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Biliary Epithelium: A Neuroendocrine Compartment in Cholestatic Liver Disease

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Abstract

Hepatic fibrosis is characterized by abnormal accumulation of extracellular matrix (ECM) that can lead to ductopenia, cirrhosis, and even malignant transformation. In this review, we examine cholestatic liver diseases characterized by extensive biliary fibrosis such as Primary Sclerosing Cholangitis (PSC), Primary Biliary Cholangitis (PBC), Polycystic Liver Disease (PLD), and MDR2$^{-/-}$ and BDL mouse models. Following biliary injury, cholangiocytes, the epithelial cells that line the bile ducts, become reactive and adopt a neuroendocrine phenotype in which they secrete and respond to neurohormones and neuropeptides in an autocrine and paracrine fashion. Emerging evidence indicates that cholangiocytes influence and respond to changes in the ECM and stromal cells in the microenvironment. For example, activated myofibroblasts and hepatic stellate cells are major drivers of collagen deposition and biliary fibrosis. Additionally, the liver is richly innervated with adrenergic, cholinergic, and peptidergic fibers that release neurohormones and peptides to maintain homeostasis and can be deranged in disease states. This review summarizes how cholangiocytes interact with their surrounding environment, with particular focus on how autonomic and sensory regulation affects fibrotic pathophysiology.

Introduction

Cholangiocytes are specialized epithelial cells lining the intra and extrahepatic bile ducts that function to modify bile composition through water and electrolyte secretion and absorption and to detoxify xenobiotics. Bile is initially secreted by the apical surface of

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hepatocytes into specialized interstitial spaces called bile canaliculi that are sealed by tight junctions. Bile flows towards the portal tracts where it first enters the small intrahepatic biliary tree lined with “small” cholangiocytes (~ 8 μm) at the Canals of Herring. The biliary tree becomes progressively larger to form large intrahepatic bile ducts, lined by “large” cholangiocytes (~ 15 μm), and ultimately extrahepatic bile ducts empties bile into the duodenum [1, 2]. Large and small cholangiocytes, lining large and small bile ducts, respectively, exhibit distinct morphological, functional, and proliferative features that vary based on the disease state [1–3].

Disease targeting cholangiocytes, termed cholangiopathies, lead to cholestasis, increased biliary pressure, biliary fibrosis, and chronic inflammation that can trigger cirrhosis or malignant transformation [4]. Cholestasis refers to the accumulation of bile in hepatic tissue following intrahepatic or extrahepatic biliary obstruction. Extrahepatic obstruction can be caused by gallstones, pancreatic ductal adenocarcinoma, strictures, or cholangiocarcinoma, whereas intrahepatic biliary diseases include Primary Biliary Cholangitis (PBC), Primary Sclerosing Cholangitis (PSC), and polycystic liver disease (PLD) [5]. Cholangiopathies are commonly characterized by four main stages of disease. Disease progression begins with portal hepatitis and inflammation with bile duct destruction. This is followed by periportal hepatitis and biliary proliferation, which can progress to fibrous septa or bridging necrosis in the liver and eventually stage four, cirrhosis [6]. During late stages of disease, the balance between proliferation and cholangiocyte death is disturbed, leading to ductopenia and further biliary fibrosis [7]. However chronic inflammation can also induce cholangiocyte hyperplasia resulting in an increased risk for malignant transformation, especially in primary sclerosing cholangitis [4]. Symptoms of cholangiopathies are reflective of the cholestatic process and eventual loss of liver function including fatigue, pruritus, portal hypertension and xanthomas [6]. Current treatment options are limited to ursodeoxycholic acid supplementation, providing the best improvement in PBC patients[7].

Cirrhosis is end-stage, irreversible liver scarring and the leading cause of liver failure for which the current mainstay of treatment is liver transplant. There is about an 80% mortality rate without transplant once full hepatic failure ensues [58]. Major consequences of cirrhosis include portal hypertension and its effects such as ascites, variceal bleeding, hepatic encephalopathy and renal failure [9]. Liver Fibrosis or scar formation is characterized by abnormal accumulation of extracellular matrix and further progression to cirrhosis involves diffuse scarring with dense fibrous septations around regenerating hepatocytes [5]. The onset of liver fibrosis begins with injury or insult to either the hepatic parenchyma or the biliary epithelium, as seen in cholestatic disease. In the face of chronic injury hepatocyte regeneration is no longer sufficient and ductular proliferation of intrahepatic bile ducts takes place. An increased population of cholangiocytes accumulates at the border of the bile ducts and hepatic parenchyma, contributing to the progression of liver fibrosis through the recruitment of fibrogenic cells [75]. The etiology of injury plays a role in the cirrhotic pattern, with biliary fibrosis inducing toxic bile acid accumulation that leads to inflammation and activation of cholangiocytes and myofibroblasts that cause a portal-portal fibrotic picture [9].

