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Paclitaxel interrupts TGF- β 1 signaling between gallbladder epithelial cells and myofibroblasts

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Abstract

Background—The cellular and molecular mechanisms of fibrogenesis in the extrahepatic biliary epithelium are not known. TGF- β 1 is a cytokine implicated in signaling pathways that mediate collagen formation. An observation that paclitaxel (PT), applied topically into the rat common bile duct, inhibited stricture formation led us to hypothesize that PT's effects might be due to interruption of TGF- β 1 signaling between biliary epithelial cells and subepithelial myofibroblasts.

Materials and methods—We tested this hypothesis using an *in vitro* cell culture model in which murine gallbladder epithelial cells (GBEC) are cultured separately or co-cultured with human gallbladder myofibroblasts (GBMF).

Results—Exposure to *E. coli* lipopolysaccharide (LPS) enhanced TGF- β 1 mRNA expression, and stimulated TGF- β 1 protein secretion into both apical and basolateral compartments in GBEC. This effect was more prominent with basolateral secretion, and was also more pronounced in the co-culture system. In GBMF, collagen I mRNA expression and protein secretion were stimulated by treatment with LPS or TGF- β 1. GBMF also expressed TGF- β 1 mRNA, whose levels were enhanced by exposure to either LPS or exogenous TGF- β 1. PT inhibited LPS-induced TGF- β 1 mRNA expression and protein secretion in GBEC in both culture systems. TNF- α mRNA expression and protein secretion were not affected by PT in GBEC, demonstrating that the effects were specific for TGF- β 1. PT also inhibited LPS- and TGF- β 1-induced collagen I mRNA expression and protein secretion in GBMF.

Conclusions—These findings support a model in which GBEC communicate with subepithelial GBMF via TGF- β 1, leading to collagen deposition and fibrosis; and in which GBMF possess autocrine mechanisms involving TGF- β 1 that could regulate collagen production. PT inhibits these fibrogenic pathways.

Keywords

biliary epithelium; cholangitis; fibrosis; gallstones

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INTRODUCTION

The cellular and molecular mechanisms of fibrogenesis in the extrahepatic bile ducts and the gallbladder are not known. Recently, transforming growth factor-beta 1 (TGF- β 1)¹ was shown to be upregulated in the gallbladders of patients with cholelithiasis compared to gallbladders removed from patients without gallstones [1]. TGF- β 1 [2–5] is regarded as a key cytokine that induces collagen synthesis and secretion by myofibroblasts [6] and is a potent chemoattractant for monocytes and fibroblasts [3, 7, 8]. TGF- β 1 mRNA and protein were detected in the smooth muscle cells and fibroblasts of the gallbladder [1], suggesting a role for this cytokine in inducing and maintaining fibrogenesis in this organ.

Fibrosis in the extrahepatic bile ducts with stricture formation can also occur in proliferative cholangitis (PC), which is associated with chronic biliary tract infection by enteric bacteria [9]. This condition is characterized by hyperplasia of bile duct epithelium and inflammatory cell infiltration. The resulting bile stasis can lead to recurrent bacterial cholangitis, intrabiliary lithiasis, and secondary biliary cirrhosis [10, 11]. The histologic features of PC can be reproduced in an *in vivo* rat model, in which the pathological process is initiated by insertion of a thread into the bile duct [12]. In this model, topical application of a single dose of paclitaxel (PT) led to a significant decrease in epithelial cell proliferation and hyperplasia.

PT, derived from the bark of the Pacific yew, *Taxus brevifolia*, is a taxene that stabilizes the cellular microtubule network by inducing polymerization of tubulins [13]. As microtubules are involved in cell division, migration, maintenance of cell integrity and intracellular trafficking of organelles, PT is anti-proliferative and cytotoxic. This feature has been exploited clinically for anti-tumor therapy.

Several lines of evidence led us to hypothesize that PT's effects in the biliary system might involve TGF- β 1. PT's effects overlap with LPS-induced signaling pathways, including those involving TGF- β 1 [14]. PT induces cytokine expression in macrophages, an effect that is abrogated by TGF- β 1 [15]. The TGF beta family, acting via Smads, interacts with the microtubular network [16]. Finally, the fact that the effects in the rat common bile duct occurred after just one dose [12] made us wonder whether PT had any effects on fibrogenesis *per se*, apart from its effects on cell proliferation and cell death.

