A Potential Profibrogenic Role of Biliary Epithelium-Derived Cardiotrophin-1 in Pediatric Cholestatic Liver Disease

Xiangwei Hua, Yuhua Shan, Dawei Li, Dongwei Xu, Jiang Zhang, Taihua Yang, Longzhi Han, Conghuan Shen, Yun Xia, Qimin Chen, Xiong Ma, Jianjun Zhang and Qiang Xia

As a cytokine of the interleukin-6 family, cardiotrophin-1 (CT-1) has been shown to be an important endogenous protector in liver injury. Our study aimed to investigate the role of CT-1 in liver fibrosis in pediatric cholestatic liver disease (PCLD). CT-1 mRNA and protein expression levels were upregulated in PCLD liver biopsy tissues compared with controls. Immunohistochemistry and confocal microscopy of liver sections showed that CT-1 was predominantly expressed by biliary epithelium cells. Serum CT-1 was elevated significantly in the children with PCLD compared with controls. Serum CT-1 levels exhibited a moderate positive correlation with the Scheuer stage of hepatic fibrosis and serum TB levels and a weak correlation with serum ALP levels. In vitro analysis indicated that LX-2 cells preconditioned with CT-1 exhibited significant increments in proliferation and accumulation of extracellular matrix components, while also positively regulating the STAT3 and p38MAPK pathways. In conclusion, biliary epithelium-derived CT-1 may exert a profibrogenic potential in PCLD.

Introduction

Cholestatic liver diseases are common and important causes of cirrhosis in children. Congenital biliary dilatation (CBD) is a relatively common congenital biliary malformation, and biliary atresia (BA) is the most common cause of pediatric liver transplantation (Iwai and others 1992; McEvoy and Suchy 1996; Chen and others 2006). Despite diverse etiologies, the common pathophysiological process of these diseases involves fibrosis. Moreover, the progressive and irreversible fibrosis often leads BA patients to cirrhosis within a few years. Hepatic stellate cells (HSCs) play a central role in liver fibrosis, and the potential factors that activate HSCs and promote fibrosis are of major interest (Friedman, 1996). In this study, a potentially influential molecule in this process, cardiotrophin-1 (CT-1), was investigated in pediatric cholestatic liver disease (PCLD).

CT-1, a cytokine of the interleukin-6 family, was named after its ability to induce cardiac myocyte hypertrophy (Pennica and others 1995a, 1995b; Richards and others 1996). This protein is highly expressed in the heart, skeletal muscle, liver, lung, and kidney (Pennica and others 1996). Based on its activation of key survival pathways such as JAK/STAT3, CT-1 has primarily been studied as a crucial endogenous protector in reperfusion/ischemic damage and fulminant liver failure after subtotal hepatectomy or induced by other nocuous factors (Ghosh and others 2000; Bustos and others 2003; Iniguez and others 2006; Garcia-Cenador and others 2013b). Recently, CT-1 was also reported to be involved in glucose and lipid metabolism (Natal and others 2008; Moreno-Aliaga and others 2011; Castano and others 2014). The ability of CT-1 to remodel the cardiovascular system or other tissues and organs has been observed (Lopez-Andres and others 2012; Schillaci and others 2013). CT-1 can promote cardiac, vascular, and renal fibrosis and has been associated with dysfunction of related organs. It has been reported to stimulate the proliferation of fibroblasts in cardiac regeneration (Tsuruda and others 2002).

In the present study, we demonstrated a significant correlation of CT-1 with hepatic fibrosis in PCLD and performed an overall analysis of its effects on the fibrotic properties of LX-2. These findings undoubtedly help us to further understand the biological function of the cytokine and provide a potential therapeutic target in liver fibrosis.
Materials and Methods

Patients

After obtaining institutional review board approval and informed consent, 33 children with pediatric cholestasis (age, 22.35 ± 2.69 months; 16 males and 17 females), including 23 children with BA and 10 children with CBD, were recruited as the experimental group. For the serum study, 9 age- and gender-matched children (age, 12.67 ± 1.77 months; 4 males and 5 females) were included as the pediatric healthy controls (PHCs). The liver tissues for the controls were obtained from 7 healthy adults (AHCs) (age, 25.86 ± 2.55 years; 3 males and 4 females) who were donors in a living donor liver transplantation. Controls were clinically diagnosed as being free from liver-associated diseases, with no symptoms or signs of liver dysfunction and no severe inflammatory or other systemic diseases. Serum samples were collected for a routine liver function test, and the CT-1 concentration was assayed. All cholestatic cases were confirmed by operation and pathological. Study participants with BA and AHCs were prospectively recruited from Renji Hospital, School of Medicine, Shanghai Jiaotong University; the participants with CBD and PHCs were recruited from Shanghai Children’s Medical Center, School of Medicine, Shanghai Jiaotong University.

