Recent advances on the mechanisms regulating cholangiocyte proliferation and the significance of the neuroendocrine regulation of cholangiocyte pathophysiology

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Abstract: Cholangiocytes are epithelial cells lining the biliary epithelium. Cholangiocytes play several key roles in the modification of ductal bile and are also the target cells in chronic cholestatic liver diseases (i.e., cholangiopathies) such as PSC, PBC, polycystic liver disease (PCLD) and cholangiocarcinoma (CCA). During these pathologies, cholangiocytes (which in normal condition are in a quiescent state) begin to proliferate acquiring phenotypes of neuroendocrine cells, and start secreting different cytokines, growth factors, neuropeptides, and hormones to modulate cholangiocytes proliferation and interaction with the surrounding environment, trying to reestablish the balance between proliferation/loss of cholangiocytes for the maintenance of biliary homeostasis. The purpose of this review is to summarize the recent findings on the mechanisms regulating cholangiocyte proliferation and the significance of the neuroendocrine regulation of cholangiocyte pathophysiology. To clarify the mechanisms of action of these factors we will provide new potential strategies for the management of chronic liver diseases.

Key Words: Biliary tree; cholangiocytes; liver; proliferation; neuroendocrine regulation

Introduction

The liver (the largest organ of the human body) is considered as a gland capable of performing endocrine-metabolic and exocrine functions (1-3). The liver plays a key role in the homeostasis of the whole body metabolism. In particular, synthesizing many proteins and enzymes, the liver plays a primary role in the regulation of the energetic metabolism and it also contributes to the detoxification and elimination of a wide variety of both endogenous and exogenous molecules. In addition, the liver secretes bile that is necessary for the digestion of fats. The bile is then delivered to the second portion of the duodenum through intrahepatic bile ducts (4), whose lumen is lined by specialized epithelial cells: cholangiocytes (5,6) that modify the final composition of bile by a number of basal and hormone-regulated events (4,5,7,8).

Features of the biliary tree

The human biliary tree originates at the level of the biliary pole of hepatocytes that are responsible for the production of canalicular bile (9). Thereafter, the bile is secreted into the lumen of bile canalicular. The bile canalicular are narrow spaces of 0.5-2 μm delimited by simple introflections of the

plasma membrane at the biliary poles of adjacent hepatocytes. The biliary pole of hepatocytes is characterized by numerous and short microvilli that protrude into the lumen and, it is isolated from the vascular pole by occluding junctions (10).

Near the biliary pole, the cytoskeleton of hepatocytes is characterized by many microfilaments predominantly formed by actin and myosin. These microfilaments that are located around the bile canalicular regulates the passage of bile along the canals themselves. The canals of Hering origin in proximity of portal spaces (10,11).

The point at which the bile canalicular continue into the canals of Hering is defined as the ductule-canalicular junction. At this level, intrahepatic bile ducts are partly lined by cholangiocytes and in part by hepatocytes. At this point of the biliary epithelium, partially undifferentiated cells have been identified: the hepatic progenitor cells (HPC) (12). HPC constitute a resident stem cell compartment in the liver and they are able to differentiate into both hepatocytes and cholangiocytes (13-15). The canals of Hering are, therefore, points of anatomical and physiological connection between the bile canalicular and interlobular bile ducts in the portal space. The canals of Hering are lined by a few (3 or 4) cubic-shaped cholangiocytes, which rest on a thin basement membrane (7,16). The canals of Hering continue into the interlobular ducts, lined by a continuous layer of cylindrical-shaped cholangiocytes (16). The interlobular ducts are characterized by a diameter of 15-100 µm and in the portal space they draw up alongside the ramifications of the hepatic artery and of the portal vein. The interlobular bile ducts then continue into ducts of progressively larger size to form two large intrahepatic ducts (right and left hepatic duct), which drain respectively the right and left hepatic lobe of the liver and, at the hilum, they give rise to the extrahepatic bile ducts (4,17,18).

According to diameter the human intrahepatic bile duct can be classified into: large ducts (>800 µm), and then gradually segmental ducts, zonal, septal, interlobular ducts (15-100 µm) and ductules (<15 µm) (16).

In rodents, the intrahepatic biliary ductal system is morphologically heterogeneous and is formed by small (<15 µm of diameter, lined by small cholangiocytes, 8 µm in size) and large (>15 µm of diameter, lined by large cholangiocytes, 15 µm in size) bile ducts (5,7).

