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Original Article

The inflammatory phenotype of the fibrous plate is distinct from the liver and correlates with clinical outcome in biliary atresia

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ABSTRACT

Biliary atresia is an inflammatory cholangiopathy of still undetermined etiology. Correlations between histologic findings and clinical outcome in this disease have largely been based on evaluation of liver parenchyma. This study aimed to characterize the pattern of inflammation within the biliary remnant and identify associations between the type and degree of inflammation and clinical outcome as reflected by the transplant-free interval. The inflammation within the fibrous plates and livers of 41 patients with biliary atresia was characterized using immunohistochemical markers and the cell populations were digitally quantified. The type and quantity of cells within the infiltrate were then correlated with length of time from Kasai portoenterostomy until transplant. Histologic and immunohistochemical features of the biliary remnant allowed stratification of patients into "inflammatory plate" and "fibrotic plate" groups. Overall there was no significant difference in transplant-free interval between the two cohorts; however, there was a trend towards a longer time to transplant among patients in the "fibrotic plate" group. In addition, the composition of the inflammatory infiltrate in the fibrous plate was distinctly different from that present in the liver and only the characteristics of the inflammation in the fibrous plate, in particular the number of Foxp3+ T regulatory lymphocytes correlated with clinical outcome. The results of this study support the view of the extra-hepatic biliary tree as the primary site of injury in BA with the changes seen in the liver as secondary manifestations of outflow obstruction. The association between specific inflammatory cell subtypes within the fibrous plate and the length of transplant-free interval also supports the role of the immune system in the initial process of bile duct damage in biliary atresia.

Introduction

Biliary atresia (BA) is an inflammatory cholangiopathy of infancy. It is the most common cause of end-stage liver disease in infants and is also the main indication for liver transplantation in children. The incidence of BA is estimated at between 1/5,000 and 1/18,000 live births, being more frequent among non-white patients and in girls [1]. Clinically, two types of BA are described: a perinatal form (~80% of cases) that manifests as jaundice in the first post-natal weeks in initially asymptomatic children and an embryonic form (~20% of cases). The embryonic form occurs without a jaundice-free interval and is associated with other congenital anomalies including those involving the cardiovascular system (interruption of inferior vena cavae with azygos continuation, pre-duodenal portal vein, hypoplastic left heart syndrome, atrial/ventricular septal defects), digestive system (intestinal malrotation), lungs (bronchial anomalies) or situs anomalies (asplenia or polysplenia) [2,3].

The etiology of BA remains uncertain, although multiple causes have been proposed involving immunologic [4,5], genetic [6,7], vascular [8] and infectious agents [9–11] resulting in a fibro-obliterative process acting upon and destroying a normally

Abbreviations: BA, biliary atresia; Treg, T regulatory lymphocytes.

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developing biliary duct system [12] rather than a primary developmental failure.

BA is suspected clinically in neonates with jaundice beyond 14 days of life with elevated conjugated bilirubin, high gamma-glutamyl transpeptidase and alkaline phosphatase. Liver biopsy is performed to confirm the diagnosis with histology demonstrating varying degrees of portal tract fibrosis, edema, bile duct proliferation and cholestasis with intracanalicular bile plug formation [13]. Early diagnosis is critical as prompt surgical intervention, typically consisting of Kasai portoenterostomy, directly correlates with long-term prognosis. The procedure involves removal of the fibrotic segment of extra-hepatic bile duct with anastomosis of a jejunal Roux loop to the cut surface of the porta hepatis with the goal of restoring adequate bile flow; however, most patients eventually require liver transplantation [14], particularly in cases with poor early response to Kasai, failure to thrive, late-onset (adolescent) cholestasis, recurrent cholangitis or portal hypertensive bleeding [15].

Only a few studies, concentrating mostly on histopathologic changes in the liver, have attempted to determine relationships between microscopic features and clinical course in patients with BA [16–19]. Further characterization of the changes within the extra-hepatic biliary tree represented in the fibrous plate, the actual target of the fibro-inflammatory process, is needed to increase our understanding of the etiology and clinical course in this disease.

This study analyzed the composition of the inflammatory infiltrate of the fibrous plates and livers of BA patients at the time of Kasai portoenterostomy to determine if any associations exist between the pattern of inflammation and clinical outcome as demonstrated by transplant-free interval or bilirubin level. The data obtained revealed differences in the type and degree of inflammation between the biliary remnant and corresponding hepatic parenchyma and uncovered additional factors that correlated with time until liver transplantation following Kasai portoenterostomy in a subset of patients.