The aim of this paper is to summarize the new and current research on the proliferative, neuroendocrine and secretory effects of cholangiocytes during cholestatic liver injury and the role they may play in initiating liver fibrosis.

Pathobiology of Liver Fibrosis

Microenvironment

The anatomical features and the microenvironment surrounding cholangiocytes play an important role in its pathophysiology. Bile ductules are formed by 4–5 ~8 μm diameter cuboidal-shaped small cholangiocytes [1, 3], whereas large, columnar-shaped cholangiocytes (~15 μm diameter), make up the increasingly larger bile ducts [1, 3]. The apical side of cholangiocytes facing the lumen possess single, primary cilia that is used to both sense the composition of passing bile and to physically help push bile along [5]. Additionally, cholangiocytes are linked by tight junctions to prevent backflow of water, solutes, and/or toxins. The epithelial barrier can become leaky over the course of injury and lead to the regurgitation of toxic substances back into the hepatic parenchyma.

Cholangiocytes sense and respond to their surrounding microenvironment to maintain homeostasis. Biliary epithelium sits atop a basement membrane that separates it from a matrix of proteoglycans and fibrous proteins produced and maintained by portal mesenchymal and fibroblast cells [10]. Proteoglycan fibers hold a large amount of water taking on a gel-like consistency that gives tissue the ability to withstand compressive forces. In addition, they also store signaling ligands for TGF-β and Wingless (Wnt) pathways that are released following injury or changes in the microenvironment such as mechanical stress or proteolytic cleavage [11]. In contrast, fibrous proteins are more rigid and provide resistance to stretching forces. The major family of fibrous proteins found in the extracellular matrix is collagen (which causes fibrosis when overproduced in disease states) however other fibrous proteins are important as well including fibronectin, elastin, and laminin [10]. While collagen and elastin provide tensility and strength to biliary architecture, a specialized membrane-embedded surface receptor, integrin, links cytoskeleton machinery to the ECM [12]. Integrins are responsible for both attaching to and migrating through the ECM as well as initiating intracellular signaling in response to extracellular stimuli. Integrins can group together to form focal adhesions to anchor cells in the ECM. However, focal adhesion kinases (FAK) can cross phosphorylate integrin-fibronectin associated structures and cause dissociation. Focal complexes are temporary, sparsely located integrin complexes that form when the cell is migrating through the ECM. The main type of integrin expressed in biliary epithelium is αvβ6 subtype (Figure 1) [13].

Cholangiocyte physiology

The main physiological role of cholangiocytes is to modify the passage of bile with water and electrolytes and to nullify xenobiotics and microbiota [7, 14]. Bile salts are secreted by salt transporters on the apical portion of cholangiocytes and are recycled via the enterohepatic system [15]. Bacteria can be recycled from the large intestine along with modified bile acids and come into contact with cholangiocytes, which express toll-like receptors and pathogen-associated molecular patterns [16]. In response, cholangiocytes secrete large amounts of IgA

and pro-inflammatory cytokines to recruit inflammatory cells and clear unhealthy bacterial presence [16]. Following food intake, the hormone secretin is released from S cells of the duodenum as well as cholangiocytes themselves [17]. Table 1 provides a complete list of signaling in cholangiocyte pathophysiology.

In response, large cholangiocytes, but not small, have enhanced intracellular cAMP level and activated the apical AE2 channel which exchanges bicarbonate anions into the lumen for chloride anion. This layer of bicarbonate anions is referred to as an “umbrella” and protects the biliary epithelium from the harsh acidity and other toxic components of bile acids [18]. Coincidentally, this mechanism can also be stimulated by acetylcholine released from vagal innervation. Secretin also downregulates miR-125b and let7a, which reduces the expression of VEGF and NGF, respectively, to increase biliary proliferation [17]. Secretin also drives TGF-β-1/TGF-β-1R autocrine signaling loop in MDR2$^{-/-}$ mouse models and human PSC cases [19]. The actions of secretin are countered by somatostatin, which downregulates cAMP, and gastrin, which downregulates expression of secretin receptor itself.

