Biliary Atresia - Translational Research on Key Molecular Processes Regulating Biliary Injury and Obstruction

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Biliary atresia is the most common cause of pathologic jaundice in young infants and results from the obstruction of the extrahepatic bile ducts by an inflammatory and fibro-obliterative process. Although the pathogenesis of the disease is multifactorial, recent patient- and animal-based studies began deciphering the molecular pathways involved in biliary injury and duct obstruction. Using large-scale genomics and immunostaining of livers from children with biliary atresia, investigators have discovered unique molecular signatures of dominant proinflammatory cytokines at the time of diagnosis. To study hypotheses generated from these patient-based studies, the anatomical and inflammatory profiles of a mouse model of rotavirus-induced biliary atresia were analyzed and found to share striking similarities with the human profiles. Then, using these mice in mechanistic studies, interferon-gamma (IFNγ) has been shown to regulate the biliary tropism of lymphocytes to the biliary system, and to play a critical role in the inflammatory obstruction of extrahepatic bile ducts. The ability to combine human studies with a laboratory model of neonatal biliary injury and obstruction opens a new era of opportunities to advance the field of biliary atresia, and to develop new therapeutic strategies to improve long-term outcome with the native liver of children with biliary atresia. *(Chang Gung Med J 2006;29:222-30)*

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Biliary atresia is the most common cause of pathologic jaundice in children and the most frequent indication for liver transplantation in the pediatric population worldwide.[1] It results from a progressive fibrosing and inflammatory cholangiopathy that begins in early infancy and rapidly progresses to the complete obstruction of the extrahepatic bile ducts. As a consequence, the young infant presents clinically as jaundice, acholic stools and hepatomegaly, features that may be shared with other causes of neonatal cholestasis. Therefore, the initial task is to differentiate biliary atresia from other forms of neonatal cholestasis. This is an important task because early surgical intervention to re-establish biliary flow offers the greatest chances for long-term outcome with the native liver in children with biliary atresia. In the absence of surgical intervention, ongoing injury leads to biliary cirrhosis, portal hypertension, and end-stage liver disease. At this stage, liver transplantation is the only therapeutic option for long-term survival.

Despite the severe consequences to child's health, research that directly addresses pathogenic mechanisms of disease has been limited. One of the main factors limiting studies of etiology and pathogenesis of biliary atresia is the multifactorial nature of the disease and the advanced stage of biliary injury at the time of diagnosis in most patients. This notwithstanding, important observations in patient- and laboratory-based studies have identified poten-

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tial etiologies and pointed to possible pathogenic mechanisms of disease. We are now entering a new phase of research in which the availability of an experimental model of biliary atresia serves as a model for mechanistic studies to directly test hypotheses regarding disease pathogenesis generated from patient-based studies. In this article, we analyze the current views on mechanisms of disease, and summarize recent work in which we used complimentary human- and mouse-based studies to demonstrate that inflammatory cytokines may play a central role in epithelial injury and duct obstruction in biliary atresia.

Proposed mechanisms of disease in biliary atresia

Any proposed mechanisms of disease in biliary atresia must take into account two important clinical features that are exclusive to biliary atresia: (1) the onset of disease is restricted to the neonatal period, and (2) the target of injury is limited to the biliary system. In addition, there is no evidence of recurrence of hepatobiliary lesions typical of biliary atresia following liver transplantation. Integrating these features with the increased incidence of non-hepatic congenital malformations in a group of infants with the embryonic type of biliary atresia, it appears that the pathogenesis of biliary atresia obeys biological rules dictated, at least in part, by prenatal and postnatal development.

Despite the variability in clinical phenotypes, the intra- and extrahepatic biliary tree of all patients undergoes an inflammatory and fibrosing process triggered by an as yet unidentified agent. Conflicting evidence exists in regards to the etiological agent, the exact timing of onset of injury, and the factors that perpetuate the persistent hepatobiliary inflammation. Based largely on epidemiologic and clinical features, evaluation of the liver and biliary tree at different phases of disease, potential predisposing genetic factors, and the pace of disease progression, five mechanisms have been proposed to be involved in pathogenesis of biliary atresia: (1) defect in morphogenesis of the biliary tract, (2) defect in fetal/prenatal circulation, (3) environmental toxin exposure, (4) viral infection, and (5) immunologic/inflammatory dysregulation (Table).[2] Integrating these concepts, a working hypothesis of disease pathogenesis can be proposed in which the atresia phenotype derives from an interplay between environmental and genetic factors that results in biliary injury and progression to complete obstruction of extrahepatic bile ducts (Fig. 1).