Vascularization and innervation of the biliary tree

The biliary epithelium is nourished by terminal branches of the hepatic artery, which constitute a complex vascular system called the peribiliary plexus (PBP). The PBP, which extends up to the interlobular ducts flows into the hepatic sinusoids, either directly through lobular branches, which indirectly through branches of the portal vein or pre-lobular branches (17,19,20). In addition, numerous studies performed on rats have shown that small and large bile ducts have a different vascularization (21). In fact, the PBP is more extended in large bile ducts. The resulting difference of blood supply strongly influences the metabolic capabilities and functions of bile ducts of different size and of cholangiocytes that line their walls (17,22). The PBP is characterized by a bilaminar vascular network near the hilus of the liver and larger portal tracts, whereas it is formed by fewer connected vessels in the smaller portal tracts (23).

From a physiological point of view, the PBP is crucial in allowing the active reabsorption and therefore the modification of bile by cholangiocytes. In particular, it allows to reabsorb substances present in the bile and to bring them back to parenchymal cells (17). The canals of Hering and part of bile ductules are not directly vascularized (23), and this could in part justify the quiescent state under normal conditions of the HPC compartment located in this tract of the biliary tree (23).

The human intra- and extra-hepatic biliary epithelium is innervated by autonomic nerves that originate from the celiac plexus (sympathetic fibers) and the vagus nerve (parasympathetic fibers). These nerve fibers are organized to form the hepatic plexus divided into: anterior plexus around the hepatic artery and posterior plexus surrounding portal vein and bile duct (24).

Hepatic plexus (anterior and posterior) nerve fibers that originate through the hilum penetrate the liver parenchyma following the branches of the hepatic artery, the portal vein and the biliary tree. This innervation is also extended to hepatocytes and intrahepatic vascular structures (19,25).

In addition to classical neurotransmitters (e.g., adrenaline, noradrenaline and acetylcholine), the nerve fibers release in the liver numerous other neuropeptides such as neuropeptide Y (NPY), the calcitonin gene-related peptide (CGRP), somatostatin, vasoactive intestinal polypeptide (VIP), enkephalin and bombesin. Many of these neuropeptides have been shown to be able to stimulate or inhibit the functional activity of cholangiocytes (26,27).

Experimental models for the study of biliary tree and cholangiocytes

In both rat and human livers, cholangiocytes are heterogeneous

in size (that can vary from 3 to 80 μm^2) (7) and line bile ducts of different sizes (5,7). For example, intrahepatic bile ducts of small dimensions (small bile ducts: diameter less than 15 μm) (5) are lined by cholangiocytes of smaller size, whose diameter is about 8 μm (small cholangiocytes) (7). In contrast, bile ducts of greater size (large bile ducts: diameter greater than 15 μm) are lined by cholangiocytes more voluminous, whose diameter is around 15 μm (large cholangiocytes) (7,18). In addition to morphological heterogeneity, cholangiocytes display ultrastructural, antigenic, functional and proliferative heterogeneous responses (7,10,18,28). In fact, small cholangiocytes possess cuboidal shape and have a larger nucleus and scant cytoplasm (10). This aspect is typical of poorly differentiated cells in which it occurs an intense synthesis of RNA messenger but lower post-transcriptional activity. Conversely, large cholangiocytes have columnar shape, a small nucleus and abundant cytoplasm (7,18). Both small and large cholangiocytes express cytokeratin-7 and -19, alkaline phosphatase, whereas only large cholangiocytes the secretin receptor, cystic fibrosis transmembrane conductance regulator (CFTR) and chloride bicarbonate anion exchanger 2 (Cl^-/HCO_3^- AE2) and respond to secretin with enhanced secretory and proliferative activities (2,5,7,29-31).

A number of experimental models have improved our understand of the mechanisms that regulate cholangiocytes proliferation, apoptosis, secretion, transport, signal transduction and the corresponding dysfunctions that lead to the diseases of the biliary tract. Three types of cholangiocytes proliferation have been described (27). Type I or "typical" cholangiocyte proliferation: represents a hyperplastic reaction that involves an increase of the number of intrahepatic bile ducts, confined to the portal space. Cholangiocytes proliferating, form well-differentiated tubular structures that show a well-defined lumen (4,13). In rodents, "typical" cholangiocyte proliferation is observed in different experimental conditions such as extrahepatic bile duct ligation (BDL) (2,4,22), partial hepatectomy (32) and chronic treatment with L-proline (33). In addition, "typical" cholangiocytes proliferation is also observed after the acute administration of carbon tetrachloride (CCl_4) (34) or chronic feeding α-naphthylisothiocyanate (ANIT) (35) or bile salts such as Taurocholate (36,37). In human liver, a typical cholangiocyte proliferation is observed during acute obstructive cholestasis and in the early stages of chronic cholestatic diseases. This type of proliferation appears to be due to the stretching of the existing bile ducts localized in the portal tracts (27,38).

