Journal of Surgical Research 154, 21–29 (2009)
doi:10.1016/j.jss.2008.05.023

ASSOCIATION FOR ACADEMIC SURGERY, 2008

Integrin $\alpha v\beta 6$ and Mediators of Extracellular Matrix Deposition Are Up-Regulated in Experimental Biliary Atresia[1]

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Submitted for publication February 13, 2008

Introduction. **Biliary atresia (BA) is a progressive obliteration of the extrahepatic bile ducts resulting in hepatic fibrosis. The underlying mechanisms have not been defined. We used an animal model of BA to evaluate mediators of extracellular matrix (ECM) processing to determine which factors may be involved.**

Methods. **Newborn BALB/c mice received an intraperitoneal injection with rhesus rotavirus or saline within 24 h of birth. Livers were harvested on days 7 and 14 for histology and immunohistochemistry (IHC). RNA expression was determined using quantitative real-time PCR. Human liver from patients with BA and those having a resection for nonfibrosing diseases was also evaluated.**

Results. **In experimental mice, mRNA expression for tissue inhibitor of metalloproteinase (TIMP)-1 and matrix metalloproteinase (MMP)-7 was increased 18-fold and 69-fold, respectively on day 7, with further increases on day 14. On day 14, mRNA expression for plasminogen activator inhibitor (PAI)-1 (38-fold), TIMP-4 (9.5-fold), and MMP-9 (5.5-fold) mRNA was also observed. Furthermore, integrin α_v β_6 mRNA expression was increased on days 7 (11-fold) and 14 (6-fold). Presence of integrin α_v β_6 protein was confirmed by IHC in both mouse and human specimens in the proliferating biliary epithelium.**

Conclusions. **Our data suggest experimental BA is associated with increased mRNA expression of ECM degradation inhibitors, TIMP-1, PAI-1, and TIMP-4. MMP-7 and MMP-9 expression is also elevated in this model. Furthermore, increased gene expression of integrin $\alpha_v\beta_6$ was demonstrated and IHC confirmed protein expression. Integrin $\alpha_v\beta_6$ or the inhibitors of ECM breakdown may be attractive targets for future treatment strategies.** © 2009 Elsevier Inc. All rights reserved.

Key Words: **biliary atresia; hepatic fibrosis; integrin α_v β_6; metalloproteinase; plasminogen-activator inhibitor; tissue inhibitor of matrix metalloproteinases.**

INTRODUCTION

Biliary atresia is a progressive obliteration of the bile ducts that affects approximately one in 10,000 children in the first 3 months of life. The current treatment, a portoenterostomy to enhance bile drainage from the liver, is effective in only about one-third of patients, with those who have surgery before significant hepatic fibrosis has developed having the best prognosis [1]. Progressive fibrosis ultimately leads to liver failure, and thus biliary atresia is the leading indication for liver transplantation in the pediatric population [2]. The factors involved in this process are unknown; however, we surmise that an imbalance between extracellular matrix (ECM) deposition and breakdown may play a central role. Indeed, differential expression of several known mediators of this process has been demonstrated in patients with biliary atresia. Recent evidence using a bile duct ligation model suggests that transforming growth factor-beta (TGF-β) and its upstream mediator integrin $\alpha_v\beta_6$ are central to the development hepatic fibrosis after biliary obstruction [3]. Therefore, we proposed to evaluate these same factors and known downstream mediators of TGF-β function, including the plasminogen-activator inhibitors (PAIs) and the tissue inhibitors of matrix metalloproteinases (TIMPs) in a model of biliary atresia [4].

[1] Presented in part at the 2nd Academic Surgical Congress, Phoenix, AZ, February 2007 and at the 38th Annual Meeting of American Pediatric Surgical Association, Orlando, FL, May 2007.

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0022-4804/09 $36.00
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An animal model of biliary atresia has been described, which utilizes administration of a rhesus rotavirus (RRV) to newborn mice which reliably mimics the human condition [5] In this model, interferon-γ seems to be a central mediator since interferon-γ knockout mice were immune to the honing of lymphocytes to the bile duct and subsequent inflammation and fibrosis. Thus, the authors postulated that interferon-γ may represent a putative target for future treatment strategies. However, in children, there is usually significant fibrosis of the biliary tree and often the hepatic parenchyma at the time of diagnosis with very little acute inflammation, suggesting antifibrotic strategies may be more useful. Thus, we used the previously described animal model to evaluate known mediators of ECM deposition as well as various promoters of hepatic fibrosis to determine which factors may be important in the pathogenesis of biliary atresia and may serve as future targets for therapy.

