RECENT ADVANCES IN PATHOPHYSIOLOGY OF BILIARY TREE: INFLUENCE OF SEX HORMONES IN CHOLANGIOCYTE GROWTH

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### Abstract

Cholangiocytes are epithelial cells that line the biliary tree (1). The intrahepatic biliary tree is characterized by interconnected ducts which starts with canals of Hering, continues into intrahepatic ducts of increasing diameter to end at the level of the extrahepatic bile ducts (2). These cells play a key role in the ductal secretion of water and bicarbonate during the process of bile formation. These cells are also the target in several chronic cholestatic liver diseases (termed cholangiopathies), including primary sclerosing cholangitis (PSC), primary biliary cirrhosis (PBC), polycystic liver disease (PCLD) and cholangiocarcinoma (CCA) (3, 4). During these conditions, the balance between proliferation/loss of cholangiocytes is lost with critical effect on the maintenance of biliary functions and intrahepatic bile ductal mass (3, 5, 6). A typical feature that occurs under specific experimental conditions, such as common bile duct ligation (BDL) (Fig.1). After BDL, there is biliary hyperplasia and secretin stimulated choleresis (a functional marker of cholangiocyte growth) (5). Cholangiocyte growth/apoptosis is regulated by several factors, including growth factors, cytokines, gastrointestinal and sex hormones, such as estrogens, prolactin, follicle-stimulating hormone (FSH), testosterone and progesterone (3, 7, 8, 9). The purpose of this review is to summarize the recent findings on the effects of sex hormones in cholangiocyte pathophysiology. To clarify the mechanisms of action of these substances will provide new potential strategies for the management of chronic liver diseases.

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I colangiociti sono cellule epiteliali che rivestono l'albero biliare (1). L'albero biliare intraepatico è caratterizzato da una rete di dotti interconnessi, inizia con i canali di Hering, continua in dotti intraepatici di diametro crescente per finire a livello dei dotti biliari extraepatici (2). Queste cellule svolgono un ruolo chiave nella secrezione di acqua e bicarbonato durante il processo di formazione della bile. Esse sono anche il bersaglio di diverse malattie colestatiche croniche del fegato (chiamate colangiopatie), tra cui la colangite sclerosante primitiva (PSC), la cirrosi biliare primitiva (PBC), la malattia policistica del fegato (PCLD) e il colangiocarcinoma (CCA) (3, 4, 5). In queste condizioni, viene perso l'equilibrio tra proliferazione/morte dei colangiociti con effetti negativi sul mantenimento delle funzioni biliari e della massa dei dotti intraepatici (4, 6, 7). Questa è una situazione che si verifica in particolari condizioni sperimentali, come ad esempio con la legatura del dotto biliare principale (BDL) (Fig.1). Dopo BDL, c'è iperplasia biliare e coleresi stimolata da secretina (un marcatore funzionale di crescita colangiocitaria) (6, 8). La crescita/morte dei colangiociti è regolata da diversi fattori, tra cui fattori di crescita, citochine, ormoni gastrointestinali e sessuali, quali estrogeni, prolattina, ormone follicolo-stimolante (FSH), testosterone e progesterone (4, 9, 10, 11). Lo scopo di questa review è quello di riassumere le recenti scoperte sugli effetti degli ormoni sessuali nella fisiopatologia dei colangiociti, cercando di chiarire i meccanismi d'azione di queste sostanze per fornire nuove potenziali strategie terapeutiche nella gestione delle malattie croniche del fegato.
Fig. 1 - Some representative immunohistochemistry for CK19, a specific marker of biliary epithelium in normal and BDL rats. On the left, a normal liver section to show a characteristic portal space, with some biliary ducts (stained in brown for the immunoreaction), a branch of portal vein (the largest blood vessel) and a branch of hepatic artery (the smallest blood vessel). On the right, a liver section from BDL rat in which is evident the hyperproliferation of the biliary ducts. Original magn., 40x.

Background
The liver is functionally modulated by sex hormones. Long-term use of oral contraceptives (OCs) and anabolic androgenic steroids (AASs) can induce both benign hyperplasia and malignant tumors.

