Molecular basis of cholestatic diseases of surgical interest

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Cholestasis constitutes one of the most common and severe manifestations of acquired or inherited liver disease. When manifest in early infancy, it is often life-threatening and usually requires surgical management. In many cases, liver transplantation is the only effective therapy. Extensive knowledge about the molecular mechanisms underlying several pediatric cholestatic disorders has been gained in recent years from studies in both experimental models and clinical forms. In this review, we focus on recent contributions to the knowledge of molecular basis of main pediatric cholestatic disorders, such as biliary atresia, Alagille syndrome, and familial intrahepatic cholestasis. For some of them, putative targets of therapeutic interest, such as interferon-γ and Farnesoid X receptor, have been proposed.

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Cholestasis results from the impaired secretion of bile from the liver to the intestine. As such, it represents a clinical and biochemical syndrome that is produced by a wide variety of disease processes that affect the liver. Individuals with cholestasis manifest jaundice, severe itching, malabsorption of fats and lipid-soluble vitamins, and, in many cases, progressive liver damage. These clinical manifestations are due to accumulation in blood and tissues of substances normally secreted in the bile, namely bilirubin, bile acids, and cholesterol, and to the absence of bile from the intestine.

There are many causes of cholestasis in early infancy ranging from normal physiologic jaundice to complete obliteration of biliary tree. Intrahepatic cholestasis may result from occlusion or paucity of intrahepatic bile ducts or from functional failure of the hepatocytes to secrete bile. Obstruction of extrahepatic bile duct is usually referred to as extrahepatic cholestasis. Progress in the molecular mechanisms underlying disorders that fall into these categories and often require liver transplantation forms the basis of this review.

Biliary atresia

Biliary atresia represents the most frequent cause of neonatal cholestasis. It is a devastating disease resulting from a fibroinflammatory obliteration of the bile duct system that leads to impaired bile flow and ongoing hepatocellular injury. For affected children to survive, the only effective treatment is surgical. The bile duct obstruction may be relieved by portoenterostomy (the Kasai’s procedure), which in most cases improves bile drainage, particularly if
it is performed within the first months of life. However, this is a temporary treatment, and the majority of patients develop progressive hepatic fibrosis and eventually require liver transplantation. In fact, biliary atresia remains the most common indication for liver transplantation in childhood. Two clinical forms of biliary atresia are currently recognized: the fetal or embryonic form and the perinatal one. In both cases, the cause of disease remains unknown. Although it typically presents soon after birth, it does not appear to be an inherited disease. The embryonic form (also termed “polyasplenia syndrome” or “biliary atresia splenic malformation syndrome”) has been related to defects in embryogenesis, since it is frequently associated with other congenital anomalies, including poly- or asplenia, situs inversus, and cardiac abnormalities. A putative role of genes regulating laterality was suggested from findings in mice carrying a mutation in the inversin (Inv) gene, which present abnormalities in organ symmetry and obliteration of extrahepatic bile ducts. However, the involvement of INV in the embryonic form of biliary atresia seems unlikely, since no mutation in this gene has been found in affected children. Heterozygous mutations in genes involved in establishing the left–right axis, such as CFC1, which encodes the CRYPTIC protein, have been detected in several individuals with biliary atresia and laterality defects. But these mutations are suggested to confer a predisposition rather to produce the disease phenotype. To date, no consistent association between mutations in genes regulating laterality and the development of biliary atresia has been reported. Recently, Zhang and coworkers have suggested that phenotypic manifestations of the embryonic form of biliary atresia are modulated by epigenetic factors, since livers from affected children exhibit an increased expression of genes involved in chromatin function and imprinted genes, as compared with livers from patients with the perinatal form. Such a possibility is highly suggestive, but has yet to be adequately investigated.

Children with the perinatal form of biliary atresia do not present other congenital anomalies. Different etiologies have been postulated for the disease, including vascular lesions, environmental toxins, aberrant immune and/or inflammatory response, and occult viral infections. The current theories suggest that biliary atresia is not a single disease, but rather a heterogeneous condition resulting from a complex interplay among genetic factors, insults that target the hepatobiliary tree, and activation of particular immunologic pathways.