We therefore embarked on a study using an *in vitro* gallbladder epithelial cell (GBEC) culture system with the goal of determining whether a signaling pathway linking TGF- β 1 and collagen secretion existed, and which could be specifically inhibited by PT. We hypothesized that exposure of GBEC to bacterial lipopolysaccharide (LPS) occurring with chronic infection would stimulate the release of TGF- β 1, which in turn would induce proliferation and activation of subepithelial gallbladder myofibroblasts (GBMF), thereby leading to collagen production. We chose to use murine GBEC cultured separately or co-cultured with human GBMF because these are normal well-differentiated non-neoplastic cells with which we have had extensive experience in our laboratory and which have proven to be excellent models for the study of gallbladder cell physiology [17].

¹Abbreviations: GBMF (gallbladder myofibroblasts), IL (interleukin), LPS (lipopolysaccharide), GBEC (gallbladder epithelial cells), PC (proliferative cholangitis), PT (paclitaxel), SFM (serum free media); TGF- β 1 (transforming growth factor- β 1), TNF- α (tumor necrosis factor- α).

MATERIALS AND METHODS

Materials

PT, purchased from Sigma Chemical Co (St. Louis, Mo.), was dissolved in dimethylsulfoxide (DMSO) at a concentration of 10 mM and stored at -20°C . Culture media was added to PT stock to yield final concentrations ranging from 1 nM to 50 μM . Final DMSO concentration was less than 0.4 %. Tissue culture supplies were from Sigma, except where noted. Vitrogen, a bovine dermal collagen, was originally from Cohesion Technologies (Palo Alto, CA), and is currently available as Purecol (Inamed, Fremont, CA). Falcon culture plates (Becton Dickinson, Franklin, NJ) were used. Transwell inserts (24 mm diameter, 3 μm pore size) were from Corning (Acton, MA).

Cell culture

GBEC were isolated from wild-type C57BL/6 mice and cultured on Vitrogen-coated Transwell inserts as previously described [17]. GBMF were isolated from the serosal surface of a normal human gallbladder obtained at cholecystectomy using a protocol approved by the local Institutional Review Board, and cultured as previously described [17, 18]. Prior to each experiment, half the Transwell inserts with confluent GBEC were transferred to new plates without GBMF (separate culture), the other half transferred to plates with GBMF cells (co-culture). Each Transwell insert contained $\sim 3 \times 10^6$ GBEC when confluent. Each feeder layer well contained $\sim 1.5 \times 10^5$ GBMF when confluent. All experiments were performed when cells were fully confluent.

Measurement of cytokines in GBEC

We measured cytokines released by GBEC, grown on Transwell inserts, into the apical and basolateral media, as well as intracellular cytokine concentrations. For each culture system, growth media were exchanged for serum free media (SFM), incubated for 1 h with PT (1 nM-10 nM) in 0.1% DMSO or with vehicle alone (control), and then LPS (2 $\mu\text{g}/\text{mL}$) was added to the upper compartment. After 5–48 h exposure to LPS, media from the upper and lower compartments were harvested separately. The cells were harvested by scraping and evaluated for mRNA by RT-PCR (in cells) and protein by ELISA (in media and cells).

RT-PCR

Total RNA was extracted using RNeasy reagent (Qiagen, Valencia, CA) or RNeasy Mini Kit (Qiagen, Valencia, CA). 1 μg of RNA was reverse transcribed according to the manufacturer's protocol (SuperScriptTM First-Strand Synthesis System for RT-PCR, Gibco BRL) using oligo (dT) as primer. PCR amplifications were performed using a PCR kit (Gibco BRL) and Gene Amp Thermal Cycler System 9600 (Perkin-Elmer). Negative controls were performed by omitting the RT step or the cDNA template from PCR amplification. For semi-quantitative PCR, target sequences for type I collagen and TGF- β 1 were amplified between 25 and 35 cycles in order to yield visible products within the linear amplification range. Table 1 shows primer sequences and expected product sizes. Cycle conditions for amplification were 94°C for 15 s and 60°C for 30s, followed by 68°C for 120 s. PCR products were analyzed in 1.2 % agarose gels, and the resulting ethidium bromide-stained bands were compared to standards of known size run on the same gel and each band quantified by image analysis (Gel Expert, Nucleo Tech). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or β -actin was used as internal controls.