In particular, the main topic of this review is biliary epithelium. It is formed by cholangiocytes, cells morphologically and functionally heterogeneous (10, 11). Small cholangiocytes have a cuboidal shape, a high nuclear/cytoplasm ratio and they are hypothesized to be committed biliary progenitors (12). Large cholangiocytes are columnar in shape, display a small nucleus and conspicuous cytoplasm. These cholangiocytes line interlobular ducts located in the portal triads and all the larger intrahepatic ducts (13). During cholangiopathies, when large cholangiocytes are damaged, small cholangiocyte proliferation is activated resulting in repopulation of bile ducts. But, the terminal stages of most cholangiopathies are characterized by a disappearance of intrahepatic bile ducts (ductopenia) (14, 15) whit an imbalance between cholangiocyte death and proliferation (16, 17) (Fig. 2).
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To better study these pathologies and their mechanisms of action, a lot of experimental models has been created, such as the bile duct ligated model (BDL), partial hepatectomy, acute CCl₄ feeding or chronic feeding of α-naphthylisothiocyanate (ANIT) and bile salts (18, 19) (Fig. 1). The BDL rat is a well-characterized model of selective and “typical” proliferation of cholangiocytes, which lead to an enlarged ductal mass and increased cholangiocyte secretory activities (6, 20). In these hyperplastic models, cholangiocyte proliferation is closely associated with increased secretin receptor gene expression and secretin-stimulated cAMP intracellular levels (21, 22).

In the last years, agents and mechanisms modulating cholangiocyte proliferation have been extensively investigated (23, 24). In particular, sex hormones have been demonstrated to play an important role in the growth of biliary epithelium (7, 25). They have been known to regulate the target cell growth positively or negatively in the receptor-dependent manner. Generally, sex hormones have been believed to support cell growth, while antihormones inhibit the hormone-dependent growth. This difference is not so obvious. In fact, antiandrogens have also been tested to increase the growth of prostate cancer (26). Tamoxifen (TMX), the most widely used therapeutic agent of estrogen-dependent breast cancer, exhibits organ- and species-dependent differences in cell growth regulation. A possible
explanation is that two pathways exist for estrogen-dependent growth, one for the stimulatory and another for the inhibitory signal (27).

In several years, our findings have shown that liver expresses estrogen and androgen receptors and experimentally both androgens and estrogens have been implicated in stimulating cell proliferation. In detail, estrogens stimulate both in vitro and in vivo cholangiocyte proliferation and they may modulate cholangiocyte proliferation by synergizing growth factors (28). In the same manner, we discovered that testosterone is important in sustaining biliary proliferation and ductal secretory activity in pathological conditions associated with functional damage of the biliary epithelium (9). Moreover, other hormones and growth factors cross talk together in normal and pathologic conditions. For that reason, it would be important try to sum up the last findings in this field to add new targets for future studies.

Female Sex Hormones

The liver is a hormone-sensitive organ. In fact both normal and pathologic liver from male and female mammals have been shown to express specific estrogen receptors (ERs). Experimentally, estrogens may act as liver tumor inducers or promoters in vivo (29), and are involved in stimulating cell proliferation in vitro (30). Moreover, anti-estrogens like TMX have been shown to reduce levels of ERs and to inhibit cell proliferation following partial hepatectomy (31).

Estrogens are formed from androgens through the action of an aromatase enzyme. Their importance in the normal liver physiology is not clear, but it has been showed that estrogens play an important role in the control of liver cell proliferation (30). Moreover, the hepatic ERs increase and are actively translocated to the nucleus after partial hepatectomy in humans and rats (31, 32).

The estrogens are well known carcinogenic agents in estrogen-responsive tissues (breast, uterus). In liver, experimental models have shown that estrogens act as tumor promoters and may induce hepatocarcinoma (HCC) and colangiocarcinoma (CCA) (33, 34, 35). Considering all these premises, HCC and CCA could be consider estrogen-dependent cancer like breast cancer and the use of anti-estrogen drugs should control the growth of these tumors.

Several studies have used TMX, an anti-estrogen drug, for the treatment of liver cancers and the results appeared to be initially encouraging. TMX has several other biologic activities that may have relevance in cancer treatment: inhibition of PKC, calmodulin, TGF-α and TGF-β1 induction; antagonism of estrogen binding to the erbB-2 oncogene; and activation of NK-mediated cytotoxicity. Some of these may be responsible for the reported effects of TMX on various cancers (36, 37, 38).

