

Toxic bile acids do not increase transforming growth factor-beta expression in bile duct epithelial cells or collagen synthesis in myofibroblasts

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Abstract

Primary sclerosing cholangitis (PSC) is a chronic, progressive hepatobiliary disorder characterized by extensive fibrosis and stricturing of the intra- and/or extra-hepatic bile ducts. In the present study we tested the hypothesis that low phosphatylcholine (PC) levels in PSC bile permit toxic bile acid induced injury of biliary tract epithelial cells resulting in enhanced transforming growth factor-beta (TGF- β) expression and increased collagen synthesis by myofibroblasts. Thus, TGF- β mRNA expression was documented in bile duct epithelial cells exposed to varying concentrations of the toxic bile acid; glycochenodeoxycholic acid (GCDC) +/- PC. In these experiments, as well as in co-culture experiments where bile duct epithelial cells were cultured with peripheral blood mononuclear cells and myofibroblasts, TGF- β mRNA expression in all cell types remained unaltered. Moreover, collagen type I α 1 mRNA expression by myofibroblasts also remained unaltered. The results of this study do not support the hypothesis that PC deficiency contributes to toxic bile acid-induced bile duct injury and/or myofibroblast activation.

Introduction

Primary sclerosing cholangitis (PSC) is a chronic, progressive, fibrotic disorder of the hepatobiliary system that often progresses to cirrhosis [1]. The disease is more common in males, often in their third or fourth decades of life. Aside from endoscopic/surgical corrections of dominant biliary strictures, no specific treatments have been identified that favorably alter the course of the disease [2].

The pathogenesis of PSC remains unclear. One common hypothesis proposes that toxic bile acids induce biliary tract epithelial cell injury, resulting in the release of profibrotic cytokines such as transforming growth factor-beta (TGF- β) that activate neighbouring myofibroblasts and enhance collagen production [3-5]. Recent data indicating that PSC bile is deficient in phosphatylcholine (PC), a phospholipid released by hepatocytes into bile, which serves to protect epithelial cell membranes from the detergent effects of toxic bile acids, would support that hypothesis [6,7].

In the present study, we documented the effects of toxic bile acids on TGF- β mRNA expression in bile duct epithelial cells and collagen type I α 1 mRNA expression in myofibroblasts in the presence and absence of exogenous PC.

Methods

Materials

KMBC bile duct epithelial cells were kindly provided by Dr. Gregory J. Gores at the Mayo Clinic School of Medicine while LX-2 myofibroblasts were donated by Dr. Scott L. Friedman at the Mount Sinai School of Medicine. All reagents for cell culture (Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), trypsin-

EDTA, non-essential amino acids, glutamine and Trizol Reagent were purchased from Invitrogen (Burlington, ON). Polymerase chain reaction (PCR) primers were designed by the Oligo 5 computer software and synthesized by Invitrogen. SYBR[®] Safe DNA gel stain were purchased from Invitrogen (Invitrogen Canada Inc., Burlington, Canada). The iScript[™] cDNA Synthesis Kit and iQ[™] SYBR[®] Green Supermix were purchased from Bio-Rad Laboratories (Canada) Ltd. (Mississauga, Ontario). Co-culture plates were purchased from Corning (Corning Incorporated, NY, USA). The toxic bile acid glycochenodeoxycholate (GCDC), L- α -phosphatidylcholine (PC) and other chemicals were purchased from Sigma-Aldrich Co. (Oakville ON).

Cell culture

KMBC cells were cultured in DMEM supplemented with 110 mg/L sodium pyruvate, 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μ g/ml streptomycin. LX-2 cells were cultured in DMEM/F12 supplemented with 0.1 mmol/L non-essential amino acid, 2 mmol/L glutamine, 110 mg/L sodium pyruvate, 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μ g/ml streptomycin. All the cells were incubated at 37°C in a humidified atmosphere of 5% CO₂ and 95% air.

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Co-culture of KMBC and LX-2 cells

KMBC and LX-2 cells were cultured in co-culture plates with inserts containing a 3.0-um porous membrane at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. For each plate, 8 × 10⁶ KMBC cells were cultured in the upper chamber and 8 × 10⁶ LX-2 cells in the lower chamber. Culture medium was removed one day before treatment and cells were incubated in the medium described above. The indicated concentrations of GCDC were added into the upper chamber bath solution for 24h prior to cell harvesting.

Co-culture of KMBC peripheral blood mononuclear cells (PBMC) and LX-2 cells

KMBC, PBMC (derived from healthy donors) and LX-2 cells were cultured in co-culture plates with inserts containing a 3.0-um porous membrane at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. For each well, 5 × 10⁶ KMBC and 3 × 10⁶ PBMC cells were cultured in the upper chamber and 8 × 10⁶ LX-2 cells in the lower chamber. Culture medium was removed one day before GCDC exposure and the cells incubated in the medium described above.

