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ORIGINAL RESEARCH

Immunohistochemical localization of transforming growth factor β-1 and its relationship with collagen expression in advanced liver fibrosis due to biliary atresia

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Christian Farrington[1]
Don Novak[1]
Chen Liu[2]
Allah B Haafiz[1]

[1]Hepatology and Liver Transplantation, Division of Pediatric Gastroenterology, Hepatology and Nutrition, [2]Department of Pathology, Immunology and Laboratory Medicine, University of Florida College of Medicine, Gainesville, FL, USA

Purpose: Biliary atresia (BA) is the most common indication of liver transplantation in children. Pathogenesis of hepatic fibrosis, which is a prominent feature of BA, remains obscure. The purpose of this work was to determine the cellular sources of transforming growth factor beta-1 (TGFβ1) and establish the relationship between TGFβ1-producing cells and extracellular matrix producing myofibroblasts (MFBs) in advanced BA.

Methods: Trichrome staining and immunohistochemistry were carried out to determine the expression pattern of collagen and TGFβ1 protein in explant liver specimens from patients with BA. The intensities of portal and lobular TGFβ1 expressions were compared. Immunofluorescence technique was carried out to determine the relationship between α-smooth muscle actin (α-SMA)-positive-MFB and TGFβ1-positve cells.

Results: Lobular TGFβ1 protein expression was significantly higher than portal (89 ± 6 versus 10 ± 1 arbitrary units, $P \leq 0.05$), whereas no difference was noted in livers used as control (10 ± 1.6 versus 19 ± 5 arbitrary units, $P = 0.11$). TGFβ1 expression was more in the center of nodules versus MFB in surrounding fibrous septa. Contrary to TGFβ1 expression, α1-SMA was mostly expressed in the portal structures and the adjacent fibrous septa enacting lobulation of the parenchyma. The results obtained by coimmunofluorescence staining showed no colocalization of α-SMA and TGFβ1.

Conclusions: TGFβ1 protein expression is mostly localized to hepatocytes in advanced BA. These findings suggest a paracrine mechanisms of TGFβ1-driven fibrogenesis in advanced BA.

Keywords: biliary atresia, liver fibrosis, transforming growth factor β-1, liver transplantation

Introduction

Untreated biliary atresia (BA) is universally fatal, mandating timely diagnosis and expedited portoenterostomy operation. This procedure, despite its appeal at the time of initial diagnosis, permits long-term survival in only about 20% of the affected infants. Liver transplantation (LT) becomes necessary for the long-term survival of most of the affected children due to the complications of cirrhosis, such as portal hypertension and poor growth. Although the exact pathogenesis of BA remains elusive, it is generally agreed that BA phenotype can result from several distinct mechanisms of liver injury, provoking a stereotypic response comprising inflammation, bile duct proliferation, apoptosis, and fibrogenesis.[1] In this context, it is not surprising that the extent of liver fibrosis is the most important predictor of the outcome of portoenterostomy procedure.[2–4] However, despite its prime significance, the assessment and management of liver fibrosis have not yet been incorporated in routine postportoenterostomy

Correspondence: Allah B Haafiz
Division of Pediatric Gastroenterology, Hepatology and Nutrition, University of Florida College of Medicine, 1600 SW Archer Road, PO Box 100296, Gainesville, FL 32610, USA
Tel +1 352 273 9350
Fax +1 352 273 9055
Email haafiab@peds.ufl.edu

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patient care, mostly due to poor understanding of the underlying mechanisms of progressive liver fibrosis. Conversely, significant progress has been made in understanding the pathogenesis of liver fibrosis due to diseases afflicting adult populations. As such, contrary to conventional dogma, liver fibrosis is now considered to be a reversible pathologic event, provided that underlying mechanisms are addressed in a timely manner.[5–8] Similarly, a consensus has emerged for the pivotal role of hepatic stellate cells (HSCs) in liver fibrosis.[9–12]

Transforming growth factor β-1 (TGFβ1) is the master fibrogenic cytokine that is well known to be involved in liver fibrosis in a variety of liver diseases.[13–15] Through its receptors on myofibroblasts (MFBs) and other cells, TGFβ1 orchestrates key fibrogenic events, most notably expansion of the MFB pool, induction of extracellular matrix proteins, and inhibition of matrix metalloproteinases.[16–20] However, disease-specific mechanisms driving TGFβ1 upregulation are not well understood. Given that downstream fibrogenic actions of TGFβ1 are relatively well understood,[10,21] elucidation of its cellular sources is a critical initial step not only for understanding possible triggers but also to delineate the march of molecular events linking ongoing liver injury to fibrosis. For example, as with any other chronic liver disease, it is quite conceivable that the cellular sources of TGFβ1 will vary according to the triggers determined by site, underlying mechanisms, and the extent of liver injury.