Defective morphogenesis

Biliary atresia may belong to a spectrum of diseases characterized by the inappropriate persistence or lack of remodeling of the embryonic ductal plate forming an anatomic variant known as “ductal plate malformation.” This potential contribution of ductal plate malformation as a contributing or causative factor for biliary atresia is supported by the persistence of the embryonic shape of interlobular bile ducts in some infants at the time of diagnosis.[3] In agreement with a prenatal onset of injury, a cystic dilatation of the biliary system was detected in three fetuses dur-

Table. Potential Mechanisms Involved in the Pathogenesis of Biliary Atresia (Adapted from Balistreri et al.[2]).

Mechanism	Supporting data
1. Defect in morphogenesis	Coexistence of non-hepatic embryologic abnormalities
	Abnormal remodeling of the “ductal plate”
	Mutations in *laterality* genes (CFC1, ZIC3) in patients with biliary atresia and *laterality* defects
	Epigenetic factors: overexpression of regulatory genes in children with the embryonic form of biliary atresia
	Inv mouse: model of biliary obstruction and situs inversus
2. Defect in prenatal circulation	Intrauterine devascularization results in abnormal extrahepatic bile ducts
3. Immunologic dysregulation	Increased expression of intercellular adhesion molecules
	Increased frequency of the HLA-B12, B8 or DR3 alleles
	Hepatic profile displaying a predominant Th1-like phenotype
	Prevention of inflammatory obstruction of bile ducts in mice deficient in IFNγ
4. Viral infection	CMV, reovirus, rotavirus, and other viruses detected in infants with biliary atresia
	Biliary obstruction in newborn mice infected with rotavirus
5. Toxin exposure	Time-space clustering of cases

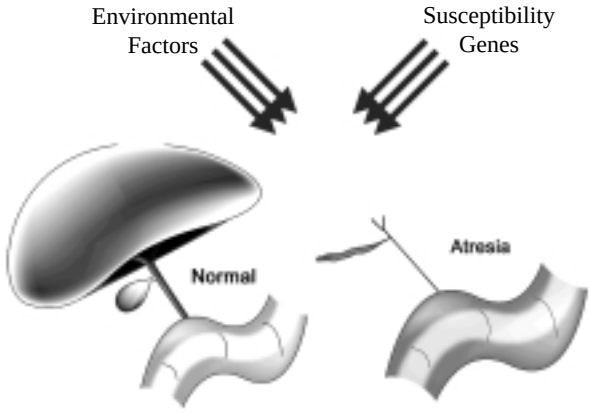

Fig. 1 Working hypothesis on the pathogenesis of biliary atresia, in which the injury and obstruction of extrahepatic bile ducts result from an interplay between environmental (viral and toxic insults) and genetic/developmental factors (abnormal prenatal circulation, genetic defects, and immunologic dysfunction)

ing routine prenatal ultrasound. Evaluation of the neonates after birth showed only duct remnants proximally and distally to the cysts, which histological features akin to typical findings of biliary atresia.[4]

