Autocrine regulation of biliary pathology by activated cholangiocytes

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The liver is comprised of two epithelia cell types: hepatocytes, which initiate secretion of bile at the bile canaliculus, and cholangiocytes, which line the bile ducts and modify ductal bile during transport to the duodenum in response to a series of spontaneous and hormone-regulated events (3, 53). The biliary system, which is lined by cholangiocytes, forms a three-dimensional network extending from the proximal branch called the canals of Hering to the extrahepatic ducts (4, 5, 54). The canals of Hering are lined by both cholangiocytes and hepatocytes along with bipotential hepatic progenitor cells (103, 107), which bridge the bile canaliculus with bile ductules that merge to form interlobular ducts that continue merging to form the ducts of larger sizes. Cholangiocytes possess specific surface-transport systems for secreting a large number of substrates such as electrolytes and bicarbonate. A number of factors have been shown to play key roles in the regulation of ductal secretion such as the autonomic nervous system, gastrointestinal hormones, and peptides (9).

In the liver, only cholangiocytes express the secretin receptor (SR) (7). The biological action of secretin on cholangiocytes occurs via a series of coordinated events (3, 5, 53). First, secretin binds to the basolateral SR of cholangiocytes causing an adenyl cyclase-dependent increase in cAMP levels and activation of protein kinase A (PKA) (5, 53). Second, PKA phosphorylates the cystic fibrosis transmembrane conductance regulator at the apical membrane of cholangiocytes triggering the release of Cl− (6, 53). The resulting Cl−/HCO3−/anion exchanger 2 to secrete bicarbonate into ductal bile (31, 53). Additionally, cAMP contributes to Cl− conductance through exchange proteins activated directly by cyclic AMP, which is a PKA-independent pathway (74).

Cholangiocytes in the adult liver are normally mitotically dormant (1). Constitutive expression of proteins involved in cell cycle, such as p27 and members of the Bcl-2 family of proteins have been shown to be important for holding cholangiocytes in a resting state (46, 101). The importance of cAMP signaling in the regulation of cholangiocyte proliferation was evidenced by administration of forskolin (an adenylate cyclase activator) to rats. In this study, forskolin increased the number of bile ducts, CAMP levels, and provided the first evidence for the cAMP-dependent signaling pathways plays also a key role in pathologies such as autosomal recessive polycystic kidney disease through exchange proteins activated
directly by cyclic AMP and PKA-dependent mechanisms (13). In addition, many forms of cell damage, disruption of cell matrix, or release of cytokines may trigger proliferation by evoking cAMP, phosphoinositide 3-kinase (PI3K)/AKT, Src and Ca\(^{2+}\) signaling pathways (13, 32). A summary of the molecular pathways regulating cholangiocyte proliferation is illustrated in Fig. 1.

Cholangiocytes are the primary targets of human diseases termed cholangiopathies (4, 59). These diseases can be genetic or acquired and lead to cholestasis, chronic liver injury, and eventually liver failure (4, 59). The progression of cholangiopathies is characterized by enhanced apoptosis and absence of proliferative responses to liver injury (4, 59). Elevated apoptosis and reduced proliferation results in loss of bile ducts (ductopenia), which is a unifying event in cholangiopathies (4, 59). Cholangiocytes respond to injury by activating compensatory proliferative and secretory mechanisms. The proliferative compensatory mechanism opposes cholangiocyte death and sustains bile ducts (61, 67). The increased proliferation of bile ductules, termed “ductular reaction,” together with an epithelial-mesenchymal transition (EMT) and periportal fibrosis leads to cirrhosis (90). It has been suggested that proliferating cholangiocytes contribute to the ductular reaction in the liver by undergoing EMT that is associated with the activation of other cell types in the liver (39). During EMT, mature epithelial cells lose their normal cell-to-cell contacts, which may be necessary for biliary proliferation (i.e., ductular reaction) to occur (39). However, controversy exists for the existence of EMT in biliary epithelia. For example, in animal models of liver fibrosis, lineage tracing did not demonstrate evidence of EMT in cholangiocytes (23, 91). Several studies have shown that cholangiocytes express EMT markers (82, 84, 86). Although, it is clear that cholangiocytes can express putative markers of EMT in models of biliary damage, there

![Fig. 1. Major molecular pathways mediating cholangiocyte proliferation. Regulation of cholangiocyte proliferation occurs through 1) G protein-coupled receptors (GPCR)-induced cAMP production and downstream PKA and/or exchange proteins activated directly by cyclic AMP (EPAC) activation; 2) GPCR activation of Ca\(^{2+}\), or PKC pathway, or inhibition by inositol 1,4,5-trisphosphate (IP\(_3\)) pathway; and 3) tyrosine kinase activation and JAK/STAT or phosphoinositide 3-kinase (PI3K)/AKT pathway. Induction of these pathways can activate transcription factors for genes involved in proliferation and the synthesis of growth factors that participate in an autocrine loop. AC, adenylyl cyclase; LKB1, liver kinase B1.](image-url)
remains uncertainty as to whether these cells undergo full phenotypic change to mesenchymal cell type in pathogenesis of human liver diseases.

