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Differential Expression of Hepatic Fibrosis Mediators in Sick and Spontaneously Recovered Mice with Experimental Biliary Atresia[1]

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Background. **Hepatic fibrosis leading to cirrhosis is the major morbidity in patients with biliary atresia (BA). This fibrosis is due to an imbalance in extracellular matrix (ECM) breakdown and deposition. We have previously demonstrated increased mRNA expression for inhibitors of ECM breakdown without increased expression for mediators of ECM deposition in our animal model of BA by d 14. However, only a mild degree of hepatic fibrosis was seen at this time. We hypothesized that expression patterns for these proteins may change once more significant fibrosis had been established, and added resuscitation to the model to improve survival. Interestingly, we found that some mice spontaneously recovered at later time points with resuscitation, and thus compared expression for inhibitors of ECM breakdown and deposition in sick and recovered mice to determine the differences.**

Methods. **Newborn Balb/c mice received an intraperitoneal injection 1.0×10^6 fluorescence forming units of rhesus rotavirus 24h after birth. Mice were monitored daily for weight gain, development of jaundice, acholic stools, and bilirubinuria. Fifty μL/g of 5% dextrose in normal saline were subcutaneously injected daily to each mouse starting on d 7 until sacrifice. Mice that survived past d 14 were sacrificed at d 21 after saline or RRV infection. Livers were then harvested post-injection d 21 for histologic and immunohistochemical analysis. RNA expression of known mediators of fibrosis was evaluated using quantitative real-time PCR. Protein expression was assessed using ELISA. Weights and normally distributed data were compared using Student's *t* test. Histologic findings were compared using Fisher's exact test. Comparisons of gene expression and skewed data were performed by the Mann-Whitney U test. Statistical significance was assigned to any *P* value less than 0.05.**

Results. **Daily resuscitation resulted in a 35% (24/68) survival rate to d 21 in our model. Mice that recovered were significantly heavier than those that remained ill on d 14 (6.15 ± 1.16 *versus* 4.94 ± 0.82, $P = 0.02$) and 21 (7.31 ± 1.41 *versus* 4.14 ± 0.53, $P < 0.001$) despite the fact that there was no difference between the groups with respect to weight on d 7 (4.29 ± 0.90 *versus* 3.89 ± 0.81, $P = 0.32$). We found that all (10/10) animals that displayed clinical signs of biliary atresia on d 21 had moderate or severe histologic findings, while only one (1/9) of the recovered animals had liver abnormalities at sacrifice ($P < 0.001$ *versus* sick group). We also found that the sick mice had statistically significant median fold-increases of mRNA expression for TIMP-1 (31.9 *versus* 9.1, $P = 0.041$), TIMP-4 (88.1 *versus* 1.8, $P = 0.022$), and MMP-7 (51.8 *versus* 11.9, $P = 0.006$) compared with those that recovered. There was a trend toward decreased mRNA expression for PAI-1, which did not reach statistical significance (median 27.7 *versus* 2.19, $P = 0.066$). Increased protein expression for TIMP-1 and PAI-1 were also found in the sick group. The mRNA expression for the fibrillar collagens, fibronectin-1, connective tissue growth factor, snail-1, TIMP-2 and -3, and MMP-2 and MMP-9 was not different in the sick and recovered groups 21 d after RRV infection, and was not elevated from baseline gene expression.**

Conclusions. **With resuscitation added to the animal model of BA, some mice spontaneously recover while others progress to more significant hepatic fibrosis. Mice with hepatic fibrosis have a continued increase in mRNA expression of TIMP-1, TIMP-4, and MMP-7, with a trend toward increased mRNA expression of PAI-1 on d 21. Protein levels for TIMP-1 and PAI-1 were also increased in the sick mice. Recovered mice display mild to no hepatic parenchymal disease**

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and a normal pattern of mRNA expression for the mediators of fibrosis tested. No increase in mRNA expression for the mediators of ECM deposition was found in either group. These data further support the notion that inhibition of ECM breakdown alone is sufficient to induce hepatic fibrosis. Modulation of this process may be a putative target for preventing liver injury in patients with BA. © 2010 Elsevier Inc. All rights reserved.