Type II or "atypical" proliferation occurs in rodents after the administration of CCl_4 (39) and it is present in humans after massive hepatic necrosis, alcoholic liver disease, focal nodular hyperplasia and chronic cholestatic diseases such as primary biliary cirrhosis (PBC) and PSC (27,40). This pathology is characterized by the proliferation of intrahepatic bile ducts not confined to portal areas but extended to the periportal area and in the adjacent liver parenchyma. This type of proliferation leads to the formation of irregular and tortuous ductular structures that do not possess a well-defined lumen and are associated with inflammation and infiltration of neutrophils; moreover, these neo-formed ducts are not functionally efficient. Unlike "typical" proliferation, in type II proliferation has been described the presence of transitional cells with phenotypic characteristics of both cholangiocytes and hepatocytes. This finding is in favor of the hypothesis that atypical proliferation is a phenomenon of metaplasia and may originate from the hepatic progenitor cell compartment rather than from the replication of pre-existing ducts (13,27).

Type III proliferation ("oval cell" proliferation) characterizes the early stages of carcinogenesis in rat liver and is caused by treatment with chemicals. This pathology is characterized by the formation of tubular irregular and disorganized structures with a not well defined lumen which protrude inside the hepatic lobule with alteration of the architecture of the entire parenchyma (27).

Oval cells, so called for their shape, represent a subpopulation of cells, located at levels of canals of Hering that are heterogeneous in the expression of proteins and cell surface markers (13,41-43). Oval cells show phenotypes of immature or neoplastic hepatocytes (e.g., expression of α-fetoprotein and albumin) and cholangiocytes (e.g., expression of cytokeratin 7 and 19) (41,44). Oval cells have the capability of differentiating into hepatocytes, cholangiocytes, enterocytes, and exocrine pancreatic cells (42,43).

BDL is the most used experimental model to study "typical" proliferation of cholangiocytes. This maneuver causes a rapid and marked proliferation of bile ducts leading to increased biliary mass (2,17). In the BDL rat model, the proliferation of cholangiocytes and the reorganization of the hepatic microvasculature occur according to a well-defined timetable. In fact, one week after BDL, the number of bile ducts increases, particularly at the edges of portal tracts. At this time-period, cholangiocytes appear to markedly proliferate. In this phase, the hepatic microcirculation is poorly changed (17,20,22,23). After two weeks, proliferation of bile ducts continues and bile ducts are characterized by a dilated lumen and lined by cholangiocytes resting

on a basement membrane. At this stage, there is an active reorganization of the PBP that follows the proliferation of the bile ducts (20,45). After four weeks, the abundant proliferation of bile ducts is accompanied by septa of connective tissue located on the edge of the liver lobules. By means of vascular corrosion casts observed by scanning electron microscope (46) is possible to see a PBP that extends around the lobules and appears to be formed by homogeneous capillaries widely distributed that originate from arterioles arising from branches of the hepatic artery (20).

The evaluation of the molecular mechanisms that regulate the "typical" proliferation of cholangiocytes in BDL rats was made possible by the development of *in vitro* models such as the isolation and characterization of small and large cholangiocytes or intrahepatic bile duct units (IBDU) (2,5,7,30,37). The isolation of cholangiocytes from livers of normal or cholestatic rats is achieved through separation techniques based on immunoaffinity, i.e. through the use of antibodies expressed on the surface of all intrahepatic cholangiocytes (2,7). The isolation and study of IBDU of different sizes is essential for the direct assessment of the proliferative capacity of bile ducts. These units retain the morphological, phenotypic and functional characteristics of the intrahepatic bile ducts in situ (2,7). These tools have allowed us to define the morphologic and functional heterogeneity of intrahepatic bile ducts in normal and pathophysiological states. For example, after BDL large (but not small) cholangiocytes proliferate through the activation of cAMP-dependent signaling, leading to enhanced intrahepatic bile duct mass by activation of cAMP signaling (2,39,47). Also, cAMP induces cholangiocyte proliferation in association with increased activity of protein kinase A (PKA) and phosphorylation of Src-Tyrosine 139 and ERK1/2 (48). This signaling cascade is also activated by factors such as estrogens (49), nerve growth factor (50), and insulin-like growth factor-1 (IGF-1) (51). Conversely, serotonin decreases biliary hyperplasia in cholestatic rats by the inhibition of cAMP/PKA/Src/ERK1/2 signaling (52).