A number of animal models of cholestasis and human cholangiopathies have demonstrated neuroendocrine-like changes in cholangiocytes during cholangiopathies (4, 9, 39). These changes represent an adaptive response to biliary damage and are necessary for survival and repair of the biliary tree. However, adaptive changes can be a major determinant for disease progression and the fibrogenic process. Chromogranin A, glycolipid A2-B4, S-100 protein, neural cell adhesion molecule, and neuroendocrine granules are phenotypic markers that have been described in proliferating cholangiocytes that display neuroendocrine phenotypes (9). These activated/proliferating cells with neuroendocrine characteristics secrete factors that affect the progression of biliary diseases. Here, the known autocrine factors and their roles in the progression of biliary disease and fibrosis are reviewed. These autocrine factors are highlighted in Table 1. With the growing numbers of recently identified autocrine factors that participate in the regulation of cholangiocyte proliferation and progression of biliary diseases, we provide a comprehensive review of each factor and the specific mechanisms they regulate.

**Peptide Hormones**

*Secretin/SR axis*. Secretin is a hormone produced by S cells that reside within the crypts of Lieberkun of the duodenum. Secretin stimulates biological actions in multiple organs including the pancreas, stomach, and biliary epithelium. The secretin/SR axis has been shown to play a key role in the regulation of biliary proliferation (7). SR is only expressed by cholangiocytes in the liver, and its expression is closely coupled to both proliferative and secretory activity. SR expression is upregulated during extrahepatic cholestasis induced by biliary duct ligation (BDL) (7). The importance of the secretin/SR axis is highlighted in recent studies that demonstrated that there is a significant reduction of large cholangiocyte proliferation in the SR(−/−) knockout mouse model with BDL (41). These effects were dependent on the activation of the cAMP/PKA/ERK1/2 intracellular signaling pathway (41). In addition, in vitro studies showed that the basal proliferative rate of cholangiocytes was decreased after stable knockdown of SR by short hairpin RNA (41), indicating autocrine regulation of biliary growth by secretin. Preliminary data (S. Glaser and G. Alpini, unpublished observations) from our laboratory demonstrates that cholangiocytes express secretin mRNA and secrete secretin, which indicates a possible autocrine role of secretin in the regulation of biliary function. This autocrine mechanism underscores the fundamental role of secretin as a trophic factor for cholangiocytes and illustrates the importance of autocrine regulation of biliary function in the pathogenesis of cholestatic liver diseases.

*Glucagon-like peptide 1*. Glucagon-like peptide 1 (GLP-1) is secreted by enteroeodocrine L cells of the gastrointestinal (GI) system. The endocrine effects of GLP-1 are stimulated through its interaction with a specific G protein-coupled receptor (GLP-1R). The finding that GLP-1 induces pancreatic ductal cells to acquire a neuroendocrine phenotype is of great interest as it provides a new function for GLP-1 in differentiation of ductal epithelia (21) and the rationale for evaluating the role of GLP-1 in the regulation of biliary function. A recent study on biliary adaptive responses to cholestasis has shown that cholangiocytes are locally regulated by GLP-1 (70). In BDL rats, GLP-1R was expressed by cholangiocytes and stimulation with GLP-1 activated the P3K, cAMP-PKA, and Ca2+/CamKII pathways. Proliferating cholangiocytes expressed the GLP-1 mRNA transcripts, which were diminished by adding the GLP-1R antagonist exendin 9–39 to the culture medium (70). The study suggests that GLP-1 signaling from the GI tract and continued GLP-1-autocrine signaling by cholangiocytes is a driving factor for the neuroendocrine transdifferentiation of cholangiocytes (70).

