adenylyl cyclase[113]. Based upon our preliminary data, we propose a model in which the overall outcome of histamine on cholangiocyte growth is represented by a balance between its stimulatory (by activation of H1 and H2 histamine receptors)[90,114] and its inhibitory (by activation of H3 and H4 histamine receptors)[115, 116] actions on small and large cholangiocyte proliferation. Specifically, we have shown that small but not large mouse cholangiocytes: (1) express the $H_1$ histamine receptors and the calcium-dependent CaMK I (but not II or IV) protein kinase; and (2) proliferate in response to $H_1$ histamine receptor agonists, proliferation that was blocked by BAPTA/AM, Gö6976 and W-7, a CAMK inhibitor[117]. $IP_3$ (but not cAMP) levels were increased in small cholangiocytes treated with HTMT dimaleate. Chronic administration of the specific H3/H4R agonist (RAMH) to BDL rats decreased large cholangiocyte proliferation and cAMP levels compared to BDL rats treated with NaCl[115,116]. This inhibition is mediated through negative regulation of the cAMP-dependent PKA/ERK1/2 pathway[115,116].

The mechanisms by which different sized ducts proliferate or are damaged in response to various liver injury/toxins (e.g., BDL, partial hepatectomy, vagotomy, feeding of ANIT, bile acids or $CCl_4$)[3,26,47-52] are unclear. Furthermore, the pathophysiology of small cholangiocytes is undefined in these models. Based upon preliminary data and unpublished observations from our laboratory, we propose that neural/hormonal-dependent (cholinergic and adrenergic) activation of the $Ca^{2+}$-dependent NFAT (Nuclear Factor of Activated T-lymphocytes) stimulates the proliferative response of small cholangiocytes, whereas neural/hormonal-dependent activation of the cAMP-dependent CREB stimulates the proliferation of large cholangiocytes. NFAT is a ubiquitous transcription factor that was initially described in T-lymphocytes. Five isoforms of NFAT have been identified. Four of these isoforms (NFATc1 to c4) are regulated by $Ca^{2+}$ signaling[118]. Preliminary data shows that $Ca^{2+}$-dependent activation of NFATc1/c4 stimulates the proliferation of small cholangiocytes after $CCl_4$-induced damage of cAMP-responsive large bile ducts[119]. Specifically, we have shown that small but not large normal rat cholangiocytes express the NFAT isoforms, NFAT c1 and c4[119]. $CCl_4$ both *in vivo* and *in vitro* increased small cholangiocyte proliferation that was blocked by BAPTA/AM and 11R-VIVIT (NFAT inhibitor peptide)[120]. Furthermore, unpublished data from our laboratory show that the *de novo* growth of small cholangiocytes is regulated via adrenergic stimulation of $Ca^{2+}$-dependent activation of NFATc1/c4 ($Ca^{2+}$/calcineurin) and Sp1 ($Ca^{2+}$/PKC). NFAT and Sp1 cooperatively interact to regulate proliferative phenotypes in other cell types[121].

Recent studies have shown that bile acids have cytoprotective effects against apoptosis in large cholangiocytes. Feeding of taurocholate to BDL rats (treated with a single dose of $CCl_4$) prevents $CCl_4$-induced damage of large cholangiocytes, whereas small cholangiocytes (which are *de novo* activated following $CCl_4$-induced damage of large ducts)[50,51] remained mitotically dormant and unresponsive to secretin (Figure 3)[122]. *In vitro*, taurocholate prevented the inhibitory effects of $CCl_4$ on apoptotic, proliferative and secretory capacity of large BDL cholangiocytes[122]. The protective effects of taurocholate against $CCl_4$-induced damage of large BDL cholangiocytes are due to the

activation of PI3-K and AKT expression[122]. Furthermore, feeding of taurocholate to BDL + vagotomy rats prevented vagotomy activation of large cholangiocyte apoptosis and inhibition of large cholangiocyte growth[123], effects that were abolished by wortmannin, a PI3-K inhibitor[124]. Functional ASBT expression as well as phosphorylation of Akt were reduced by vagotomy but restored by taurocholate feeding[123]. Chronic feeding of taurocholate prevented the increase in cholangiocyte apoptosis and the damage of large cholangiocyte proliferation induced by adrenergic denervation by 6-OHDA administration[125]. Taurocholate effects are mediated by the PI3K pathway, since the simultaneous administration of wortmannin reverses such effects[125]. In addition, the feeding of ursodeoxycholate and tauroursodeoxycholate to BDL + vagotomy rats prevented the activation of apoptosis and the loss of proliferation of large cholangiocytes observed in this model[126]. In this study[126], the protective effects of these two bile acids were neutralized by the simultaneous administration of BAPTA/AM (an intracellular $Ca^{2+}$ chelator)[72] or Gö6976 (a PKC inhibitor)[65]. Both ursodeoxycholate and tauroursodeoxycholate increased $IP_3$ and $Ca^{2+}$ levels, together with enhanced phosphorylation of PKC-$\alpha$[126]. The data suggests that bile acids are important in modulating large cholangiocyte proliferation in denervated livers.