ELISA assays for TNF- α , IL-1 β , IL-6 and TGF- β 1

At the end of each incubation, 200 μL each of media from the upper and lower compartments of the Transwells were removed and immediately frozen at -70°C . The cells were lysed

with 1% NP-40 buffer (in 150 mM NaCl, 50 mM Tris, pH 8.0) containing 100 µg/mL phenylmethylsulfonyl fluoride and 1 µg/mL aprotinin, and the lysates frozen at -70°C . TNF- α , IL-1 β , and IL-6 were measured in thawed cell lysates and undiluted media, using mouse immunoassay kits (R&D Systems, Minneapolis, MN).

TGF- β 1 protein levels were measured using the human TGF- β 1 Immunoassay Kit (R&D Systems). 400 µl of harvested media were mixed with 0.2 mL of 1N HCl, incubated at 25°C for 10 min, and neutralized by 1.2 N NaOH and 0.5 mol/L HEPES. The limit of detection is 5 pg/mL with a linear response over the range of 30–2,000 pg/mL.

Measurement of TGF- β 1 and type I collagen in GBMF

The media overlying confluent GBMF were exchanged for fresh media, and PT (200 nM), or the 0.1% DMSO vehicle was added. After 1 h, LPS (2 µg/mL) or TGF- β 1 (5 ng/mL; R&D Systems) was added and cultures were incubated for 48 h, either in the presence (co-culture), or absence (separate culture) of an overlying Transwell insert containing GBEC. GBMF cells and media were then harvested for measurement of type I collagen protein by collagen specific electrophoresis (cf. below). Total RNA was extracted from the cells for RT-PCR.

Collagen-specific electrophoresis

Collagen was extracted from previously frozen and lysed GBMF and spent media as previously described [19]. In brief, cell lysates were centrifuged at 3500 rpm and the pellet was digested overnight at 4°C in 1.0 mL 3 % acetic acid containing 0.5 mg pepsin. Digestion was arrested by addition of pepstatin (1:30 ratio of pepstatin to pepsin), the sample centrifuged, and collagen precipitated from the supernatant by addition of NaCl to a concentration of 1.0 M. After stirring overnight at 4°C , the collagen was sedimented at 14,000 rpm, and the pellet retained. Spent media was concentrated 20-fold with a Centricon microconcentrator (Amicon, Beverly, MA) and collagen extracted as described above. Protein content was measured by a modified Lowry method (Sigma). Collagen pellets were resuspended in 2X SDS loading buffer and resolved by PAGE using a 6% separating gel. Standards of 10 µg of human type I collagen (Southern Biotechnology Assoc., Birmingham, AL) or rat-tail type I collagen (Collaborative Biomedical Products, Bedford, MA) were loaded on each gel. Collagen bands stained with Coomassie blue were quantified by densitometry (Gel Expert, Nucleo Tech), and normalized to the optical density of the standards.

Assessment of the anti-proliferative effects of PT

GBEC: Cells were plated onto 24-well plates coated with 0.25 mL of a 1:1 mixture of Vitrogen and growth media, and cultured with 0.5 mL of conditioned media (which was harvested from GBMF plates after 48 h, passed through a 0.45-µm filter (Nalgene, Rochester, NY), and mixed with the same volume of fresh growth media prior to use. After reaching >50 % confluency, SFM containing DMSO (0.1–5 %) or PT (1 nM–50 µM) in 0.1% DMSO was added. **GBMF:** Cells were cultured onto 24-well plates, and fed with 0.5 mL growth media. After reaching >50 % confluency, fresh media containing PT (1 nM–50 µM) and DMSO (0.1 %), or the DMSO solution at 0.1 to 5 % final concentration were added. For both cell types, after 48 h incubation with DMSO $\pm$ PT, cell proliferation was assessed using the Cell Titer 96™ Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI). Each concentration of PT or DMSO was studied in triplicate wells in three separate experiments.

LDH release assay

The effects of PT and DMSO were also assessed by assay of LDH release into the media, a marker of cell membrane disruption and loss of cell viability. LDH release has been validated as a marker of cell viability in cultured GBEC [20]. GBEC and GBMF were cultured as described above, then treated with 1 or 10 nM PT (for GBEC) or 200 nM PT (for GBMF) for 48 h. LDH release was measured as described [21], with results calculated as units of LDH activity/min, expressed as percent LDH release of control cells.