Sample collection and preparation

Surgically obtained liver tissues that would be used to detect the mRNA and protein expression of CT-1 were immediately snap-frozen in liquid nitrogen and stored at −80°C until analysis. The liver tissues were fixed in 10% neutral buffered formalin, embedded in paraffin, and 4-μm-thick sections were cut from each paraffin block. The slides were stained with hematoxylin–eosin, and the fibrotic stages were determined according to the Scheuer grading and staging system. Sera for the study were obtained from blood samples by centrifugation at 3,000 rpm for 10 min and stored at −80°C until analysis.

Quantitative real-time polymerase chain reaction and Western blot

A standard TRIzol method (Invitrogen) was used to extract total RNA from tissues and cultured cells and to synthesize complementary DNA. Real-time polymerase chain reaction (RT-PCR) was performed using SYBR Premix Ex Taq™ (Takara) in an ABI PRISM® 7900HT sequence detector. The β-actin gene was used as an endogenous control. The primer sequences were as follows: β-actin forward 5′-TTG AGC GAA GGG CAC CAC CAG-3′; β-actin reverse 5′-GCACCCAC CA CCAACGG ATCG-3′; CT-1 forward 5′-GGGAGGGGA AATCGGACC-3′; CT-1 reverse 5′-CCCGAAGGGTCT CCTTG-3′; α-SMA forward 5′-TTG AGC GAA GGG CAC CAC CAG-3′; α-SMA reverse 5′-TTG AGC GAA GGG CAC CAC CAG-3′; COL1α1 forward 5′-TTG AGC GAA GGG CAC CAC CAG-3′; COL1α1 reverse 5′- TTG AGC GAA GGG CAC CAC CAG-3′; COL3α1 forward 5′-GCT GCC ATC AAA GGA CAT CG-3′; COL3α1 reverse 5′-GAG AGC CCT CAG ATC CTC TT-3′; TIMP-1 forward 5′-TTG AGC GAA GGG CAC CAC CAG-3′; TIMP-1 reverse 5′-TTG AGC GAA GGG CAC CAC CAG-3′. Liver tissue or LX-2 extracts were prepared for immunoblotting analyses, as previously described (Logan and others 2013). Immunoblots were probed with antibodies against CT-1 (Abcam), β-actin (Santa Cruz Biotechnology); Stat3, p-Stat3(Ser727), p38, and p-p38(Thr180/Tyr182) (Cell Signaling Technologies) at the recommended dilutions.

Immunohistochemistry and confocal microscopy

Immunohistochemistry (IHC) and confocal microscopy with formalin-fixed, paraffin-embedded liver tissues were performed, as previously described, for human liver sections (You and others 2012) using primary antibodies for CT-1 (1:100; Abcam) and CK7 (1:100; Abcam). The intra-assay variation among the triplicates for all samples was <15%.

Enzyme-linked immunosorbent assay

Serum samples were divided from the sample within 1 h after the venous blood was collected. A human CT-1 enzyme-linked immunosorbent assay (ELISA) kit (R & D) was used to measure the serum CT-1 concentration according to the manufacturer’s instructions (R & D Antigenix America for human CT-1), as previously described (Natal and others 2008).

Cell maintenance

LX-2, a human HSC line, was a gift from Dr. Ma X (Division of Gastroenterology and Hepatology, Ren Ji Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai Institute of Digestive Disease, China) and maintained in DMEM that contained 10% FBS and 1% penicillin/streptomycin. At a density of 50%-70%, LX-2 cells were starved in serum-free DMEM for 24 h. The cells were then treated with recombinant human CT-1 (Peprotech) in serum-free DMEM.

Cell proliferation assay

LX-2 cells were seeded in 96-well plates at 1,000 cells per well and cultured overnight. The cells were starved for 24 h by replacing the medium with the serum-free medium, followed by stimulation with CT-1(100 ng/mL) or Stattic (Selleck Chemicals). Cell culture was continued for 24 h and assessed using the Cell Counting Kit 8 (Dojindo). The absorbance value of each well was determined at 450 nm using a microplate reader (Molecular Devices).