Regardless of the initiating insult, there is increasing evidence pointing to the activation of a cell-mediated immune response as a determinant factor in the pathogenesis of biliary atresia. Earlier studies showed that bile duct damage in children with biliary atresia was associated with lymphocytic infiltration into bile duct epithelium. The cell infiltrates were subsequently demonstrated to be predominantly Kupffer cells (resident liver macrophages), and CD4+ (helper), CD8+ (cytotoxic), and natural killer lymphocytes. A functional commitment of lymphocytes has been supported by gene-profiling analyses performed in liver biopsies from infants with biliary atresia at early stages of disease and age-matched diseased control subjects. Samples from children with biliary atresia exhibited a coordinated overexpression of genes regulating lymphocyte differentiation, such as osteopontin, a regulator of cell-mediated immunity, and interferon-γ (IFN-γ). Whether hepatic inflammation of children with biliary atresia represents a specific immune process involved in the pathogenesis of the disease or a secondary response to cholestasis has been recently addressed. Mack and coworkers have found that portal tracts of children with biliary atresia at the time of diagnosis (3-12 weeks of age) are infiltrated with a characteristic inflammatory cell population (CD4+ and CD8+ T cells, and Kupffer cells), with local production of proinflammatory cytokines, such as IL-2, IFN-γ, and tumor necrosis factor-α. This pattern was not found in liver from patients with other neonatal cholestatic disorders (including extrahepatic bile duct obstruction by a choledocal cyst), therefore suggesting that portal tract inflammation in biliary atresia is not secondary to the presence of cholestasis, but rather it is an event involved in the pathogenesis of the disease. Further experimental evidence has been provided by Shivakumar and coworkers in a murine model of rotavirus-induced biliary atresia. Rotavirus infection of neonatal mice specifically targeted bile duct cells and triggered an immediate infiltration of the hepatobiliary system by neutrophils, followed by IFN-γ-producing T lymphocytes, which resulted in obstruction of extrahepatic bile ducts. IFN-γ was demonstrated to play a key regulatory role in the pathogenesis of bile duct injury and obstruction, since loss of expression of this cytokine prevented the inflammatory and fibrosing occlusion of bile ducts. Although, as noted above, an increased expression of IFN-γ has been found in infants at early stages of disease, it is difficult to assess whether IFN-γ also regulates biliary obstruction in humans. Nevertheless, altogether these findings offer the encouraging prospect that selective blockage of IFN-γ action may be an approach to management of this disease.

### Alagille syndrome

Alagille syndrome is an autosomal dominant disorder characterized by developmental abnormalities of the liver, heart, face, eye, kidney, and skeleton. This disease exhibits extremely variable expressivity ranging from apparent normal phenotype to severely affected cases; some patients present only congenital cardiovascular defects such as tetralogy of Fallot and pulmonary artery hypoplasia or stenosis. The hepatic manifestations result from a paucity of intrahepatic bile ducts and vary from mild to severe cholestasis. Liver transplantation is eventually necessary in 30% to 50% of patients who have hepatic symptoms in infancy. Alagille syndrome is caused by mutation or deletion of one copy of JAG1 (Jagged1) gene, which probably leads to
protein haploinsufficiency.\textsuperscript{23, 24} \textit{JAG1} encodes a cell surface protein that functions as a ligand for the Notch transmembrane receptors. Interaction of Notch receptors (Notch 1 to 4) with their ligands (JAG1, JAG2, Delta-like1, Delta-like3, Delta-like4) represents an evolutionary conserved cell-to-cell communication system that plays a critical role in cell fate determination and differentiation. See the text for details.

In the liver, \textit{JAG1}/Notch signaling pathway appears to be involved in bile duct morphogenesis and/or the maintenance of the differentiated phenotype of biliary epithelium.\textsuperscript{25} A putative role in the postnatal development of intrahepatic bile ducts is suggested by findings showing that bile ducts are not congenitally lacking in patients with Alagille syndrome, but that the ductal paucity develops progressively after birth.\textsuperscript{19} Consistent with this, it has been recently reported that Notch signaling pathway is activated in mice during neonatal period by interaction between Jag1 expressed in the periporal mesenchyme and Notch2 receptor located in the adjacent biliary epithelial cells.\textsuperscript{27} However, expression patterns of \textit{JAG1} and Notch receptors in the developing human liver have raised the possibility that \textit{JAG1}/Notch signaling also influences duct formation during embryogenesis. Thus, in the human fetal liver, at the time of ductal plate formation, Notch3 receptor has been detected in mesenchymal cells located in the vicinity of ductal plate cells that expresses \textit{JAG1}.\textsuperscript{28} Further studies are needed to fully define the temporal and spatial specific interactions between \textit{JAG1} and Notch receptors and the stage(s) at which Notch signaling pathway is involved in biliary development. Nevertheless, it is clear that the molecular outcome of \textit{JAG1}–Notch interaction is the activation of key genes involved in cell differentiation. The chain of intracellular events triggered on binding of \textit{JAG1} to Notch receptors expressed on adjacent cells is illustrated in Figure 1. In the absence of Notch signaling, transcription of primary target genes is silenced by a corepressor complex, which includes histone deacetylases, recruited by the transcription factor RBP-J (also known as CSL). The receptor/ligand interaction induces the proteolytic cleavage of the receptor that releases its intracellular domain. This domain migrates into the nucleus and displaces the corepressor complex from the RBP-J protein, leading to transcriptional activation of the target genes.\textsuperscript{25, 29, 30}