Statistical analysis

For experiments where three or more groups were compared, statistical analysis was performed using one-way ANOVA, combining data from triplicate wells of three separate experiments. For experiments where two groups were compared, unpaired student's t-test was used, combining data from three separate experiments. Significance was set at $P < 0.05$. Statistical analyses were performed using GraphPad Prism v.4.0 (San Diego, CA).

RESULTS

Effects of LPS on expression of TGF- β 1 mRNA expression and protein secretion by GBEC

We first measured the effects of LPS on TGF- β 1 mRNA levels in GBEC cultured separately. LPS increased expression of TGF- β 1 mRNA in GBEC to approximately 2.5 times control levels (Figure 1, lane 1 vs. 2). Various concentrations of LPS were tested, with the response plateauing by 2 μ g/ml. All subsequent experiments were performed using this concentration. We then measured TGF- β 1 protein secretion by GBEC cultured separately or co-cultured with GBMF. At baseline, GBEC and media contained no measurable TGF- β 1 after 24 h of culture. Compared with control (untreated) cells, separately cultured or co-cultured GBEC treated for 5–24 h with LPS caused no significant release of TGF- β 1 into the apical or basolateral media (data not shown). Significant amounts of TGF- β 1 were measured, however, after 48 h treatment with LPS (Table 2). In both types of cultures, LPS stimulated release of TGF- β 1 into basolateral media that was ~3–6 times greater than control values; stimulation of release into apical media was lower (up to 2.6 times control values). TGF- β 1 concentrations in the basolateral media of control and LPS-stimulated cells were strikingly higher in the co-culture system, as compared with separately cultured GBEC. This difference was not seen in the apical media. Thus, in GBEC, LPS stimulated the release of TGF- β 1 predominantly into the basolateral media; this effect was more striking with co-cultured compared with separately cultured cells. In addition, co-culture with GBMF induced TGF- β 1 secretion by unstimulated GBEC. GBEC did not secrete IL-1 β or IL-6 despite exposure to LPS for up to 48 h (data not shown).

Effects of LPS or TGF- β 1 on expression of type I collagen mRNA expression and protein secretion by GBMF

Subepithelial myofibroblasts would be the most likely cell type to mediate collagen secretion. In the gallbladder, cells in the subepithelium express TGF- β 1 [1]. We therefore examined the response of GBMF to TGF- β 1 or LPS. GBMF exhibited constitutive expression of type I collagen mRNA; this was stimulated almost three-fold by treatment with exogenous TGF- β 1 or LPS (Fig. 2; lane 1 vs. 2 or 3). We tested a range of doses of TGF- β 1, and found that the response plateaued at 5 ng/ml; this dose was therefore used in all subsequent experiments. We also looked for evidence of type I collagen secretion. Type I collagen levels in cells and media of cultured GBMF were measured by protein electrophoresis (Figs. 3A, cells; 3B, media) and quantified by densitometry. The concentration of type I collagen in untreated cells was 7.8 μ g/mL (Fig. 3A; lane 1); this was stimulated 63% by TGF- β 1 (lane 2) and 68% by LPS (lane 3). Media showed similar results

(Fig. 3B): Type I collagen in control media was 10.4 $\mu\text{g/mL}$ (lane 1), and was stimulated 36% by TGF- β 1 (lane 2) and 63% by LPS (lane 3).

Effects of LPS or TGF- β 1 on expression of TGF- β 1 mRNA by GBMF

The responsiveness of GBMF to TGF- β 1 suggested that signaling pathways between GBEC and GBMF involving TGF- β 1 were present. In addition, as TGF- β 1 is expressed in the subepithelial layer of the gallbladder [1], GBMF could also be regulated by TGF- β 1 via an autocrine mechanism. In support of this concept, TGF- β 1 mRNA was expressed in GBMF; expression increased 2.5-fold above control values following treatment with either exogenous TGF- β 1 or LPS (Fig. 4; lane 1 vs. 2 or 3).

Effects of PT on GBEC and GBMF cell proliferation

We also investigated the effects of PT on the parameters described above. We wished to determine whether PT's effects would occur at doses that did not cause cytotoxicity. First, we determined the dose response of the two cell types to the anti-proliferative effects of PT. Exposure to PT for 48 h caused dose-dependent inhibition of cell proliferation of GBEC and GBMF (Fig. 5). The greater sensitivity of GBEC to PT as compared to GBMF (or other cell lines [22–24]) was reflected by their IC₅₀ doses of 0.01 μM and 2 μM , respectively. The PT vehicle DMSO at concentrations 0.4 % had no anti-proliferative effects; therefore, the final DMSO concentration was 0.4 % in all studies.