## HETEROGENEITY IN CHOLANGIOPATHIES

Chronic cholestatic liver diseases (cholangiopathies), which target intrahepatic and extrahepatic bile ducts, are characterized by the coexistence of cholangiocyte growth/apoptosis, inflammation and fibrosis[3,127]. Cholangiopathies differentially target the biliary epithelium with heterogeneous proliferative and apoptotic responses of different sized ducts[3,47,50,128-130]. Primary biliary cirrhosis is characterized by the selective proliferation/loss of small interlobular bile ducts[3,131]. Some studies demonstrated that damage of interlobular bile ducts is immune mediated[3,132]. The origin of primary sclerosing cholangitis (PSC), which is associated with inflammation and fibrosis of bile ducts, originates from multiple factors including autoimmune, bacterial, congenital, drug, or viral agents[3,73]. PSC affects mainly extrahepatic and interlobular or septal bile ducts although smaller bile ducts can be affected[3,73]. Patients with small duct PSC seem to have a good prognosis in terms of survival and development of cholangiocarcinoma[133]. Cholangiocarcinoma occurs frequently in patients with PSC and targets mainly the major bile duct bifurcation[3,134]. Peripheral cholangiocarcinoma occur within the liver rather than within large bile ducts may arise from small bile ducts[3,134]. Mutations in the CFTR gene are responsible for causing the human biliary disease, cystic fibrosis, due to defective transport of water and chloride presumably by large cholangiocytes expressing CFTR[135]. Our previous studies in rodent liver has shown that CFTR is expressed principally in large cholangiocytes and in bile ducts greater than 15 $\mu$M diameter[12,13] but in studies of human liver of cystic fibrosis patients, CFTR was expressed in both large and small ducts[136].

Defective chloride transport and chloridemediated bile secretion by large cholangiocytes may be responsible for the reduced fluidity and alkalinity of bile, leading to bile duct damage. $Ca^{2+}$-dependent $Cl^-$ channels[137,138] (presumably expressed by both small and large cholangiocytes) may be able to secrete bile, thus compensating for loss of CFTR functional activity of CFTR in large cholangiocytes[54]. In polycystic kidney liver disease (PKLD), the genetic defect results in the growth of multiple epithelial cysts within the renal, liver parenchyma and intrahepatic bile ducts[139]. The disease targets presumably large bile ducts since the cystic ductal cells also secrete $Cl^-$ and $HCO_3^-$ (as normal large cholangiocytes)[2,3,54,71,73] but the secretion is diminished, likely due to reduced $Cl^-/HCO_3^-$ exchanger activity in cystic ductal cells as compared with normal cholangiocytes[139]. Biliary atresia, which is the most common reason of cholestasis in infants and children, is a destructive, inflammatory process of the extrahepatic bile ducts but as the disease progresses smaller intrahepatic bile ducts are also involved[140]. The pathogenesis of biliary atresia is unknown but infections or toxic agents combined with genetic/immunologic susceptibility have been proposed[3, 141, 142].

## SUMMARY

In this review, we have summarized the findings demonstrating that the intrahepatic biliary epithelium is heterogeneous regarding: (1) morphological characteristics, vascularization and innervation; (2) secretory activity in response to gastrointestinal hormones/peptides, nerve receptor agonists and bile salts; and (3) apoptotic and proliferative responses to liver injury/toxins and gastrointestinal hormones/peptides. Specifically, the intrahepatic biliary epithelium is formed by bile ducts of different sizes with small ducts lined by small cholangiocytes, whereas larger ducts are lined by larger cholangiocytes[12-14]. Following a general background on cholangiocyte functions, we discussed the *in vivo* and *in vitro* experimental models that allowed us to demonstrate that the biliary epithelium is morphologically and functionally heterogeneous. Following a brief review on the heterogeneous distribution of non-transport related proteins, we discussed the secretory functions of small and large cholangiocytes. While large cholangiocytes secrete water and electrolytes[12,13,48] by changes in cAMP/PKA/CFTR/$Cl^-/HCO_3^-$, small cholangiocytes may secrete bile by a transduction pathway (different from that observed in large cholangiocytes)[12,13,48] involving activation of $IP_3$/$Ca^{2+}$/PKC. We have presented data demonstrating that small and large cholangiocytes differentially proliferate or are damaged in response to liver injury/toxins. Small and large ducts also differ regarding the proliferative and apoptotic responses to liver injury/toxins[2,71,73]. We propose that activation of the $Ca^{2+}$-dependent NFAT stimulates the proliferation of small cholangiocytes, whereas neural/hormonal-dependent activation of the cAMP-dependent CREB stimulates the proliferation of large cholangiocytes. In the last part of the review, we have briefly outlined the heterogeneity of the biliary epithelium in relationship to chronic cholestatic liver diseases targeting different sized ducts.

## FUTURE PERSPECTIVES

The concept that the biliary epithelium is functionally heterogeneous is clinically relevant since in chronic cholestatic liver diseases cholangiocyte proliferation/damage is an event restricted to a specific duct size. Further studies are needed for understanding the pathophysiology of small cholangiocytes in the overall contribution of the functions of the biliary epithelium. However, some preliminary studies from our laboratory suggest that small cholangiocytes secrete bile (by a $IP_3/Ca^{2+}$/PKC-dependent mechanism) and proliferate by activation of the $Ca^{2+}$-dependent transcription factor, NFAT. Further studies are necessary to evaluate the role of the nervous system in the regulation of the heterogeneous secretory, apoptotic and proliferative responses of different sized bile ducts to gastrointestinal hormones, injury/toxins and viruses. Since PBP proliferation is observed only in large proliferating cholangiocytes from BDL rats, we propose that blood supply and circulating factors (e.g., vascular endothelial growth factor and placental growth factor) may be important in the regulation of the heterogeneous response of cholangiocytes to liver injury/toxins.

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