Regarding the function of small cholangiocytes, several studies have shown that the activation of D-myo-inositol 1,4,5-triphosphate (IP$_3$)/Ca^{2+}-dependent signaling regulates the function of these cells (53-56). Small mouse cholangiocytes proliferate after H1 histamine receptor stimulation by activation of the IP$_3$/CaMK I/CREB pathway (53). Further, histamine stimulates the proliferation of small and large cholangiocytes by activation of both IP$_3$/Ca^{2+} and cAMP-dependent signaling mechanisms, respectively (56). For example, a study has shown that activation of alpha[1]-adrenergic receptors stimulate the growth of small mouse cholangiocytes via Ca^{2+}-dependent activation of nuclear factor of activated T cells 2 and specificity protein 1 (54). A recent study has demonstrated the existence of a P2 signaling axis along the length of the biliary tree with the "upstream" small cholangiocytes releasing ATP, which can serve as a paracrine signaling molecule to "downstream" large cholangiocytes stimulating Ca^{2+}-dependent secretion (55). Following functional damage of large cholangiocytes by apoptosis (e.g., after treatment acute administration of CCl$_4$ and chronic treatment with gamma-aminobutyric acid (GABA), small cholangiocytes *de novo* proliferate and replenish the damaged biliary tree by amplification of Ca^{2+}-dependent signaling and *de novo* acquisition of large cholangiocyte phenotypes (34,39,57).

One of the functions of cholangiocytes in humans is to modify the composition of bile. The bile produced by hepatocytes is particularly rich in bile salts, glutathione, lipids, proteins and other organic compounds and its flowing into bile ducts allows cholangiocytes to change its composition through mechanisms of reabsorption and secretion (58,59). Cholangiocytes in the adult liver are quiescent (34,39), since they express factors such as cyclin dependent kinase inhibitors p27, bcl2 and Bclx$_L$ that are responsible for this mitotically dormant state (60). However, in pathological states cholangiocytes become mitotically activated both in experimental cholestasis (e.g., BDL and A feeding) (4,35) and in human cholangiopathies such as PBC and PSC classified as "vanishing bile duct syndrome" (58,59). The evolution of these cholangiopathies is characterized by an initial balance between cholangiocyte apoptosis with reduction of the number of bile ducts and compensatory cholangiocyte proliferation. By contrast, the end stage is characterized by the collapse of the proliferative capacity of cholangiocytes, resulting in the reduction of the number of bile ducts (vanishing bile duct syndrome). In these cases the compensated (non-ductopenic) stages of disease are characterized by a similar rate of apoptosis and proliferation, whereas in the terminal ductopenic stages apoptosis predominates favoring the evolution toward ductopenia. Therefore, in these pathologic conditions, the mechanism of repair based on the proliferation of residual cholangiocytes is not sufficient to avoid the evolution toward ductopenia. In this case then, stimulation of cholangiocyte proliferation could be a strategy for the management of human ductopenic pathologies affecting the intrahepatic biliary tree. On the other hand, reduction of cholangiocytes proliferation can be considered as the
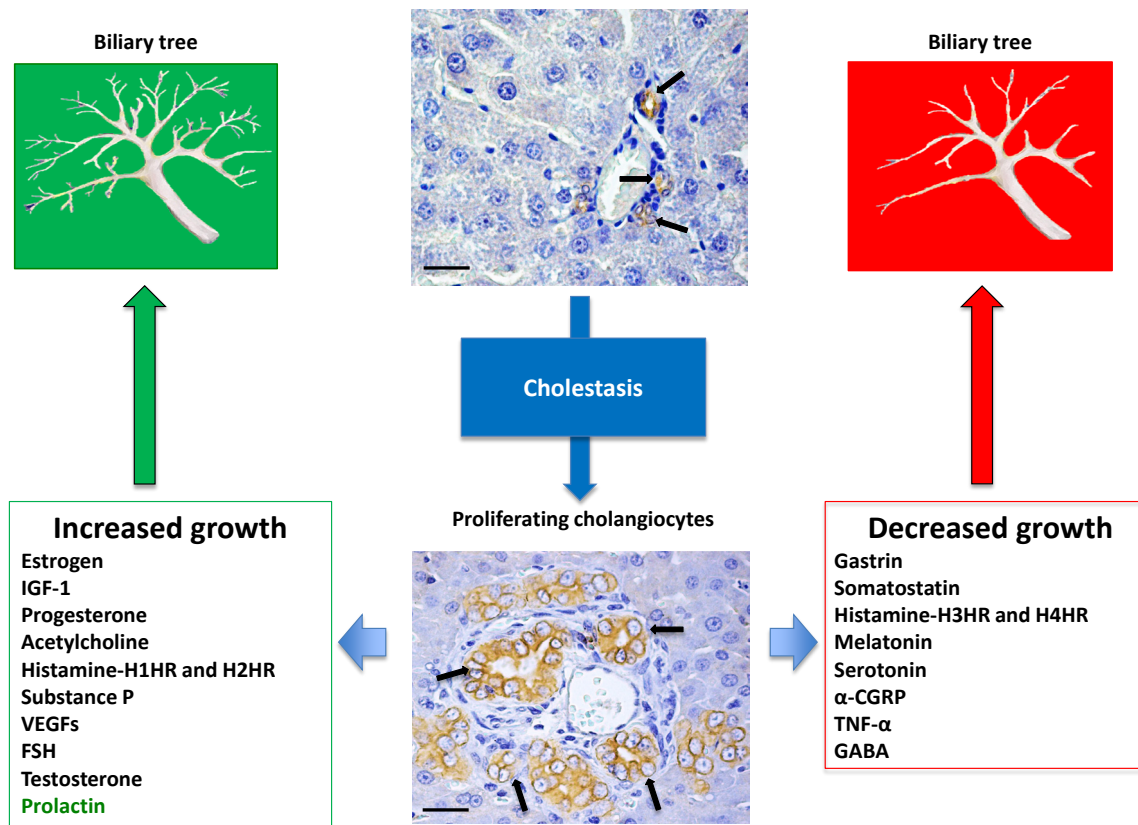
Figure 1 Schematic illustration of the neuroendocrine compartment. Top. Immunohistochemistry for CK19, a specific marker of biliary epithelium in liver section from normal rat. Bar =40 μm. In normal condition cholangiocytes are quiescent (black arrows) but since they are target cells in several chronic cholestatic liver diseases (termed cholangiopathies), they start to proliferate as happen for example in the experimental model of bile ducts ligation (bottom. Immunohistochemistry for CK19 in liver section from BDL rat in which is evident the high proliferation of the biliary ducts (black arrows). Bar =40 μm. Proliferating cholangiocytes acquire the phenotype of neuroendocrine cells, and start to secrete different cytokines, growth factors, neuropeptides, and hormones to modulate cholangiocytes proliferation (on the right in red) or apoptosis (on the left in green) trying to reestablish the balance between proliferation/loss of cholangiocytes