Much of these processes are regulated by bile acid sensing within the cholangiocytes. Surface receptor TGR5, and nuclear farnesoid X receptor (FXR) sense accumulation of bile acids and respond accordingly. TGR5 responds to bile acid sensing by increasing intracellular cAMP thus bolstering the bicarbonate umbrella, by modulating cholangiocyte proliferation and by exerting anti-inflammatory effects [2021]. FXR agonism has similar outcomes. TGR5 agonism and increased cAMP signaling is one mechanism of cholangiocyte hyperproliferation following cholestatic liver injury [22]. For example, TGR5 is overexpressed in polycystic liver disease (PLD) and blocking it with an antagonist reduces proliferation and cyst size [22]. This outcome is logical as somatostatin, which counteracts secretin signaling and is a cAMP downregulator, is the only currently available therapy for PLD.

Proliferation

Following cholestatic liver injury, cholangiocytes become reactive and adopt a neuroendocrine-like phenotype by secreting and responding to a number of peptides in both autocrine and paracrine fashions. Almost all models of biliary injury trigger cholangiocyte proliferation, a process deemed “ductular reaction.” There are multiple cell types involved in this process, and the type of liver injury determines how ductular reaction takes shape. For example, following bile-duct ligation (BDL) a mouse model for extrahepatic cholestasis, large cholangiocytes respond by undergoing mitosis and proliferating while small cholangiocytes transdifferentiate into large cholangiocytes. Distinct signaling mechanisms regulate these disparate outcomes. In this model, large cholangiocytes respond to increased secretin-secretin receptor cognate interactions to boost intracellular cAMP levels and trigger the PKA/Src/MEK/ERK1/1 pathway [23]. In contrast, small cholangiocytes are characterized by activation of the IP_3/Ca^{2+}/calmodulin pathway [24]. Since new ducts arise from pre-existing ducts, growth is usually constrained to portal areas of injury. However, some cholangiopathies, including PSC and PBC, can involve progenitor cell populations to grow *de novo* bile ducts. These progenitor cells, sometimes termed “oval” cells, are located in the

Canals of Herring where hepatocytes that form bile canaliculi meet with small cholangiocytes of the terminal bile ductules.

The Neurohormonal and Mechanical Basis for Hepatic Fibrosis

A key step in the initiation of liver fibrosis is activation of cells that transform into myofibroblasts [9]. In certain models of biliary injury, activated cholangiocytes can recruit inflammatory cells and myofibroblasts to the site of injury where they secrete ECM components and pro-inflammatory, pro-fibrotic cytokines that causes scarring and fibrosis [5, 8]. Several sources of myofibroblasts have been identified, including hepatic stellate cells (HSCs) and portal fibroblasts. While epithelial cells (i.e. cholangiocytes) may not undergo epithelial to mesenchymal transition (EMT) based on some reports, they can assume a pro-fibrogenic, non-cuboidal phenotype [25].

TGF-β plays a major role in fibrosis, proliferation, EMT, and ECM turnover. TGF-β lies latent within the extracellular matrix until it is activated by mechanical stress and/or proteolytic cleavage in response to injury or cellular signaling. Once it binds to its cognate receptor (TGF-β receptor), it induces phosphorylation of its associated transcription factor, SMAD. Phosphorylated SMADs then translocate to the nucleus where it controls gene expression. A direct outcome of TGF-β signaling is increased expression, synthesis, and deposition of ECM such as collagen, fibronectin, and proteoglycans. Furthermore, TGF-β inhibits matrix metalloproteinases (MMP) and increases the activity of tissue inhibitors of proteinases (TIMPS) to decrease ECM breakdown. [58] An imbalance between MMPs and TIMPS is a major characteristic of fibrosis, with increased TIMP activity greatly increasing ECM deposition.

Recently, the role of mechanical-induced stress and TGF-β activation, and subsequent fibrosis, has been clarified through its relationship with αvβ6 integrin. As mentioned above, integrin-fibronectin complexes can be activated by increased mechanical stress. Increased binding affinity of αvβ6 integrin associates with latency-associated peptide, which releases latent TGF-β and triggers SMAD phosphorylation and nuclear translocation. Wang et al demonstrated that αvβ6 integrin expression is highly upregulated in cholangiocytes following acute biliary obstruction, and that lack of αvβ6 integrin expression ameliorated fibrotic phenotype. Additionally, αvβ6 integrin is implicated in potential cholangiocyte EMT, presumably towards pro-fibrotic fibroblasts [1326].

Angiogenesis and sinusoidal remodeling also accompanies fibrogenesis with an increase in pro-angiogenic molecules such as vascular endothelial growth factor (VEGF). Angiogenesis occurs along with ductular reaction in order to meet the increased nutritional needs of the proliferating epithelial cells. When cholangiocytes proliferate, VEGF-induced angiogenesis of the peribiliary plexus (PBP) takes place. Cholangiocytes secrete VEGF, allowing for an autocrine regulation of cholangiocyte proliferation and a paracrine regulation of PBP proliferation. [27] VEGF also plays a role in fibrosis, acting to activate HSC and collagen production. Other players in liver fibrosis include Kupffer cells and inflammatory cells such as lymphocytes and mast cells that release pro-fibrogenic molecules [5].

