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Long-term outcome and necessity of liver transplantation in infants with biliary atresia are independent of cytokine milieu in native liver and serum

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ABSTRACT

Purpose: Biliary atresia (BA) is a rare disease of unknown pathogenesis in infants characterized by an inflammatory, progressive destruction of the biliary system and deterioration of liver function. The standard treatment for BA is a Kasai-hepatoportoenterostomy (KPE). However, liver transplantation (LTX) becomes necessary in about 50–80% of cases. Therefore, some authors advocate for primary LTX in BA, but this would require early markers to predict which children would benefit from KPE or to show rapid progression to liver cirrhosis (RLC) instead.

Methods: Snap-frozen liver biopsies and sera samples of 57 infants with BA were collected during KPE. Clinical and follow-up data were assessed via the biliary atresia and related diseases registry (BARD-online.com). Protein-levels of 25 pro- and anti-inflammatory mediators of 49 infants were assessed via multiplex protein-immunoassay and analyzed by *t*-test as well as multidimensional principal component analysis.

Results: 22 different immunomodulatory mediators were detectable in livers of children with BA, while serum protein levels were very low to undetectable. Following KPE, 33 BA patients showed RLC that required early LTX, while 24 had favorable course of disease with long-term survival with native liver (SNL). There were no significant differences between RLC and SNL in terms of local (from liver samples) nor systemic (from sera) immunomodulatory mediators. Protein levels were much lower in sera than in livers without statistical correlation.

Conclusion: Our data suggest that local or systemic immunomodulatory mediators are unsuitable for predicting the disease course of BA. Thus, no deduction for optimal treatment strategy can be drawn. Collectively, we conclude that in BA, the degree of inflammation and protein microenvironment in the liver at the time-point of KPE are dismissible factors for the future course of disease.

1. Introduction

Biliary atresia (BA) is a rare disease of the neonatal liver leading to a progressive destruction of the biliary system and subsequent liver cirrhosis and failure [1]. In the western hemisphere, there is approximately 1 case per 15,000–20,000 live births [2]. The pathogenesis of BA is

Abbreviations: BA, biliary atresia; BARD, Biliary Atresie and Related Diseases; Beta-NGF, beta-nerve growth factor; Beta-FGF, beta Fibroblast growth factor; BDNF, brain-derived neurotrophic factor; CCL, CC-chemokine ligand; CXCL, (C–X–C motif) ligand; EGF, epidermal growth factor; ELISA, enzyme linked immunosorbent assay; FGF, fibroblast growth factor; FIG, figure; FIGF, C-fos-induced growth factor; GM-CSF, granulocyte macrophage colony-stimulating factor; GRO-alpha, growth related oncogene-alpha; HGF, hepatocyte growth factor; IFN, interferon; IL, interleukin; IL-1RA, interleukin-1 receptor antagonist; IP, interferon-gamma induced protein; KPE, Kasai-Portoenterostomy; LIF, Leukemia inhibitory factor; LTX, liver transplantation; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory proteins; MP, Medical Products; NCBI, National Center for Biotechnology Information; PCA, principal component analysis; PDGF-bb, platelet-derived growth factor receptor; PIGF, placenta growth factor; RANTES, regulated and normal t cell expressed and secreted; RLC, rapid liver cirrhosis; SCF, stem cell factor; SD, standard deviation; SDF, stromal cell-derived factor; SNL, survival with native liver; TGF, tumor growth factor; TNF, tumor necrosis factors; VEGF-a, vascular endothelial growth factor alpha

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unknown, although clinical observations and evidence gathered from experimental models point toward progressive inflammation due to an autoimmune process, possibly triggered by a viral infection [1,3]. If untreated, BA has a fatal prognosis. Causative treatments and medication are not available. Instead, a temporary improvement of the bile release can be achieved by a Kasai-hepatoportoenterostomy (KPE) [4]. The surgically-accessible, extrahepatic bile ducts are removed, and a temporary bile release is achieved in most patients by Y-Roux Hepaticojejunostomy [5,6]. Despite successful KPE, liver failure may still result from progressive intrahepatic obliteration in 50–80% of cases [7,8]. Thus, BA has been the most common indication for liver transplantation (LTX) during childhood for the past 20 years [9,10]. Although the mortality of BA has been significantly reduced by LTX during recent decades, the donor liver supply is restricted and up to 20% of BA patients die while still on waiting lists for transplantation [11].

Occasionally, primary LTX without prior Kasai-procedure is indicated, as some data point to fewer complications after LTX if a Kasai procedure is not performed before the transplant [12–16]. However, LTX in early infancy is technically more difficult and risky due to the sensitivity of the very young organism; LTX is therefore associated with increased post-transplant mortality [17]. Thus, KPE, performed as early as possible, is still the gold standard for children with BA. After surgery, some patients show a favorable, jaundice-free course of disease, enabling growth and a sufficient quality of life for many years [18]. Some children who have already undergone KPE are later recommended for secondary LTX. In children aged two years or older, secondary LTX has a significantly reduced post-transplant mortality compared to infants who undergo LTX [17]. In other cases, however, a rapid progression of the disease and deterioration of the liver function occur despite successful KPE, and early LTX is necessary to ensure the child's survival. In these cases, KPE may even have been disadvantageous for the child since secondary LTX has more technical challenges in the setting of preoperated situs (e.g. abdominal adhesions) and requires longer anesthesia. In addition, KPE itself harbors significant risks associated with any abdominal surgery [14,15]. Thus, it would be ideal to know in advance whether and for how long infants with BA would benefit from KPE, and whether a primary liver transplant may be considered.