Extraction of RNA

TRIzol[®] reagent was employed to extract total RNA from cells according to the manufacturer’s instructions. Briefly, cells were dissolved in 1 ml TRIzol[®] reagent and incubated at room temperature for 5 min to permit complete dissociation of the nucleoprotein complex. After adding 0.2 ml chloroform, tubes were shaken vigorously by hand for 15 sec, and incubated for 2 min at room temperature. After centrifuging the sample at 12,000xg for 15 min at 4°C, the mixture separated into a lower red phenol-chloroform phase, a white interphase, and a clear upper aqueous phase. The aqueous phase which contained RNA was transferred into new Eppendorf tubes and RNA was precipitated using 0.5 ml isopropyl alcohol. Samples were incubated at room temperature for 10 min and then centrifuged at 12,000xg for 10 min at 4°C. RNA pellets were washed with 1 ml 75% ice-cold ethanol and centrifuged at 7,500xg for 5 min at 4°C. After being air-dried for 10 min, RNA pellets were resuspended in DEPC water. RNA concentration was assessed by Multi-Mode Microplate Reader (BioTek Instruments, Inc., Winooski, Vermont, USA). Absorbance of each diluted RNA sample was measured at wavelengths 260 nm and 280 nm. RNA was then stored at -80°C until used in further experiments.

Reverse transcriptase PCR (RT-PCR)

The first strand cDNA was synthesized by the iScript[™] cDNA Synthesis Kit as per the manufacturer’s manual. PCR was performed using the iQ[™] SYBR[®] Green Supermix and the oligonucleotides synthesized by Invitrogen. The specific primers for the present study were designed with the respective sequences from GenBank by Oligo 7 program and listed in Table 1. PCR amplification was carried out by applying 28-30 cycles comprising: denaturation at 94°C for 1 minute,

different annealing temperatures for 30 seconds, elongation at 72°C for 2 minutes, followed by a final elongation at 72°C for 8 minutes using a MiniOpticon[™] Real-Time PCR Detection System (Bio-Rad Laboratories (Canada) Ltd. Mississauga, Ontario). PCR products were also analyzed by electrophoresis on a 1.0% agarose gel. Identity of PCR products was confirmed by DNA sequencing at the DNA sequencing facility of the Manitoba Institute of Cell Biology.

Results

In the first series of experiments, KMBC cells were exposed to varying concentrations of GCDC (5-500 μM) alone or 50 μM GCDC + PC (100 μM). As shown in Figure 1, TGF-β mRNA expression in KMBC cells remained unaltered in the presence of GCDC and GCDC + PC.

Because an autocrine loop has been described for TGF-β and myofibroblasts (8), we also exposed LX-2 cells to GCDC (5-500 μM) alone and GCDC (50 μM) + PC (100 μM) but again, TGF-β mRNA expression remained unaltered (Figure 2).

To exclude the possibility that GCDC exposure results in the release of pro-fibrotic cytokines other than TGF-β, which in turn stimulate LX-2 fibrogenic activity, we cultured GCDC (50 μM) +/- PC (100 μM) exposed KMBC cells in the upper chamber and LX-2 in the lower chamber of co-culture plates. Collagen Ia1 mRNA expression

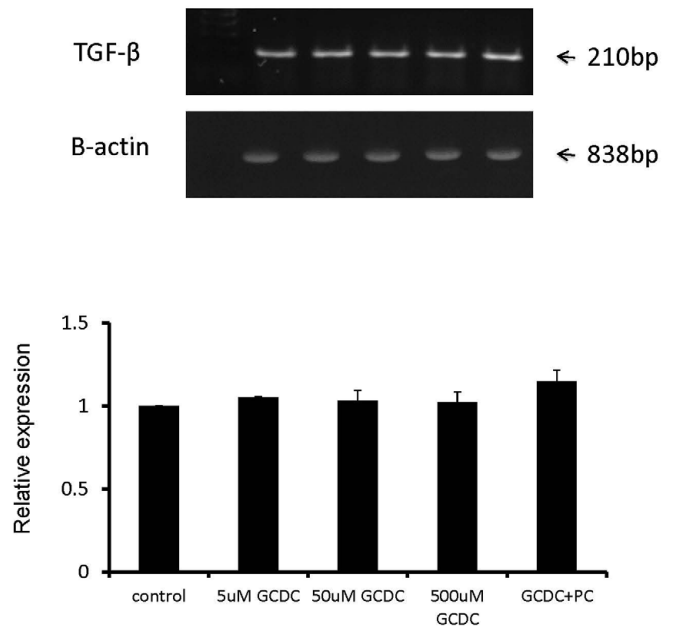


Figure 1. No induction or reduction of TGF-β mRNA expression after GCDC +/- PC treatment in KMBC cells. KMBC cells were treated by 5uM, 50uM, 500uM of GCDC alone or 50uM GCDC with 100uM PC. The mRNA abundances of TGF-β in KMBC cells were examined and remained unaltered in the presence of GCDC and GCDC + PC.