Reconstruction of the topology of events linking injury to repair (fibrosis) therefore requires successive examinations, which is an enterprise that is hampered by ethical restrictions on liver biopsies after portoenterostomy, thus limiting the scope of fibrosis assessment to explant specimens only. In this work, using explant specimens, we sought to determine whether TGFβ1 is predominantly produced in portal and periportal structures, which are the main sites of liver injury or hepatic lobular cells, situated distant from the site of injury, thereby indicating paracrine mechanisms of TGFβ1-driven fibrogenesis in advanced BA.

Materials and methods

To accomplish our goal, using explant specimens, we carried out trichrome staining and immunohistochemistry to determine the hepatic expressions of collagen and TGFβ1 proteins. To determine whether TGFβ1 is expressed by collagen-producing MFB, immunofluorescence was performed to determine colocalization of TGFβ1 and α1 smooth muscle actin (α-SMA). α-SMA is commonly used as a marker of collagen-producing MFB, the main fibrogenic cell type with diverse origins, including HSCs.

Patient population and liver samples

After approval from the Institutional Review Board of the University of Florida (UF), FL, USA, patients with BA who required LT were identified from the UF liver transplant database. To determine the expression of markers of fibrosis in BA, liver sections were obtained from explant specimens archived from LT done between the years 2000 and 2008 (N = 30; 16 males, 14 females). The mean age at the time of LT was 1.8 ± 0.369 years (range 2–17 years). Liver sections from archived sections of living related liver donors were used as controls. The alternative approach, using margins from hepatoblastoma resections as controls, was not pursued because our preliminary work indicated extensive and inconsistent expression of fibrogenic cytokines in these 'healthy margins'. Therefore, albeit age-related limitations, healthy human liver sections were used as controls in this study. All biopsied specimens were 4 μm thick, fixed in phosphate-buffered formalin, and embedded in paraffin. Serial adjacent sections were used for hematoxylin and eosin staining, trichrome staining, immunohistochemistry, and immunofluorescence.

Expression and distribution of collagen

Excessive collagen deposition in the extracellular matrix is a hallmark of liver fibrosis. Collagen distribution was determined by trichrome staining using a kit from ScyTek Laboratories (TGG500, West Logan, UT, USA) according to the directions of the manufacturer. The stage of liver fibrosis was determined in a blinded fashion by using trichrome-stained slides according to the METAVIR scoring system.[22] We used the METAVIR system because, through extensive intraobserver variation studies, this system was designed to be simple, consistent, and reproducible.[23] Similarly, this system has been applied to a variety of chronic liver diseases,[24] including biliary disorders such as primary biliary cirrhosis[25] and primary sclerosing cholangitis.[26] Moreover, it is noteworthy that the staging components of the most commonly employed scoring systems, especially when applied to advanced liver disease, yield similar results.

TGFβ1 immunohistochemistry

After rehydration, 4-μm paraffin-embedded sections on glass slides were incubated in 3% hydrogen peroxide to block the endogenous peroxidase activity. Sections were

then blocked by incubation in 3% bovine serum albumin in phosphate-buffered saline (PBS). A monoclonal mouse antihuman TGFβ1 antibody (Abcam 27969, Cambridge, MA, USA) that reacts to both monomeric and dimeric forms of TGFβ1 was applied at a dilution of 1:500 (diluted in 3% bovine serum albumin) and incubated at 4°C overnight. After a 5-min wash in PBS, the slides were treated with goat antimouse IgG horseradish peroxidase-labeled secondary antibodies (Bio-Rad, Cat#17065, Hercules, CA, USA) at a dilution of 1:500 (diluted in 3% bovine serum albumin) for 30 min at room temperature. Bound antibodies were detected with an ABC kit (Vector Laboratories, Burlingame, CA, USA). The slides were stained with diaminobenzidine, washed, counterstained with Mayer's hematoxylin, dehydrated, treated with xylene, and mounted. As a control to immunostaining, separate sections from both BA and normal human liver tissues were incubated with and without primary and appropriate isotyped antibodies.