Early prognostic biomarkers are necessary to optimize patient care by offering supportive therapies to maintain quality of life and early registration on transplant waiting lists. As periductal inflammation is a hallmark of BA, numerous pro-inflammatory cytokines have been implicated in the pathogenesis of BA [3]. However, current data on cytokine profiles in human BA patients are based primarily on a comparison of serum markers between BA patients and healthy or cholestatic control groups [19,20]. Information on intra-hepatic inflammatory profiles in BA patients is sparse and has not been correlated to the clinical course so far. We hence hypothesized that the levels of immunomodulatory proteins in liver or sera of infants with BA may predict the course of disease and thus indicate whether the individual patient is a good candidate for KPE, LTX, or a combination of both measures.

2. Methods

2.1. Patients and follow up

The study protocol is in accordance with the declaration of Helsinki and approved by the local ethics committee. Written informed consent was obtained from each patient's guardian.

Between 2004 and 2014, a total of 119 patients with isolated BA presented in our hospital and were eligible for inclusion. Follow-up data for more than 2 years and sufficient parallel bio-banked sera and liver tissue specimens were available for 57 patients. 49 of these infants were determined to have either a favorable prognosis (jaundice free (bilirubin < 20 μmol/l) survival with own liver > 2 years) or an unfavorable prognosis (death or LTX) based on a chart review and the biliary atresia and related diseases registry (www.bard-online.com). One patient underwent a Re-KPE 48 days following the first operation on day 42. The samples retrieved at the second KPE were not included in group analyses, but were compared separately to the samples from the first KPE. None of the BA patients included in this study had any associated non-hepatic congenital anomalies.

2.2. Sera and liver tissue retrieval and sample preparation

During the Kasai-procedure, sera and an approx. 0.5×0.5 cm specimen of liver tissue was obtained, immediately snap-frozen in liquid nitrogen, and subsequently stored at -80 °C. Liver tissue was thawed and homogenized in the presence of lysis buffer with 5 mm stainless steel beads for 30 sec at 25 Hz using a homogenizer FastPrep 24 (MP Biomedical). Protein concentrations were measured (Pierce BCA Assay Kit, Thermo Scientific) and adjusted to 500 μg/ml per sample.

2.3. Multiplex measurement of immunomodulatory proteins

45 biomarkers were measured simultaneously using ProcartaPlexTM Multiplex Immunoassays (Affymetrix, eBioscience) according to the manufacturer's protocols in duplicates, as previously described [21,22]. All pro- and anti-inflammatory ($n = 25$) mediators were included in the analysis. In brief, this assay quantifies proteins based on the principles of a sandwich ELISA with specific antibodies recognizing epitopes on a total of 45 different proteins. The proteins were classified into 3 groups (growth factors, pro-inflammatory mediators, and anti-inflammatory mediators) based on NCBI protein and gene searches (www.ncbi.nlm.nih.gov). All pro- and anti-inflammatory mediators were included in this study. Most proteins could be classified into more than one group due to overlapping pleiotropic functions (Table 1).

2.4. Data analysis

Statistical analysis was performed using GraphPad Prism software version 6.0 (GraphPad Software, San Diego, USA) to calculate correlation coefficients, and an unpaired *t*-test was used to analyze SNL vs. RLC groups. Data are displayed as means and standard deviations (SD). $P < 0.05$ was considered statistically significant. The quantified results were subsequently analyzed with multidimensional principal component analysis (PCA), and individual cytokines were compared with the Qlucore Omics Explorer data analysis software (Qlucore, Lund, Sweden).

Table 1
Classification of investigated mediators.

Function		Protein
Growth factor		βNGF, BDNF, CXCL12/SDF-1, bFGF/FGF2, GM-CSF, HGF, IL-9, IL-15, IL-21, LIF (negative regulator), PDGF-bb, PIGF-1, SCF, VEGF-α, VEGF-d/FIGF
Pro-inflammatory cytokines, chemokines		CCL2/MCP-1, CCL3/MIP-1α, CCL4/MIP-1b, CCL5/RANTES, CCL11/Eotaxin, CXCL12/SDF-1, IL-1, IL-2, IL-4, IL-6, CXCL8/IL-8, IL-10, IL-12 IL-15, IL-17, IL-18, IL-21, IL-25, IL-31, CXCL10/IP-10, TNF-α, TNF-β, IFN-α, IFN-β, IFN-γ
Anti-inflammatory	Th2-associated	IL-4, IL-5, IL-10, IL-13, IL-31, IL-1RA, TGF-β