Research Article

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Perforin and granzymes work in synergy to mediate cholangiocyte injury in experimental biliary atresia

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Background & Aims: Biliary atresia represents obstructive cholangiopathy in infants progressing rapidly to cirrhosis and end-stage liver disease. Activated NK cells expressing Nkg2d have been linked to bile duct injury and obstruction by establishing contact with cholangiocytes. To define the mechanisms used by cytotoxic cells, we investigated the role of perforin and granzymes in a neonatal mouse model of rotavirus (RRV)-induced biliary atresia.
Methods: We used complementary cell lysis assays, flow cytometric analyses, quantitative PCRs and *in vivo* systems to determine the mechanisms of bile duct epithelial injury and the control of the tissue phenotype in experimental biliary atresia.
Results: RRV-infected hepatic NK and CD8 T cells increased the expression of perforin and injured cholangiocytes in short-term culture in a perforin-dependent fashion. However, the loss of perforin *in vivo* delayed but did not prevent the obstruction of bile ducts. Based on the increased expression of granzymes by perforin-deficient cytotoxic cells in long-term cytolytic assays, we found that the inhibition of granzymes by nafamostat mesilate (FUT-175) blocked cholangiocyte lysis. Administration of FUT-175 to perforin-deficient mice after RRV infection decreased the development of jaundice, minimized epithelial injury, and improved long-term survival. However, the inhibition of granzymes alone in wild-type mice was not sufficient to prevent the atresia phenotype in newborn mice. In infants with biliary atresia, hepatic *Granzymes A and B* mRNA, but not *Perforin*, increased at the time of portoenterostomy.
Conclusions: Perforin and granzymes have complementary roles mediating epithelial injury by NK and CD8 T cells. The prevention of experimental biliary atresia can only be achieved by inhibiting both granules.

Keywords: Cholestasis; Cholangiocyte; Immunity; Jaundice; Liver; Children; Neonates.
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Abbreviations: PKO, perforin knockout; ffu, fluorescence-forming units; RRV, *Rhesus* rotavirus type A; WT, wild type.

Introduction

Biliary atresia is a progressive liver disease of infancy resulting from an inflammatory and fibrosing obstruction of extrahepatic bile ducts. Although early diagnosis and surgical intervention by Kasai portoenterostomy may restore continuity of bile flow from liver to duodenum, the majority of patients die from end-stage biliary cirrhosis in early childhood. Studies using livers and/or biliary remnants from infants with biliary atresia reported an enhanced proinflammatory footprint and an infiltration by $CD4^+/CD8^+$ T lymphocytes, natural killer (NK) and dendritic cells [1–8]. In mechanistic experiments using a neonatal mouse model of *Rhesus* rotavirus (RRV)-induced biliary atresia, disruption of the adaptive immune response by loss of IFNγ or CD8 T cells reduced bile duct obstruction and improved the cholestasis phenotype [9,10]. Recently, we ascribed a key function for NK cells in the initiation of epithelial injury by engaging and lyzing cholangiocytes through the Nkg2d receptor [11]. The effector mechanisms used by NK cells to target cholangiocytes, however, remain largely unknown.

Innate immune lymphocytes are critical for early host defenses against viral infections and exert cytotoxic effects against virus-infected cells primarily by granule exocytosis [12]. This potent cytolytic process is housed within the cytoplasmic granules rich in perforin, granzymes and other effector molecules. In addition, binding of stimulatory receptors like Nkg2d on cytotoxic cells by ligands of target cells activates a cascade of intracellular signaling events resulting in the secretion of IFNγ and TNFα, and in the polarization and exocytosis of cytolytic granules [13,14]. Chief among these granules are perforin and granzymes that work in concert to clear virus-infected cells [15]. Based on the central role of NK and CD8 T cell signaling in cholangiocyte injury and on the increased expression of perforin and granzymes in livers of patients with biliary atresia [10,11], we hypothesized that the perforin-granzyme system is required for epithelial injury of bile ducts. Testing this hypothesis using complementary *in vitro* and animal approaches, we found that the individual loss of perforin or inhibition of granzymes had minimal impact on the development of bile duct injury after RRV. However, the simultaneous loss/inhibition of both granules prevented cholangiocyte lysis and bile duct obstruction, and improved the phenotype of experimental biliary atresia.

Materials and methods

Experimental model of biliary atresia

BALB/c mice were purchased from Charles River Laboratories (Wilmington, MA) and Balb/c *Perforin* knockout (PKO) mice were a kind gift from Dr. John T. Harty (University of Iowa, Iowa City, IA). Newborn PKO and WT mice were injected intraperitoneally with 1.5×10^6 fluorescence-forming units (ffu) of RRV in 20 μl volume within 24 h of birth to induce experimental biliary atresia as described previously [9]. In granzyme blocking studies, the protease inhibitor nafamostat mesilate (FUT-175, Enzo Life Sciences, Inc., Farmingdale, NY) was administered intraperitoneally at a dose of 15 μg/g body weight in 20 μl 1X phosphate buffered saline (PBS) soon after birth followed by RRV infection 24 h later; control mice received 20 μl of PBS [9–11]. Thereafter, FUT-175 was administered daily until 14 days of life. Groups of mice were sacrificed between 3 and 14 days and the extent of duct injury was determined [16]. The Institutional Animal Care and Use Committee (IACUC) of the Cincinnati Children's Research Foundation approved all the animal experiments and protocols.

Human livers

Liver RNA was isolated from 1 to 3 month old infants at the time of diagnosis of biliary atresia. Control biopsies were obtained from livers of deceased donors aged 2–3.5 years being used for transplantation; age-matched donors from healthy subjects were not pursued due to ethical considerations. These subjects were described in a previous publication [3,11]. Written informed consent was obtained from the patients' guardians.

Liver function tests

Serum total bilirubin (Total Bilirubin Reagent Set; Pointe Scientific, Inc. Canton, MI) and alanine transaminase (DiscretPak ALT Reagent Kit; Catachem, Inc. Oxford, CT) were quantified according to the manufacturer's instructions.

Flow cytometric analysis and cytotoxicity assays

Mononuclear cells were isolated from livers of neonatal mice and subjected to antibody-staining for flow cytometric analysis and cytotoxicity assays for NK and CD8 T cells as described previously [11]. Description of the assays and a list of antibodies and reagents are provided as Supplementary Materials and methods.

Protein and gene expression

Supernatants from co-culture experiments were used to quantify granzyme B, Il-1α, Il-10, Il-12p40, Il-15, and Il-17 by ELISA or protein Milliplex™. Gene expression was quantified by real-time PCR using specific primers and a Stratagene Mx3005P thermocycler (Agilent Technologies, Inc., Santa Clara, CA), as described previously [9]. Description of the assays for protein and gene expression is provided as Supplementary Materials and methods.

Statistical analysis

The numbers of mice or tissues used in each experiment are presented in the text or figure legends. All *in vitro* experiments were performed in 6 wells for each condition. Values are expressed as mean ± standard deviation (S.D.) and statistical significance was determined by unpaired *t* test with or without Welch's correction with significance set at $p < 0.05$. Survival curves were created by Kaplan-Meier analysis using the GraphPad Prism version 5.00 Software (GraphPad Software, San Diego, CA).

Results

Increased perforin expression and requirement for cell lysis

To test the hypothesis that the perforin-granzyme system is required for epithelial injury of bile ducts, we quantified the