Effects of PT on LDH release in GBEC and GBMF

Next, we assessed whether the doses at which cell proliferation was inhibited correlated with cytotoxicity by measuring LDH release into the media. No significant LDH release was detected in GBEC following exposure to 1 nM and 10 nM PT after 48 h of incubation ($n=3$; $P>0.05$ for control compared to the two treatment groups). Furthermore, we found no significant LDH release from GBMF following exposure to 200 nM PT after 48 h of incubation ($n=3$; $P>0.05$). Assessment of cell morphology by light microscopy showed no detectable changes between cells treated with PT compared to cells treated with the vehicle, or with untreated cells, in both cell types. The PT doses, incubation times, and culture conditions were identical to those used in the experiments described below.

Effects of PT on LPS-mediated TGF- β 1 mRNA expression in GBEC

LPS-mediated TGF- β 1 mRNA expression was inhibited approximately 20% by pre-treatment with either 1 nM or 10 nM PT (Fig 1, lanes 5 and 6); conversely, PT alone at these two doses (Fig 1, lanes 3 and 4) had no significant effect compared with control.

Effects of PT on LPS-mediated TGF- β 1 protein secretion in GBEC

In both culture systems, pretreatment for 1 h with 1 nM PT alone caused an approximate doubling of TGF- β 1 concentrations in the basolateral media, but no change in the apical media. However, pretreatment with the same dose of PT inhibited by about half the LPS-induced stimulation of TGF- β 1 secretion into the basolateral media in both culture systems (Table 2). While PT alone induced TGF- β 1 release, this effect appeared to be due to a specific effect, as preferential secretion into the basolateral compartment was preserved.

Effects of PT on LPS- or TGF- β 1- mediated type I collagen mRNA expression and protein secretion by GBMF

For these studies, we used a higher concentration of PT, given the higher doses needed to show anti-proliferative effects of PT on this cell type (Fig. 5). We chose 200 nM as at this concentration PT alone had no significant effect on type I collagen mRNA expression. Pretreatment with PT inhibited TGF- β 1- or LPS-stimulated type I collagen mRNA

expression by 28% and 23%, respectively (Fig 2; lane 2 vs. 5; lane 3 vs. 6). Similarly, stimulation by TGF- β 1 or LPS of type I collagen levels in GBMF were inhibited almost completely by pretreatment with PT (Fig 3A; lane 2 vs. 5; lane 3 vs. 6). Collagen release into the media that was stimulated by TGF- β 1 or LPS was inhibited almost completely by pretreatment with PT (Fig. 3B; lane 2 vs. 5; lane 3 vs. 6).

Effects of PT on LPS- or TGF- β 1-mediated TGF- β 1 mRNA expression by GBMF

Pretreatment with PT suppressed TGF- β 1-stimulated expression by about one-fifth (Fig. 4; lanes 2 vs. 4) and LPS-stimulated TGF- β 1 mRNA expression by about one-third (Fig 4; lanes 3 vs. 5). PT treatment alone had no effect on TGF- β 1 mRNA levels (data not shown).

Effects of LPS $\pm$ PT on TNF- α secretion by GBEC

In order to provide additional evidence that cytotoxicity was not the explanation for PT's effects, we investigated the effects of this compound on the mRNA expression and protein secretion of another cytokine. TNF- α is a key pro-inflammatory cytokine that is produced by murine GBEC [25]. We first investigated TNF- α secretion by GBEC cultured separately or co-cultured with GBMF. GBEC secreted less than 30 pg/mL of TNF- α into the apical media over 24 h, whether cultured separately or co-cultured with GBMF; this was unaffected by PT treatment. Similar to previously studies [25], LPS (2 μ g/mL) induced time-dependent release of TNF- α by GBEC cultured separately. After 24 h of LPS exposure, GBEC co-cultured with GBMF secreted more TNF- α into the apical (270 ± 1.8 pg/mL) and basolateral media (240 ± 2.0 pg/mL) compared to untreated cells (29.9 ± 1.5 pg/mL and < 5 pg/mL in the apical and basolateral media, respectively) ($P < 0.05$ vs. control). The apical secretion of TNF- α by GBEC cultured separately was similar to co-cultured cells, but basolateral release was about 30% lower in co-cultured GBEC ($P < 0.05$). Pretreatment with PT (1 nM) had no effect on LPS-stimulated TNF- α release into either compartment in either culture system. We conclude that PT at a dose that exhibits no significant LDH release has negligible effects on TNF- α secretion in cultured GBEC.

