Biliary System Architecture: Experimental Models and Visualization Techniques

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Summary
The complex architecture of the liver biliary network represents a structural prerequisite for the formation and secretion of bile as well as excretion of toxic substances through bile ducts. Disorders of the biliary tract affect a significant portion of the worldwide population, often leading to cholestatic liver diseases. Cholestatic liver disease is a condition that results from an impairment of bile formation or bile flow to the gallbladder and duodenum. Cholestasis leads to dramatic changes in biliary tree architecture, worsening liver disease and systemic illness. Recent studies show that the prevalence of cholestatic liver diseases is increasing. The availability of well characterized animal models, as well as development of visualization approaches constitutes a critical asset to develop novel pathogenetic concepts and new treatment strategies.

Key words
Biliary system • Mouse model • Cholestasis • Visualization • Morphology

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Introduction
The biliary system is a complex 3-dimensional network of tubular structures connecting the hepatocellular parenchyma of the liver with the lumen of the small intestine. Bile is produced by the hepatocytes and is secreted into the bile canaliculi which are delineated by the apical membrane of the neighboring hepatocytes. Bile canaliculi are not tubular structures, but rather, the dilated intercellular spaces sealed by tight junctions. The bile canicular surface is bristled with innumerable villi which are actively involved in bile secretion. The bile flows from canaliculi to the network of bile ducts which are lined with cuboidal epithelial cells called cholangiocytes. Cholangiocytes are responsible for the secretion, reabsorption, and regulation of bile constituents. Bile ducts anastomose into larger ductules and larger ducts eventually forming the common bile duct which enters the gall bladder where the bile is stored.

The biliary architecture does not exist in a static state; rather it forms a highly dynamic network that actively adapts to changing requirements. For instance, various pathological conditions lead to adaptive remodeling of the biliary tree. This involves the complex process “ductular reaction”, the proliferation of cells residing in the biliary compartment. Ductular reactions represent a common repair mechanism and can be observed in response to both acute and chronic hepatotoxic insults or in cholangiopathies characterized by cholestatic condition. Epithelial injury leads to activation of cytokeratin-19-positive cholangiocytes comprising liver stem cells (termed hepatic progenitor cells or oval cells in rodents). Expansion of these transiently amplifying cells in “reactive ductules” correlates with the extent of injury and gives rise to strands of cholangiocytes of newly formed ductules.
These reactions are accompanied by inflammation and an increase in matrix deposits leading to periportal fibrosis. Ductular reaction yields diverse phenotypes not only in dependency on type of injury but also within any liver pathology, however, it always results in rearranged architecture of biliary tree with more pronounced ramification and anastomoses.

Longitudinal studies in diverse cholangiopathies have been restricted by relatively low incidence of the disease and by the limited accessibility of the human biliary tract. The availability of well-defined rodent models can aide the understanding of the disease and address specific questions, difficult to address in human studies. The invasive nature of human examinations and the necessity for multiple assessments at various stages of pathology constitutes limitations for human patient studies. In contrast, mouse models are a valuable tool to conduct stage-dependent examinations, multiple sampling studies and employment of more informative visualization techniques. In addition, mouse models with targeted inactivation of specific genes are instrumental to determine the role of these genes in the development and progression of biliary system pathologies.

The classical histomorphological characterization and two-dimensional (2D) morphology and immunohistochemistry in sample sections provide limited ability to study the three-dimensional (3D) architecture of the biliary tree. Increasing evidence on the importance of bile duct organization and its dynamic remodeling in response to diverse pathologies requires visualization techniques enabling detailed 3D studies to better understand the beneficial or deleterious outcomes after liver injury.

The purpose of this review article is to provide an overview of the most frequently used rodent models of obstructive cholestasis and detail some of the of the most recent advances in the study of biliary system in these models, and state of the art visualization techniques for description of biliary tree architecture.

**Experimental models**

Surprisingly, despite the rapid technological development in modeling various complex diseases, there are only a few well defined models of biliary epithelia damage recapitulating the morphological changes observed in their human counterparts. Rodents are the most common animal models of hepatobiliary damage induced by obstructive cholestasis. Rodents are also well characterized with respect to biliary tree remodeling.