Studies have demonstrated that it is possible to attenuate liver fibrosis, depending on the level of progression. [9] Scar formation can be reversed if the insulting stimulus is removed. HSCs can cease to drive fibrotic reaction if they are inactivated by apoptosis, senescence, or reversion back to their pre-myofibroblast phenotype and by the degradation of collagen via matrix metalloproteinases. [5825]. Future therapies aimed at inhibiting pro-fibrotic and/or stimulating anti-fibrotic pathways have the potential to ameliorate difficult-to-treat diseases such as PSC or PBC and pre-empt the need for liver transplantation.

The Role of the Neuroendocrine System

Structure and Function of Hepatic Innervation

The unique neural environment of the hepatobiliary system has been extensively studied in the setting of hepatic fibrosis. The liver contains both efferent and afferent nerves that can be influenced by catecholamines, acetylcholine and neuropeptides. Furthermore, these nerves possess specific locations and differing densities throughout the hepatic architecture. Efferent nerves originate from hypothalamic nuclei and control autonomic output to the liver. Afferent sensory nerves travel either via the vagal pathway to sense and relay information about circulating cytokines and metabolites, or the lower thoracic pathway to relay pain information to the CNS. [28] The nerves follow the pathway of hepatic vasculature within the portal triad. Thus, both hepatic innervation and blood supply are in close contact with biliary microenvironment and ECM. Human liver sections have shown increased intralobular innervation compared to non- human subjects [29].

The autonomic nervous system plays a complex role in liver function including glucose metabolism, fluid balance, regulation of blood and bile flow as well as hepatic regeneration and fibrosis [30]. It functions as a two-neuron system consisting of a preganglionic and postganglionic neuron. Sympathetic and parasympathetic nerves both release acetylcholine from preganglionic neurons that synapse with nicotinic acetylcholine receptors on postganglionic nerves.

Sympathetic Nervous System

The sympathetic efferent autonomic innervation originates from the celiac or mesenteric ganglion and release norepinephrine in the liver that interacts with adrenergic receptors. Adrenergic receptors consist of α-1, α-2, β-1, β-2 and β-3 subtypes, each with different receptor mechanisms and downstream effects. A-1 receptors are G protein coupled receptors that activate phospholipase C and intracellular calcium signaling. A-2 receptors inhibit adenylyl cyclase and lower intracellular cAMP levels whereas β-1, β-2 and β-3 receptors stimulate adenylyl cyclase and intracellular cAMP levels.

The sympathetic nervous system and its role in hepatic fibrosis have been well characterized. HSCs express adrenergic receptors and secrete and respond to catecholamines, such as norepinephrine, in an autocrine fashion to increase proliferation [31]. α-1 adrenergic receptor stimulation increases collagen deposition from HSCs, presumably in a calcium signaling-dependent manner [28]. The total number of HSCs and activated HSCs, as well as liver fibrosis progression, was reduced by chemical sympathectomy and treatment with

adrenergic blocking drugs[3233]. Furthermore, cholangiocytes express all adrenergic receptor subtypes and activation of α-1 receptors by catecholamines stimulate the growth of small cholangiocytes by activating the IP_3/Ca^{2+}/calmodulin pathway [34].

The "Local RAS"

The sympathetic nervous system is amplified during times of stress with many downstream effects, including influences on the renin-angiotensin-aldosterone system (RAS) due to its strict control over renin production. The RAS is responsible for regulating blood pressure and maintaining fluid homeostasis. Decreases in kidney perfusion or direct sympathetic stimulation via B2 adrenergic receptors are potent stimuli for renin release. Renin converts angiotensinogen to angiotensin I in the liver, with subsequent conversion to angiotensin II via the ACE converting enzyme in the lungs. Angiotensin II has many important downstream effects, including regulation of fluid balance by stimulating aldosterone release, increasing tubular reabsorption of sodium, stimulating release of arginine vasopressin and acting as a vasoconstrictor [35]. Angiotensin II mediates it effects through two receptor types, AT1 and AT2. The regulatory mechanisms of fluid balance including sodium balance, vasopressin and aldosterone release, and vasoconstriction are mediated by the AT1 receptor. The AT1 receptor has also been implicated to be involved in pro-fibrogenic mechanisms such as cell proliferation, hypertrophy and endothelial dysfunction [36].