Table 1. Primers and conditions of polymerase chain reaction.

Genes		Primers	Tm (°C)	Size (bp)
TGF-β1	sense	5'-TACCTGAACCCGTGTGCTCT-3'	59.5	210
	anti-sense	5'-TTTCCCTCCACGGCTCAACC-3'		
Collagen	sense	5'-CTCCTGGCAAAGATGGACTCA-3'	60.8	544
	anti-sense	5'-ATGCTCTCGCCGAACCAGA-3'		
B-actin	sense	5'-GCACCACCTTCTACAATG-3'	60	838
	anti-sense	5'-TGCTTGCTGATCCACATCTGS-3'		

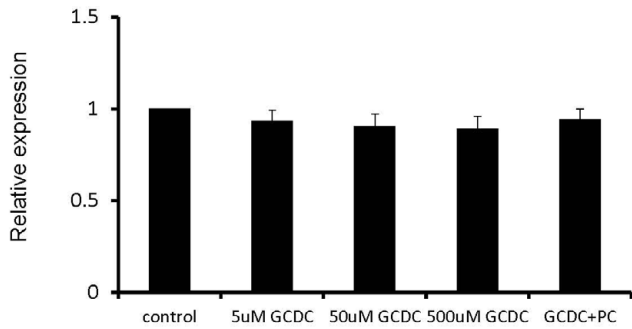
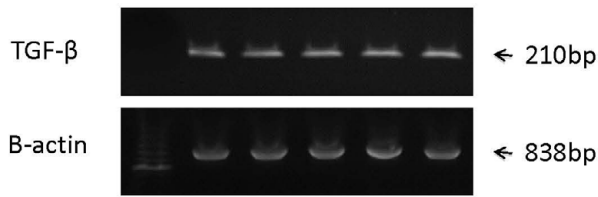
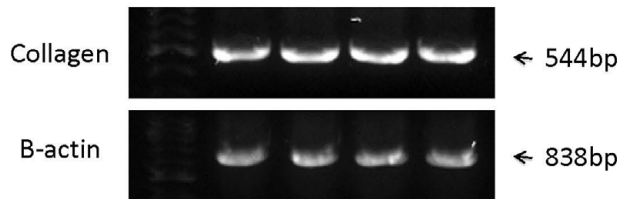


Figure 2. There was no alteration of TGF-β mRNA expression after GCDC +/- PC treatment in LX-2 cells. LX-2 cells were treated by 5uM, 50uM, 500uM of GCDC alone or 50uM GCDC with 100uM PC. The mRNA abundances of TGF-β in LX-2 cells were examined and remained unaltered in the presence of GCDC and GCDC + PC.



50uM GCDC	-	+	-	+
100uM PC	-	-	+	+

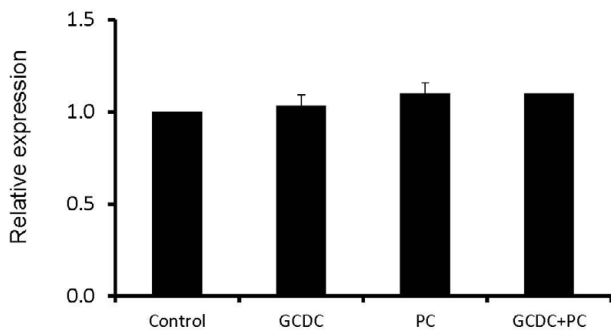


Figure 3. No induction or reduction of collagen mRNA expression in LX-2 cells in the lower chamber after GCDC +/- PC treatment of KMBC cells in the upper chamber. KMBC cells were treated by 50uM of GCDC, 100uM of PC and 50uM GCDC with 100 uM PC. The mRNA abundances of collagen in LX-2 cells were examined and there were no significant differences between GCDC, PC and GCDC+PC treatments.

by LX-2 cells was then determined by RT-PCR. As shown in Figure 3, collagen mRNA expression remained unaltered in these experiments.