**Common bile duct ligation (CBDL)**

Common bile duct ligation has been a highly reproducible experimental model for biliary epithelial injury for the last three decades (Kountouras et al. 1984). This model reliably induces proliferation of cholangiocytes, activation of oval cells, portal myofibroblasts and subsequent development of ductular reaction characterized by an increased number of ductules (Fig. 1A-F). Histopathological signs include cholestasis, portal inflammation, and extensive fibrosis closely resembling human biliary cirrhosis (Fig. 1F) (Kountouras et al. 1984). The biliary tree architecture in response to CBDL adapts to optimize the intraluminal surface area by way of corrugation and branching (Vartak et al. 2016). This adaptive remodeling leads to formation of a significantly denser duct meshwork around the portal vein to increase the capacity to drain hepatocyte-generated biliary fluid. The classical protocol is simple and relatively fast, the surgical procedure consists of a midventral laparotomy and isolation of the common bile duct above the duodenum, followed by double ligation of the bile duct and dissection between the ligatures (Starkel and Leclercq 2011, Tag et al. 2015). The original CBDL protocol was developed for rats; however it has been successfully adapted for mice (Tag et al. 2015). The relatively high mortality rates (due to rupture of the gall bladder in mice or biliary cyst in rats) can be circumvented using the microsurgical technique (Aller et al. 2008).

The CBDL model is useful for dissecting the molecular mechanisms of hepatic inflammation and fibrogenesis (Iredale 2007, Iredale et al. 2013) and to test novel antifibrotic approaches and drugs (Arias et al. 2003, Buryova et al. 2013, Patsenker et al. 2008). CBDL is also instrumental for assessment of stability of the bile duct epithelial barrier (Rao and Samak 2013). Thus the critical role of junctional complex constituents (e.g. ZO-1/2, claudin, occludin, and E-cadherin), cytoskeletal components (cytokeratin 19) or cytolinkers (epiplakin) has been delineated over the last few years (Chen et al. 2015, Fircincieli et al. 2013, Maly and Landmann 2008, Szabo et al. 2015).

**3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) diet**

In DDC fed mice, injury of the biliary epithelium due to formation of intraductal porphyrin plugs (Fickert et al. 2007) are constantly observed. The
developing cholestasis leads to injury and activation of cholangiocytes with increased expression of profibrogenic and proinflammatory cytokines (MCP-1, PDGF, TNFα, and TGFβ) and adhesion molecules (VCAM, osteopontin), infiltration of neutrophils, proliferation of periductal myofibroblasts and extensive ductular proliferation (Fig. 1G-I). DDC-induced injury also leads to hepatocellular necrosis, activation of Kupffer cells and compensatory parenchymal proliferation (Miyao et al. 2013). The DDC diet was originally established as a model for the formation of Mallory-Denk bodies, keratin filament-containing inclusions, typical for nonalcoholic steatohepatitis, metabolic liver diseases, and chronic cholestatic liver diseases (Strnad et al. 2008). Due to development of cholangitis with onionskin-type periductal fibrosis, and biliary fibrosis, DDC was suggested as a model of human primary sclerosing cholangitis (Fickert et al. 2007). Although the classical protocol requires 4-8 weeks of DDC feeding (Fickert et al. 2007), increased liver damage markers (Chalupsky et al. 2013) and pronounced alterations in canalicular structures (dilatation, meandering and microvilli loss) were observed as early as 1-7 days on DDC (Miyao et al. 2013). The DDC diet leads to ductular obstruction in the terminal branches of the biliary tree and unlike CBDL, allows the investigation of the regenerative processes in biliary epithelium after cholestatic injury (Boulter et al. 2012).

![Fig. 1. Ductular reaction and biliary fibrosis in CBDL and DDC diet models of obstructive cholestasis.](image)

DDC diet-based model is widely used to investigate processes associated with chronic cholangiopathies (e.g. cholestasis and biliary fibrosis) and to test novel therapeutic strategies. Along with CBDL, DDC diet represents the first choice model in studies of mechanical stability and resilience of bile duct epithelial...
barrier. In addition, this model is attractive for in vivo studies on the crosstalk between extracellular niche and hepatic progenitor cells in cholangiopathies (Kim et al. 2015, Peng et al. 2016, Pi et al. 2015).

