The Role of Hepatic Stellate Cells and Transforming Growth Factor- β_1 in Cystic Fibrosis Liver Disease

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Liver disease causes significant morbidity and mortality from multilobular cirrhosis in patients with cystic fibrosis. Abnormal bile transport and biliary fibrosis implicate abnormal biliary physiology in the pathogenesis of cystic fibrosis-associated liver disease (CFLD), yet the mediators linking biliary events to fibrosis remain unknown. Activated hepatic stellate cells (HSCs) are the pre-eminent mediators of fibrosis in a range of hepatic disorders. The dominant stimulus for matrix production by HSCs is the cytokine transforming growth factor (TGF)- β_1 . In CFLD, the role of HSCs and the source of TGF- β_1 have not been evaluated. Liver biopsy tissue obtained from 38 children with CFLD was analyzed. Activated HSCs, identified by co-localization of procollagen $\alpha_1(\text{I})$ mRNA and α -smooth muscle actin, were demonstrated as the cellular source of excess collagen production in the fibrosis surrounding the bile ducts and the advancing edge of scar tissue. TGF- β protein and TGF- β_1 mRNA expression were shown to be predominantly expressed by bile duct epithelial cells. TGF- β_1 expression was significantly correlated with both hepatic fibrosis and the percentage of portal tracts showing histological abnormalities associated with CFLD. This study demonstrates a definitive role for HSCs in fibrogenesis associated with CFLD and establishes a potential mechanism for the induction of HSC collagen gene expression through the production of TGF- β_1 by bile duct epithelial cells. (*Am J Pathol* 2002, 160:1705–1715)

Hepatobiliary fibrosis is the most clinically relevant liver complication of cystic fibrosis (CF) and was responsible for 5% of deaths in the CF population before the advent of liver transplantation. Significant morbidity from liver disease occurs in ~20% of patients with CF¹ and develops during childhood, usually before or around puberty.² De-

spite significant medical and scientific advances in the field of CF, our understanding of the clinical significance, pathogenesis, and management of liver disease in these patients is only just emerging. The localization by Cohn and colleagues,³ of the CF transmembrane regulator (CFTR) to the apical domain of bile duct epithelial cells (BDECs) supports the prevailing hypothesis of abnormal biliary physiology leading to inflammation and focal biliary fibrosis. The common ultrastructural finding of periductular collagen deposition further supports this hypothesis⁴ without providing clues to the mechanisms by which this may occur. The link between biliary CFTR defect, cholestasis, and the development and progression of fibrosis is still missing.

The hepatic stellate cell (HSC) is widely regarded as the key fibrogenic cell co-ordinating hepatic extracellular matrix formation. HSC activation, and maintenance in the active state is the basic prerequisite for hepatic fibrosis.⁵ Indeed, the role of HSCs in hepatic fibrosis has been well documented by our group and others in a range of fibrosing conditions of the liver.^{6–9} Yet the role of HSCs in CF-associated liver disease (CFLD) has not been characterized. Activated HSCs express an intracellular microfilament protein, α -smooth muscle actin (SMA), widely used as a marker protein of the activated phenotype. Activated HSCs also express a number of different cytokine receptors including transforming growth factor- β_1 (TGF- β_1) receptor. TGF- β is a pleiotrophic cytokine involved in tissue growth, differentiation, and the immune response.¹⁰ TGF- β_1 is recognized as the dominant stimulus to extracellular matrix production by HSCs.⁵ The cellular source of this vital stimulus to collagen production by HSCs can vary in different disease states.¹⁰ We have previously shown that in biliary atresia, an example of accelerated biliary fibrosis in childhood, TGF- β_1 is produced by hepatocytes, HSCs, and BDECs.⁶ The present study was designed to demonstrate whether activated HSCs are indeed the cellular source of increased collagen production in children with CFLD and to establish a potential mechanism for fibrogenesis by establishing the primary source of the profibrogenic cytokine TGF- β_1 .