Intensity of TGFβ1 immunostaining

The intensity of the TGFβ1 immunostaining was determined by using ImageJ software (http://rsb.info.nih.gov/ij). From each slide, five random portal and lobular areas were analyzed with a color histogram using a color deconvolution plug-in routinely used for diaminobenzidine staining.[27]

Immunofluorescence

After rehydration, 4-μm sections of paraffin-embedded tissue on glass slides were blocked by incubation in 4% bovine serum albumin in PBS. A monoclonal TGFβ1 mouse antihuman antibody (Abcam 27969) and a polyclonal rabbit anti-α-SMA (Abcam 5694) were applied to the slides at the dilution of 1:100 (diluted in 4% bovine serum albumin) and incubated at 4°C overnight. After being washed for 5 min in PBS, the slides were treated with fluorescently labeled secondary antibodies at a dilution of 1:500 (diluted in 4% bovine serum albumin) for 45 min at room temperature. Secondary antibodies used included donkey antimouse IgG (Alexa Fluor® 488, Invitrogen A21202, Carlsbad, CA, USA) and donkey antirabbit IgG (Alexa Fluor 594, Invitrogen A21207). Afterward, the tissue sections were washed with PBS and mounted with UltraCruz® medium (sc-24941) containing 4′,6-diamidino-2-phenylindole (DAPI) for nuclear staining. As a control for the technique and nonspecific Fc receptor binding, BA and normal human liver tissue sections were incubated with and without primary or secondary antibody as well as isotyped rabbit (ab27478) and mouse (ab18447) antibodies.

Data analysis

Student *t*-test was applied by using GraphPad software (La Jolla, CA, USA) to compare the means of color pixel histograms (representing the intensity of TGFβ1 expression) and METAVIR scores. Values are presented as mean ± standard error of the mean. A P value of <0.05 was considered statistically significant.

Results

Expression and distribution of collagen in advanced BA

The purpose of trichrome staining was two-fold: 1) to determine the distribution of collagen to serve as a reference for the distribution of TGFβ1 and 2) to determine the extent of fibrosis by the METAVIR scoring system. In this system, F0 represents no fibrosis; F1, portal fibrosis without septa; F2, portal fibrosis and few septa; F3, numerous septa extending to adjacent portal tracts or terminal hepatic venules; and F4, cirrhosis. The mean METAVIR score was 3.8 ± 0.8 (N = 30, minimum 3, maximum 4), consistent with advanced cirrhosis, a typical feature of end-stage liver disease due to BA. A representative trichrome-stained slide is shown in Figure 1A showing thick collagenous bands interspersed between nodular liver tissues, thus producing portal-to-portal bridging fibrosis, a histological pattern typical for biliary cirrhosis. In contrast, healthy control livers (Figure 1B) had only minimal collagen staining in portal areas, consistent with noted F0 METAVIR scores.

Hepatic distribution of TGFβ1 and α1-SMA in advanced BA

In explant liver specimens, as shown in Figure 2A, heterogeneous overexpression of TGFβ1 is evident in the hepatic

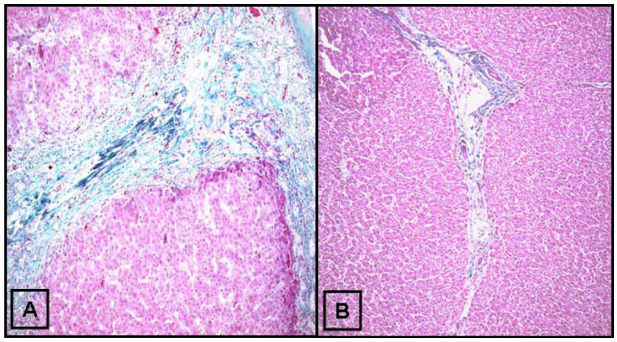

Figure 1 Trichrome staining for collagen in human biliary atresia. Trichrome staining was done as described in the Materials and methods section. **A**) Thick bands of collagen septa (green) separating nodular liver tissue. **B**) Trichrome staining of a healthy control liver (original magnification ×10).
Abbreviation: BA, biliary atresia.