Bile acids-based diets

The effect of bile acids (BA), both primary (synthesized from cholesterol in the liver) and secondary (further metabolized by bacteria in the intestine) on the pathophysiology of biliary epithelia has been studied over the past 50 years. In general, the hepatotoxicity of BA is considered to be associated with their degree of hydrophobicity.

Lithocholic acid (LCA) is the most potent bile acid for inducing hepatocellular damage through intrahepatic cholestasis. Mice fed a LCA-supplemented diet show precipitation of poorly soluble LCA and its metabolites in bile canaliculi and ductuli and the plugging of the biliary tree (Fickert et al. 2006). The obstruction leads to increased biliary pressure, epithelial rupture and leakage of bile into parenchyma, which in turn results in destructive cholangitis, periductal edema and activation of biliary epithelial cells and proliferation of periductal myofibroblasts with subsequent development of fibrosis in a focal manner resembling CBDL model. Besides intrahepatic cholestasis, LCA has been proposed to act by altering the canalicular membrane and impairing the function of biliary export pumps (Kubitz et al. 2004). Recent studies have demonstrated that LCA disrupts phospholipid and sphingolipid homeostasis following changes in the expression of enzymes involved in their synthesis (Matsubara et al. 2011) in a TGFβ-SMAD3 dependent manner (Matsubara et al. 2012). It has also been proposed that the pathogenesis observed in LCA-induced cholestasis is directly caused by toxicity to cholangiocytes and hepatocytes (Woolbright et al. 2014), which is opposed by detoxification enzymes (e.g. CYP3A, sulfotransferase2A) and regulated by nuclear receptors (Staudinger et al. 2001). Mice fed a 1 % LCA-supplemented diet show morphological alterations within 1-3 days, however, long-term maintenance on this diet is not possible due to LCA toxicity. The LCA model is therefore best suited for studies of pathogenetic mechanisms involved in acute phase of cholangiopathies.

An attractive alternative to LCA is the more soluble and less toxic cholic acid (CA). CA feeding in mice leads to deregulation of bile acid homeostasis, increase of hepatic and serum bile acid levels and subsequent hepatocellular damage (Song et al. 2011). CA also acts as an important negative regulator of bile acid synthesis (Li-Hawkins et al. 2002). As such, CA aggravates the effects of targeted inactivation of BA transporters (e.g. Abcb4 and 11) in various mouse models (Lamireau et al. 2007, Wang et al. 2003) and has been instrumental for dissecting the molecular pathways of BA metabolism and transport. When combined with 1 % cholesterol and 15-19 % fat, 0.5 % CA contributes to diet lithogenicity in gallstone formation models (Kuba et al. 2015).

Visualization techniques

Plastination

Visualization of complex biliary system has mainly used the technique of 2D examination, which fails to provide a complete and quantifiable representation of the ductal structures they are intended to elucidate. Plastination, preparation of 3D plastic resin casts is a widely used technique for visualizing and quantifying various 3D structures and networks and represents an attractive solution to overcome the limitations of 2D approaches. The routine protocol, based on injecting methacrylate resins (e.g. Mercox II) and chemical tissue maceration is simple, provides highly reproducible results, but requires advanced technical proficiency (Walter et al. 2012). Resulting 3D casts (Fig. 2A) provide a permanent snapshot allowing time-independent analysis, which yields quantitative information on branch number, diameter, and volume when combined with microcomputed tomography (Muller et al. 2016, Sparks et al. 2011). Recently, plastination has been successfully used to analyze the 3D architecture of biliary system in mouse models, showing that Notch signaling is required for post-natal maintenance of its intact structure (Sparks et al. 2011). Analysis of resin casts is also recommended to visualize bile duct alterations in mouse models of primary sclerosing cholangitis (PSC) (Fickert et al. 2014).