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Table 1. Patient Clinical and Histopathological Details

Patient no.	Sex	Age years	Meconium ileus?	Insulin dependent?	Clinical hepato-splenomegaly?	Portal hypertension?	Scheuer fibrosis score	LPD (0-3)	%PTI
1	f	7.9					0	0	0
2	f	6.5					0	0	0
3	f	6.7					0	0	0
4	m	18.7					0	0	0
5	f	8.9					*0	0	0
6	m	10.8	Yes		Yes		*0	0	0
7	f	11.3	Yes		Yes		*0	0	0
8	f	15.6			Yes		*0	0	0
9	f	17		Yes	Yes		*0	0	0
10	f	2.4			Yes		1	0	0
11	f	10.5			Yes		1	0	0
12	m	10	Yes				1	1	100
13	m	2.7			Yes		1	1	10
14	m	9.1			Yes		1	0	0
15	f	8.7					1	2	50
16	f	15	Yes	Yes			1	0	0
17	f	14					2	2	100
18	f	9					2	2	60
19	f	1					2	3	100
20	f	11.4		Yes	Yes		2	0	0
21	m	1.3					2	1	100
22	m	13.4	Yes		Yes	Yes	2	1	100
23	m	12.1			Yes	Yes	2	1	30
24	f	10.9					2	1	25
25	m	15.9					2	2	30
26	m	9.9			Yes	Yes	2	1	60
27	m	15.4			Yes	Yes	2	2	40
28	f	8.9	Yes		Yes	Yes	2	2	100
29	m	12	Yes		Yes	Yes	3	1	100
30	f	11	Yes		Yes	Yes	3	1	75
31	f	16			Yes	Yes	3	3	20
32	f	14.8			Yes	Yes	3	2	100
33	m	15.6	Yes		Yes		3	1	66
34	f	9.2			Yes	Yes	3	3	100
35	f	9.9	Yes		Yes		3	2	30
36	m	8.4	Yes	Yes	Yes	Yes	3	2	50
37	f	16.4		Yes	Yes	Yes	4	1	66
38	f	15.6	Yes	Yes	Yes	Yes	4	1	100

LPD, limiting plate disruption; an assessment of LPD in the most affected portal tract as a marker of disease severity; % PTI, the percentage of portal tracts involved in the histological abnormalities associated with CFLD.

*, Indicates those with steatosis as the only histopathological abnormality.

Materials and Methods

Patient Details and Biopsy Collection

Thirty-eight children (14 males, 24 females; median age, 10.9 years; range, 1.0 to 18.7 years) with CF confirmed by sweat test were suspected as having CFLD. All children had evidence of chronic liver disease with at least two of the following: 1) clinical hepatomegaly and/or splenomegaly; 2) persistent elevation of serum alanine aminotransferase (ALT $>1 \times$ upper limit normal) longer than 6 months; 3) abnormal ultrasound scan with abnormal echogenicity or nodular edge suggestive of cirrhosis. No child had end-stage disease and all had normal synthetic function. Only one child was pancreatic sufficient, the others were on replacement enzymes in recommended doses. None had undergone hepatobiliary surgery. There was clinical evidence of hepatomegaly and/or splenomegaly in 24 (63%) children; definite portal hypertension in 13 (34%); a history of meconium ileus at birth in 12 (32%); and 6 were insulin-dependent. Subnor-

mal serum vitamin A levels were detected in 15 (45%) of 33 tested. Genotyping was available in 22 patients revealing 14 (63%) df508 homozygotes, 7 (32%) df508 heterozygotes, and 1 untypable. All children were from families of northern European descent. Clinicopathological details are summarized in Table 1.

Control tissue was not available from children with CF and no liver disease. Normal control tissue was available from two transplantation donor livers (one male, 13 years; one female, 10 years) and from the healthy margin of a liver resection for epithelioid cyst from a 10-year-old male. The study protocol was approved by the ethics committee of the Royal Children's Hospital, Brisbane, Australia.

All children underwent percutaneous liver biopsy under general anesthetic. It has been recent practice to perform an ultrasound scan before biopsy to confirm a safe site and this was performed in 31 of the 38 patients. All children with evidence of fibrosis on biopsy were subsequently offered ursodeoxycholic acid (20 mg/kg/day).