The fibrotic effects of the RAS are mediated by a "local RAS" consisting of functional components of the RAS system found to be up-regulated in organs undergoing fibrotic change. In the liver, RAS system components are expressed by hepatic stellate and Kupffer cells. Activation of the AT1 receptor in hepatic stellate cells increases TGF-β1 expression and consequently hepatic fibrosis [37]. Cholangiocytes express local RAS components as well and its expression is increased in the cholestatic BDL mouse model. Biliary mass, proliferation, and fibrosis increases following AngII treatment but is reduced with losartan treatment, an AT1R antagonist. *In vitro*, AT1 receptor activation increases cholangiocyte proliferation in a PKA/ERK1/2/pCREB-dependent fashion[38]. In $MDR2^{-/-}$ mice, a model of PSC, treatment with propranolol, a non-selective β-adrenergic blocker, decreased hepatic inflammation and fibrosis, including expression of angiotensin [39].

AVP

Arginine vasopressin (AVP), a neurohormone released by the posterior pituitary, is increased in times of pain, stress, trauma, and increased concentrations of angiotensin II. The main role of AVP is to maintain fluid homeostasis in response to changing plasma osmolarity and effective arterial volume [35]. Its effects are regulated by two G protein coupled receptors V1 and V2. V2 receptor is upregulated in liver samples from both BDL mice *and* human polycystic disease. Furthermore, *in vitro* stimulation of AVP increased cellular proliferation and cAMP levels in both small cholangiocytes and cell lines taken from cystic biliary epithelium [40]. Given that the biliary epithelium in both BDL mouse models and human polycystic liver disease experience increased mechanical stress, and AVP functions in part to regulate the mechanical properties in its target organ, the role of AVP in response to increased biliary mechanical stress remains to be elucidated.

Parasympathetic Nervous System

The parasympathetic nervous system signals the liver through the vagus nerve and releases acetylcholine that reacts with both nicotinic *and* muscarinic acetylcholine receptors. Muscarinic acetylcholine receptors are metabotropic G-protein coupled receptors composed of five subtypes, M1–M5, which vary in tissue location [41]. Nicotinic acetylcholine receptors (nAChR) are ionotropic receptors composed of a 4 subunits around a central pore [42]. Cholangiocytes primarily express M3 muscarinic receptors and α7 nicotinic receptors (α7nAChR) [43]. Acetylcholine is quickly degraded by acetylcholinesterase upon release. Nicotinic receptors, but not muscarinic receptors, can also be stimulated by nicotine, the principal addictive component of tobacco smoke. In contrast to acetylcholine, nicotine cannot be degraded by acetylcholinesterase and has an approximate half-life of two hours. In addition, nicotine's lipophilic profile allows for its accumulation in tissue [44]. Cholinergic stimulation appears to have a pro-proliferation, pro-survival impact on biliary growth. BDL mice who underwent vagotomy exhibited decreased biliary mass and M3 muscarinic receptor expression as well increased cholangiocyte apoptosis. In addition, deactivated cAMP signaling leads to a decrease in ductal secretion [45]. HSCs also express muscarinic receptors and activated HSCs upregulate the M3 subtype. HSCs secrete and respond to acetylcholine in an autocrine and paracrine fashion to increase their proliferation and expression of fibrotic markers albeit in a PI3-K and MEK dependent fashion [46].

Nicotine has been shown to be involved in many pro-fibrotic mechanisms, including initial damage to epithelium and contributing to the development of fibrosis through recruiting inflammatory mediators, producing reactive oxygen species and activating the cells responsible for collagen deposition [47]. Nicotine has been shown to up-regulate fibrosis in the hearts of rat embryos and decrease cardiac function as seen by decreased blood volume pumped from the left ventricle and ejection fraction [48]. Female mice treated with nicotine showed significantly increased heart and liver weight with fat deposition around portal veins and increased necrosis, congestion and fibrosis [49]. Nicotine also causes increased activation of HSCs and pro-fibrotic players such as TGF-B and collagen 1-α-2, correlating with increased liver fibrosis [50].

Nicotinic receptors can be classified into neuronal and muscle subtypes. Genome studies demonstrated that neuronal subtypes have been found in non-neuronal tissues including the lung, liver, colon and intestine [51]. The neuronal α7nAChR is expressed in macrophages, HSCs, and cholangiocytes and is implicated in inflammation and fibrosis [5250]. Nicotine administration to xenograft cholangiocarcinoma (CCA) mouse models increased CCA proliferation *and fibrosis* in an α7nAChR-dependent manner [53]. Furthermore, chronic nicotine exposure in rats increased biliary proliferation and fibrosis also in an α7nAChR-dependent manner. Specific α7-nAChR agonism acts through the Ca^{2+}/ERK1/2 pathway [54]. More studies need to be done in order to categorize other nicotine receptors found in the hepatobiliary system and their functions, as well as whether inhibition of the α 7-nAChR can attenuate proliferation and fibrosis.