Although the implication of \textit{JAG1} in Alagille syndrome has been widely evidenced and tissue distribution of \textit{JAG1} is consistent with clinical abnormalities observed in affected patients,\textsuperscript{31, 32} there is no clear genotype–phenotype correlation and, as stated earlier, individuals carrying identical mutations may have highly variable manifestations. This has been best exemplified in a case of monozygotic twins with discordant Alagille phenotypes: one twin presented severe pulmonary atresia and mild liver disease; the other had tetralogy of Fallot and severe cholestasis that required liver transplantation.\textsuperscript{33} Therefore, identification of a particular \textit{JAG1} mutation does not seem to offer any prediction of the severity of the disease. The variable phenotypes could most likely be explained by the existence of either genetic or environmental modifiers, or the interaction of both. This is supported by studies with mouse models. Mice heterozygous for \textit{Jag1} mutation, whose genotype mimics that of patients with Alagille syndrome, exhibit only ocular defects, and do not manifest other phenotypes associated with Alagille syndrome in humans.\textsuperscript{34} However, mice doubly heterozygous for a \textit{Jag1} null allele and a \textit{Notch2} hypomorphic allele exhibit the developmental abnormalities characteristic of Alagille syndrome.\textsuperscript{35} Thus, \textit{Notch2} gene appears to act as a genetic modifier to interact with a \textit{Jag1} mutation in mice. This raises the possibility that polymorphisms in particular \textit{Notch2} alleles, or in genes encoding other components of the Notch signaling pathway, may influence the severity of Alagille syndrome.

Progressive familial intrahepatic cholestasis

Progressive familial intrahepatic cholestasis (PFIC) is a clinical term that encompasses a subset of hereditary disorders caused by impaired bile flow without anatomic obstruction.\textsuperscript{36} Affected children typically present chronic intrahepatic cholestasis early in infancy, which leads to end-stage liver disease. Jaundice, severe pruritus, and failure to thrive are the common and predominant symptoms.\textsuperscript{37} This heterogeneous group is currently separated into different genetic diseases, inherited in an autosomal-recessive fashion. They include BSEP disease and FIC disease, which are characterized by low serum concentrations of \(\gamma\)-glutamyl transpeptidase activity and cholesterol despite conjugated hyperbilirubinemia and by decreased concentrations of bile salts in bile.\textsuperscript{38, 39} BSEP disease is caused by mutation in \textit{ABCB11} gene, which encodes the primary bile salt export pump (BSEP). It is localized in the canalicular membrane of hepatocytes and functions in transporting bile acids out of the hepatocyte into the canaliculus.\textsuperscript{40, 41} Hepatocellular retention of bile...
FXR coordinately regulates bile acid metabolism and enterohepatic circulation. On binding to bile acids or their conjugates, FXR induces in the liver the expression of the canalicular transporters BSEP, multidrug resistance protein 3 (MDR3; ABCB4), and multidrug resistance-related protein 2 (MRP2; ABCC2), while it represses the basolateral sodium taurocholate cotransporting polypeptide (NTCP; SLC10A1). In addition, FXR also inhibits transcriptional activity of cholesterol 7α-hydroxylase (CYP7A1), the rate limiting step in the conversion of cholesterol to bile acids. In the intestine, FXR positively regulates the ileal bile acid binding protein (I-BABP) and down-regulates the apical sodium-dependent bile salt transporter (ASBT; SLC10A2).

FXR-mediated bile acid transport and metabolism can be now understood as a putative involvement of this or other bile acid-activated transcription factor in the pathogenesis of some idiopathic cholestatic liver diseases will be under study. In FIC1 disease, the use of FXR agonists could have potential therapeutic implications.

Treatments for BSEP disease and FIC1 disease have included ursodeoxycholic acid therapy, partial external biliary diversion, ileal exclusion, and liver transplantation. Partial biliary diversion has been reported to be more effective than ileal exclusion for the management of patients with PFIC. It has also been shown that, following biliary diversion, hepatic ultrastructural appearance, bile acid composition, and biliary excretion improve in PFIC patients. However, this procedure might not work equally well in the different PFIC subtypes. A recent study conducted in a large group of children with genetically documented FIC1 disease and BSEP disease has revealed that the outcome of biliary diversion is better in patients with BSEP disease; in FIC1 disease patients, response to this intervention is poor. Interestingly, the outcome of partial biliary diversion in BSEP patients appears to depend on mutations found.

BSEP disease is readily corrected by liver transplantation. In contrast, a number of clinical problems persist or arise in some patients with FIC1 disease after liver replacement, including pancreatitis and intractable diarrhea. Some of these complications could be ascribed to persistent alterations in ileal bile acid transport owing to FXR downregulation, with increased presentation of bile salt to the ileum after liver transplantation leading to exacerbation of diarrhea. In this scenario, and taking into account the predominant role of FIC1 in the regulation of intestinal bile acid absorption, it is suggested that ileal bypass procedure might be an efficacious alternative approach for the management of FIC1 disease.

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