Statistical analyses

Statistical analyses were performed using SPSS version 15.0 (SPSS, Inc.). Data are expressed as the mean ± standard deviation.
deviation (SD). Quantitative data were analyzed using the independent samples t-test, as appropriate. Receiver operating characteristic (ROC) analysis was used to assess the diagnostic utility of elevated serum CT-1 levels in identifying children with pediatric cholestasis. Associations between the CT-1 measures, histological fibrosis stage, and liver function index were conducted using Spearman’s correlation. For all statistical analyses, a 2-tailed $P < 0.05$ was considered to be significant.

Results

**Elevated hepatic CT-1 mRNA and protein expression levels in infants with pediatric cholestasis**

To investigate CT-1 mRNA expression in liver biopsy tissues, we detected CT-1 transcription levels by quantitative RT-PCR. The CT-1 mRNA levels were significantly higher in the infants with pediatric cholestasis compared with the healthy controls ($P < 0.05$) (Fig. 1A). Next, CT-1 protein levels were evaluated in the infants with pediatric cholestasis and the healthy controls. Western blot analysis revealed increased intracellular protein expression of CT-1 in infants with pediatric cholestasis compared to that in the healthy controls (Fig. 1B).

**Cellular localization of CT-1 expression in liver tissue samples**

In cholestatic infants and in liver sections from healthy controls, IHC indicated that biliary epithelium cells (BECs) were the predominant cell type that expresses the CT-1 protein. In normal liver tissues, the stains were restricted to portal areas, whereas in cholestatic sections, the staining was also widely present in cholangiocytes within proliferating bile ductules among the interlobular regions (Fig. 2A). Using fluorescent confocal probes, we determined that the green fluorescence demonstrating a positive CT-1 stain overlapped with the biliary duct epithelium cells marked by CK-7; these findings further support the hypothesis that in fibrosis of cholestatic pathogenesis, CT-1 may exert an important effect through cholangiocytes (Fig. 2B).

**Serum CT-1 in children with pediatric cholestasis compared with healthy controls**

The plasma CT-1 concentration was significantly higher in the children with pediatric cholestasis compared with the healthy controls ($P < 0.01$) (Fig. 3A). The ROC curve analysis showed that a serum CT-1 of 292.6 pmol/mL had 81.8% sensitivity and 100% specificity for distinguishing between the children with pediatric cholestasis and the healthy controls (Fig. 3B). Thus, CT-1 appears to be elevated early in cholestasis in PCLD.

**In vivo association between CT-1 and liver fibrosis in PCLD**

Hepatic fibrosis has been shown to be an important feature for cholestatic liver injury. We explored whether CT-1 was associated with liver fibrogenesis. Serum CT-1 levels were plotted against the stage of hepatic fibrosis in cholestatic children and demonstrated a strong positive correlation ($r = 0.632$, $P < 0.001$) (Fig. 4).

**In vivo association between CT-1 and liver functions in PCLD**

Further analysis of the linear correlation between the CT-1 serum concentration and liver function index indicated...
that TB was the best candidate, with a moderate linear trend ($r=0.689$, $P<0.001$), while ALP demonstrated a mild trend ($r=0.366$, $P<0.05$) (Fig. 5). Besides, we did not find a significant correlation between the CT-1 serum concentration and ALT serum levels, although previous studies had suggested CT-1 as an important hepatocyte survival factor (data not shown).

**In vitro CT-1 regulates the function of LX-2**

Because of the significant correlation between CT-1 and liver fibrosis in PCLD, we investigated whether CT-1 could promote the fibrogenesis ability of HSCs. First, the stimulation of CT-1 was succeeded by significantly enhancing proliferation of LX-2 cells *in vitro*, which could be blocked by Stattic, an inhibitor of STAT3 (Fig. 6A). Then, the incubation of LX-2 cells with CT-1 resulted in remarkable increases in $\alpha$-SMA, COL1z1, COL3z, and TIMP-1 expressions (Fig. 6B).

**In vitro CT-1 activates STAT3 and p38MAPK pathways**

In the previous section, the STAT3 pathway was proved to play an important role in HSC proliferation. It was also reported that the p38MAPK pathway regulated TIMP-1 expression in HSCs. So, we performed western blot analysis to investigate whether CT-1 could activate the mentioned 2 routes. Our study confirmed that CT-1 exposure upregulated the expression of Phospho-Stat3 (Ser727) and Phospho-p38 MAPK (Thr180/Tyr182) (Fig. 7).