Scanning electron microscopy

Higher resolution analysis, resolving also canals of Hering or bile canaliculi, is enabled by scanning electron microscopy (SEM) imaging. In comparison to conventional 2D confocal microscopy, SEM reveals more subtle changes in canalicular morphology (Fig. 2B) such as dilatation, meandering and loss of microvilli, which are characteristic for the early phase of sclerosing cholangitis in the DDC mouse model (Miyao et al. 2013).
SEM imaging also facilitates the visualization of intraluminal walls of bile ducts with distinguishable structures of microvilli and primary cilia at the apical membrane of cholangiocytes (Fig. 2C). Therefore, SEM, when used in conjunction with other microscopic techniques, can provide clear information on 3D microarchitecture.

**In vivo imaging**

Until recently, minimal effort was undertaken to visualize biliary tree architecture of mouse models in vivo. Prospective cohort studies provide the possibility of repeated individual examinations, which increases the experimental efficiency and reduces the number of animals in an experiment. For standard examination of patients with various hepatobiliary diseases, magnetic resonance cholangiography (MRC) represents the primary imaging modality. In PSC patients, MRC allows visualization of strictures and dilatations of large and medium-sized bile ducts (Fickert et al. 2014). MRC is non-invasive, does not require a contrast agent and yields superior soft tissue contrast (Maccioni et al. 2010). MRC has been adapted to mouse models where it provides clear images of the gallbladder, extra-, and intrahepatic bile ducts and can be improved with intrabiliary gadoxetate disodium administration for better contrast. In the CBDL mouse model, MRC was sufficient to visualize cholangiographically distinct progressive dilation of the biliary tree (Tabibian et al. 2013). However, application of MRC in the mouse has several limitations. Image acquisition is prone to motion artifacts, producing suboptimal image quality and provides with limited visualization of the diminutive intrahepatic ducts. Computed tomography cholangiography has been proposed to overcome these limitations, but direct comparison revealed MRC has given superior image quality to date (Tabibian et al. 2013).

An extremely attractive approach for visualization of biliary tree would be based on near-infrared (NIR) imaging and lipophilic fluorescent probe which has been recently tested in mouse models of bile duct injury (Figueiredo et al. 2010). However, at the current stage of development, NIR imaging provides only reliable visualization of extrahepatic bile ducts and the liver does not appear to be conducive to in vivo 3D fluorescence imaging modalities.

**3D confocal microscopy and reconstruction**

Studies of biliary tree architecture using confocal microscopy have so far been confined to 2D mode which provides unsatisfactory results for description of both, ductular and canalicular structures (compare Fig. 2D with 2E). Several easy-to-apply protocols have recently become available providing the possibility of detailed morphometrical analysis of intrahepatic bile ducts. ‘Liver architectural staining’ protocol (Hammad et al. 2014) enables simultaneous

**Fig. 2.** Visualization of biliary tree architecture. Resin cast of liver left lobe of adult mice (A). Resin casts of adult mice was performed by manual, retrograde injection of a modified acrylic into the common bile duct, followed by tissue maceration. Scale bar=3 mm (1 mm, magnified inset). SEM images of bile canaliculi (B) and bile ducts (C). Asterisk, hepatic sinusoid; arrows, bile canaliculi; arrowheads, microvilli in B. Arrow, primary cilium; arrowheads, microvilli in C. Scale bars=5 µm (2 µm, magnified insets). Immunofluorescence staining of bile canaliculi with antibody for ZO-1 (D) and example of 3D reconstruction of bile canalicular architecture from 10-µm-thick liver slice (E). Scale bar=30 µm.
visualization of bile canaliculi, sinusoids and central veins by immunolabeling their markers. 3D reconstruction of 100-μm tissue sections and image analysis software-based quantification yields key architectural parameters (e.g. radius, branching angle, branching density and canalicular volume) for both networks in the same sample. This approach has been recently adopted to visualize and reconstruct adaptive remodelling of interlobular bile ducts in CBDL mouse model (Vartak et al. 2016) showing changes in the intraluminal surface area through corrugation and branching. Other recent confocal 3D study of intrahepatic bile ducts describes their de novo formation during embryonic development (Takashima et al. 2015).

Conflict of Interest
There is no conflict of interest.

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