The earlier onset of disease and the presence of non-hepatic malformations in a group of infants with biliary atresia also suggest a prenatal onset and a pathogenesis that differs, at least in part, from that of infants with the perinatal form. The main associated malformations, poly- or asplenia, cardiovascular defects, abdominal situs inversus, intestinal malrotation, and anomalies of the portal vein and hepatic artery, point to potential defects in embryogenesis and asymmetric left-right determination of visceral organs.[5] In support of this concept, abnormalities in organ symmetry and biliary drainage have been identified in the *inv* transgenic mouse. In this transgenic mouse, a recessive insertional mutation of the *inversin* gene results in complete abdominal situs inversus, severe jaundice, poor weight gain, and death within the first week of life in 100% of homozygous mice.[6] In a detailed morphological analysis of the hepatobiliary system in the *inv* mouse, a defect in patency of the extrahepatic ductular system was identified by trypan blue cholangiography and absent excretion of ^{99m}technetium-labeled tracer.[7] Interestingly, although the livers displayed ductular proliferation, there was no inflammation or necrosis within the hepatic parenchyma or portal tracts. This histological discrepancy with the typical features of infants with biliary atresia suggests that the biological basis of the obstruction in extrahepatic bile ducts may differ in both settings. In agreement with this concept, the full nucleotide sequence and mutational analyses in children with laterality defects and biliary atresia failed to identify mutations in the *inversin* gene.[8] The identification of loss-of-function mutations in the *CFC1* gene, which encodes the CRYPTIC protein, in patient with heterotaxy and biliary atresia maintains alive the potential role of laterality genes in contributing to the phenotypic determination of biliary atresia.[9]

Another gene that may participate in pathogenesis of biliary atresia is *Jag1*. Mutations in the *Jag-1* gene cause the Alagille syndrome, an important cause of intrahepatic cholestasis in children.[10,11] Interestingly, sequence analysis of the *Jag-1* gene in children with biliary atresia identified a high frequency of single nucleotide polymorphisms in those infants with biliary atresia with poor outcome.[12] Taken together, these data suggest that genetic factors governing morphogenesis of the biliary system may play an important role in development and/or progression of liver disease in biliary atresia.

Defective fetal/prenatal circulation

Impaired blood flow through the hepatic artery, which supplies the intra- and extrahepatic biliary system, in early development has been proposed to be an initiating factor for duct injury in biliary atresia. This is an attractive concept based on the presence of vascular abnormalities associated with biliary atresia, and the arterial hyperplasia and hypertrophy described in liver specimens of affected infants.[13] Additional data from humans or the development of experimental models to study the impact of blood flow on biliary development will be necessary to further validate a potential role of impaired circulation in the pathogenesis of biliary atresia.

Toxin exposure

Toxin exposure may play a potential role in pathogenesis of biliary atresia. To date, the only supportive data in humans is the time-space clustering of cases.[14,15] In animals, unusual outbreaks of hepatobil-

iary injury in lambs and calves in New South Wales, Australia occurred in 1964 and 1988, with pathological specimens displaying features akin to the pathology seen in humans with biliary atresia. Despite the localized geographical distribution of the outbreaks, an extensive investigation for causative phytotoxins or mycotoxins did not prove fruitful in revealing the toxic agent.[16]

Viral infection

The isolation of different viruses in livers of infants with biliary atresia points to a potential role as an initiating factor in pathogenesis of disease. The types of virus vary with the geographic region and patient population. For example, hepatitis B virus (HBV) antigens were detected in livers of infants with biliary atresia in Japan, but these findings were not reproduced in the U.S.[17-19] Likewise, there is little evidence to support the role of HAV or HCV in spite of histological findings suggesting the presence of non-A, non-B viruses in the liver.[17,20] Other reports using a variety of techniques and substrates for viral detection have implicated cytomegalovirus, retrovirus, human papiloma virus, human herpes virus-6, reovirus, and rotavirus in specific groups of patients with biliary atresia, but also with neonatal intrahepatic cholestasis and choledochal cyst.[21-31] The etiologic role for one single agent, however, has not been validated due to the inability to reproduce the association in other patient populations.[31-35] Nevertheless, among these viruses, reovirus type 3 and rotavirus type C continue to emerge as potential triggering agents for biliary atresia.