### Table 1. Autocrine factors secreted by cholangiocytes and their role in proliferation or fibrogenesis

<table>
<thead>
<tr>
<th>Autocrine Factor</th>
<th>Mechanism/Outcome</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proliferation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Secretin</td>
<td>cAMP</td>
<td>↑</td>
</tr>
<tr>
<td>Glucagon-like peptide</td>
<td>cAMP, Ca2+, P3K</td>
<td>↑</td>
</tr>
<tr>
<td>Leptin</td>
<td>JAK/STAT/ERK1/2</td>
<td>↑</td>
</tr>
<tr>
<td>Insulin-like growth factor 1</td>
<td>P3K</td>
<td>↑</td>
</tr>
<tr>
<td>Progesterone</td>
<td>Ca2+/PKC</td>
<td>↑</td>
</tr>
<tr>
<td>Prolactin</td>
<td>cAMP, JAK/STAT, PKC, Src</td>
<td>↑</td>
</tr>
<tr>
<td>Follicle-stimulating hormone</td>
<td>cAMP, PKC</td>
<td>↑</td>
</tr>
<tr>
<td>Testosterone</td>
<td>cAMP</td>
<td>↑</td>
</tr>
<tr>
<td>Serotonin</td>
<td>Inhibits proliferation through IP3</td>
<td>↑</td>
</tr>
<tr>
<td>Met-enkephalin</td>
<td>Inhibits proliferation through IP3</td>
<td>↑</td>
</tr>
<tr>
<td>Nerve growth factor</td>
<td>P3K, ERK1/2</td>
<td>↑</td>
</tr>
<tr>
<td>CGRP</td>
<td>cAMP</td>
<td>↑</td>
</tr>
<tr>
<td>VEGF</td>
<td>PKC, ERK 1/2</td>
<td>↑</td>
</tr>
<tr>
<td>Fibrogenesis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Renin-angiotensin</td>
<td>TGF-β1, NADPH oxidase, TNF-α, procollagen</td>
<td>↑</td>
</tr>
<tr>
<td>Platelet-derived growth factor</td>
<td>EMT, stellate cell activation</td>
<td>↑</td>
</tr>
<tr>
<td>Transforming growth factor-β</td>
<td>αVβ6 integrin, collagen, laminin, fibronectin, others</td>
<td>↑</td>
</tr>
<tr>
<td>Connective tissue growth factor</td>
<td>TGF-β1, collagen, activates fibroblasts</td>
<td>↑</td>
</tr>
<tr>
<td>Endothelin 1</td>
<td>collagen, apoptosis, decreases VEGF</td>
<td>↑</td>
</tr>
<tr>
<td>Hedgehog</td>
<td>osteopontin, cc116, EMT, activates MF-HSCs</td>
<td>↑</td>
</tr>
<tr>
<td>Substance P</td>
<td>collagen, α-SMA</td>
<td>↑</td>
</tr>
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</table>

CGRP, calcitonin gene-regulated peptide; VEGF, vascular endothelial growth factor; P3K, phosphoinositide 3-kinase; IP3, inositol 1,4,5-trisphosphate; EMT, epithelial-mesenchymal transition; MF-HSCs, myofibroblast-hepatic progenitor cells; SMA, smooth muscle actin.
Leptin. Leptin is a peptide hormone secreted by adipocytes that acts on the hypothalamus to regulate satiety and caloric homeostasis. Serum leptin is increased in obesity and a wide range of cancers including cholangiocarcinoma (33, 109). Studies have shown that the interaction of leptin with its receptor OBRI stimulates the JAK/STAT pathway through STAT3 and downstream ERK1/2, resulting in angiogenesis and cancer growth. The expression of leptin and the leptin receptor was demonstrated in normal cholangiocytes as well as cholangiocarcinoma cells (25). This study demonstrated that leptin stimulates cholangiocarcinoma growth and that local leptin secretion is involved in cholangiocyte growth (25).

Insulin-like growth factor 1. Insulin-like growth factor 1 (IGF1) is a circulating hormone and local growth factor that is secreted primarily from the liver (3). Through its interaction with the specific receptor IGF1-R, IGF1 is important for cell survival and prevention of apoptosis in a number of epithelia. Recent studies exploring the effect of local secretion of IGF1 in bile ducts have demonstrated that selective siRNA silencing of local IGF1 secretion potentiated the cytotoxic effects of the bile acid glycochenodeoxycholic acid more potently than circulating IGF1 (10, 34). Furthermore, IGF1 mRNA was expressed by both cholangiocytes and hepatocytes. A paracrine mechanism may also play a role in injury/repair because both hepatocytes and cholangiocytes secrete and respond to IGF1.

Reproductive and Steroid Hormones

Estrogen. Steroid hormones (i.e., sex hormones) regulate cell growth in a variety of organs. The most well-known of these, estrogen, has been shown to have a profound effect on cell growth. Estradiol (E2), the primary estrogen, targets the uterus, cardiovascular, nervous, and digestive systems (11). Recent studies have shown that estradiol is a mitogen for the liver. One study demonstrated that chronic administration of estradiol causes an increase in total liver mass (27). Another study reported that estradiol caused a rapid but transient growth in the liver and suggested that estrogens stimulate at a converging point for different growth factor signal-transduction pathways such as hepatocyte growth factor, transforming growth factor-α, epidermal growth factor, and acidic fibroblast growth factor (79). However, these mitogenic effects in the liver have not been fully elucidated. Cholangiocytes are among the cells targeted by estrogens as evidenced by studies showing that estrogen affects proliferation, repair, fibrosis, and angiogenesis. Each of these processes is regulated by autocrine/paracrine factors released from cholangiocytes. Data suggest that estrogen is a potent activator of an autocrine/paracrine loop involving vascular endothelial growth factor (VEGF)-A/C (8, 69). An autocrine loop involving nerve growth factor (NGF) and its receptor tyrosine kinase receptor A (TrkA) is also stimulated by estrogens (37). It is likely that other autocrine/paracrine factors are released in response to estrogen, but this remains to be determined.