possible strategy for the management of pathologies characterized by uncontrolled proliferative conditions such as cholangiocarcinoma (61-63) and polycystic liver diseases (60,64,65). We know that progenitor cells are activated when injury is associated with impaired regeneration of the mature cell types involved (66,67). Several factors such as viruses and alcohol can cause acute and chronic human liver disease through damage and loss of hepatocytes and/or cholangiocytes. The degree of progenitor cell activation, and the number of intermediate hepatocytes is directly proportionate with the degree of inflammatory activity (67,68), and with the stage of the disease (67,69). This aspect indicates that the HPC compartment activation is linked with the evolution of the disease. At the ductopenic stage of cholangiopathies,

the functionality of bile ducts is strongly altered due to the change in bile flow and subsequent biliary stasis (58,59). Therefore, the fine regulation of secretion and proliferation of cholangiocytes is critical for the normal and physiological function of the liver. These aspects are influenced and controlled by many factors, including bile acids, autonomic nervous system, neuroendocrine substances, growth factors and hormones (*Figure 1*).

Regulation of cholangiocyte function and proliferation by hormones and neuropeptides

Sex hormones

Sex steroid hormones are able to influence the development

and course of chronic liver diseases (70,71). It has been shown that cholangiocytes are estrogen-sensitive cells able to express both the estrogen receptors (ER-α and ER-β) in contrast to hepatocytes that express only the receptor ER-α (45). Our findings have shown that liver expresses estrogen and androgen receptors and experimentally both androgens and estrogens have been implicated in stimulating cell proliferation. Specifically, estrogens stimulate both *in vitro* and *in vivo* cholangiocyte proliferation and ERs may modulate cholangiocyte proliferation. In fact cholangiocyte proliferation, after 3 weeks of BDL, is associated with increased expression of both ER-α and ER-β (45,49,72). When tamoxifen (anti-estrogen) or ICI 182,780, a specific ER antagonist, were used to treat BDL rats, the intrahepatic bile duct mass markedly decreases in comparison with control BDL rats (45). These data were confirmed by ovariectomy of which effects reduce cholangiocyte proliferation in BDL (72). *In vitro* experiments showed that 17β-estradiol induces cholangiocytes proliferation by activation of the ER/Src/Shc/ERK1/2 pathway (49). In humans, it has been shown that at late stages of PBC (73) the biliary expression of ER is decreased and ER-α positivity in cholangiocytes is markedly lower than that of cholangiocytes in primary sclerosing cholangitis and alcoholic cirrhosis (73). It has been shown that the low expression of ER-α in late stages of PBC promotes the development of PBC toward ductopenia (73). The relevance of estrogens in sustaining biliary growth is also supported by the fact that in middle-aged women (mainly affected by PBC), estrogen and progesterone levels are decreased (74,75).

In patients with cholangiocarcinoma (CCA) the expression of ER-α is higher than in normal condition, whereas a similar expression of ER-β was observed (73,76). In ovary, prostate, colon, and breast cancers it has been shown that, neoplastic transformation and tumor progression is associated with upregulation of ER-α and downregulation of ER-β (77,78). These data confirm the hypothesis that ER-α promotes cell proliferation whereas the opposite is observed for ER-β (77-79).