Proliferation of the biliary epithelium occurs in several human pathologies (cholestasis and infectious and toxic liver injury) in which it may affect the outcome of the disease (39). It has been of particular interest to discover whether estrogens affect cholangiocyte pathophysiology in relation to the development of cholangiopathies (40). Male and female biliary epithelium express ER-α and ER-β subtypes (Fig. 3A).
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Fig. 3A - Immunohistochemistry for estrogen receptor-β (ERβ) in normal and pathologic biliary epithelium. On the left, normal rat cholangiocytes in which the immunopositivity for the subtype of estrogen receptor is weak. On the right, a liver section from BDL rat in which the expression of ERβ is considerably increased.

In particular, ER-β is overexpressed in cholangiocytes proliferating after BDL with enlarged bile duct mass associated with enhanced protein expression of total and p-ERK1/2 and of the adapter protein Shc (34). The Src/Shc/ERK1/2 cascade is typically activated by several growth factors (41), thus suggesting that estrogens could potentiate the effects of growth factors by sharing similar intracellular signalling mechanisms. Consistently, in cholangiocytes, estrogens exert additive effect with NGF, IGF-1 and VEGF on modulating proliferation (3, 24, 28, 42, 43). To get more insights into the role of estrogens in the modulation of cholangiocyte proliferation, we also evaluated the effect of: i) two estrogen antagonists (TMX and ICI 182,780) (7), ii) ovariectomy (OVX), and iii) estrogen replacement treatment (25). Cholangiocyte proliferation during BDL is closely in relationship with enhanced expression of ER. It is known that ER-α and ER-β have different responses to the two antiestrogens (44). ER modulators produce distinct effects depending on species and tissues (45). Studies report that TMX has mixed agonistic and antagonistic action on ER-α but only antagonistic effects on ER-β, whereas ICI 182,780 acts as a pure antiestrogen on both receptor subtypes (46). In liver, both TMX and ICI 182,780 induce a decrease in bile duct mass, suggesting that the antiestrogenic effect is the predominant mechanism of action for both compounds (7). In breast cancer and hepatocellular carcinoma, TMX induces apoptosis by a multifactorial mechanism, including blocking the mitogenic effect of estrogens and induction of apoptosis (47). In biliary epithelium, Fas antigen, a marker of apoptosis weakly present in cholangiocytes of normal and BDL rats, is strongly increased by TMX and ICI 182,780, suggesting that when the mitogenic effect of estrogens is
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Blocked, Fas antigen overexpression occurs. This dysregulation of apoptosis could play an important role in the progression of a cholestatic disease (48). Certainly, the maintained or even increased estradiol serum levels allow exclusion of the possibility that the two antiestrogens decrease cholangiocyte proliferation in BDL by impairing estrogen production (49). OVX impairs the proliferative response of intrahepatic cholangiocytes during BDL with a decreased expression of ER-α and ER-β. This decreased of intrahepatic bile duct mass is associated with enhanced Fas-mediated apoptosis of proliferating cholangiocytes. Exogenous administration of 17-β estradiol normalizes the proliferative response of the intrahepatic bile ducts by restoring proliferation and decreasing apoptosis (25).

These results suggest that estrogens and their metabolites can have a pathogenic role in diseases which preferentially affects women (50). This is true for primary biliary cirrhosis (PBC), where an estrogenic functional deficiency has been hypothesized (51). The disease, in fact, predominantly affects females (52). Estrogens may influence the course of PBC by directly modulating the pathophysiology of cholangiocytes, which are the primary target in the disease (53). Cholangiocytes of PBC patients, but not normal subjects, express both ER-α and ER-β (Fig. 3B).

**Fig. 3B - Immunohistochemistry for estrogen receptor-β (ERβ) in normal and pathologic biliary epithelium.**

On the left, normal human hepatic tissue negative for ERβ. On the right, a section from PBC in which the presence of ERβ is enhanced in cholangiocytes. Original magn., 40x.