The Role of Neuropeptides and Neurohormones

During cholestatic injury, cholangiocytes secrete and respond to neuropeptides and neurohormones, adopting a neuroendocrine phenotype. This is demonstrated by cholangiocyte expression of neuroendocrine cell markers such as chromogranin A and S-100. Neuropeptides are short chain polypeptides that function as neurotransmitters or neurohormones. They can be secreted from peptidergic nerves but are often secreted along with epinephrine or acetylcholine from adrenergic and cholinergic fibers, respectively. Nerve fibers that contain peptides are found in association with hepatic vasculature and bile ducts, including the portal vein and hepatic artery. [29] Neuropeptides serve many functions including vasoconstriction, vasodilation and functioning in efferent sensory pathways with current research focusing on their role in cholangiocyte proliferation. The role these markers play in proliferation, fibrosis, and progression of cholestatic disease is a subject of active research [55].

Melatonin

Melatonin is a neurohormone synthesized in the pineal gland by serotonin N-acetyltransferase (AANAT). It is secreted in bile in high concentrations, indicating a role in the hepatobiliary system. It is thought that it provides antioxidant effects in the hepatobiliary tract by neutralizing reactive oxygen species [56]. Synthesis of melatonin is normally increased after prolonged exposure to darkness. In mouse models of BDL and PSC, prolonged exposure to darkness increased melatonin synthesis to reduce biliary hyperplasia and liver fibrosis [5758]. Directly enhancing levels of AANAT and melatonin also attenuate liver fibrosis, with decreased biliary proliferation, improved liver morphology and a decrease in pro-fibrotic cytokines [57]. Melatonin acts on melatonin receptor type I (MT1) and downregulates cAMP/PKA levels to reduce biliary proliferation and ductular secretion [59]. The role of other melatonin receptors (MT2 and MT3) on biliary pathophysiology remains unclear. Melatonin modulates angiogenesis following cholestatic liver injury and biliary proliferation through control of VEGF levels. Increased angiogenesis plays a crucial role in cholangiocyte proliferation, as increased mass leads to increased nutritional needs. The proliferation of the peribiliary plexus meets these needs by responding to pro-angiogenic factors secreted by proliferating cholangiocytes, such as VEGF. Melatonin plays an anti-angiogenic role, as increases in VEGF were seen when AANAT expression was decreased [60]. Furthermore, the reduction in angiogenesis is paralleled by decreased liver fibrosis as well. In mouse models of PSC, miR200b antagonizes AANAT and melatonin expression leading to increased biliary proliferation, angiogenesis, and fibrosis. Overexpressing AANAT or inhibiting miR-200b ameliorated these processes [58].

A-CGRP

A-calcitonin gene-related peptide (α-CGRP) is a 37-amino acid neuropeptide that acts through G protein coupled receptor calcitonin-like receptor (CLR) to upregulate cAMP/PKA/CREB activity. It is most commonly found in capsaicin-sensitive dorsal root ganglia of spinal afferent nerve pathway and thus plays a role in sensing pain. It is also released into peripheral organs in response to stimuli to regulate vasodilation and inflammatory cytokines. In the liver, CGRP-positive nerves are located primarily in the

periportal areas in close complex with hepatic vasculature and biliary tree. CLR expression was demonstrated in hepatocytes, Kupffer cells, and B-lymphocytes. $CGRP^{-/-}$ mice exhibited increased hepatic damage in immune-mediated liver injury. Exogenous α-CGRP administration rescued this phenotype and reduced hepatic levels of IL-6 and TNF-α [61].

Additionally, CLR expression was measured in isolated cholangiocytes, and α-CGRP was found in cholangiocyte supernatant, implying the prospect of α-CGRP regulation of biliary growth in an autocrine and paracrine loop. Paradoxically, both exogenous administration of CGRP *and* CGRP−/− mice exhibited decreased cholangiocyte proliferation following BDL [62]. This is in line with previous reports that α-CGRP stimulation can have opposite effects on macrophage activation [61]. CGRP plasma levels increase following BDL, and pretreatment of *in vitro* cholangiocytes with CGRP attenuated the pro-proliferative effects of BDL supernatant [62]. While CGRP plays a role in cholangiocyte proliferation, it clearly must also work in concert with other neuropeptides.