parenchyma, more in the center of the liver nodules, although hepatocytes close to collagen in fibrous septa are also noted to express a low level of TGFβ1 protein. Conversely, in livers used as control, the distribution of TGFβ1 is virtually all interstitial and quite homogeneous throughout the hepatic parenchyma (Figure 2B). Contrary to TGFβ1 expression, α-SMA is expressed mostly in the portal structures and the adjacent fibrous septa enacting lobulation of the parenchyma (Figure 2C). As expected, in liver specimens used as control, α-SMA expression is restricted to portal structures (Figure 2D). Consistent with the TGFβ1 expression in hepatic parenchyma, quantitative analysis of TGFβ1 protein content was significantly higher in lobular versus portal areas (89 ± 6 versus 10 ± 1 arbitrary units, $P \leq 0.05$) in BA (Figure 3). In contrast, in livers used as control, lobular versus portal TGFβ1 expression was not significantly different, although a trend of more portal expression was noted (10 ± 1.6 versus 19 ± 5 arbitrary units, $P = 0.11$; Figure 3). These results were in agreement with the coimmunofluorescence staining, which showed no significant localization of α-SMA and TGFβ1 (Figure 4C and D). Once again, most of the TGFβ1 expression is seen in parenchymal cells (Figure 4A, C and D), whereas α-SMA expression is mostly restricted to the area of fibrous septa (Figure 4B). The summary of these findings is that in advanced BA, TGFβ1 is mainly expressed by hepatic parenchymal cells.

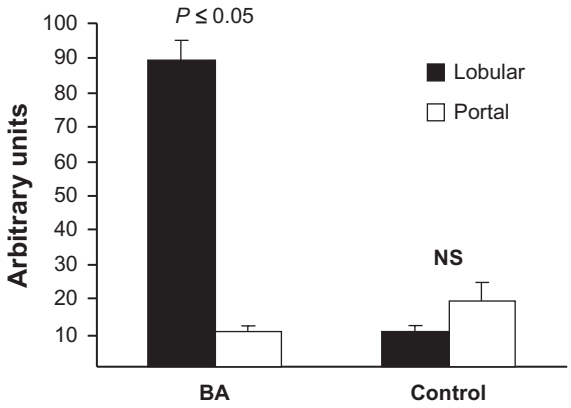

Figure 3 Pattern of hepatic distribution of TGFβ1 protein in human BA. Liver sections were obtained from archived explant specimens from patients who required LT due to BA (N = 9). The control sections were procured from the biopsies of living related liver donors. The expression of TGFβ1 was performed by immunostaining, which was quantified by using ImageJ software. From each slide, five random portal and lobular areas were analyzed. GraphPad software was used to analyze the data using *t*-test to compare the means of color pixel histograms from portal and lobular areas. A *P* value ≤ 0.05 was considered significant.
Abbreviations: BA, biliary atresia; NS, not significant; TGFβ1, transforming growth factor beta-1; LT, liver transplantation.