The ER expression varies according to different stages of disease and correlates with the proliferation and the death. Especially in the last stage (IV) where the maximal degree of ductopenia is reached, cholangiocytes are negative for ER-α (53). For that reason, the modulation of ERs could delay the progression of PBC toward ductopenia. Regarding cholangiocarcinoma, a tumor with increasing incidence and prevalence, we found a higher expression of ER-α and increased ERα/Erβ ratio (54). Interestingly, estrogens and IGF-1 exert an additive proliferative effect blocked by both ER antagonists and IGF-1 blocking antibody triggering to apoptosis in Fas-positive cells (55, 56, 57). In the end, in vitro, we have also demonstrated the direct stimulatory effect of estrogens on proliferation of cholangiocytes isolated from normal rats in association with enhanced protein expression of p-ERK1/2, Src, and Shc. In basal conditions,
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Cholangiocytes are dormant cells, but proliferation is significantly increased when cells were exposed to 17β-estradiol (31) and blocked by TMX, ICI 182,780 and by inhibitors of MEK and Src (55). In addition, another important female sex hormone has been studied, progesterone, a steroid hormone synthesized by the ovaries, adrenal glands, central and peripheral nervous system, and by the corpus luteum during pregnancy (58). Its biosynthesis consists in the conversion of cholesterol to pregnenolone, the precursor of all steroid hormones (59). The steroidogenic acute regulatory protein (STAR) mediates the translocation of cholesterol from the outer to the inner mitochondrial membrane (60). In the inner mitochondrial membrane, P450scc catalyzes the transformation of cholesterol to pregnenolone (61). It moves from the mitochondria to the microsomal compartment where it is converted to progesterone by 3β-hydroxysteroid dehydrogenase (3β-HSD) (62). We have recently demonstrated that progesterone stimulates the proliferation of both male and female cholangiocytes (8). Chronic administration of progesterone increased the number of bile ducts of normal rats (8). Whereas, treatment with an anti-progesterone antibody inhibited cholangiocyte growth stimulated by BDL (8). Interestingly, normal and BDL cholangiocytes expressed the biosynthetic pathway (i.e., steroidogenic acute regulatory protein or STAR, 3β-HSD, and cytochrome P450 side-chain cleavage) and secrete progesterone (8). These findings provide further support for the concept that endocrine autocrine and paracrine mechanisms play an important role as a repair and compensatory mechanism for bile duct loss during cholangiopathies, therefore, counteracting the evolution of the disease toward the terminal ductopenic stage (53).

Male Sex Hormones

Testosterone and dihydrotestosterone (17-beta-hydroxy-5-alpha-androstan-3-one) are the most potent androgens in mammals (63). Testosterone is secreted in the testes of males, the ovaries of females, and a small amount is also secreted by the adrenal glands. Like the other steroid hormones, androgens exert their effects through the activation of specific hormone receptors, the androgen receptors (ARs), which function as a ligand inducible transcription factor (64). Receptors specifically activated by testosterone and DHT have been identified on the cytoplasm and the nucleus of the hepatocyte. These receptors are present in the normal liver tissue from both male and female mammalians, but their expression and activation is reported to be increased in the tumor tissue and in the surrounding liver tissue of individuals with HCC (65-67). The presence of androgen receptors (ARs) has also been associated to an increased risk of tumor recurrence and to a reduced survival after hepatic resection for HCC. According to a study of Nagasue et al., individuals who had AR negative tumors showed a survival of 55% at 5 years after surgery, while those with AR positive tumors had a survival rate of 0% (68). Considering these data, liver is an androgen-sensible organ with a biological behavior similar to that of the prostate; thus, HCC might be a hormone-sensible tumor like prostate cancer and therefore should respond to an anti-androgen treatment. Several studies have utilized different anti-androgenic compounds in an attempt of treating the progression of liver cancer. However, most of the published studies showed a complete lack of effect of this therapeutic approach (69). Grossmann et al. showed that low testosterone levels are common in men with severe liver disease such as PBC (52) and predict mortality independent of the Model for End Stage Liver Disease (MELD) score, the standard score used to prioritize the allocation of liver transplants (70). Moreover, androgens have an important role in glucose homeostasis and lipid metabolism and exert a primary role in the liver. In fact, it has been shown that low testosterone levels are associated with an increased risk of type 2 diabetes mellitus, particularly evident in men with prostate carcinoma, after androgen deprivation (71). The role of androgens on biliary epithelium has been poorly investigated, and it was interesting to discover the role of testosterone and its receptors in the regulation of cholangiocyte growth and ductal secretory activity in cholestasis. In our studies we showed that i) cholangiocytes express testosterone receptors (Fig. 4A), 17β- hydroxysteroid dehydrogenase 3 (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 could be prevented by exogenous administration of testosterone (9); These data suggest an autocrine compensatory role of testosterone in sustaining cholangiocyte proliferation in cholestasis, a pathological condition characterized by testicular atrophy and lowered serum testosterone levels (52, 72).
According with our findings, other studies have shown the presence of functional ARs in liver cells, including hepatocytes and bile ducts from PBC patients (73). In particular, the expression of ARs was low in normal patients and increased in patients with PBC (Fig. 4B) (73).