Histological Assessment

All 38 liver biopsies were assessed for histopathological changes. Liver tissue was fixed in 10% buffered formalin and embedded in paraffin. At least 10 levels of each biopsy were examined after staining with hematoxylin and eosin, hematoxylin/van Gieson, Perls', periodic acid-Schiff-diastase, orcin, and a silver-impregnated method for reticulin fibers. All specimens had at least six (range, 6 to 13) portal tracts available for assessment. Severity of fibrosis was assessed using the grading system of Scheuer.¹¹ A semiquantitative assessment of ongoing hepatic injury was performed by assessing limiting plate disruption by bile duct proliferation with associated inflammation.¹¹ Limiting plate disruption was modified for use in CFLD in recognition of the variability in severity within the same tissue core, by scoring the worst affected portal tract. A score of 1 to 3 was assigned based on whether the limiting plate was disrupted less than one-third of the circumference of the portal tract/septa (score 1), between one- to two-thirds of the circumference (score 2), or more than two-thirds of the circumference (score 3). All portal tracts in the liver biopsy were assessed and the percentage of portal tracts involved (%PTI) in the histological abnormalities associated with CFLD (including bile ductular proliferation, inflammation, limiting plate disruption, and fibrosis) was calculated.

Immunohistochemistry

SMA

To evaluate whether activated HSCs were present in CFLD, liver sections were subjected to immunohistochemistry for SMA. All 38 liver sections were deparaffinized in xylol and rehydrated by gradient alcohol before exposure to 3% hydrogen peroxide in water to quench endogenous peroxidases. They were then incubated with a mouse monoclonal anti- α -SMA primary antibody (1:400, clone 1A4; Sigma Chemical Co., St Louis, MO). The secondary antibody was a biotinylated rabbit anti-mouse immunoglobulin (1:400; DAKO, Glostrup, Denmark) as previously described.⁶ The detection system used was a streptavidin-biotin complex/horseradish peroxidase kit (DAKO) and the chromogenic substrate was 3,3'-diaminobenzidine tetrahydrochloride (Sigma Fast DAB; Sigma Chemical Co.). The negative control used was nonimmune mouse IgG (1:50; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Sections were counterstained with eosin.

TGF- β

Liver tissue sections were available from 30 of the 38 patients enrolled in the study. Liver sections were subjected to antigen retrieval by heating in a microwave oven on high power for 8 minutes in a 0.01 mol/L trisodium citrate buffer (pH 6.0) then blocked in 5% skim milk in Tris-buffered saline (pH 7.6) for 20 minutes at room temperature. The primary antibody was an anti-TGF- $\beta_{1,2,3}$ antibody (150 μ g/ml; R&D Systems Inc., Minneapolis,

MN) for the cellular localization of TGF- β protein.⁶ The negative control used was nonimmune mouse IgG (1:50, Santa Cruz Biotechnology, Inc.). The sections were then subjected to the same detection methodology as for SMA.

Cytokeratin-19

To confirm the cellular localization of TGF- β , the expression of a specific marker for BDECs, cytokeratin-19 (CK-19), was assessed by immunohistochemistry. Non-specific binding was blocked in liver sections by 3% H₂O₂ for 10 minutes. Antigen retrieval was performed by incubating sections in 50 mg/ml of Protease type VIII (Sigma Chemical Co.) for 5 minutes at 37°C. Sections were incubated with a mouse monoclonal antibody to CK-19 (1:150, clone b170, IgG1, Novocastra NCL-CK19; Novocastra, Newcastle, UK) at room temperature for 1 hour, followed by 30 minutes in DAKO Envision+ (goat anti-mouse immunoglobulin-peroxidase conjugate, DAKO). The DAKO AEC+ high-sensitivity substrate-chromogen detection system was applied for 15 minutes. Sections were counterstained with hematoxylin.

In Situ Hybridization

To identify whether activated HSCs are the cellular source of increased type I collagen production, liver sections were subjected to *in situ* hybridization for procollagen $\alpha_1(I)$ mRNA followed by immunohistochemistry for SMA as previously described.⁶

For detection of procollagen $\alpha_1(I)$ mRNA, a 1500-bp fragment of human procollagen $\alpha_1(I)$ cDNA was subcloned into pGEM 11Z vector (Promega, Madison, WI). For detection of TGF- β_1 mRNA, a 308-bp fragment was generated by polymerase chain reaction (PCR) using nested primers, which added the transcription initiation sites Sp6 and T7 to the 5' and 3' ends of the PCR product, respectively.

Digoxigenin-labeled riboprobes for sense (control) and anti-sense were produced for both procollagen $\alpha_1(I)$ and TGF- β_1 by *in vitro* transcription with SP6 and T7 polymerases. *In situ* hybridization was performed on 5- μ m human liver sections as previously described.⁶ Unbound complex was removed by washing and sections were subjected to immunohistochemistry for SMA as previously described to co-localize procollagen $\alpha_1(I)$ mRNA to activated HSCs.