DISCUSSION

Our results in an *in vitro* model system composed of normal, well differentiated murine GBEC, cultured separately or co-cultured with human GBMF, provide insights into the cellular mechanisms involved in fibrogenesis in the extrahepatic biliary epithelium. The *in vitro* model used in our experiments eliminates the presence of other cells types (such as inflammatory cells), which would contribute to the cytokine profile and complicate the results. By these means, we could focus on the roles of two key cell types, GBEC and GBMF, in the fibrogenic process. Our results support a model in which TGF- β 1 serves as a key pro-fibrogenic cytokine that is secreted by both cell types in a manner suggesting that paracrine and autocrine modes of stimulation of collagen I synthesis and secretion by GBMF are operative. Furthermore, the observation that PT interrupts the effects on TGF- β 1 and collagen I secretion opens the way for future investigations into the potential therapeutic uses of topically-delivered formulations of this compound for the prevention of fibrosis in the extrahepatic biliary system.

GBEC produce cytokines, including IL-6, IL-8, TNF- α , and MCP-1, and express IL-6 and TNF- α receptors at the cell surface [25, 26], particularly in the presence of pro-inflammatory mediators such as bacterial LPS. We have extended these observations by showing that TGF- β 1 synthesis and polarized secretion by GBEC are enhanced by LPS. In both culture systems, the increased levels of TGF- β 1 protein in response to LPS were markedly inhibited by pretreatment of GBEC with PT. However the inhibition by PT of the

LPS-induced increase in TGF- β 1 was significantly less for mRNA than protein, suggesting a major role for post-transcriptional regulation of TGF- β 1 in the inhibitory effect of PT.

We also studied changes in synthesis and release of TGF- β 1 and type I collagen by GBMF in response to LPS and TGF- β 1. This assessed whether GBMF could respond to LPS that might have leaked through damaged epithelium in the setting of inflammation, and/or to the TGF- β 1 released basolaterally by GBEC stimulated by bacterial products. The altered responses of GBEC, when cultured alone vs. co-cultured with GBMF, indicate that subepithelial GBMF can also modulate the responses of GBEC via the enhanced release of TGF- β 1. Based on these findings, we postulate that LPS, or TGF- β 1 released by GBEC, mediate release of TGF- β 1 from GBMF, which can then further enhance release of TGF- β 1 from GBEC resulting in a positive feedback loop. Raised TGF- β 1 levels would trigger collagen production by GBMF leading to the accumulation of extracellular matrix and subsequent fibrosis.

The effects of PT support a model in which the anti-fibrogenic effects of this agent occur at multiple levels, being due to inhibition of the release of TGF- β 1 by GBEC, inhibition of TGF- β 1 release by GBMF, and possibly direct inhibition of collagen I synthesis by GBMF. The net effect would be decreased collagen synthesis by myofibroblasts in the subepithelial layers of the gallbladder wall. Such a multi-faceted effect of PT is consistent with the wide variety of effects reported in other cell types. PT is an anticancer agent [13] which, by stabilizing polymerized microtubules and enhancing microtubular assembly, arrests the cell cycle in the G₀/G₁ and G₂/M phases, leading to cell death [27, 28]. PT also affects immune responses, expression of cytokines, and multiple other cellular functions [13, 14, 27–30]. In addition, PT suppresses the migration and proliferation of tumor cell lines [23], epithelial cells [22], fibroblasts [24], and vascular smooth muscle cells [31]. The lipophilic PT molecule is rapidly taken up by cells [32], and has a long duration of action, even after a single dose applied topically [29, 33]. Its effectiveness after local treatment has been demonstrated by inhibition of proliferation and migration of vascular smooth muscle cell *in vitro* and *in vivo* [31, 34], and suppression of epithelial proliferation in a rat model of PC [12]. The inhibitory effects of PT on fibrogenesis in the biliary system raise the prospect that stents eluting this compound might be an effective means to prevent recurrent biliary strictures, a concept that has recently been demonstrated in a porcine model [35]. A similar application has proven useful for the prevention of coronary restenosis [36].