Similarly, testosterone is important in sustaining biliary proliferation and ductal secretory activity in pathological conditions associated with functional damage of the biliary epithelium (80). Specifically, we demonstrated that: (I) cholangiocytes express testosterone receptors, 17β-hydroxysteroid-dehydrogenase3 (HSD3, the key enzyme regulating testosterone synthesis), and secrete testosterone; (II) castration influences the biliary growth in normal and BDL rats decreasing bile duct mass and secretin-stimulated cAMP levels, effects that were prevented by the exogenous administration of testosterone (80); These data suggest an autocrine role of testosterone in sustaining cholangiocyte proliferation during cholestasis. Parallel to these findings, other studies have shown the presence of functional ARs in liver cells, including hepatocytes and bile ducts from PBC patients (81). In particular, the expression of androgen receptors (ARs) was low in normal patients and increased in patients with PBC (81). The fact that testosterone prevents the loss of biliary growth and function supports the concept that androgens can be important for the management of ductopenic conditions associated with decreased testosterone levels as occurs in PBC (80). There is limited information regarding the role of progesterone in the regulation of biliary functions. We have recently demonstrated that: (I) progesterone stimulates proliferation of both male and female cholangiocytes (82); and (II) normal and BDL cholangiocytes expressed the biosynthetic pathway (i.e., steroidogenic acute regulatory protein or STAR, M3β-Hydroxysteroid dehydrogenase (M3β-HSD), and cytochrome P450 side-chain cleavage) for the synthesis of progesterone and secrete progesterone (82). These findings support the concept that endocrine, autocrine and paracrine mechanisms play an important role as compensatory mechanism for bile duct loss during cholangiopathies (73).

Cholangiocytes also express the long and short form of prolactin receptor (83). Also, chronic *in vivo* administration of prolactin to normal rats increases cholangiocyte proliferation and intrahepatic ductal mass (83). The proliferative effect of prolactin is associated with the activation of the $IP_3/Ca^{2+}/PKC/Src/MAPK$ pathway and the phosphorylation of the JAK2/STAT5 signalling pathway (83). These findings demonstrate the ability of proliferating cholangiocytes to secrete prolactin, thus regulating their own proliferation by an autocrine loop.

Follicle stimulating hormone (FSH) is produced in the anterior pituitary gland of the brain (84). Several studies demonstrated that liver cirrhosis is associated with endocrine dysfunction, notably in the gonadal axis (85). In fact, gonadotropin deficiency occurs with liver damage and in some patients with hemochromatosis (86). We found that cholangiocytes expressed the FSH receptor (FSHR) and secreted FSH (86). Treatment of normal rats with FSH increases cholangiocyte growth via cAMP/ERK1/2/Elk-1 signalling mechanism, whereas administration of antide (a gonadotropin releasing hormone antagonist that blocks FSH secretion) or an anti-FSH antibody decreases cholangiocyte proliferation and ductal secretory responses (87).

Moreover, other hormones and growth factors cross

talk together in normal and pathologic conditions. Indeed, the IGF1 has been shown to regulate cholangiocytes proliferation. In fact, the IGF1 is a circulating peptide (of which the liver is the main source of production) that acts locally as a growth factor with multiple endocrine, paracrine and autocrine functions (51,88,89). The IGF1 is synthesized by the liver under the control of pituitary GH (Growth Hormone) which is tied to specific receptors (GH-R), induces the synthesis and release of circulating IGF-1 that play a key role in postnatal growth of many organs. Therefore, in cholangiocytes there is a complex interaction between estrogen and IGF1, which together are involved in the modulation of proliferation, apoptosis and cell differentiation promoting the processes of tissue repair (51,90).

Secretin

Secretin is one of the main factors responsible of the regulation of biliary secretion (4,8). Specifically, in normal rodents, secretin stimulates ductal secretion in large cholangiocytes (the only cholangiocyte subpopulation that expresses secretin receptor) (2,5,7,30). The interaction of secretin with the specific receptor determines the activation of the enzyme, adenylyl cyclase, with a consequent increase in the synthesis of cAMP capable of acting on PKA. Phosphorylation of PKA leads to the opening of the chloride channel, CFTR, and subsequently the activation of the ion pump Cl^-/HCO_3^- with secretion of HCO_3^- and, for osmotic gradient, H_2O (28,31). In pathologic conditions of cholangiocyte hyperplasia there is enhanced secretin receptor expression (2,31,32) and secretin-stimulated cAMP PKA/CFTR/Cl^-/HCO_3^- (2), leading to enhanced bicarbonate secretion in ductal bile (32). Instead in case of damage of large bile ducts there is decreased proliferation and down-regulation of secretin and secretin-stimulated pathway, with decreased bicarbonate secretion in bile (39,57). Secretin has also been shown to stimulate biliary proliferation both *in vivo* and *in vitro* models (29). On the contrary, secretin has been shown to inhibit cholangiocarcinoma growth both *in vitro* in cholangiocarcinoma cells and *in vivo* in athymic mice (91). The inhibitory effects of secretin on cholangiocarcinoma growth via dysregulation of the cAMP-dependent signaling mechanisms of the secretin receptor that coupled to Galpha(i) rather than Galpha(s) (91).