Quantitation of TGF- β Protein: Correlation with Histopathological Changes

Because of the focal nature of the disease process in CFLD and the consequent nonhomogeneous expression of both TGF- β protein and mRNA, it was difficult to adequately quantitate TGF- β levels. We have used a semi-quantitative scoring system to assess the immunohistochemical expression of TGF- β protein in BDECs. This scoring system was similar to that used to assess the

percentage of portal tracts involved in the histological abnormalities associated with CFLD (see Histological Assessment). The percentage of portal tracts in each biopsy, which demonstrated positive staining for TGF- β protein within either established bile ducts and/or proliferating bile ductules, was calculated (TGF- β %PTI). This parameter was then correlated to %PTI to demonstrate an association between bile duct TGF- β expression and biliary injury in CFLD.

Quantitation of TGF- β_1 mRNA by Real-Time Reverse Transcriptase (RT)-PCR

RNA Extraction and cDNA Synthesis

To assess the involvement of TGF- β_1 in the cholestatic injury associated with CFLD, we quantitated the expression of TGF- β_1 mRNA using real-time RT-PCR technology and correlated expression with fibrosis in CFLD patients. Frozen liver tissue was available from 11 patients with CFLD. These patients consisted of three with grade 0 fibrosis, three grade 1, two grade 2, one grade 3, and two grade 4 fibrosis. At the time of biopsy a small 3- to 4-mm piece of liver was cut from the core and immediately snap-frozen in dry-ice. RNA was extracted from frozen sections using Total RNA Isolation Reagent (Advanced Biotechnologies Ltd., Surrey, UK) according to the manufacturer's instructions. Control RNA was also extracted from age-matched donor liver tissue ($n = 2$). RNA integrity was assessed by ethidium bromide staining and $OD_{260\text{ nm}}/OD_{280\text{ nm}}$ absorption ratio >1.8 .

Two μg of total RNA was reverse-transcribed with Superscript II (Life Technologies, Inc., Gaithersburg, MD) using oligo dT primers, as described by the manufacturer. The resulting cDNA was diluted 2.5-fold before PCR amplification.

Primer Design

Primers for the analysis of human TGF- β_1 and the housekeeping gene β -actin, were designed using Primer Express software (Perkin Elmer, Foster City, CA). The TGF- β_1 forward primer was TCACCATAGCAACACTCTGAGATG and the reverse primer was CCTTAACCTCTCTGGGCTTGTTT, resulting in an amplicon 84 bp in length. The β -actin forward primer was CAGGCAC-CAGGGCGTTG and the reverse primer was GCCCA-CATAGGAATCCTTCTGA, resulting in an amplicon 52 bp in length.

Real-time RT-PCR

Real time PCR was performed using a Rotorgene thermal cycler (Corbett Research, Sydney Australia). Reactions were performed in a 20- μl volume with 1 $\mu\text{mol/L}$ of forward primer, 1 $\mu\text{mol/L}$ of reverse primer, and 2 μl of cDNA. Nucleotides, MgCl_2 , Taq polymerase, and SYBR green were included in the SYBR Green PCR master mix (Perkin Elmer). Additional SYBR Green (Fisher Biotech, Perth, Australia) was added at 1:1000 dilution. Each sam-

ple was amplified in triplicate. The PCR conditions included incubating at 50°C for 5 minutes to activate uracil-*N*-glycosylase (UNGase), which removes any carry-over contamination from previous PCR products, followed by a denaturation step at 94°C for 5 minutes. Amplification was performed for 40 cycles (denaturation at 94°C for 15 seconds, annealing at 60°C for 15 seconds, and extension at 72°C for 30 seconds). Detection of the fluorescent product was measured at the end of the 72°C-annealing step. Cycling was followed by a melt curve analysis (50 to 90°C with a heating rate of 1°C per second) to confirm the amplification of specific PCR products (either TGF- β_1 or β -actin) and the absence of nonspecific amplification or primer dimer.

The expression of TGF- β_1 mRNA was standardized to the expression of the housekeeping gene β -actin in each CFLD biopsy and control liver specimen in triplicate. The mean standardized TGF- β_1 mRNA expression for each CFLD biopsy was then expressed relative to the TGF- β_1 mRNA in controls. TGF- β_1 mRNA expression was then correlated with fibrosis in each CFLD patient.