Progesterone. Progesterone is a steroid hormone secreted by the ovaries, adrenal glands, and the corpus luteum. The two main receptors of progesterone, PR-A and PR-B, are expressed in multiple organ systems such as the reproductive and nervous systems and mammary tract, where they modulate cell growth. A recent immunohistochemical study in paraffin-embedded gallbladder sections has demonstrated that many more patients (46%) with secondary hepatolithiasis show positivity for PR of gallbladder specimens than patients with primary hepatolithiasis (93). Unfortunately, in this study the immunohistochemical expression of PR was not evaluated in healthy control specimens (93). Progesterone has been shown to impair gallbladder emptying in response to cholecystokinin (105). Cholangiocytes are also the target of progesterone. In liver sections from normal and BDL rats, cholangiocytes were positive for PR (38). Chronic administration of progesterone increased the number of bile ducts of normal rats, whereas an antiprogesterone antibody inhibited cholangiocyte hyperplasia during BDL, suggesting that progesterone stimulates cholangiocyte proliferation during disease and nondisease states (38). In addition, cholangiocytes were shown to express the key proteins required for steroidogenesis (StAR, P450sec, and 3β-HSD) and synthesized and secreted progesterone (38).

Prolactin. Prolactin (PRL) is a hormone secreted by the anterior pituitary that promotes cellular differentiation and survival in a number of cell types. PRL receptors are well characterized in the mammary glands and ovaries but are also present in the adrenal glands, gonads, and the central nervous system (CNS). PRL receptors are expressed as either short or long isoforms, having different tissue distribution, and may signal through similar or different STATs. Binding of PRL to its receptors results in dimerization and activation of JAK/STAT-1, MAPK and Src signaling, whereas the long form also is associated with Ca2+. Recent studies by Bogorad et al. (18, 19) have shown that the long isoform of PRL predominates in rat intrahepatic bile ducts and its expression increases under obstructive cholestasis. We have recently shown that normal cholangiocytes express both isoforms (short and long) of PRL, expression that was increased by BDL (102). The difference between the expression of long and short forms of PRL in cholangiocytes is likely due to the different strain of rats used in our studies (female Fischer 344) (102) and the studies (albino mongrel) from Bogorad et al. (18). Administration of PRL stimulated proliferation of normal and BDL female cholangiocytes through a Ca2+/PKC pathway (102). Our studies did not establish which isoform of PRL mediates the stimulatory effects of PRL on cholangiocyte growth (102). Because PRLs activate different signaling pathways (see above), further studies are necessary to pinpoint the specific PRL isoform regulating biliary proliferation and to determine whether these receptor subtypes regulate other biliary functions such as secretion and response to damage. It is also necessary to establish whether these receptors differentially target small and/or large cholangiocytes that function by Ca2+ - and cAMP-dependent mechanisms, respectively (1, 5, 6, 29, 30, 54). Similar to other cell types (44), normal cholangiocytes expressed the message and protein for PRL and secrete PRL that was increased in cholestatic rats. Administration of an anti-PRL antibody to BDL female rats decreased cholangiocyte proliferation (102). Taken together, the data suggest that PRL stimulates biliary growth by autocrine mechanisms. One study found high PRL levels in three of five patients with nonalcoholic chronic liver diseases (50). Understanding how PRL is induced and how it regulates cholangiocyte growth may be useful in the future management and evaluation of various liver diseases and clearly warrants further exploration.

Follicle-stimulating hormone. Another hormone of the anterior pituitary and the central hormone of reproduction is...
follicle-stimulating hormone (FSH). The primary function of FSH is to stimulate germ cells. A recent study has shown that FSH induces cholangiocyte proliferation via an autocrine mechanism (68). The mRNA for FSH was found in cholangiocytes, and the protein was secreted into culture media (68). Administration of anti-FSH antibody to BDL rats decreased ductal mass, and secretin-induced cAMP-dependent phosphorylation of ERK1/2 and ELK-1. There is a striking parallel between the findings that both FSH and PRL regulate cholangiocyte growth in an autocrine fashion. In the normal pituitary gland, autocrine signals from growth factors induce gonadotrophs to produce FSH and lactotrophs to produce PRL. These initial autocrine stimulators include gonadotropin-releasing hormone, angiotensin II, leptin, vasoactive intestinal peptide, and IGF. Intriguingly, most of these autocrine factors are discussed in this review and may act as early factors in the neuroendocrine transdifferentiation of cholangiocytes. We speculate that release of these autocrine factors in a step-wise manner allows new intracellular pathways to be activated and contribute to the growth and antiapoptotic phenotype seen in cholangiocytes in chronic biliary disease and cholangiocarcinoma (Fig. 2).

Testosterone. Testosterone is an anabolic steroid hormone secreted by the testes in males. Testosterone stimulates development of the Wolffian ducts during male embryogenesis and spermatogenesis in male adults through binding to the androgen receptor (AR). ARs are expressed in a number of metastatic adenocarcinomas, hepatocellular carcinoma, and cholangiocarcinoma (47, 81, 94). We recently evaluated the effects of testosterone on cholangiocyte growth in rats and found that testosterone stimulated cAMP-dependent biliary proliferation and secretin-stimulated ductal secretion. Chemical castration using flutamide or immunoneutralization of testosterone with an antitestosterone antibody in vivo reduced testosterone levels and decreased biliary proliferation and intrahepatic bile duct mass (111). In vitro, basal proliferative rates of cholangiocytes were inhibited by flutamide (AR antagonist). Decreased testosterone serum levels have been observed in models of cholestasis, which is thought to be the result of hypotrophy of seminal vesicles (55, 113). In fact, testicular atrophy and lowered testosterone serum levels have been shown in patients with primary biliary cirrhosis (PBC) and cirrhosis (28, 106). In our study, the synthesis of testosterone by cholangiocytes was also assessed. Expression of 17β-HSD3, the enzyme regulating testosterone synthesis, was present in cholangiocytes from rats and in normal rat intrahepatic cholangiocyte cultures in culture, and testosterone hormone was present in the supernatant of cholangiocytes in culture (111). Cholangiocyte expression of 17β-HSD3 increased when testosterone was administered to rats, indicating testosterone may induce its own synthesis via an autocrine mechanism. Increased biliary secretion of testosterone is postulated to compensate locally for lower testosterone levels systemically during cholestasis (111).