Substance P

Substance P (SP) co-localizes with CGRP within the spinal afferent nerve pathway and the periportal nerve plexus and is often released alongside CGRP to mediate hepatic vasodilation [63]. SP is part of the tachykinin family and specifically binds to the tachykinin receptor neurokinin-1 (NK-1R). SP is elevated following cholestatic liver injury and drives large cholangiocyte proliferation in a cAMP/PKA/ERK1/2 dependent manner. Loss of NK-1R reduced biliary hyperplasia and expression of fibrotic markers following BDL while increasing biliary apoptosis [64]. Normal $NK\text{-}1R^{-/-}$ mice also exhibited increased hepatocyte apoptosis and elevated serum transaminases indicating liver damage. Similarly to CGRP, Kupffer cells express NK-1R and respond to SP stimulation by secreting increased levels of IL-6 and TNF-α [65]. The SP/NK-1R axis is activated in human PSC samples and is a driver of liver fibrosis in the setting of cholestatic disease. While SP may increase biliary proliferation and ductular mass in normal setting, activating the SP/NK-1R axis in cholestatic settings results in increased large cholangiocyte senescence. Senescence is the process by which cells enter growth arrest and secrete inflammatory cytokines and fibrogenic markers. Additionally, NK-1R is expressed on HSCs and secrete and respond to SP in an autocrine/paracrine fashion to drive proliferation and activation while decreasing senescence. Blocking NK-1R reversed large cholangiocyte senescence while provoking HSC senescence and reduced hepatic fibrosis [66].

Neuropeptide Y (NPY)

NPY is a neuropeptide that is richly expressed in hepatic nerves and counteracts the vasodilatory effects of SP and CGRP [67]. Cholangiocytes and HSCs express at least some of the Y1–Y5 receptors. NPY and Y2R cognate interaction activates HSCs into a myofibroblastic state and increases proliferation and production of fibrogenic factors [3168]. While cholangiocytes secrete NPY into their supernatant, NPY actually has an anti-proliferative effect on biliary epithelium. NPY secretion decreases following BDL and exogenous NPY administration decreased biliary duct mass [69]. Additionally, NPY is upregulated in CCA models and slows tumor growth and invasion in a IP_3/Ca^{2+}/PKC-dependent manner [70].

Histamine

Histamine, an aminergic molecule, is produced by histidine decarboxylase (HDC) in pre-formed granules and most abundantly carried by mast cells. Mast cell activation includes, but is not limited by, G-protein coupled histamine receptors 1–4 (H1–4R) binding which causes intracellular signaling changes and degranulation, or release, of histamine. Small cholangiocytes proliferate following H1R activation in a IP_3/CaMK I/CREB dependent pathway whereas large cholangiocytes proliferate following H2R activation through cAMP upregulation. H3–4R are thought to be anti-proliferative. Following cholestatic liver injury, mast cells are recruited to the liver and hepatic and serum histamine levels increase. Animal knockout models or cromolyn sodium treatment that reduces mast cell recruitment and histamine levels reduces biliary growth, fibrosis, liver damage and angiogenesis[71]. It is important to note that mast cells release other factors such as TGF-β1 and VEGF that can affect biliary growth, fibrosis, and angiogenesis[72].

Intrahepatic Cholangiopathies

Primary biliary cholangitis is an autoimmune cholangiopathy most common in females over the age of 40 that is characterized by only small intrahepatic bile duct destruction. [5] Presence of antimitochondrial antibodies and reactive t-lymphocytes supports an autoimmune etiology with the characteristic presence of self-reactive cytotoxic T-cells against pyruvate dehydrogenase complex antigens. [73] It is characterized histologically by the florid duct lesion, which involves destruction of interlobular bile ducts often accompanied by granulomas or the absence of bile ducts. [5] Diagnosis requires at least two positive diagnostic criteria, which include a persistent increase in alkaline phosphatase levels for greater than 6 months, a positive anti-mitochondrial antibody titer, or a positive liver biopsy. [74]

In contrast, primary sclerosing cholangitis is diagnosed mostly in men in the third through fifth decades of life. Etiology is unclear but studies have shown that both genetic and environmental factors play a role. The strong association between ulcerative colitis and primary sclerosing cholangitis supports a theory that alterations in the normal gut microbiome may be involved. [75] It is characterized histologically by large duct inflammation that involves infiltration by neutrophils and lymphocytes leading to the development of strictures and eventual scarring. Small duct involvement is often characterized by concentric onion skin fibrosis. [5] Fibropolycystic liver disease is a type of congenital cholangiopathy that includes congenital hepatic fibrosis, biliary hamartomas, autosomal dominant polycystic kidney disease, Caroli disease and choledochal cysts. All are characterized by abnormal embryologic development of the cell layer surrounding the portal vein, which give rise to biliary ducts. Consequences include cholangitis, portal hypertension or possible obstruction. [76] Choledochal cysts often predispose to obstructive liver disease such as gallstones, strictures or stenosis and are characterized by congenital dilation of the common bile duct and increased pressure across the biliary tree that can present with symptoms such as jaundice or biliary colic [5].