Statistical Analysis

Correlations between TGF- β %PTI and %PTI were determined using Pearson rank correlation. Correlations between TGF- β_1 mRNA expression and the grade of histological fibrosis were determined using Spearman rank correlation for discontinuous variables. For all statistical analyses, a P value <0.05 was considered to be significant.

Results

Histopathological Assessment of CFLD

Histopathological findings for each of the 38 patients are listed in Table 1. Nine biopsies scored zero for fibrosis, of which five revealed steatosis only (indicated with an asterisk). The remainder revealed the usual spectrum of cholestasis, bile duct proliferation, steatosis, portal inflammation, and fibrosis ranging from Scheuer grades 1 to 4 (Figure 1A). Nineteen patients (50%) had fibrosis of moderate severity (Scheuer grades 1 to 2) and 10 patients had severe fibrosis (Scheuer grades 3 to 4). The severity of limiting plate disruption was variable and was not associated with the severity of established fibrosis. The predominant inflammatory cells present were neutrophils and accompanied the proliferating bile ductules indicative of biliary obstruction. No other histopathological diagnoses were found to account for the liver disease in these patients. The control tissue was histologically normal.

Identification of Activated HSCs

Cellular Source of Procollagen $\alpha_1(I)$ mRNA Expression

Activated stellate cells were demonstrated by the expression of SMA and increased expression of SMA was

Figure 1. Serial sections of liver tissue from a child with CFLD and biliary fibrosis demonstrating activated HSCs as the cellular source of collagen production. **A:** Hematoxylin/van Gieson stain demonstrating a portal tract with bile duct proliferation and fibrosis (pink) extending into the parenchyma. **B:** Immunohistochemistry for SMA (brown) and *in situ* hybridization using the anti-sense probe for procollagen $\alpha_1(I)$ mRNA (blue) demonstrating activated HSCs around bile ducts and in the advancing edge of the fibrosis. **C:** Immunohistochemistry for SMA (brown) and *in situ* hybridization using the sense (control) probe for procollagen $\alpha_1(I)$ mRNA, confirming specificity of *in situ* hybridization seen in **B**. Activated HSCs (brown) are more clearly seen around bile ducts and in the advancing fibrosis. **D:** Co-localization of procollagen $\alpha_1(I)$ mRNA expression (blue) to SMA-positive-activated HSCs (brown), showing stellate-shaped morphology of HSCs. Original magnifications: $\times 200$ (**A–C**); $\times 1000$ (**D**).

detected in all CFLD biopsies. SMA expression ranged in distribution from mild-periportal to pan-lobular. Activated HSCs were seen particularly in the extracellular matrix surrounding both portal tracts and hyperplastic bile ducts, and within fibrous septa bridging between portal tracts (Figure 1; A to C). Furthermore procollagen $\alpha_1(I)$ mRNA expression was shown to co-localize to SMA-positive HSCs (Figure 1, B and D, and Figure 2A) demonstrating that activated HSCs are the cellular source of increased collagen leading to hepatic fibrosis in CFLD. Of interest, the predominant area of HSC procollagen $\alpha_1(I)$ mRNA expression was at the growing margin of the scar tissue formation, where the stellate-shaped morphology of HSCs is clearly seen (Figure 1D). Procollagen $\alpha_1(I)$ mRNA expression was not seen in hepatocytes, BDECs, smooth muscle cells, or the portal tract vasculature. Procollagen $\alpha_1(I)$ mRNA signal specificity was demonstrated by the absence of signal in SMA-positive HSCs, using the sense probe (Figure 1C). There was no significant expression of either perisinusoidal or periportal SMA or procollagen $\alpha_1(I)$ mRNA in normal control liver biopsies (results not shown).

Co-Localization of Activated HSCs and Increased Collagen Protein Deposition

Liver biopsies were examined histologically for collagen protein deposition using hematoxylin/van Gieson stain. Figure 2B demonstrates an enlarged bile duct surrounded by excessive collagen protein deposition. Figure 2A demonstrates increased numbers of activated HSCs surrounding the same enlarged bile duct, showing co-localization of SMA and procollagen $\alpha_1(I)$ mRNA in the identical region of increased collagen protein deposition.

Presence of Activated HSCs in CFLD without Histological Fibrosis

Increased expression of SMA was also observed in the nine CFLD biopsies without evidence of histological fibrosis (Figure 2, C versus D). This suggests that the presence of activated HSCs may be an early marker for the development of hepatic fibrosis in these patients.