Neuropeptides and Neurotransmitters

Serotonin. Serotonin is a neuroendocrine hormone, which in the GI tract acts as a potent messenger to nearby epithelial cells (64). Physiologically, serotonin induces fluid and ion secretion from the intestinal mucosa and potentiates the effect of intestinal hormones on pancreatic secretion (64). Serotonin has been found to modulate cell proliferation in a number of cells including kidney epithelial cells and hepatocytes (12). Serotonin has been shown to abolish fasting-induced bile flow in canine by acting directly on the biliary epithelium (58). We have recently demonstrated the role of serotonin modulation of cholangiocyte proliferation (72). In this study, the receptors for serotonin were expressed on the basolateral domain of cholangiocytes and upregulated in BDL rats (72). Stimulating the serotonin receptor with the serotonin agonists 8-hydroxy-DPAT and aniprotline, decreased cholangiocyte proliferation through increased inositol 1,4,5-trisphosphate, and down-

Fig. 2. Cholangiocyte progression through the course of biliary damage. Cholangiocytes are activated by injury or cholestasis resulting in their proliferation and secretion of primary autocrine factors. Stimulation by these factors invokes a neuroendocrine change and the production of other (secondary) autocrine factors eventually leading to an epithelial-mesenchymal transition. In time, activated cells may progress into cancer.
stream inactivation of the effector molecules, Src and ERK1/2 (72). Strong evidence points toward the role of serotonin in autocrine regulation of biliary function. Serotonin is synthesized and secreted by normal rat cholangiocytes. However, there was an upregulation in serotonin secretion in rats with extrahepatic cholestasis induced by BDL (72). Immunoneutralization of the endogenous serotonin secreted by cholangiocytes using an antiserotonin antibody was shown to enhance the growth of the biliary epithelium (72). These findings suggest that in normal cholangiocytes serotonin has an important role in neutralizing physiological levels of proliferative signals such as secretin and certain bile acids and may curtail the strong proliferative signals from high levels of secretin, bile acids, and growth factors in the cholestatic liver (72). It is worth noting, however, that, although serotonin decreased the proliferation of noncancerous cholangiocytes, the opposite effect was found in cholangiocarcinoma (2). We speculate that cholangiocytes may escape the inhibitory action of serotonin through a change in the expression pattern of serotonin receptor subtypes.

**Opioids.** Endogenous opioids (endorphins, enkephalins, dynorphins, endomorphins) are found in high concentration in the CNS, where they are involved in processes such as pain and addiction, but are also found peripherally (62, 97). In the gastrointestinal tract, endogenous opioids are known to stimulate receptors on a variety of different cell types (49). In fact, studies in the mouse and human have shown that endogenous opioids modulate proliferation in peripheral organs such as pancreas, colon, and bile ducts (71, 114, 115). Furthermore, fetal human and rat livers and the liver of rats with cholestasis express the preproenkephalin mRNA that encodes for the endogenous opioid peptide Met-enkephalin. Also, Met-enkephalin immunoreactivity is detected in hepatocytes and in proliferating bile ductules in cholestatic liver. Nicoll and colleagues (80) suggested that endogenous opioids may have an autocrine effect during cholestasis. A recent study has shown that cholangiocytes secrete and respond to opioids during cholestasis (71). This study demonstrated the presence of three opioid receptors on cholangiocytes: δOR, μOR, and κOR (71). Stimulating δOR strongly diminished proliferation, whereas activation of μOR slightly increased proliferation (71). Additionally, expression of Met-enkephalin was found in the bile of normal rats, and the levels were tripled when cholangiocytes were stimulated with secretin (71). It was suggested that this increase in Met-enkephalin secretion was a regulatory mechanism to limit the excessive growth of the biliary tree by interacting with the δOR (71). The concept that liver cells including bile ducts are sources of endogenous opioids during cholestasis is supported by the fact that hepatic Met-enkephalin immunoreactivity is enhanced in patients with PBC (16). Hepatic concentrations of proenkephalin-derived opioids are also increased in cholestatic rats (17). In cholangiocarcinoma, the autocrine production of endogenous opioids was shown to inhibit the growth and migration of malignant cholangiocytes (73). This study outlined a self-limiting mechanism, whereby the secretion of endogenous opioids protects cholangiocytes from malignant transformation (73).