METHODS

Experimental Design

The experimental protocol was approved by the Institutional Animal Care and Use Committee at the New York University School of Medicine. Pregnant time-dated BALB/c mice were purchased. After spontaneous vaginal delivery, the neonates were randomized to either receive an intraperitoneal (i.p.) injection with saline control (0.02 mL) or 1.5×10^6 fluorescence forming units (ffu) of rhesus rotavirus (RRV) of serogroup 3 (diluted in 0.02 ml of saline) within 24 h of birth. The mice were returned to their mothers, maintained in their normal environment, and housed in a room with a standard 12-h dark-light cycle. Mice that died within the first 2 d of injection or that were not fed by their mothers after injection were excluded from the study. The mice were weighed daily and examined for the development of icterus in their skin not covered with fur, the development of acholic stools, and the development of bilirubinuria. Those mice in the experimental group which did not display clinical signs of biliary atresia were also excluded. Starting on day 7, 0.05 mL of normal saline was injected subcutaneously into each animal as resuscitation on a daily basis until sacrifice. Groups of mice (n = 6 for each group) were sacrificed at days 7 and 14 after saline or RRV infection. The gross appearance of the liver and bile ducts were recorded. These organs were then harvested for protein and RNA isolation, and histological and histochemical analysis.

Virus

We obtained commercially available RRV strain MMU 18006 which was then cultivated in MA-104 cells (embryonic African greenmonkey) using minimal essential medium with Earle's salts and 10% fetal calf serum. Penicillin 100 IU/mL and streptomycin 100 μg/mL were added to prevent bacterial contamination. The virus was isolated, concentrated, and then titrated into 1.5×10^6 ffu aliquots to be injected intraperitoneally into the mice. This dose of virus was chosen due to its high rate of inducing cholestasis and its low rate of early death [5, 6].

Histology and Immunohistochemistry

For hematoxylin and eosin (H and E) and immunohistochemical staining, a specimen of liver, gallbladder, and extrahepatic bile duct from each experimental and control mouse were isolated, fixed in formalin, and imbedded in paraffin. Each block of paraffin was cut in five random sections and stained or probed. The interpretation of the sections was performed by a pediatric pathologist (MAG) who was blinded to the treatment groups. The H and E sections were graded into one of three groups: normal liver; indeterminate; and biliary atresia. Immunostaining was performed with an antibody to detect protein expression of integrin $\alpha_v\beta_6$ (Biogen Idec, Cambridge MA; ch6.2A1 diluted to 0.5 μg/mL) [7]. The antibody for integrin $\alpha_v\beta_6$ was a generous gift from Biogen Idec. The immunohistochemistry (IHC) was similarly graded into one of four groups: no expression; mild expression; moderate expression; and extensive expression. Comparisons between the treatment and control groups was carried out using χ^2 analysis or Fisher's Exact test if the expected frequency of any cell was less than five. Statistical significance was assigned to any P value less than 0.05.

Real-Time Polymerase Chain Reaction (RT-PCR)

Standard methods for quantitative RT-PCR (qRT-PCR) have been published elsewhere and were used for each mouse in both the control and experimental groups [8]. Power SYBR green PCR Master Mix (Applied Biosystems, Foster City, CA) and an ABI 7900HT Sequence Detection System was used to measure a threshold cycle (C_T) value for each sample with the following profile: 1 cycle of 50 C for 15 s, 95 C for 10 min, 40 cycles of 95 C for 15 s, and 60 C for 1 min. After amplification was complete, a final melting curve was performed according to the dissociation protocol of the ABI 7900HT instrument. RNA content from each sample was normalized to its eIF4B and GAPDH content. Relative quantitation of gene expression was based on the comparative C_T method. Comparisons of gene expression between groups was performed by one-way analysis of variance (ANOVA) followed by Bonferroni correction. Statistical significance was assigned to any P value less than 0.05.

Human Studies

The experimental protocol was approved by the Institutional Review Board at the New York University School of Medicine and Mount Sinai School of Medicine. We compared specimens from five patients undergoing liver biopsy for biliary atresia (BA) at the time of Kasai portoenterostomy with explant specimens from five patients undergoing liver transplantation for progression of BA. Liver specimens from five patients having a resection for nonfibrosing liver diseases served as controls. The diagnoses for these five patients included: hepatoblastoma (n = 2), Crigler-Najjar syndrome, urea cycle defect, and ornithine transcarbamylase deficiency. An attempt was made to age-match the controls, although this was limited by the availability of tissue. The median age of Kasai biopsy and BA liver explant patients was 7 wk and 9 mo, respectively, while the age range for control tissue samples was from 5 to 17 mo. All specimens had been obtained during the routine clinical care of the patients, and were permanently fixed and paraffin-embedded at the time of surgery.

Immunohistochemical Labeling of Human Tissue

The paraffin-embedded blocks were newly processed and fresh slides were made at the time of the current study. Each block was cut in random sections to be evaluated, and the interpretation was performed by a pediatric pathologist (MAG) who was blinded to the groups. Immunostaining was performed with antibodies to detect protein expression of integrin $\alpha_v\beta_6$ (Biogen Idec, 6.2 A1 diluted to 0.5 μg/mL) and cytokeratin 19 (CK19) (Ventana Medical Systems, Tucson AZ, prediluted by the manufacturer). The antibody for integrin $\alpha_v\beta_6$ was a generous gift from Biogen Idec. Staining for CK-19 was performed by standard automated processes at the NYU Cancer Center Immunohistology core facility. The procedure for integrin $\alpha_v\beta_6$ immunoreactivity was performed as follows. Liver sections probed for integrin $\alpha_v\beta_6$ were de-waxed and treated with 0.3% hy-

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