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Original contribution

Elaboration of tubules with active hedgehog drives parenchymal fibrogenesis in gestational alloimmune liver disease☆,☆☆

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Summary Gestational alloimmune liver disease (GALD) produces severe neonatal liver disease that is notable for paucity of hepatocytes, large numbers of parenchymal tubules, and extensive fibrosis. Liver specimens from 19 GALD cases were studied in comparison with 14 infants without liver disease (normal newborn liver; NNL) to better understand the pathophysiology that would produce this characteristic histopathology. GALD liver parenchyma contained large numbers of tubules comprising epithelium expressing KRT7/19, EPCAM, and SOX9, suggesting biliary progenitor status. Quantitative morphometry demonstrated that in GALD, the area density of KRT19+ tubules was 16.4 ± 6.2 versus 2.0 ± 2.6 area% in NNL ($P < .0001$). Functional hepatocyte mass was markedly reduced in GALD, 16.3 ± 6.2 versus 61.9 ± 11.0 area% of CPS1+ cells in NNL ($P < .0001$). A strong inverse correlation was established between CPS1+ area density and KRT19+ area density ($r^2 = 0.66$, $P < .0001$). Tubules showed active hedgehog signaling as determined by SHH and nuclear GLI2 expression and expressed the profibrogenic cytokine SPP1. SPP1 protein content and *SPP1* expression were greater in GALD than NNL (15- and 13-fold respectively; $P = .002$). GALD liver contained large numbers of activated myofibroblasts and showed greater than 10-fold more fibrosis than NNL. The extent of fibrosis correlated with the area density of KRT19+ tubules ($r^2 = 0.387$, $P = .001$). The data support a pathogenic model in which immune injury to fetal hepatocytes provides a stimulus for expansion of parenchymal tubules, which, by way of Hh activation, produce fibrogenic signals leading to vibrant fibrosis.

1. Introduction

Severe fibrotic liver disease in the newborn indicates the onset of liver injury during fetal life. Such disease has been called "congenital cirrhosis" and has been associated with the neonatal hemochromatosis phenotype, wherein there are iron overload and tissue siderosis in a pattern similar to that seen in

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hereditary hemochromatosis [1]. The most frequent cause of fetal liver injury leading to congenital cirrhosis and the neonatal hemochromatosis phenotype is gestational alloimmune liver disease (GALD) [2]. GALD-related alloimmunity is specifically directed at fetal hepatocytes. No nonhepatocyte elements in the liver appear to be injured, and tissues outside the liver appear to be unaffected by the primary immune process. The mechanism of alloantibody-induced hepatocyte injury appears to involve the fetal innate immune system. The terminal complement cascade is activated by the classical pathway and results in the formation of membrane attack complex. Immunohistochemical staining for C5b-9 complex (the neoantigen created during terminal complement cascade activation and culmination with formation of membrane attack complex) shows nearly all hepatocytes in cases of GALD to have complement-mediated injury [3]. The immediate result of such injury may be liver failure in the fetus and may lead to fetal death [4]. However, in most cases, the process moves more slowly, starting in midgestation and resulting in liver failure and often cirrhosis by term [2,5]. Newborns with GALD typically show clinical liver failure usually within the first days of life.

The immune injury in GALD is specifically directed at hepatocytes and results in liver disease confined to the lobule. The liver histopathology includes severe depletion of hepatocytes relative to normal newborn liver (NNL) [5]. Remaining hepatocytes appear as giant cells and pseudoacini, which are constructs of hepatocytes surrounding a central space often containing bile. Another epithelial structure appears in abundance in the livers of GALD cases: namely, tubular forms, which consist of epithelial cells surrounding a narrow central lumen that is usually devoid of bile. They are usually narrow and elongated in contrast to the generally round pseudoacini. They are morphologically similar to the "ductular reaction" observed in humans with massive and submassive necrosis associated with acute liver failure [6,7]. It is thought that this represents a regenerative effort and that the ductules may be derived from stem cells residing in the canals of Hering and/or ductal plate remnants [7]. Further expansion of regenerative elements results in the "ductular reaction" in which neoductules (also known as *neocholangioles*) expressing biliary progenitor markers extend from the ductal plate/canals of Hering into the lobule. Biliary atresia and other cholestatic liver diseases of infancy are notable for "ductular reaction" along the limiting plate of the portal triad. In the case of biliary atresia, it appears that a repair response to biliary injury leads to a hyperactive hedgehog (Hh)–driven regenerative signal with resultant biliary dysmorphogenesis [8]. The liver histopathology in GALD and biliary atresia are in stark contrast; however, the 2 diseases are similar in that they exhibit extensive fibrosis very early in life.

In GALD, fibrosis is in the lobule, whereas in biliary atresia, it is portal. Tubules that appear throughout the lobule in GALD share several morphologic features, with reactive ductules appearing along the limiting plate in biliary atresia. In addition, GALD is a disease of the fetal liver wherein Hh and other developmental pathways are maximally active. We hypothesized that formation of parenchymal tubules drives the lobular fibrogenesis in GALD, in analogy to the role of reactive ductules in portal fibrogenesis in biliary atresia [8,9]. Hence, we examined the relationships among tubule formation, Hh activity, osteopontin production, myofibroblast expansion and activation, and fibrosis in GALD. The results suggest that expanded formation of tubules is likely central to lobular fibrogenesis in GALD and that active Hh signaling by tubules is involved.

2. Materials and methods

2.1. Cases and reference materials

GALD cases: liver specimens from 19 GALD cases were included in the study. The subjects of this study were newborns with liver failure and the NH phenotype. All 19 liver specimens showed evidence of complement-mediated hepatocyte damage and were among cases previously reported [3]. Their postpartum ages ranged from 1 to 12 weeks. Of these, 14 specimens were collected by autopsy and 5 were hepatic explants. All 19 specimens were used for histology and immunohistochemistry studies, whereas the 5 hepatic explants were in addition snap-frozen at the time of harvest and processed for RNA and protein expression analyses. Reference cases (referred to as *NNL* throughout) comprised the following. Liver specimens from 8 newborns who died from perinatal asphyxia were obtained at autopsy. These cases showed no histologic liver damage and have been similarly used in other published studies [3,5,10]. Liver specimens from 6 children aged 4 months to 5 years were snap-frozen at harvest and processed for RNA and protein analysis. These cases included 4 deceased organ donors, 1 infant undergoing laparotomy, and the disease-free liver of 1 child undergoing hepatoblastoma resection. The specimens all showed normal histology and have been used for gene and protein expression analyses in comparison with neonatal and infant liver disease in other published studies [8,10]. This study was approved by the institutional review board of Lurie Children's Hospital of Chicago.

2.2. Molecular techniques

The expressions of the various genes listed in the Table were determined using real-time reverse transcription polymerase chain reaction, as previously described [8,10]. The detailed methods and primer sequences used are presented in Supplementary Table S1. The expression of each gene was normalized to the expression of GAPDH in each sample. The results for individual genes are listed according to how they appear in the following results and will be presented as supporting evidence for the other studies performed. The expressions of genes relative to GAPDH in GALD liver and NNL are provided in Supplementary Table S2. In the Table, the normalized expression of each gene in GALD and NNL was computed as the fold-expression relative to the median expression in the NNL cohort. The statistical significance of