Finally, to determine whether macrophage activation (resulting from epithelial cell damage following exposure to toxic bile acids) is required to stimulate myofibroblast activity, KMBC cells exposed to

GCDC (5-500 μM) +/- PC (100 μM) were co-cultured with PBMCs in the upper chamber and LX-2 cells in the lower chamber. Once again, collagen Ia1 mRNA expression in LX-2 cells remained unaltered in these experiments (Figure 4).

Discussion

One of the principal features of PSC is the progressive fibrosis that eventually encases much of the extra- and intra-hepatic biliary tract and liver [1]. Although the mechanism responsible for the enhanced fibrosis remains to be elucidated, certain previously described findings may be relevant. These include low concentrations of PC (a phospholipid that protects epithelial cells from toxic bile acid-induced injury) within PSC bile; increased concentrations of toxic bile acids in cholestatic livers; high levels of TGF-β mRNA and protein expression in PSC liver and improved outcomes with TGF-β lowering agents in animal models of PSC [9-12]. However, the results of the present study are not supportive of the hypothesis that PC deficiency in PSC bile permits toxic bile acid-induced biliary tract epithelial injury which in turn, results in enhanced TGF-β expression (by bile duct epithelial cells and/or tissue macrophages) resulting in activation of adjacent myofibroblasts. The results also argue against toxic bile acids directly altering TGF-β mRNA expression and collagen synthesis by myofibroblasts. Finally, the incorporation of co-culture experiments served to exclude the possibility that cytokines other than TGF-β (and/or the presence of macrophages) are required for toxic bile acid-induced activation of myofibroblasts.

Notwithstanding the above findings, there are a number of limitations to the study that warrant emphasis. First, the cells employed included bile duct epithelial and myofibroblast cell lines rather than primary cells derived from human livers. Whether these cell lines are less sensitive to the toxic effects of bile acids and pro-fibrogenic cytokine stimulation than primary cells remains to be determined. Second, the concentration range of toxic bile acids employed was derived from previous reports documenting concentrations of GCDC in human blood [13]. Whether higher concentrations are present in human bile and in particular, the bile of PSC patients, is unclear. Third, the co-culture experiments physically separated bile duct epithelial

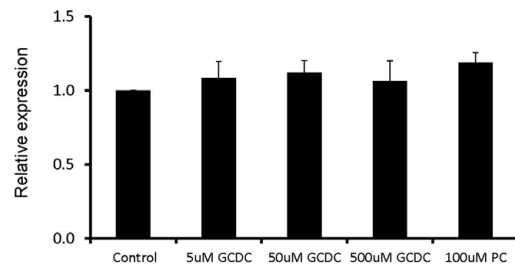
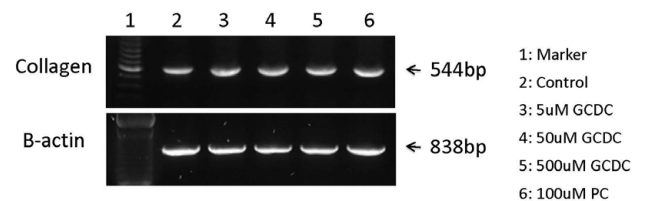


Figure 4. No induction or reduction of collagen mRNA expression in LX-2 cells in the lower chamber after GCDC +/- PC treatment in KMBC and PBMC cells in the upper chamber. KMBC and PBMC cells were treated by 50uM of GCDC, 100uM of PC and 50uM GCDC with 100uM PC. The mRNA abundances of collagen were examined and there were no significant differences between GCDC, PC and GCDC+PC treatments.

and peripheral blood mononuclear cells from myofibroblasts thereby preventing cell-cell contact and possible intercellular communication. Fourth, perhaps longer periods of cell exposure to toxic bile acids were required to induce bile duct epithelial cell injury. However, the rapid biochemical and histologic changes associated with acute bile duct ligation models argue against that possibility [14]. Fifth, it should be noted that PBMCs consist of peripheral blood monocytes and not tissue macrophages. Perhaps the additional features of the latter cell population are essential for the expression and release of pro-fibrogenic cytokines in this setting [15]. Finally, we did not explore the possibility that restoring bile PC concentrations to normal levels may favorably alter the course of PSC by means other than protecting biliary tract epithelial cells from toxic bile acid-induced injury.

In conclusion, the results of this study do not support the hypothesis that PC deficiency permits toxic bile acid-induced injury of biliary tract epithelial cells and subsequent activation of adjacent myofibroblasts, resulting in the enhanced fibrosis seen in PSC. Thus, at this time, restoration of low PC levels to normal values in PSC bile does not appear to be a worthwhile therapeutic approach to the treatment of PSC.

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