Cellular Source of TGF- β_1 in CFLD

Immunohistochemistry for TGF- β

Immunohistochemistry for TGF- β protein revealed increased expression in 19 of 30 (63%) liver biopsies assessed. Expression was seen predominantly in BDECs within both established bile ducts (whether surrounded by fibrosis or not) and hyperplastic bile ductules, and also by occasional HSCs within the periportal regions of the acinus (Figure 3A). TGF- β expression was also demonstrated in hepatocytes within the regenerative nodule and at the scar interface (Figure 3, C and D). Significant TGF- β expression was demonstrated in 2 of 5 (40%) biopsies graded zero for fibrosis, 9 of 16 (56%) biopsies graded 1 to 2, and 8 of 9 (89%) biopsies graded 3 to 4.

There was no TGF- β expression detected in negative controls using nonimmune IgG as the primary antibody (results not shown) or in normal control liver biopsies (Figure 3B).

In Situ Hybridization for TGF- β_1

In situ hybridization demonstrated that TGF- β_1 mRNA was predominantly expressed in BDECs within hyperplastic ducts and also by occasional HSCs within the scar (Figure 3E). TGF- β_1 mRNA signal specificity was demonstrated using the sense (control) probe (Figure 3F). There was no significant TGF- β_1 mRNA expression in normal control liver biopsies (results not shown).

CK-19 Staining

Using immunohistochemistry, we have demonstrated that TGF- β was expressed in BDECs (Figure 4, A and C) identified by the expression of CK-19 in serial liver sections (Figure 4, B and D). When detected, TGF- β expression was present in both established bile ducts (Figure 4A), whether surrounded by fibrosis or not, and also in hyperplastic bile ductules (Figure 4C).

Role of TGF- β_1 in Biliary Fibrosis

Correlation Between TGF- β Protein Expression and Histopathological Parameters of Injury

Because of the focal nature of the disease process in CFLD and the consequent nonhomogeneous expression of both TGF- β protein and mRNA, it was difficult to adequately quantitate TGF- β levels in the liver. Of the 19 biopsies that demonstrated TGF- β expression, 12 showed TGF- β immunohistochemistry within BDECs. We have used a semiquantitative scoring system (see Materials and Methods section for TGF- β immunohistochemistry) to assess the percentage of portal tracts in each biopsy that demonstrated positive staining for TGF- β protein (TGF- β %PTI) within either established bile ducts and/or proliferating bile ductules. TGF- β %PTI was significantly correlated with %PTI, the percentage of portal tracts showing histological abnormalities associated with CFLD ($r = 0.65$, $P < 0.03$; Figure 5). This suggests that TGF- β protein is most likely to be expressed in BDECs in portal tracts showing histological abnormalities and thus correlates with markers of disease activity.

Correlation Between TGF- β_1 mRNA Expression and Fibrosis

TGF- β_1 mRNA expression was quantitated in snap-frozen liver tissue that was available from biopsies from 11 patients with CFLD and 2 controls. TGF- β_1 mRNA expression was significantly increased by 8.2 ± 3.4 -fold in CFLD patients compared to controls ($P = 0.05$). In addition, there was a significant correlation between TGF- β_1 mRNA expression and the histological grade of fibrosis ($r = 0.78$, $P < 0.005$), demonstrating a definitive

Figure 2. Activated HSCs in the presence of periductal fibrosis (**A** and **B**) and in the absence of histological fibrosis (**C** and **D**). Serial sections of liver tissue from a child with CFLD demonstrating fibrogenic activity and fibrosis around a bile duct viewed at high power. **A:** Activated HSCs demonstrated by immunohistochemistry for SMA (brown) surrounding an expanded bile duct. Current fibrogenic activity is seen in an activated HSC demonstrated by co-localization of SMA (brown) and procollagen $\alpha_1(I)$ mRNA (blue) (asterisk). **B:** Hematoxylin/van Gieson staining of a serial section demonstrating collagen deposition (pink) co-localized to the area of activated HSCs surrounding the expanded bile duct. HSCs expressing procollagen $\alpha_1(I)$ mRNA in **A** is identified (asterisk). **C:** Increased expression of SMA was also observed in CFLD liver without histological evidence of fibrosis. Perisinusoidal-activated HSCs demonstrated by immunohistochemistry for SMA (brown). **D:** Serial section stained with hematoxylin/van Gieson graded zero for fibrosis. Original magnifications: $\times 1000$ (**A**, **B**); $\times 100$ (**C**, **D**).