Materials and methods

Subjects: Information on 41 BA patients enrolled in this study (1/1/1981 – 12/31/2008) was collected under the approval of The Children's Hospital of Philadelphia Institutional Review Board. In three instances, BA occurred in association with other congenital anomalies: one case of heterotaxy, one case with an unspecified fatty acid oxidation disorder and another with global developmental delay. All 41 patients underwent Kasai portoenterostomy and had slides of the biliary remnant (fibrous plate) for review. In 34 cases, a corresponding liver biopsy obtained at the time of Kasai or shortly before surgery was also available for review. All patients subsequently had liver transplantation after varying lengths of time. The minimal follow-up period after transplantation was 4 years.

Histology and immunohistochemistry: Biliary remnants obtained at the time of Kasai were oriented and serially sectioned during macroscopic examination with alternative sections submitted for microscopy. The tissue from the fibrous plates and livers were then routinely processed, embedded in paraffin, sectioned at 4 microns and stained with hematoxylin and eosin (H&E) for light microscopy. All cases were reviewed to confirm the histologic impressions at the time of diagnosis and the most representative section from each case was stained with primary antibodies directed against T lymphocytes (CD4, Leica, 1:100 and CD8, Vector, 1:100); B lymphocytes and plasma cells (CD79A, DAKO 1:100), natural killer (NK) cells (CD56, Cell Marque, 1:400), macrophages (CD163, Vector 1:100) and T-regulatory (Treg) cells (Foxp3, Biolegend, 1:200). Antigen retrieval was performed for 20 min with either epitope retrieval 2 reagent (ER2, Leica, for CD4, Foxp3, CD8, CD79A, CD163) or epitope retrieval 1 reagent (ER1, Leica, for CD56). Visualization was obtained with BOND Polymer Refine Detection System and DAB Enhancer. Due to tissue exhaustion, Foxp3 staining was performed on 38 (out of 41) biliary remnant and 29 (out of 34) liver specimens.

Image acquisition and digital image analysis: Immunostained slides of the fibrous plates and livers were scanned using the Aperio ScanScope® CS slide scanner (Aperio Technologies, Vista, CA) and the entire digitized sections were analyzed using the Aperio ImageScope software (version 10.0.1346.1807; Aperio Technologies, Vista, CA; http://www.aperio.com/download.asp). For liver sections, all portal tracts available were thus analyzed. Output of the scan analysis consisted of the raw numbers and also the percentage of all cells with membranous (CD4, CD8, CD79A, CD56, CD163) or nuclear (Foxp3) positivity from the total number of cells present on the slide (including fibroblasts, bile duct epithelium, endothelial cells and hepatocytes). The sum of all positive cells was calculated and designated as "percentage of total inflammatory cells". In some cases, the percentage of total inflammatory cells was greater than 100%, which could be explained by the differences in detection/intensity thresholds used for each antibody as well as non-specific staining of non-inflammatory cells (for example focal CD56 reactivity of bile duct epithelium). However, these cell types were present in all cases, their number was low and it was not felt to contribute to significant differences between the cases. In some cases, the Foxp3+:CD4+ cells ratio was greater than 1, which again can be explained by the differences in the detection threshold between the antibodies. One-third of all immunostained slides for each cell subtype were also reviewed by two pathologists (NA, TRB) blinded to clinical features and digital image analysis results to insure accuracy of the digital system. No significant differences in the quantitation of positive cells using the digitized versus manual method were noted.

Statistical analysis: Data generated by digital image analysis had a non-parametric distribution. Spearman correlation coefficient was used to determine the association between the percentage of total inflammatory cells or specific types of inflammatory cells and transplant-free interval (defined as the difference, in days, between the date of transplantation and date of portoenterostomy) or age at Kasai. The Mann – Whitney *U* test was used to assess the differences in the inflammatory infiltrate composition between the fibrous plate and the liver. The probability of early or late transplant based on the amount of fibrous plate inflammation was determined by Fisher exact probability test. The bilirubin level (<2 or >2 mg/dl at 90 days post Kasai) was also compared between patients with different types of biliary remnant histology. A p value <or = 0.05 was considered statistically significant.

Results

Histologic and immunohistochemical analysis of the biliary remnant allows stratification of patients into "inflammatory plate" and "fibrotic plate" groups. Routine microscopic examination of H&E stained sections of the biliary remnant revealed two primary histologic patterns. One group was characterized by robust inflammation infiltrating around a few residual bile ducts lined by degenerated epithelium (Fig. 1A and B). A second group demonstrated a sparse inflammatory infiltrate and scattered remaining bile ducts of smaller caliber in a more abundant fibrotic background (Fig. 1C and D). The impression was confirmed on review of immunostained slides which also revealed a mixed inflammatory infiltrate composed of mature lymphocytes, macrophages and plasma cells, although the infiltrate was sparse in the latter group with abundant fibrotic stroma (Fig. 1E). Based on quantification