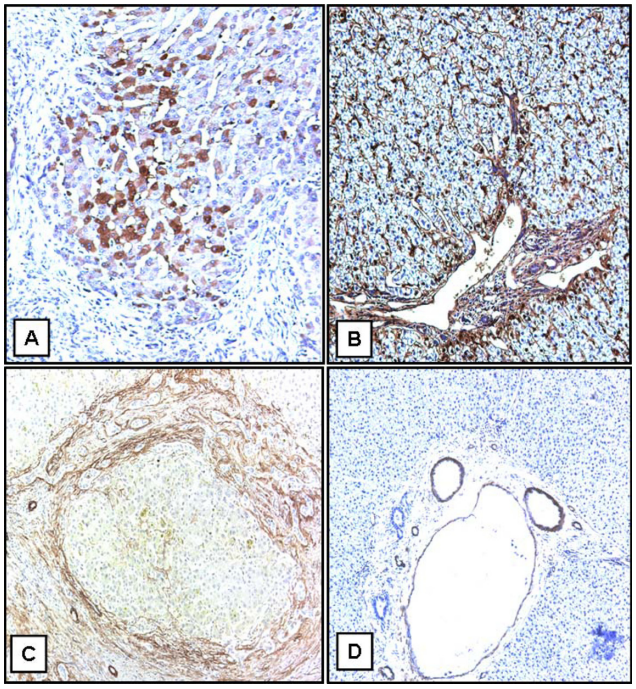

Figure 2 Immunohistochemical localization of TGFβ1 protein in human BA. Specific staining of TGFβ1 protein is represented by brown coloration. **A**) The expression of TGFβ1 in BA, which is mostly in lobular areas. **B**) TGFβ1 expression in a liver used as control. **C**) Expression of α1-SMA in BA. **D**) α1-SMA from a liver used as control (original magnification ×10).
Abbreviations: TGFβ1, transforming growth factor beta-1; BA, biliary atresia; SMA, smooth muscle actin.

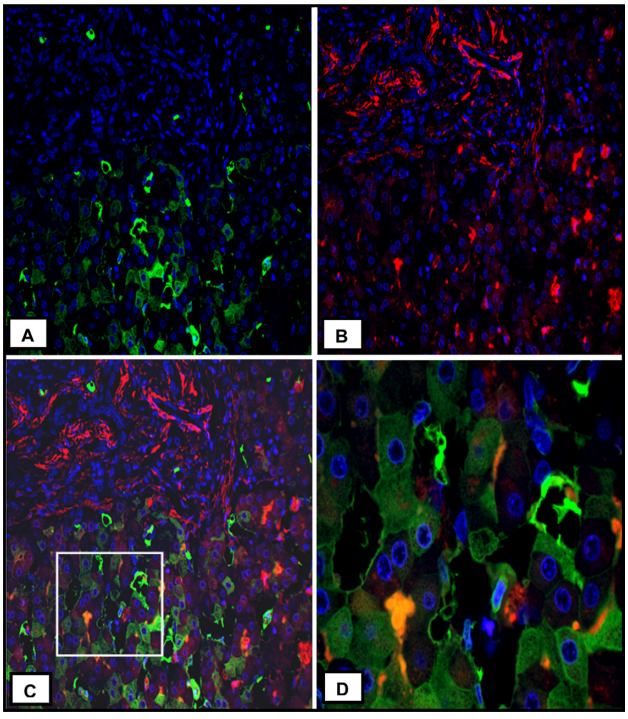

Figure 4 Characterization of hepatic cellular sources of TGFβ1 in human BA. **A**) Immunofluorescence staining for TGFβ1 (green, original magnification ×20). **B**) Immunofluorescence staining for α-SMA (red, original magnification ×20). **C**) Merge image of A and B. **D**) The original magnification ×60 of the box in C. Heterogeneous expression of TGFβ1 is seen in hepatocytes.
Abbreviations: TGFβ1, transforming growth factor beta-1; BA, biliary atresia; SMA, smooth muscle actin.

Discussion

The present report documents the novel observation that hepatic TGFβ1 is principally immune-localized to parenchymal cells in advanced liver disease due to BA. Additional novel observations are as follows: 1) TGFβ1 expression is homogenous, interstitial, and quantitatively more portal than lobular in normal human liver; 2) in contrast to normal human liver, heterogeneous lobular expression of TGFβ1 becomes more significant in advanced BA; and 3) more TGFβ1 is expressed in the center of nodules, away from fibrous septa, suggesting the existence of paracrine interactions between parenchymal cells and fibrogenic MFB in advanced cirrhosis due to BA.