TNF- α release was not affected by the presence or absence of co-cultured GBMF, whereas TGF- β 1 secretion was greatly influenced by the presence of GBMF. A lack of inhibitory effect of PT on TNF α release was also noted, suggesting that the effect of PT on TGF- β 1 was specific, as opposed to a non-specific effect due to cytotoxicity. After 5 to 12 h of LPS treatment of GBEC, there was no definite increase in TGF- β 1 protein with or without co-culture with GBMF. After 48 h of co-culture, however, LPS prominently increased the release of TGF- β 1 protein into the basolateral media. Since GBEC released TNF- α soon after being treated with LPS (5–24 h), it is possible that the basolaterally secreted TNF- α might have stimulated the release of TGF- β 1 from the underlying GBMF that was observed after 48 h. This is compatible with reports on pulmonary inflammation and intestinal fibrogenesis, which show that TNF- α contributes to the induction of TGF- β 1 in myofibroblasts [37].

We took care to delineate whether the effects of PT were due to cytotoxicity or to more specific cellular effects. To this end, we used a cell proliferation assay to determine the dose response to PT for the two cell types. GBEC proliferation was more sensitive to PT, with an IC₅₀ of ~10 nM. When we treated GBEC with PT at this concentration and at a dose that was 1/10th of this, there was little or no difference in the outcomes measured and there was

no evidence of cytotoxicity as assessed by LDH release. Furthermore, outright cell death would be expected to lead to non-specific and generalized effects on cytokine and collagen production. On the contrary, we observed differential effects, such as between TNF- α and TGF- β 1, and between apical and basolateral release of TGF- β 1. Similarly, the 200 nM concentration of PT used for the experiments with GBMF did not lead to significant LDH release. Certainly, PT has a myriad of cellular effects that can be considered “toxic”, but our results point to more subtle and nuanced effects on cellular functions. In this respect, our results are in accord with the effects of low dose PT on other cell types such as macrophages and vascular smooth muscle cells [14, 15, 34].

Our findings support the hypothesis that TGF- β 1 is secreted by GBEC and myofibroblasts. The accumulated TGF- β 1 in subepithelial tissue likely causes subepithelial fibroblasts to change into a myofibroblast phenotype. Activated myofibroblasts produce both TGF- β 1 mRNA/protein and type I collagen mRNA/protein. TGF- β 1 could further enhance secretion of epithelial TGF- β 1 by a positive feedback loop. Therefore GBEC may communicate with myofibroblasts via TGF- β 1, and the persistent activation of myofibroblasts by this cytokine may cause excessive collagen deposition leading to fibrosis. While our data do not prove this, the results are nevertheless consistent with such a model. The effects of PT on these two cell types therefore has potential clinical relevance for the treatment of biliary diseases in which fibrosis plays a role, such as cholecystolithiasis, PC and primary sclerosing cholangitis [38].

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Figure 1. TGF-β1 mRNA expression by GBEC

Lane 1: Control; Lane 2: LPS 2 μg/mL; Lane 3: PT 1 nM; Lane 4: PT 10 nM; Lane 5: LPS 2 μg/mL + PT 1 nM; Lane 6: LPS 2 μg/mL + PT 10 nM. The top portion of the figure shows the results of a typical experiment; the bottom portion shows densitometry values normalized to the signal from the housekeeping gene GAPDH from n = 3 experiments.

*P<0.001; **P=0.002; #P=0.01.

Type I Collagen→

β-actin→

Figure 2. Effects LPS or TGF-β1 and /or PT on type I collagen mRNA expression in GBMF

Lane 1: Control; Lane 2: TGF-β1 5 ng/mL; Lane 3: LPS 2 μg/mL; Lane 4: PT 200 nM; Lane 5: TGF-β1 5 ng/mL + PT 200 nM; Lane 6: LPS 2 μg/mL + PT 200 nM. The top portion of the figure shows the results of a typical experiment; the bottom portion shows densitometry values normalized to the signal from the housekeeping gene β-actin from n = 4 experiments.

*P=0.003; **P=0.01; #P=0.0002.