Conclusion and future perspectives

This review focuses on the role of sensory innervation in cholestatic liver disease and the pathophysiology of liver fibrosis. It highlights the complex interplay of cholangiocyte proliferation, fibrosis, and angiogenesis as well its interaction with the surrounding ECM and stromal cell types. The role of mechanical stress in extrahepatic biliary obstruction and PLD remain an area of active research and have the potential to shed new insights into these disease processes. Furthermore, how hepatic innervation and neurohormonal/peptide signaling affects cholangiopathies could lay the groundwork for comprehensive therapies in the future. Deeper understanding of these overlapping and often redundant pathways is necessary in order to make progress on disease management.

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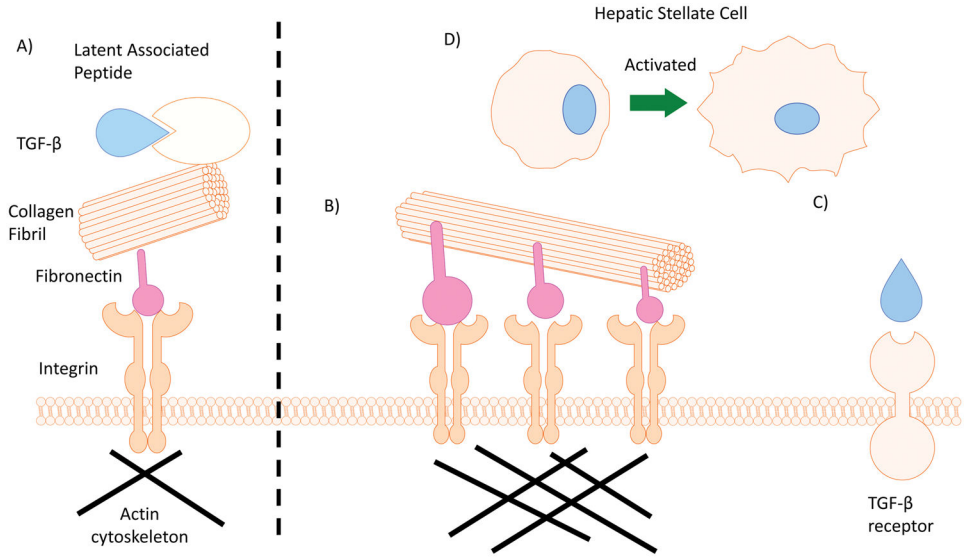

Figure 1.

Table 1

A list of extracellular hepatic neurohormone, neuropeptides, and autonomic innervation signaling with corresponding changes in intracellular signaling and functional outcome.

Extracellular Signal	Intracellular Signal	Functional Outcome
Secretin	↑ cAMP	↑ large cholangiocyte proliferation/fibrosis/ductular secretion
	↑ Ca^{2+}	↑ small cholangiocyte proliferation
Gastrin	↓ cAMP	↓ ductular secretion
TGR5/FXR	↑ cAMP	↑ ductular secretion
α1	↑ PLC/Ca^{2+}	↑ proliferation/activation of HSCs and small cholangiocytes
α2	↓ cAMP	
β 1–3	↑ cAMP	↑ in lammation/fibrosis
AT1	↑ PKA/ERK1/2/pCREB	↑ cholangiocyte proliferation
AVP	↑ cAMP	↑ cholangiocyte proliferation
M3	↑ cAMP	↑ cholangiocyte proliferation
	↑LJ3K/MEK	↑ HSC proliferation
α7nAChR	↑ Ca^{2+}/ERK1/2	↑ cholangiocyte proliferation
Melatonin-MT1	↓ cAMP/PKA	↓ proliferation/ductular secretion
α-CGRP	↑ cAMP/PKA/CREB	↓ in lammation
SP	↑ cAMP/PKA/CREB	↑ cholangiocyte proliferation/fibrosis ↓ apoptosis
NPY	-	↑ HSC/myo ibroblast proliferation/activation
	IP3/Ca^{2+}/PKC	↓ cholangiocyte proliferation
H1	IP3/CAMK1/CREB	↑ small cholangiocyte proliferation
H2	↑ cAMP	↑ large cholangiocyte proliferation