**NGF.** NGF is a member of the neurotrophin family, which acts on cells expressing the specific tyrosine kinase receptors of the Trk family. NGF is known for its vital role in the maintenance of sympathetic and sensory nerve growth but recently has been shown to regulate proliferation, differentiation, and remodeling in experimental models of various diseases affecting nonnervous tissues (63, 95). NGF is a putative growth factor and acts in an autocrine fashion in tissues such as the pancreas and in a number of cancers (78, 108). During cholestasis, NGF has been shown to modulate the proliferative responses of bile ducts through ERK 1/2 and PI3K pathways (37). Interestingly, stimulating cholangiocyte growth with estrogen had an additive effect on NGF-induced proliferation and increased autocrine signaling of the NGF-TrkA loop (37). Because stellate cells also produce NGF and express TrkA, it is possible that cholangiocytes and stellate cells cross talk through a NGF-paracrine mechanism. NGF-β overexpression is associated with lymph node metastasis and nerve infiltration in human hilar cholangiocarcinoma (110). The paracrine and autocrine roles of NGF have also been suggested in promoting the development of liver cirrhosis and hepatocellular carcinoma (89).

**Sensory neuropeptides.** Autonomic and sensory neural pathways regulate the biliary proliferation and function during cholestatic states (42). Both types of neurons possess efferent functions and can release sensory neuropeptides, calcitonin gene-regulated peptide (CGRP), and substance P (SP), locally in the organs that they innervate (42, 48, 76). In rodent liver, CGRP-positive nerves are present as dense networks in the fibromuscular layer of the biliary epithelium, surrounding the portal vein, and in the stromal compartment of portal areas (43). Circulating levels of sensory neuropeptides such as CGRP and SP are increased in human and animal models of cirrhosis (15, 60). CGRP signals via calcitonin-like receptors (CLR). CLR requires a receptor activity-modifying protein (RAMP) to be functionally active. Both α- and β-CGRP signal through the CLR/RAMP1 receptor. We have recently evaluated the role of CGRP on cholangiocyte proliferation (42). Cholangiocytes expressed α-CGRP, β-CGRP, and CGRP receptor components. CGRP activated proliferation of murine cholangiocytes through cAMP/PKA/CREB-dependent signaling (42). The supernatant of isolated cholangiocytes from 3-day CGRP-KO mice had reduced proliferation compared with wild-type mice, suggesting that CGRP may act via an autocrine mechanism (42). In support of this, a CGRP-receptor antagonist reduced the proliferation of cholangiocytes in culture (42). Another sensory neuropeptide, SP, is a member of the tachykinin peptide family, which is made up by SP, neurokinin A, neurokinin B, neuropeptide K, neuropeptide γ, and hemokinin. The tachykinin receptor family consists of neurokinin-1, -2, and -3 receptors (NK-1R, NK-2R, and NK-3R). SP binds and signals via the NK-1R (40). Our recent study provided evidence that 1) cholangiocytes express NK-1R and 2) the SP-NK-1R axis sustains the proliferation of cholangiocytes by activation of cAMP signaling. Knockdown of the NK-1R gene in cholestatic mice decreases intrahepatic bile duct mass concomitant with reduced PKA phosphorylation compared to controls (40).

**Angiogenic Factors**

**VEGF.** The VEGF family is well known for its role in angiogenesis. Members of this family include the cytokines VEGF-A, -B, -C, -D, -E, angiopoietin-1 (Ang-1), and placenta growth factor. These peptide signals function as mitogens for vascular endothelial cells by regulating vascular dilation, per-
meability, migration, and survival. In the liver, VEGF-A has been shown to prevent bile-duct damage caused by hepatic artery ligation (HAL) (35). We provided the first evidence that cholangiocytes express the message and secrete VEGF-A/C and VEGF-A/C stimulates biliary growth by interacting with VEGF-R2/R3 by activation of autocrine signaling (36). Blocking VEGF expression/secretion in vivo by anti-VEGF-A or anti-VEGF-C antibodies decreased biliary hyperplasia during cholestasis (36). These findings suggest that VEGF mediates the adaptive proliferative response of cholangiocytes to cholestasis (36). In BDL rats, HAL induced the disappearance of the peribiliary vascular plexus, increased biliary apoptosis, decreased cholangiocyte proliferation, and increased cholangiocyte angiogenesis (35). HAL-mediated effects were prevented by administration of recombinant VEGF-A, which suggested that VEGF stimulated biliary growth by an autocrine mechanism (35). Release of VEGF-A from cholangiocytes in primary culture was decreased after HAL in BDL rats, which was restored during VEGF-A administration (35). Furthermore, Fabris et al. (24) have shown that VEGF and Ang-1 supports biliary growth via autocrine mechanisms in cholangiocytes from polycystic liver disease (ADPKD) (24). VEGF stimulated proliferation in cholangiocytes and had a paracrine effect on the portal vasculature (24). It was postulated that this mechanism plays a role in promoting the growth of liver cysts and their vascular supply in ADPKD (24). A recent study has also shown that autocrine and paracrine VEGF signaling promotes the growth of liver cysts in polycystic-2-knockout (Pkd2KO) mice (96). Treatment with the mTOR inhibitor, rapamycin, significantly slowed the progression of cystic disease in Pkd2KO mice by decreased cyst area, and liver/body weight ratio (96).