Figure 3. Effects of PT on type I collagen synthesis in GBMF

Cells were treated with the indicated compounds and cells (A) and media (B) were assayed for type I collagen by collagen specific electrophoresis. Lane 1: Control; Lane 2: TGF-β1 5 ng/mL; Lane 3: LPS 2 μg/mL; Lane 4: PT 200 nM; Lane 5: PT 200 nM + TGF-β1 5 ng/mL; Lane 6: PT 200 nM + LPS 2 μg/mL. The top portions of the figures show the results of typical experiments; the bottom portions show the densitometry values from n=3 experiments. Collagen I standards, run in parallel lanes, displayed a distinct band at 150 kDa (data not shown). *P=0.006; **P=0.02; #P=0.002.

TGF- β 1→

β -actin→

Figure 4. Effects of treatment with LPS or TGF- β 1 and /or PT on TGF- β 1 mRNA expression in GBMF

Lane 1: Control; Lane 2: TGF- β 1 5 ng/mL; Lane 3: LPS 2 μ g/mL; Lane 4: PT + TGF- β 1 5 ng/mL; Lane 5: PT + LPS 2 μ g/mL. The top portion of the figure shows the results of a typical experiment; the bottom portion shows densitometry values normalized to the signal from the housekeeping gene β -actin from n=3 experiments. *P<0.0001; #P=0.002.

Figure 5. Anti-proliferative effects of PT in GBEC and GBMF

Cell proliferation measured after 48 h exposure to a single-dose of PT (1 nM-50 μ M). Each data point represents the mean $\pm$ SE of three different experiments using triplicate wells.

Table 1

Primer sequences used for PCR amplification

Primers (mRNA)		Sequences	Size (bp)	Cycles
m TGF- β 1	sense	5'- GCCTCCGCATCCCACCTT-3'	1031	25
	antisense	5'- CAGAAGTTGGCATGGTAGCC-3'		
h TGF- β 1	sense	5'-CTATCGACATGGAGCTGGTG-3'	810	25
	antisense	5'-TGCGGAAGTCAATGTACAGC-3'		
h type I collagen	sense	5'-ACGTCCTGGTGAAGTTGGTC-3'	984	25
	antisense	5'-ATGTTCTCGATCTGCTGGCT-3'		
m GAPDH	sense	5'-ATGTCAGATCCACAACGGATACAT-3'	270	25
	antisense	5'-ACTCCCTCAAGATTGTCAGCAAT-3'		
h GAPDH	sense	5'-TCACATATTCTGGAGGAGCC-3'	177	25
	antisense	5'-GGCTCACCATGTAGCACTCA-3'		
h β -Actin	sense	5'-CCATGTACGTTGCTATCCTGGC-3'	289	25
	antisense	5'-ATCTCTTGCTCGAAGTCCAGGG-3'		

m: mouse, h: human

Table 2TGF- β 1 secretion by GBEC

Treatment	Separate Culture		Co-Culture with GBMF	
	Apical media (pg)	Basolateral media (pg)	Apical media (pg)	Basolateral media (pg)
Control	32 $\pm$ 5	28 $\pm$ 6	60 $\pm$ 6	184 $\pm$ 9 ^{b,c}
LPS (2 μ g/mL)	85 $\pm$ 9 ^a	159 $\pm$ 15 ^{a,b}	103 $\pm$ 13 ^a	501 $\pm$ 44 ^{a,b,c}
PT (1 nM)	51 $\pm$ 4	58 $\pm$ 9	44 $\pm$ 3	369 $\pm$ 31 ^{a,b,c}
PT (1 nM) + LPS (2 μ g/mL)	60 $\pm$ 7	97 $\pm$ 10	60 $\pm$ 8	212 $\pm$ 16 ^{a,b,c}

TGF- β 1 was measured by ELISA and calculated as the total pg in the apical and basolateral media. Values are means $\pm$ SE from three assays, with each treatment performed in duplicate. Statistical differences (*P<0.05) between groups were assessed by one-way ANOVA. In all treatment groups, basolateral secretion of TGF- β 1 was significantly greater than apical secretion (P<0.05). Under each condition, basolateral secretion of TGF- β 1 was greater in the co-culture system than in the separate culture system (P<0.05). Data are from 48 h incubations.

^aP<0.05; LPS vs. control; PT vs. control, PT + LPS vs. LPS in each column.

^bP<0.05; basolateral media vs. apical media for each type of culture in each row

^cP<0.05; co-culture vs. separate culture for each media in each row.