Reovirus type 3. Prevalence of antibodies against reovirus type 3 and the detection of the virus in hepatobiliary specimens of patients with biliary atresia have varied according to patient population and laboratory techniques. This is demonstrated by a high prevalence of immunoglobulin (Ig) G and IgM to reovirus in infants with biliary atresia,[28-30,36] but at least two studies could not find such an association.[33,37] More recently, the use of virus-specific amplification by reverse transcription-polymerase chain reaction identified reovirus in hepatobiliary samples of 55% of patients with biliary atresia and 78% with choledochal cyst, while the virus was present in tissues of only 8-21% of appropriately matched controls.[24] The putative association between reovirus and biliary atresia was initially suspected based on studies in young mice, which showed that reovirus infection in the weanling period resulted in the "oily fur syndrome", marked by growth failure, jaundice and oily fur. Histologically, reovirus induced hepatitis and intra- and extrahepatic biliary epithelial necrosis with surrounding edema and inflammation.[38-40] With repeated intraperitoneal injections, weanling mice develop fibrosis of the extrahepatic biliary tree, but do not progress to irreversible luminal obstruction.[38] The analysis of the cytokines mounting the inflammatory response in this animal model or in infants with biliary atresia infected with reovirus has not yet been performed.

Rotavirus. Rotavirus was reported in a cohort of infants with biliary atresia.[22] Administration of *rhesus* rotavirus-type A to newborn mice orally or intraperitoneally produces a notable phenotype resembling biliary atresia, with progressive jaundice, acholic stools, bilirubinuria, and growth failure, eventually culminating in death in many infected animals.[41-43] The histological appearance of the liver and biliary tree in late stages of biliary obstruction shows similarity with the histological features of biliary atresia. Analysis of the extrahepatic bile ducts before and after the onset of jaundice shows inflammation and edema of the duct wall, progressing to sloughing of the biliary epithelium, closure of the duct lumen by inflammatory cells and other cellular debris, and finally concentric fibrosis of the extrahepatic bile ducts (Fig. 2). This processes results in a segmental or continuous obstruction of the extrahepatic duct lumen. The precise mechanisms of viral-induced injury have not yet been established, but recent studies have begun elucidating cellular and molecular pathways regulating duct injury and obstruction (reviewed below).[41-44]

Inflammatory/immunologic dysregulation

Cellular phenotyping and molecular studies of liver samples from infants/children with biliary atresia suggest that the inflammation observed in the biliary system is not simply a non-specific response to an injury, but rather it may play an important effector role in the biliary injury. For example, cholangiocyte pyknosis and necrosis have been associated with infiltration of mononuclear cells into the walls of interlobular bile ducts, as well as lymphocytic infiltration into portal tracts, the duct walls at the portahepatis, and common hepatic duct remnants of

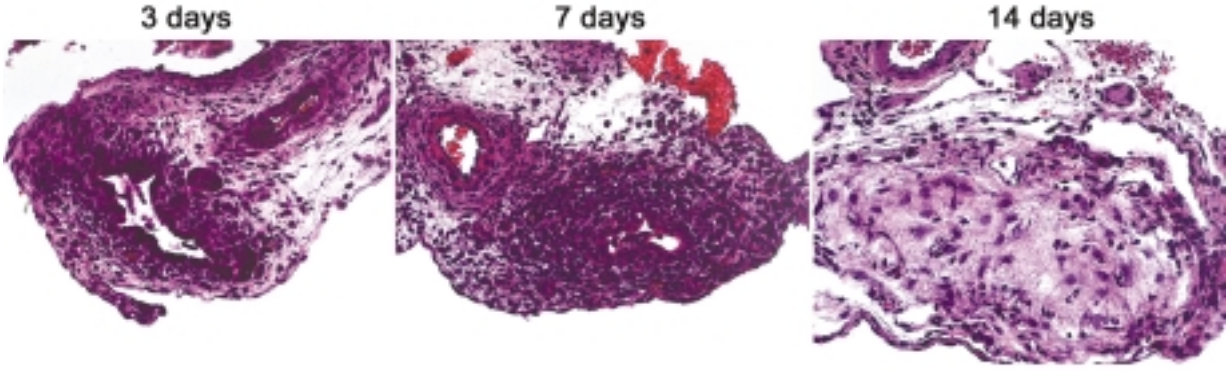

Fig. 2 Hematoxylin-eosin staining of cross sectional views of the extrahepatic bile ducts at 3, 7, and 14 days after the intraperitoneal administration of rotavirus into mice in the first 24 hours of life.