**Fig. 4A** - *Immunohistochemistry for androgen receptor (AR) in normal and pathologic biliary epithelium.*

On the left, normal cholangiocytes from rat with AR slightly expressed. On the right, a section from BDL rat in which the presence of AR is considerable increased.

**Fig. 4B** - *Immunohistochemistry for androgen receptor (AR) in normal and pathologic biliary epithelium.*

On the left, normal human tissue almost negative for the androgen receptor. On the right, a liver section from PBC patient in which the expression of AR is increased. Original magn., 40x.
The fact that testosterone increases biliary hyperplasia and prevents the loss of biliary growth and function (following castration) supports the concept that androgens can be important for ameliorating the cholestatic conditions associated with testicular hypotrophy and ductopenic conditions associated with decreased testosterone levels as occurs in PBC. These findings and the fact that the administration of neutralizing antitestosterone antibody reduces testosterone serum levels and biliary hyperplasia introduce the concept that the administration of testosterone receptor antagonists or antitestosterone antibodies may be new therapeutic approaches for decreasing the aberrant growth of cholangiocytes.

Other Sex Hormones
An other group of substances secreted by the pituitary gland and studied by our group in the last years can be defined sex hormones. They include the follicle-stimulating hormone (FSH) and the prolactin (Prl). FSH, also called gonadotropin because it stimulates the gonads, is produced in the anterior pituitary gland of the brain (74). Several studies demonstrated that liver cirrhosis is associated with endocrine dysfunction, notably in the gonadal axis (75). Patients with liver damage have low levels of LH and FSH (76). In fact, gonadotropin deficiency occurs with liver damage and in some patients with hemochromatosis (77). We found that cholangiocytes expressed the FSH receptor (FSHR) and secreted FSH (77). Treatment of normal rats with FSH increases cholangiocyte growth via cAMP/ERK1/2/Elk-1 signaling mechanism. While, administration of antide (a gonadotropin releasing hormone antagonist that blocks FSH secretion) or of an anti-FSH antibody decreases cholangiocyte proliferation and secretory responses (78).

Lastly, cholangiocytes also express the long and short form of prolactin receptor (79). We observed that chronic in vivo administration of prolactin to normal rats increases cholangiocyte proliferation and intrahepatic ductal mass and did not induce changes in lobular damage or apoptosis (79). The proliferative effect of prolactin is associated with the activation of the IP$_3$/Ca$^{++}$/PKC/Src/MAPK pathway and the phosphorylation of the JAK2/STAT5 via (79). These findings demonstrate the ability of proliferating cholangiocytes to secrete prolactin, thus regulating their own proliferation by an autocrine loop. In summary, the presented data suggest that sex hormones are a key autocrine factor regulating biliary mass and they have important pathological implications since modulation of the expression and secretion of these substances may be used to modulate cholangiocyte proliferation during cholestatic liver diseases.

Conclusions
All this amount of data induces to conclude that both estrogens and androgens have important effects in controlling the replication rate of hepatic cells. They can have effect on inducing or at least promoting the growth of liver tumors, including HCC and CCA. In addition to the study of anti-hormones, we can suggest an other approach to study the effects of sex hormones on biliary proliferation. For example, the role of enzymes, such as aromatases (that converts androgens into estrogens) may be better investigate in the hyperplastic growth of hepatic cells. In fact, first observations show hepatic tumor tissues with elevated aromatase activity and consequently higher estrogen formation rates than in non-tumoral liver tissues (80). It could improve the knowledge on estrogens to induce cell growth via nonreceptor pathways, sustaining a strategy to reduce estrogen concentration in liver diseases with the use of aromatase inhibitors.
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References

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