Figure 3. Sections of liver tissue from a child with CFLD demonstrating TGF- β_1 expression most prominently in BDECs and to a lesser degree by hepatocytes at the scar interface. Immunohistochemistry for TGF- β protein expression (brown) in BDECs (A), and along the fibrous edge of a regenerative nodule in hepatocytes (C) and BDECs (D). B: No significant expression of TGF- β protein was seen in control liver tissue. E: *In situ* hybridization for TGF- β_1 mRNA expression (blue) demonstrated in BDECs using the anti-sense probe. F: *In situ* hybridization control using the TGF- β_1 mRNA sense probe, confirming specificity seen in C. Original magnifications: $\times 400$ (A, D); $\times 200$ (B, C, E, F).

role for TGF- β_1 in the pathogenesis associated with CFLD (Figure 6).

Discussion

This study has demonstrated that activated HSCs produce the increased type I collagen seen in the fibrotic liver disease associated with CF. This study has also shown that the predominant source of the profibrogenic cytokine TGF- β_1 in CFLD is bile duct epithelium and that hepatocytes also produce increased levels of TGF- β within the regenerative nodule and at the scar interface.

The progression of CFLD is often slow, irregular in timing, and nonhomogeneous. Consequently, the demonstration of TGF- β in CFLD in our study was variable in both distribution and levels of expression. This is in contrast to our findings in accelerated models of fibrosis such as biliary atresia⁶ in which biliary inflammation and consequent fibrogenesis can progress rapidly and TGF- β staining is much more intense and homogeneous, reflecting protein quantum. However, in the present study we have demonstrated a significant correlation between the percentage of portal tracts showing histological abnormalities and the percentage of portal tracts demonstrating increased expression of TGF- β protein in BDECs. This confirms TGF- β protein is most likely to be expressed in portal tracts showing histological evidence of current disease activity. Further to these results, we have demonstrated a strong correlation between the expression of TGF- β_1 mRNA, by real-time RT-PCR, and the

histological grade of fibrosis. These observations provide strong evidence for a definitive role for TGF- β_1 in the fibrogenesis associated with CFLD.

In this study, we demonstrated that a number of liver biopsies showed no histological evidence of hepatic fibrosis, however, increased numbers of activated HSCs were present in all of these biopsies. Although it was not possible to obtain liver from CF patients without liver disease as a comparison, it is tempting to speculate that the presence of activated HSCs in CFLD may have a potential role as a marker for the earliest evidence of fibrogenesis. However, the significance of this finding requires long-term follow-up in these same patients.

The development and progression of CFLD remains an enigma. There is no correlation with CFTR genotype¹² or any other current clinical marker.² More recently, workers have postulated development of CFLD to be modulated by genetic factors different from the CFTR region.¹³ The contribution of co-morbid genes to phenotypic expression was highlighted by the discovery of the CF modifier locus predisposing for the complication of meconium ileus on chromosome 19q14.¹⁴ The role of co-morbid genes in CFLD has been strengthened by the association of allelic variants for mannose-binding lectin, an immunoregulatory protein, with development of liver cirrhosis.¹⁵ Since the earliest pathological descriptions of biliary fibrosis in CFLD,¹⁶ the subsequent localization of the aberrant CFTR to cholangiocytes by Cohn and colleagues³ and the further demonstration of aberrant cytoplasmic immunolocalization of CFTR in BDECs of patients

Figure 4. Serial liver sections from a child with CFLD demonstrating that CK-19-positive BDECs are responsible for the increased expression of TGF- β protein in expanded portal tracts. Immunohistochemistry for TGF- β protein expression (brown) in BDECs within established bile ducts surrounded by areas of fibrosis (**A**) and hyperplastic bile ductules in expanded portal tracts and at the growing margin of the scar tissue formation (**C**). Immunohistochemistry for CK-19 expression (red) in serial liver sections of **A** and **C**, respectively, in BDECs within established bile ducts surrounded by areas of fibrosis (**B**) and hyperplastic bile ductules in expanded portal tracts and at the growing margin of the scar tissue formation (**D**). Original magnifications, $\times 200$.

with CFLD by Kinman and colleagues,¹⁷ the challenge remains to link biliary events to the development of fibrosis. Current theories of pathogenesis in CFLD hold that abnormal biliary chloride transport causes defective hy-

dration of bile, impaired bile flow, and the resulting cholestasis initiates fibrogenesis. The CFTR chloride channel is the principal transporter creating an electrochemical gradient to facilitate flux of sodium ions. The resulting