infants with biliary atresia.[45-47] Phenotypic characterization of these inflammatory cells has identified $CD8^+$ T cells infiltrating proliferated bile ducts, although the cells did not express perforin or granzyme B, markers of activated cytotoxic T lymphocytes.[48] In general, however, the lymphocytes infiltrating portal tracts in biliary atresia are $CD4^+$ rather than $CD8^+$ T cells. These cells express markers of T helper (T_H) lymphocyte activation and proliferation, such as the interleukin-2 receptor (CD25) and the transferrin receptor (CD71).[49-51]

Differentiation of T_H lymphocytes to a proinflammatory phenotype (T_H1) often requires $CD4^+$ T cells to encounter exogenous antigens complexed with an MHC class II molecule on the surface of an antigen-presenting cell (APC). In the context of biliary atresia, cholangiocytes, which normally express MHC class I but not class II antigens, are induced to aberrantly express HLA-DR (a major MHC class II molecule) and act as APCs.[49,52,53] Furthermore, intercellular adhesion molecule-1 is expressed on bile duct cells of patients with biliary atresia, while one of its ligands, the leukocyte functional antigen-1, is expressed on infiltrating mononuclear cells.[51,54] Interaction between these two molecules is one of the mechanisms necessary for inflammatory cell recruitment and perpetuation of the immune response. The immune response may also be influenced by Kupffer cells (resident hepatic macrophages). In biliary atresia, Kupffer cells have been shown to express MHC class II antigen HLA-DR, and comprise a higher population of nonparenchymal cells.[51,55,56] A potential pathway that would explain the interaction of Kupffer cells and lymphocytes in the pathogenesis of biliary atresia involves infiltration of portal tracts by $CD14^+$ Kupffer cells, which are induced to express IL-18, a pro-inflammatory cytokine that promotes interferon-gamma production and T_H1-differentiation of lymphocytes.[55,56]

Molecular basis of neonatal biliary obstruction

Patient-based studies

The potential interplay of genetic, toxic and infectious agents in the pathogenesis of biliary atresia underscore the multifactorial basis of disease. Therefore, we interrogated the liver-specific transcriptional program in search for dominant molecular pathways in early phases of disease. In these studies, we compared the levels of gene expression in the livers of children with biliary atresia at different phases of disease using gene chips. For controls, we used liver biopsies from age-matched subjects with intrahepatic cholestasis. Analysis of over 12,000 genes using highly stringent statistical parameters revealed a unique transcriptional footprint for age-matched patients with biliary atresia. Interestingly, this footprint contained a significant number of the genes that were functionally related to a proinflammatory activation of lymphocytes, with an increased expression of osteopontin, a regulator of T_H1 immunity, and suppression of immunoglobulin genes. Notably, these findings were associated with the production of IFNγin 65% of infants with biliary atresia, and no detectable IFNγin disease-controls.[57] Histologically, the degree of inflammatory infiltrates was similar in

both groups, implying differential activation states of similar cell types.

Further support for the role of the hepatic inflammation as a key mediator of biliary injury was gained by a recent detailed immunohistochemical analysis and gene expression studies in livers of children with biliary atresia at the time of diagnosis (3-12 weeks of age), age-matched disease controls (neonatal giant cell hepatitis, choledochal cyst, and total parenteral nutrition-induced cholestasis), and healthy control subjects. Liver samples from subjects with biliary atresia had a greater infiltration of portal tracts by CD4 and CD8 lymphocytes as well as CD68 (Kupffer) cells, with no obvious differences in the number of cells stained with antibodies against CD20 and NK1 (markers of B and NK lymphocytes, respectively). Consistent with a proinflammatory response, there was also an increased expression of IL-2, IL-12, IFNγ, TNFα, with a distinct pattern of cytokine expression in the portal environment.[58] Although circumstantial, the collective data from patient-based studies clearly support a working hypothesis in which a T_H1, proinflammatory commitment of lymphocytes is an important effector of epithelial injury in biliary atresia. Testing this hypothesis, however, required the use of in vivo and in vitro experimental models that permitted mechanistic studies.