Autocrine/Paracrine Factors Regulating Biliary Fibrogenesis

Renin-Ang system. The renin-Ang system (RAS) is well known for its functions in regulating blood pressure, electrolyte balance, and fluid homeostasis. When blood volume is low, juxtaglomerular cells in the kidney secrete renin, which is cleaved by the liver enzyme angiotensinogen to form Ang I, which is further cleaved by Ang-converting enzyme (ACE) to form Ang II, the major physiologically active component of the RAS. The two receptors for Ang II (AT1 and AT2) are expressed in virtually all organs and often induce pathways that counteract the actions of each other. An abundance of animal and human studies on liver fibrosis have looked into the activation of the RAS after ACE/Ang receptor blocker (ARB) inhibition and point to the RAS as an important step in the pathogenesis of liver fibrosis (14, 51, 88, 112). In rats, ACE/ARB inhibitors decreased a number of fibrogenic processes such as collagen deposition, inflammation, oxidative stress, transforming growth factor (TGF)-β1 expression, matrix metalloproteinase (MMP)2/9 activity, and accumulation of myofibroblasts. In patients treated with ACE/ARB inhibitors, a decrease in collagen expression, fibrotic stage, and activation of hepatic stellate cells (HSCs) was seen. Munshi et al. (77), in a comprehensive review, discussed these findings and provided a mechanistic explanation for the RAS in liver fibrosis (77). The authors stated that proliferating biliary epithelium serves as a neuroendocrine compartment for autocrine/paracrine signaling during liver diseases by secreting RAS components. These cause downstream activation of TGF-β1, NADPH oxidase, TNF-α, and procollagen production. It is important to note that these downstream signaling pathways involve either the production or activation by reactive oxygen species-linking the fibrogenic and inflammatory processes.

Platelet-derived growth factor. Platelet-derived growth factor (PDGF) drives the differentiation of mesenchymal cell growth and differentiation during development and is released by platelets during coagulation. PDGF-β is involved in the fibro-proliferative response of chronic inflammatory disorders such as scleroderma, rheumatoid arthritis, and atherosclerosis. One study reported that, in rats, cholangiocytes responded to BDL by secreting PDGF-β (45). BDL livers had increased expression of PDGF-β mRNA and protein demonstrated by in situ hybridization and immunohistochemistry. PDGF-β secretion by the biliary epithelium was thought to induce the fibrogenic response and participate in EMT. The study suggests that PDGF-β secretion acts by autocrine/paracrine mechanisms because the PDGF-β chain and its receptor increased after BDL and expression of PDGF-R was demonstrated on nearby stellate cells. PDGF-mediated chemoattraction of hepatic progenitor cells by bile ducts has been shown in cholestatic rats (57). It is proposed that peribiliary cells distinct from HSC undergo a PDGF-mediated conversion into myofibroblasts, thus contributing to the initial formation of biliary-type liver fibrosis (56).

TGFs and integrins. TGF-β1 and -β2 are important cytokines for controlling proliferation and differentiation in a broad spectrum of tissues and extracellular matrix (ECM)-synthesis in the mesenchyme. Their activity increases the synthesis of ECM proteins such as laminin, collagen, fibronectin, and tissue inhibitors of metalloproteinases (TIMPs) while decreasing the expression of ECM degradative enzymes. In the case of wound healing and fibrosis, TGF-β cooperates with αVβ6 integrin-a profibrogenic integrin that is notable for its profibrogenic role in the activation of latent TGF-β1. In the normal liver, TGF-β cytokines and αVβ6 integrin are not expressed at high levels but increase during cholestasis. Several studies have analyzed the relationship between liver fibrosis, αVβ6 integrin, and TGF-β (75, 87, 99, 100). The expression of αVβ6 integrin transcript levels increased 100-fold in proliferating cholangiocytes during advanced liver fibrosis (87). Inhibition of the αVβ6 integrin gene and its receptor retarded progression of biliary fibrosis by reducing activation of TGF-β1 and collagen synthesis and adhesion of cholangiocytes to fibronectin. It is worth noting that, although αVβ6 integrin induces the expression of TGF-β1, the reverse pathway is also present. Sullivan et al. (99) demonstrated in human MMNK-1 cholangiocytes that the mRNA expression of the αVβ6 integrin gene (Itgβ6) is induced by TGF-β1/p38/MAPK pathway through activation of transcription factors SMAD and AP-1 (99). One study compared the normal and fibrotic liver expression of RNA transcripts for TGF-β1 and TGF-β2 (75). In normal liver tissue, expression of the TGF-β1 transcript was high in HSCs, myofibroblasts, inflammatory cells, but not in cholangiocytes. However, the levels of TGF-β1 increased significantly during fibrosis. In contrast to TGF-β1, TGF-β2 was expressed in high levels in cholangiocytes.