Key Words: **biliary atresia; metalloproteinases; plasminogen-activator inhibitor; tissue inhibitor of matrix metalloproteinases.**

INTRODUCTION

Hepatic fibrosis leading to cirrhosis and eventual liver transplantation is the major morbidity in patients with biliary atresia (BA), and this fibrosis may be due in part to an imbalance in extracellular matrix (ECM) breakdown and deposition [1, 2]. We have previously demonstrated increased mRNA expression for inhibitors of ECM breakdown without increased expression for mediators of ECM deposition on d 7 and 14 in our animal model of BA, suggesting that inhibition of remodeling precedes collagen deposition in this model [3]. However, only a mild degree of hepatic fibrosis was seen at these time points after rhesus rotavirus (RRV) inoculation, and we speculated that expression patterns may differ once more significant fibrosis had been established. To achieve this aim, we added saline and dextrose resuscitation to our model in an attempt to extend the survival of the animals to 21 d. Surprisingly, we found that a significant number of mice spontaneously recovered by the later time point, and thus we were interested in comparing expression for inhibitors of ECM breakdown and deposition in sick and recovered mice to evaluate the disparity. Spontaneous recuperation of mice in this model has been previously reported [4], but prior to this study there has been no characterization of the differences between mice that remain ill and those that convalesce. We hypothesized that increased mRNA expression for inhibitors of ECM breakdown would continue with possible increases in the expression for mediators of ECM deposition in the mice that remained ill, but not in the clinically improved mice. Furthermore, we surmised that if there was increased expression of mediators for ECM deposition, the cells most likely responsible in the liver, the hepatic stellate cells (HSC), would show activation in the sick sub-group.

METHODS

Experimental Design

The experimental protocol was approved by the Institutional Animal Care and Use Committee at the New York University School of Medicine. Balb/c mice were bred using a polygamous breeding system. After spontaneous vaginal delivery, neonates received an intraperitoneal injection with 1.0×10^6 fluorescence forming units (ffu) of 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. 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. Fifty μL/g of 5% dextrose in normal saline were subcutaneously injected daily to each mouse starting on d 7 until sacrifice. Mice that survived past d 14 were sacrificed at d 21 after RRV infection. The livers were then harvested for RNA and protein isolation, and histologic and histochemical analysis. Weights were compared using Student's *t*-test after confirmation of normally distributed data in each group using the one-sample Kolmogorov-Smirnov test. Statistical significance was assigned to any P value less than 0.05.

Virus

We obtained commercially available RRV strain MMU 18006, which was then cultivated in MA-104 cells (embryonic African green-monkey) 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 aliquots to be injected into the mice.

Histology and Immunohistochemistry

For hematoxylin and eosin (H and E), trichrome, and immunohistochemical staining, a specimen of liver from each mouse was isolated, fixed in formalin, and imbedded in paraffin. Each block of paraffin was cut in 5 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 four groups: normal liver, or mild, moderate, or severe disease. Immunostaining was performed with an antibody to detection protein expression of α-smooth muscle actin (Novus Biological, Inc., Littleton, CO) to assess HSC activation [5]. The immunohistochemistry (IHC) was graded into one of two groups: expression or no expression. Comparisons between the sick and recovered groups was carried out using χ^2 analysis or Fisher's Exact test if the expected frequency of any cell was less than 5. Statistical significance was assigned to any P value less than 0.05.

Real-time Polymerase Chain Reaction (RT-PCR)

Standard methods for quantitative real time polymerase chain reaction (qRT-PCR) have been published elsewhere and were used for each mouse in both the sick and recovered groups [6]. 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 60C 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 glyceraldehyde-3-phosphate dehydrogenase (GAPDH) content. Relative quantitation of gene expression was based on the comparative C_T method. Comparisons of gene expression between the two groups were performed by the Mann-Whitney U test. Statistical significance was assigned to any P value less than 0.05.

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