This report unveils the significance of parenchymal cells in the injury-repair interactions at advanced stages of BA. In progressive cholestatic disorders such as BA, extracellular matrix deposition typically starts in periportal areas, extends to produce bridging, and ultimately leads to frank cirrhosis. Primary events executing fibrogenesis entail recruitment and proliferation of specialized collagen-producing MFBs. Because of their well-known phenotypic evolution to become MFBs, HSCs are known as prototypic profibrogenic cells in the hepatic parenchyma. Depending on the type of liver injury, other cell types such as resident portal fibroblasts,[28] biliary duct cells,[29] and even hepatocytes[30] can also contribute to the pool of extracellular matrix-producing MFBs. TGFβ1 plays an overarching role orchestrating key fibrogenic events such as MFB recruitment and proliferation as well as upregulation of extracellular matrix protein synthesis. Fundamental questions emerging from the main finding of this work remain: what are the triggers for TGFβ1 expression in hepatocyte; what are the roles of HSC and Kupffer in TGFβ1 expression from hepatocyte; and what are molecular mechanisms of fibrogenic interactions between hepatocytes and other parenchymal and nonparenchymal cells? Given that cholestasis and oxidative stress are the most important pathophysiologic mechanisms of liver injury in BA,[23] elucidation of the fibrogenic role of bile acids and oxidative stress will be the next most logical step in the investigation of hepatic overexpression of TGFβ1. Hepatic retention of hydrophobic bile acids due to biliary obstruction plays an important role in the pathogenesis of cholestatic liver injury.[31–33] This is exemplified by bile acid-induced liver injury in children with progressive familial intrahepatic cholestasis type 2. In this disorder, homozygous mutations in the gene encoding the canalicular membrane bile salt export pump impair hepatocellular secretion of conjugated bile acids, resulting in elevated hepatic bile acid concentrations and progressive cholestasis, fibrosis, and liver failure.[34] Similarly, hydrophobic bile acids are known to cause oxidative stress[35] and apoptosis,[36] causing perpetual hepatocyte loss and fibrosis.[37]

TGFβ1 is best known as a fibrogenic cytokine, a role well documented in both human diseases and transgenic mouse models of hepatic TGFβ1 overexpression.[38,39] However, further work is required to determine the precise biological or pathological significance of the observations presented in this report. Several biological traits peculiar to TGFβ1 need careful consideration for the appropriate interpretation of these results. First, apart from its well-known profibrogenic actions, TGFβ1 also controls diverse cellular functions such as proliferation, differentiation, adhesion, migration, matrix expression, matrix degradation, and immune surveillance. Determined by the context, a stringent balance of these actions is required for normal tissue homeostasis. Second, although TGFβ1 transcription occurs in many cell types, its translation and secretion appear to be under strict regulation, a phenomenon explaining the well-documented discrepancy between TGFβ1 protein levels and corresponding mRNA expression.[40,41] Therefore, in addition to knowledge of pathophysiological context, temporally stacked examinations are necessary to determine which pathological events underpin parenchymal TGFβ1 expression in BA. A major impediment in this regard is the ethical restriction on interval liver biopsies after Kasai operation. Therefore, the interpretation of single point examinations (mRNA or protein-based) such as those adopted in this and earlier reports[42,43] is somewhat limited. Nonetheless, this work provides a framework for the synthesis of testable hypotheses.

For example, although parenchymal cells can produce extracellular matrix proteins,[44] the lack of pericellular extracellular matrix in these livers and the presence of characteristic portoportal bridging (Figure 1) strongly support paracrine fibrogenic interactions between hepatocytes and MFBs in advanced BA. Similarly, because cholestasis and oxidative stress are common pathophysiologic mechanisms of liver injury in advanced BA, it is reasonable to postulate that coupled actions of toxic bile acids and mediators of oxidative stress could be the underlying triggers for hepatic upregulation of TGFβ1. Our unpublished data from tissue culture studies support this hypothesis. Similarly, Kupffer cell activation, a frequent source of oxidative stress, is known to amplify the TGFβ1 signal pathway,[45] supporting the hypothesis that Kupffer cells may have a role in hepatocyte overexpression of TGFβ1. These hypotheses are the focus of our ongoing investigations. Similarly, questions pending resolution through further research concern the

impact of oxidative stress and toxic bile acids on expression of noncollagenous matrix proteins and matrix metalloproteinases; whether noncanonical TGFβ1 signaling pathways intersect with canonical pathway; and, if so, what the pathological and molecular determinants of these intersections are.

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Disclosure

The authors report no conflicts of interest in this work.

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