Studies using the experimental model of biliary atresia

To circumvent experimental difficulties in performing mechanistic studies in humans, we used the mouse model of rotavirus-induced biliary atresia in neonatal mice. Studying the liver and biliary system of these mice, we found that rotavirus has a unique tropism to bile duct cells, as demonstrated by the detection of rotavirus in the biliary epithelium 3 days after viral inoculation.[59] In vitro, we found that a murine cholangiocyte cell line is susceptible to rotavirus infection, but does so at a 100-lower multiplicity of infection when compared to MA104 cells (monkey kidney epithelial cells known to be susceptible to rotavirus). Following infection of cholangiocytes, recovery of live virus was only 11-fold lower than in MA104 cells. Collectively, these data supported the concept that the ability of rotavirus to destroy cholangiocytes was not the sole mechanism of injury to the epithelium and lumenal obstruction. Therefore, we investigated whether the inflammatory response to rotavirus contributed to the tissue-specific injury.

IFNγ as a key mediator of duct obstruction. First, we used flow cytometric analysis of hepatic mononuclear cells to determine the phenotypic adaptations of lymphocytes following rotavirus challenge. We found that rotavirus-infected mice displayed a 3-fold increase in CD3+ lymphocytes above controls at 7 and 14 days, which consisted of both CD4+ and CD8+ cells.[59] These cells created a hepatic environment rich of proinflammatory cytokines, with an increased expression of IFNγ and IL-12, and the complete clearance of rotavirus by 10-14 days after viral challenge. This increased production of proinflammatory cytokines was also reported by another laboratory recently, which showed the overexpression of IFNγ, TNFα, and inducible nitric oxide synthase (iNOS) in the same murine model of rotavirus-induced biliary atresia.[60] To directly examine the role of IFNγ in biliary injury, we applied the same model of rotavirus infection to mice deficient in IFNγ due to a mutation in the IFNγ gene. We found that mice lacking IFNγ developed jaundice after infection, but the symptoms gradually disappeared and were followed by improved weight gain and long-term survival.[59] Morphological analysis of the biliary tree showed that the extrahepatic bile ducts were patent and maintained continuity between the liver and duodenum. In addition, there was a marked suppression of the inflammatory infiltration by T lymphocytes, with complete prevention of the inflammatory and fibrosing obstruction of extrahepatic bile ducts.

To more directly demonstrate the critical role of IFNγ on duct obstruction, we administered recombinant IFNγ to IFNγ-deficient mice after rotavirus infection. We found that reconstitution of IFNγ resulted in the timely development of cholestasis in more than 80% of the mice, with the extrahepatic bile duct displaying recurrence of the obstruction in a fashion indistinguishable from the duct injury observed in wild-type mice. Collectively, these data demonstrate that IFNγ plays a critical role in the inflammatory obstruction of extrahepatic bile ducts, and may constitute a therapeutic target to stop disease progression in children. These data also suggest that the pathogenic mechanisms of biliary atresia obey a biological continuum previously not recognized.[2,61,62] The initiating events of this continuum,

namely the immediate jaundice and inflammation, were not dependent on IFNγ. In contrast, the progression to duct obliteration by lymphocytes and fibrosis was prevented by the loss of IFNγ.

The challenges ahead

One obvious implication of the findings that loss of IFNγ prevented bile duct obstruction is whether the timely removal of IFNγ has the potential to restore duct patency or to decrease the intrahepatic inflammation and fibrosis that are typically seen following portoenterostomy. Before these concepts are extended to clinical trials, pre-clinical studies must be carried to test whether the removal of IFNγ at or after the onset of symptoms restores patency to bile ducts and improves long-term survival in experimental biliary atresia.

Since IFNγ plays such a dominant regulatory role in duct obstruction, it is possible that other proinflammatory cytokines such as IL-2, IL-12, and TNFα and other soluble mediators (such as iNOS) work in synergism to drive the pathogenesis of biliary atresia. In this context, the nuclear factor-kB (NFkB) was recently shown to increase after rotavirus challenge in neonatal mice. Interestingly, blocking of this expression by an NFkB inhibitor decreased the inflammation in the liver and extrahepatic bile ducts.[63] The rapid succession of mechanistic studies reviewed above clearly show an expansion of the field, with an increasing interest to apply state-of-the art technology to decipher the pathogenic mechanisms of disease, and to ultimately identify agents that serve as therapeutic targets to stop disease progression in children with biliary atresia.

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