**Discussion**

Elevated expression of CT-1 has been shown to exert an important effect on multiple medical complications such as hypertension, heart failure, obesity, and renal dysfunction (Moreno-Aliaga and others 2011; Rendo-Urteaga and others 2013; Gkaliagkousi and others 2014; Lopez and others 2014). Although studies have demonstrated a key role for CT-1 in hepatocyte survival in acute liver injuries (Bustos and others 2003), CT-1 has not previously been investigated as the cause of childhood cirrhosis. This study is the first to demonstrate that increased CT-1 levels were significantly associated with the development of liver fibrosis in 2 diverse causes of pediatric cholestasis, BA and CBD.

In our study, we first demonstrated higher CT-1 mRNA and protein levels in liver tissues obtained from children with pediatric cholestasis compared with AHCs. As an important survival mechanism (Garcia-Cenador and others 2013a), CT-1 was expressed by both parenchymal and nonparenchymal liver cells (Bustos and others 2003). In the IHC study, we found that CT-1 was primarily localized in the plasma of cholangiocytes in the livers of pediatric cholestasis. Furthermore, the biliary tract could express CT-1 in fundamental conditions. In previous studies, hepatocytes have been shown to upregulate the expression of CT-1 under the stress of hypoxia or starvation or when stimulated by TGF-$\beta$ (Bustos and others 2003; Iniguez and others 2006). CT-1 has also been expressed by cardiomyocytes following stimulation by hypoxic stress, mechanical stretch, and humoral factors, such as norepinephrine and angiotensin II (Hishinuma and others 1999; Pan and others 1999; Sano and others 2000). We are the first to find that BECs are the major cells excreting CT-1. We propose that CT-1 secreted by cholangiocytes may play a fundamental role in maintaining normal liver physiological stability under normal physical conditions. However, regarding cholestasis, CT-1 would be overexpressed by biliary cells other than hepatocytes because of the proliferation of the biliary tracts and other unrevealed factors. The overexpression of CT-1 could protect hepatocytes from cholestatic injury; however, it may also exert other effects on the cholestasis liver. In this study, we found that CT-1 could promote liver fibrosis in pediatric cholestasis.

The serum CT-1 levels were also elevated in pediatric cholestasis patients compared with the healthy controls. In the present study, the ROC curve analysis of serum CT-1 in pediatric cholestasis suggests that serum CT-1 levels are elevated early in the disease process, which was verified by the strong and significant positive correlation with the hepatic fibrosis.
The sensitivity reached 81.8% and the specificity was 100% when the concentration of serum CT-1 was 292.6 pg/mL, which indicated the diagnostic significance of CT-1 in pediatric cholestasis. However, further studies with larger patient cohorts are required to assess the potential clinical utility of serum CT-1 in the prediction of early cholestasis.

Progressive fibrosis is the common progression to cirrhosis, which can result in a detrimental outcome. It has been reported that CT-1 was associated with myocardial fibrosis in hypertensive patients and experimental animals (Lopez-Andres and others 2011; Lopez and others 2014). CT-1 has been correlated with the expression of fibrosis factors, such as collagen type I and III in myocardial tissues and N-terminal pro-brain natriuretic peptide, carboxyterminal propeptide of procollagen type I, and amino-terminal propeptide of procollagen type III in plasma obtained from hypertensive patients (Lopez and others 2014). Another interesting study demonstrated that the arterial media thickness and wall fibrosis were lower in CT-1−/− mice (Lopez-Andres and others 2013). The present study demonstrated that plasma CT-1 was positively correlated with the development of hepatic fibrosis in pediatric cholestasis. Furthermore, plasma CT-1 was also positively correlated with total bilirubin and alkaline phosphatase, which primarily reflect the progression of cholestasis. In BA and CBD, obstructive cholestasis leads to fibrogenesis and its progression. Thus, the correlation between CT-1 and ALP and TB indirectly indicates the important role of CT-1 in the progression of liver fibrosis. What is more, the serum CT-1 level is a potential parameter to evaluate the hepatic fibrosis level, which helps to decrease the risk brought by biopsy. Previous studies have demonstrated that CT-1 could protect against hepatocellular damage in various models of liver injury (Bustos and others 2003; Iniguez and others 2006). However, we did not find the correlation between serum CT-1 and ALT levels in our pediatric cholestatic patients. This may be due to the application of hepatic protectants in clinics.