Gastrin

Gastrin, by interacting with its receptor cholecystokinin-B (CCK-B) located in the basolateral membrane of cholangiocytes, gastrin inhibits secretin-stimulated cAMP levels and bile secretion (4) by downregulation of cAMP signaling (92). Gastrin has also been shown to inhibit cholangiocyte hyperplasia in cholestatic rats and cholangiocarcinoma growth by Ca^{2+}-dependent activation of protein kinase C-alpha (47,93).

Somatostatin

Rat and human cholangiocytes as well as cholangiocarcinoma cell lines express somatostatin receptor subtypes, SSTR2 (4,94). Somatostatin has been shown to inhibit secretin-induced ductal hypercholeresis and exocytosis by interacting with SSTR2 on cholangiocytes by downregulation of cAMP levels (94). Somatostatin by selectively interacting with SSTR2 receptors inhibits the proliferation of large cholangiocytes in BDL rats by decreasing intracellular cAMP levels (2). Somatostatin and its analogues also inhibit the growth of human cholangiocarcinoma cell lines expressing SSTR2 receptor subtypes (95).

Histamine

Recent studies showed that small and large cholangiocytes express H1-H4HRs. Histamine and the H1HR agonist increased small bile duct mass, whereas histamine and the H2HR agonist increased large bile duct mass. H1HR agonists stimulated IP_3 levels, as well as PKCα phosphorylation and cholangiocytes proliferation, whereas H2HR agonists increased cAMP levels, as well as PKA phosphorylation and cholangiocytes proliferation (56). Agonists of H1HR stimulate small (but not large) cholangiocyte proliferation by activation of IP_3/CaMK I/CREB signaling (53). Indeed, activation of H3HR induces inhibition of large biliary growth of BDL rats by down-regulation of the cAMP-dependent PKA/ERK1,2/Elk1 pathway (96).

It has been shown that histamine increases cholangiocarcinoma growth by an autocrine mechanism, and that the inhibition of H1HR and of histamine synthesis by blocking his precursor histidine decarboxylase (HDC) significantly decreases cholangiocarcinoma growth (97).

Melatonin

Melatonin is secreted from pineal gland as well as extrapineal tissues; it regulates cell mitosis by interacting with melatonin receptors (MT1 and MT2) by modulating

cAMP and clock genes expression. In fact, in recent studies we have shown that cholangiocytes express MT1 and MT2 receptors, and clock genes such as Circadian Locomotor Output Cycles Kaput (CLOCK), Aryl hydrocarbon receptor nuclear translocator-like (ARNTL or BMAL1), cryptochrome 1 (CRY1), and Period circadian protein 1 (PER1) that were all up-regulated following BDL. Both *in vivo* and *in vitro*, melatonin decreased cholangiocytes proliferation in cholestatic rats by reducing the expression of clock genes by downregulation of cAMP levels and PKA phosphorylation in cholangiocytes (98). In the liver, melatonin has been shown to reduce oxidative damage and hepatic proliferation, and to stimulate apoptosis of hepatocytes in rats undergoing partial hepatectomy (99). Other studies have shown that melatonin ameliorates liver fibrosis (100), and reduces BDL-induced systemic oxidative stress in cholestatic rats (101).

In CCA there is dysregulation of aralkylamine N-acetyltransferase (AANAT)/N-Acetylserotonin-O-methyltransferase (ASMT) (key enzymes regulating melatonin secretion) → melatonin → melatonin receptor axis, which inhibited melatonin secretion and subsequently enhanced CCA growth (102).

Growth factors and cytokines

A number of studies have shown that epidermal growth factor (EGF), hepatocyte growth factor (HGF), IGF1, interleukina-6 (IL-6), interleukin-1α, tumor necrosis factor α (TNF-α) and Vascular Endothelial Growth Factor (VEGF) stimulate both *in vitro* and *in vivo* cholangiocyte proliferation (27,51,103). In particular about VEGF it has been shown that cholangiocytes secrete VEGF and express VEGFR-2 and VEGFR-3, all of which are amplified in BDL cholangiocytes. VEGF induces cholangiocyte proliferation by activation of IP_3/$[Ca^{2+}]_I$/protein kinase C alpha and phosphorylation of Src/ERK1/2 and it mediates the adaptive proliferative response of cholangiocytes to cholestasis via autocrine and paracrine mechanisms (22).