Connective tissue growth factor. Connective tissue growth factor (CTGF) is a hormone that exhibits chemotactic and mitogenic actions on fibroblasts. CTGF is induced by TGF-β
in endothelial cells and fibroblasts and shares biological actions on target cells such as fibrosis, chemotaxis, and proliferation. During wound healing and fibrosis, CTGF stimulates epithelial cells and fibroblasts to produce ECM components via autocrine/paracrine mechanisms (20, 65). Several studies have evaluated the production of CTGF in BDL rats and animal models of biliary fibrosis (92, 98). One study reported a sevenfold increase in CTGF mRNA expression in the bile duct epithelial cells of rats with biliary fibrosis. Another study evaluated a DNA microarray of genes expressed in early fibrogenesis as a time-course following BDL and found increased expression of TGF-β, collagen-I, CTGF, and TIMP-1 and a decrease in MMPs 42 days after BDL-induced liver fibrosis (98).

Endothelin. Endothelin (ET-1) is an important mediator of vascular function and is well known for its potent effects on vasoconstriction. During normal conditions, ET-1 is tonically inhibited by nitric oxide (NO), but during low NO bioavailability (as in oxidative stress and inflammation) ET-1 production increases. In animal models of liver disease, ET-1 has been shown to be an important mediator of liver injury and collagen synthesis (22, 104). In rats with cholangiocarcinoma, ET-1 increased collagen synthesis, apoptosis, and decreased VEGF (26). One study evaluated the role of cholangiocyte ET-1 production in hepatopulmonary syndrome (66). After BDL, hepatic ET-1 levels in tissue increased, and staining for ET-1 coincided with the cholangiocyte marker CK19. After 1-wk BDL, isolated cholangiocytes increased expression of ET-1 mRNA 3.5-fold compared to hepatocytes, which remained unchanged. A relationship between ET-1 and TGF-β1 was demonstrated as administering TGF-β1 stimulated promoter activity, expression and production of ET-1 in normal rats.

Hedgehog signaling. Hedgehog (Hh) signaling regulates remodeling and migration during embryogenesis and wound healing in adults. Cells expressing the receptor patched are responsive to Hh ligand through intracellular Smo and the Gli family of transcription factors. Activation of these transcription factors causes tissue remodeling and chemotaxis of nearby cells through the production of specific chemokines. Several studies on rats have evaluated the involvement of the activation and production of Hh ligand in the bile duct epithelium (52, 83, 85, 86). In the bile duct epithelium, IHC demonstrated expression of IHH (member of Hh family) and Gli2 (52). Hh was shown to accumulate and promote cholangiocyte EMT and chemokine production through an autocrine/paracrine mechanism involving myofibroblastic HSC (84, 85). On the other hand, neutralizing Hh with an anti-Hh antibody decreased cholangiocyte proliferation and expression of the EMT marker S100A4 (84). Furthermore, Hh induced the activation of divergent autocrine pathways including cxcl16 and osteopontin (85). This cxcl16, produced in cholangiocytes, increased the chemotaxis of natural killer T cells in culture and recruited them to the portal tracts (85).

Summary

In this comprehensive review, we highlighted the various autocrine factors that participate in the regulation of cholangiocyte proliferative and adaptive responses during the progression of chronic cholestatic liver diseases. During the early stages of biliary disease, cholangiocytes respond to both biliary pressure and other contributing factors induced by cholestasis through the activation of proliferation and the switching off of apoptosis. Activation of biliary proliferation triggers the production of a variety of autocrine factors by cholangiocytes resulting in the development of a neuroendocrine phenotype. These activated cholangiocytes begin secreting autocrine factors normally seen in development, sexual reproduction, cell migration, and the nervous system. Together these autocrine factors regulate biliary proliferation in a concerted fashion during cholestasis and also the activation of cells (i.e., hepatic stellate cells, vascular endothelial cells, hepatocytes, immune cells) in the nearby microenvironment, which results in the activation of angiogenesis, fibrogenesis, and inflammatory process and cumulates as biliary fibrosis and subsequent liver cirrhosis. In the late stages of disease, neuroendocrine cells participate in the irreversible stages of biliary fibrosis although the precise mechanism is still unknown. The autocrine/paracrine factors secreted by cholangiocytes are clearly important for orchestrating not only the proliferation of cholangiocytes but also the pathogenesis pathways that result in biliary fibrosis.

Future Perspectives

As our understanding of the critical autocrine factors that regulate biliary proliferation and the potential role of these factors in the cross-talk between neighboring cells in the portal tract microenvironment expands, so to will the potential to develop novel therapeutic interventions for chronic cholestatic liver diseases such as primary sclerosing cholangitis, PBC, and biliary atresia. Clearly additional studies are warranted to determine the translational potential of many of the factors discussed in this review. In particular, future studies are needed to evaluate mechanistically the cross-talk that occurs between cholangiocytes and other cells in the portal microenvironment, which is certainly an important aspect of the pathogenesis of cholestatic liver diseases. In addition, studies are needed to determine the cohort of factors that might be beneficial for controlled stimulation of biliary proliferation during conditions of ductopenia and perhaps in biliary atresia.

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REFERENCES