In the above parts, we found that CT-1 could be overexpressed in livers and its plasma level was positively correlated with the development of hepatic fibrosis in pediatric cholestasis. Then, we tried to explore whether CT-1 could promote fibrogenesis in in vitro studies. It is widely accepted that HSCs play a central role in liver fibrogenesis following liver injury, such as cholestasis (Friedman, 1996; Ramm, 1998). Cytokines, such as transforming growth factor-1 and MCP-1, have been demonstrated as key factors in activating and recruiting HSCs in the fibrosis of pediatric cholestasis (Ramm and others 2009; Dooley and ten Dijke 2012). CT-1 has been shown to stimulate several inflammatory cytokines, such as intercellular adhesion molecule-1 and monocyte chemoattractant protein-1, in human aortic endothelial cells (Ichiki and others 2008). Moreover, CT-1 could promote fibrogenesis in other systems (Tsuruda and others 2002; Tokito and others 2013; Lopez and others 2014). Our in vitro studies first found that CT-1 induced the increased proliferation of LX-2. Also, HSC proliferation is a key physiological process in liver fibrosis. In this way, the increased CT-1 level might promote liver fibrosis by stimulating the proliferation of HSCs in vivo. As a member of the IL-6 family of cytokines, CT-1 binds to the common receptor gp130 and activates its downstream STAT3 pathway. The gp130-STAT3 pathway has been found to be extensively involved in cell proliferation. In our study, the

FIG. 6. The effects of CT-1 on LX-2 cells. (A) LX-2 cells were treated with CT-1 (50 or 100 ng/mL) for 24 h followed by RT-PCR analysis, n = 3. (B) LX-2 cells were treated with CT-1 (100 ng/mL) for 24 h followed by CCK8 cell proliferation analysis, n = 3 (∗P < 0.05, ∗∗P < 0.01, and ∗∗∗P < 0.001).

FIG. 7. The effects of CT-1 on LX-2 cells. Western blot showing phosphorylation on Stat-3 and p38 MAPK in whole-cell lysate after treatment with CT-1 (100 ng/mL) at the indicated time. The blots are representative of 3 experiments with similar results.
STAT3 pathway inhibitor Stattic significantly prevented the proliferation potential of CT-1 on LX-2. However, IL-22, another member of the IL-6 cytokine family, was shown to induce HSC senescence through the STAT3 pathway (Kong and others 2012). It means that the STAT3 pathway might be involved in different physiological processes in HSCs when confronted with various stimulators. For CT-1 and IL-22, this may be due to the different subunits of their receptors despite the common subunit gp130 (Robledo and others 1997; Kotenko and others 2001).

The cytoskeleton protein α-Sma and collagen synthesis are usually considered as key markers for HSC activation. In our in vitro study, CT-1 could promote the gene expression of α-SMA and the synthesis of COL1α1 and COL3α1 mRNAs in LX-2. Matrix remodeling forms an integral part of liver fibrosis, where TIMP-1 plays a critical role by inhibiting members of a large family of MMPs (Chirco and others 2006). In this study, CT-1 could upregulate TIMP-1 gene expression of LX-2. Quite the contrary, IL-22 down-regulates TIMP1 expression and induces HSC senescence. As an important signaling pathway of CT-1, the p38 MAPK pathway has been proved to mediate TIMP-1 expression in HSCs (Nieto, 2006). In the present study, CT-1 could also phosphorylate p38 MAP kinase in LX-2. Therefore, CT-1 might promote HSC proliferation and extracellular matrix accumulation through the STAT3 and p38 MAPK pathways in liver fibrogenesis.

In summary, these findings suggest that biliary epithelium-derived CT-1 may play a potential profibrogenic role in pediatric cholestasis, thus making CT-1 a potential novel serum marker and a new therapeutic target for liver fibrosis.

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Author Disclosure Statement

The authors have reported no conflicts of interest.

References


Address correspondence to:
Dr. Qiang Xia
Department of Liver Surgery and Liver Transplantation Center
Ren Ji Hospital
School of Medicine
Shanghai Jiao Tong University
1630 Dongfang Road
Pudong New District
Shanghai 200127
People’s Republic of China
E-mail: xiaqiang@shsmu.edu.cn

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