Figure 5. Correlation between the percentage of portal tracts that demonstrated positive staining for TGF- β protein within BDECs (TGF- β %PTI) and the percentage of portal tracts involved (%PTI) in the histological abnormalities associated with CFLD. TGF- β %PTI was significantly correlated with %PTI ($r = 0.65$, $P < 0.03$; $n = 12$).

osmotic gradient facilitates the hydration of luminal contents. The normal CFTR channel also provides chloride ions to exchange with bicarbonate ions for the alkalization of bile. Various mechanisms have been proposed to connect abnormal hydration and pH of bile to the development of liver disease in CF. Workers have tried, unsuccessfully, to link this bile acid transport defect to liver damage via a change in the bile acid pool per se.¹⁸ Others have postulated that accumulation of toxic bile acids, with an increased glycine conjugation, in bile ducts blocked with inspissated bile may mediate CFLD.¹⁹ *In vitro* studies demonstrate that bile acids can cause hepatocyte apoptosis²⁰ and can be implicated in cholangiocyte dysfunction.²¹ Structural abnormalities of the biliary tree, both intrahepatic and extrahepatic, have been noted in patients with CF²² both with and without liver disease and it is not known how significantly they contribute to the pathogenesis of CFLD.

The present study has established a link between biliary events and the expression of procollagen $\alpha_1(I)$

Figure 6. Quantitation of TGF- β_1 mRNA expression in CFLD and correlation with the histological grade of hepatic fibrosis. TGF- β_1 mRNA expression was standardized to the expression of the housekeeping gene, β -actin, in each of the CFLD biopsies and controls. The expression of TGF- β_1 mRNA in each CFLD biopsy was then expressed as a -fold increase *versus* controls. TGF- β_1 mRNA expression was significantly correlated with the histological grade of fibrosis ($r = 0.78$, $P < 0.005$; $n = 11$).

mRNA by activated HSCs in patients with CFLD, namely the production of the profibrogenic cytokine TGF- β_1 by BDECs and its correlation with histological fibrosis. Further elucidation of the role of TGF- β_1 in CFLD is particularly pertinent. Respiratory physicians caring for patients with CF have become interested in TGF- β in light of the recent demonstration that certain TGF- β_1 genotypes are associated with more rapid deterioration in lung fibrosis and function.²³ TGF- β is a multifunctional peptide growth factor with a wide range of effects on growth, differentiation, extracellular matrix deposition, and the immune response.¹⁰ It is the dominant stimulus to extracellular matrix deposition in a wide variety of fibrosing liver disorders,⁵ including cholestasis, by stimulating collagen gene expression at the level of transcription in activated HSCs.²⁴ It also has an important role in cholestatic liver disease by causing apoptosis of hepatocytes²⁵ and apoptosis of HSCs in resolution of biliary fibrosis in rat models.²⁶ The key role of TGF- β_1 in liver fibrosis is highlighted by the finding that inflammatory responses in the liver do not lead to fibrogenic stimuli and collagen deposition in the absence of TGF up-regulation.²⁷ In studies of cirrhotic human liver tissue, mRNA for both TGF- β_1 and connective tissue growth factor, a recently identified downstream mediator of TGF- β , were localized to activated stellate cells, endothelial cells, and BDECs, and the degree of expression was shown to correlate with severity of fibrosis.²⁸ The role of TGF- β in pediatric cholestatic liver disease was first addressed by our group demonstrating that TGF- β_1 expression was particularly localized to the BDECs in biliary atresia.⁶ Subsequently, Rosenzweig and colleagues²⁹ demonstrated that the hepatic expression of TGF- β_1 protein, as assessed by immunohistochemistry, is increased in children with hepatic fibrosis secondary to CF and biliary atresia in contrast to normal liver tissue. However, this study did not examine the cellular localization of TGF- β_1 . Our study has not only confirmed increased TGF- β expression, but has demonstrated that BDECs are the predominant cellular source of TGF- β_1 in CFLD. In this respect it is similar to biliary atresia, highlighting the cholestatic influence on pathogenesis. It remains to be determined whether the mechanism that stimulates BDECs to produce TGF- β_1 occurs via retention of hydrophobic bile salts, via co-stimulation by infectious agents in the context of impaired immune modulation, or via another as yet undefined stimulus.

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