Neuropeptides

Several studies have shown that cholinergic, adrenergic and dopaminergic nerve fibers modulate cholangiocytes functions (104). It has been recently shown that cholangiocyte proliferation is positively modulated by parasympathetic and sympathetic innervation but negatively modulated by the serotoninergic system. Acetylcholine by acting on M3 subtype receptor expressed by cholangiocytes, promote proliferative and secretory activities of cholangiocytes (3). In support of these data it has been shown that vagotomy induces the disappearance of M3 acetylcholine receptor in BDL but not in normal rats, reducing cholangiocyte proliferation, and activating apoptotic cell death together with decreased cAMP intracellular levels (3). Maintenance of cAMP levels by forskolin prevented the vagotomy-induced biliary damage (3).

Adrenergic innervation also regulates hepatic proliferation. It has been shown that proliferating cholangiocytes in BDL rats express α1 and α2 adrenergic receptors (19). Adrenergic innervation affects cholangiocytes proliferation by modulating intracellular levels of cAMP, as demonstrated by the chemical sympathetic denervation of the liver by 6-hydroxydopamine (6-OHDA). In fact the treatment with 6-OHDA increased cholangiocyte apoptosis and decreased cholangiocyte proliferation in association with decreased cAMP levels, and reduced phosphorylation of PKA, ERK1/2, and AKT (19). The changes induced by 6-OHDA in cholangiocyte proliferation were prevented by dobutamine a β1 adrenergic receptor agonist, and clenbuterol a β2 adrenergic receptor agonist (19). 6-OHDA induced effects on biliary hyperplasia were also prevented by taurocholic acid feeding (105), a finding that suggests that bile acids may influence the proliferation of bile ducts. In fact, cholangiocytes in BDL rat are very sensitive to variations in composition of bile salts. It has been shown that while litocholate, taurocholate and taurolitocholate favor the proliferation of cholangiocytes, ursodeoxycholate and tauroursodeoxycholate inhibit proliferation (106). Cholangiocytes also express D2 dopamine receptor, whose activation by quinelorane induces inhibition of secretin-stimulated choleresis by increased PKC-gamma expression and decrease of PKA activity (107).

Recently, we have shown that cholangiocytes express the serotonin 1A and 1B receptors (52). Their activation inhibits the growth of the intrahepatic biliary tree in the BDL model, by activation of the IP_3/Ca^{2+}/PKC signaling pathway. Cholangiocytes are able to secrete serotonin, neutralization of which enhances cholangiocyte proliferation in the course of cholestasis (52).

Also, sensory innervation may regulate the hyperplasia of the biliary epithelium in cholestatic states (108). In fact high levels of the sensory neuropeptide α-CGRP promote biliary hyperplasia in the BDL cholestatic model. *In vitro*, both α and β-CGRP increased biliary proliferation by the

activation of cAMP-dependent PKA (108).

A recent study has also established that cholangiocytes express the neurokinin-1 receptor (NK-1R) which expression is up-regulated following BDL. *In vitro*, Substance P, a member of the tachykinin peptide family, increases cAMP levels, proliferation, and PKA phosphorylation of cholangiocytes (109).

Also, we demonstrated that GABA has heterogeneous effects on the apoptotic, proliferative, and secretory functions of small and large cholangiocytes in BDL rats. In fact we showed that chronic administration of GABA to BDL rats: induces apoptosis, reduces proliferation and decreases Ca^{2+}-dependent adenylyl cyclase 8 (AC8) expression and secretin-stimulated choleresis in large cholangiocytes. Instead after GABA administration: small cholangiocytes *de novo* proliferate leading to an increased number of small ducts; show membrane translocation and phosphorylation of Ca^{2+}-dependent PKCβII; and *de novo* express SR, CFTR, Cl^-/HCO_3^- and AC8, and secrete water and electrolytes in response to secretin. During damage of large cholangiocytes, small ducts replenish the intrahepatic biliary tree by amplification of both Ca^{2+}-dependent signaling and the acquisition of large cholangiocyte phenotypes (57).

Conclusions

In conclusion, as widely demonstrated, during chronic liver diseases, cholangiocytes are not quiescent but strongly activated cells constituting a neuroendocrine compartment, which is regulated by several and different factors including hormones, neuropeptides, cytokines, and growth factors. This neuroendocrine compartment produces and secretes several factors through which it regulates its own function and proliferation and also those of cells, which constitute the surrounding environment. Understand how to manage the neuroendocrine compartment and the several factors that this compartment is able to produce and secrete can be very important for the comprehension of progression of cholestatic liver diseases.

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