Autocrine release of TGF-β by portal fibroblasts regulates cell growth

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Abstract Portal fibroblasts (PF) are a newly isolated population of fibrogenic cells in the liver postulated to play a significant role in early biliary fibrosis. Because transforming growth factor-β (TGF)-β is a key growth factor in fibrosis, we characterized the response of PF to TGF-β. We demonstrate that PF produce significant amounts of TGF-β2 and, unlike activated hepatic stellate cells (HSC), express all three TGF-β receptors and are growth inhibited by TGF-β1 and TGF-β2. Fibroblast growth factor (FGF)-2, but not platelet derived growth factor (PDGF), causes PF proliferation. These data suggest a mechanism whereby HSC eclipse PF as the dominant myofibroblast population in biliary fibrosis.
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1. Introduction

Portal fibroblasts (PF) are a newly identified and isolated population of fibrogenic cells found in the portal region of the liver [1–8]. Their periportal localization as well as rapid proliferation and acquisition of α-smooth muscle actin expression immediately after bile duct ligation suggest that they are distinct from hepatic stellate cells (HSC). They express procollagen-1 mRNA and are postulated to be the early mediators of hepatic fibrosis after biliary injury, with HSC comprising a later cellular response [6–9].

Transforming growth factor-β (TGF-β) is one of the most important mediators of fibrosis in the liver. Multiple studies have demonstrated that TGF-β is highly expressed in fibrotic regions, that overexpression of TGF-β in animal models results in fibrosis, and that TGF-β antagonists can prevent experimentally induced fibrosis [10–14]. Although TGF-β1 is the major TGF-β isoform in the liver, TGF-β2 is of special interest because one report suggests that it is the primary TGF-β isoform expressed by proliferating bile duct epithelia in the fibrotic liver [15]. This raises the possibility that TGF-β produced by biliary epithelial cells, in particular TGF-β2, might play an important role in biliary fibrosis and might have important effects on the adjacent population of PF.

We sought to characterize the TGF-β response of PF in culture, looking in particular at growth inhibition and production of extracellular matrix material in response to both TGF-β1 and TGF-β2. We also examined similarities and differences between PF and in vitro activated and passaged HSC, which have a well-characterized response to TGF-β [16,17].

2. Materials and methods

2.1. Materials

Cell culture media, fetal calf serum (FCS), and antibiotics were obtained from Gibco BRL (Carlsbad, CA, USA). Chemicals and enzymes for cell isolation were obtained from Sigma (St. Louis, MO, USA), with the exception of pronase, which was from Calbiochem (San Diego, CA, USA). 100 mm pore mesh was obtained from Selfar America, Inc. (Kansas City, MO, USA). Growth factors were obtained from R&D Systems (Minneapolis, MN, USA). TGF-β receptor kinase inhibitor NPC-34016 was a generous gift from David Liu (Scios, Inc., Sunnyvale, CA, USA). Radiochemicals were obtained from New England Nuclear (Boston, MA, USA). Antibodies against TGF-β receptors were obtained from Santa Cruz (V22 and L21 for the type I TGF-β receptor (TβRI) and the type II TGF-β receptor (TβRII), respectively; Santa Cruz, CA, USA) and R&D Systems, Inc. (betaglycan); the antibody against fibronectin was from Invitrogen (Carlsbad, CA, USA).

2.2. Cell isolation and culture

Primary rat PF were isolated as described [1]. Briefly, rat non-parenchymal cell (NPC) fractions were obtained by collagenase and pronase digestion of rat livers followed by serial mesh filtration to mechanically disrupt cells. NPC were plated in Dulbecco's modified Eagle's medium (DMEM)/F-12 medium containing 2% penicillin–streptomycin, 3% FCS, 0.3% gentamicin, and 0.1% fungizone. Cells were observed at 96 h, at which point all cells remaining were PF. For all experiments reported here, cells were used at passages 2–4.

HSC were isolated as described [17], grown on tissue culture plastic, and used at passages 24.

2.3. Receptor affinity labeling and immunoprecipitation

PF and HSC in culture were labeled with 250 pM [^{125}I]TGF-β1, as described [18]. Cells were lysed and equal fractions were immunoprecipitated with antibodies against the type I receptor, the type II receptor, or betaglycan. Immunoprecipitants were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and visualized by autoradiography.

2.4. Matrix assays

Fibronectin secretion was measured as described [17]. For measurement of plasminogen activator inhibitor (PAI)-1 deposition, cells were treated with 100 pM TGF-β1 or -β2 or with 0.5 μM TGF-β receptor kinase inhibitor NPC-34016 for 2 h in DMEM lacking cysteine, methionine, and glutamine (ICN, Irvine, CA, USA). ^{35}S-Express labeling mix was added to 33 μCi/ml, and cells were labeled for 3 h. Plates were rinsed with phosphate-buffered saline (PBS) one time, with 10

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Abbreviations: TGF-β, transforming growth factor-β; PF, portal fibroblasts; HSC, hepatic stellate cells; FGF, fibroblast growth factor; PDGF, platelet derived growth factor; TβRI, type I TGF-β receptor; TβRII, type II TGF-β receptor

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mM Tris pH 8.0/0.5% deoxycholic acid/1 mM phenylmethylsulfamethoxazole three times, then with 2 mM Tris pH 8.0 two times. After a final rinse with PBS, the remaining matrix was scraped into reducing SDS sample buffer, then boiled and separated by SDS–PAGE. PAI-1 appears as a characteristic 47 kDa band. Autoradiographs were scanned and quantitated with ImageJ software (NIH).

2.5. [^{3}H]thymidine incorporation

[^{3}H]thymidine uptake was measured as described [17]. In short, cells were placed in DMEM/0.3% fetal bovine serum with growth factors for 4 h. [^{3}H]thymidine was added to 2 μCi/ml for an additional 22 h, at which point cells were rinsed, lysed in 2 M NaOH, and counted. TGF-β was used at 10 and 100 pM as noted, fibroblast growth factor (FGF)-2 at 1.2 nM, and platelet derived growth factor (PDGF)-BB at 0.1 μg/ml. Some cells were treated with 0.5 μM TβRI kinase inhibitor (solubilized in dimethyl sulfoxide (DMSO)) or with the equivalent volume of DMSO. Significance was determined by paired Student's *t*-test.

2.6. Quantitation of TGF-β release

Release of biologically active TGF-β1 and TGF-β2 was quantitated using commercially available immunoassays according to the manufacturer's instructions (TGF-β1 E_{max} and TGF-β2 E_{max}, Promega, Madison, WI, USA). TGF-β1 and TGF-β2 immunoassays were performed in parallel. PF were grown in six-well culture plates to near confluence. For a positive control, HepG2 human hepatoblastoma cells were grown under identical conditions. Cells were counted, then incubated overnight with TGF-β coat antibody at 4°C. Non-specific binding was inhibited with a blocking buffer for 35 min at 37°C. Cells were treated with secondary antibody for 2 h at room temperature followed by tertiary horseradish peroxidase (HRP)-conjugated antibody for 2 h at room temperature. After adding HRP reagent for 15 min at room temperature, color change was detected using a multi-well plate absorbance spectrophotometer (PowerWare 340, Bio-Tek Instruments, Winooski, VT, USA) at 450 nm. Results were calibrated to a standard curve of known either TGF-β1 or TGF-β2 concentrations done at eight dilutions (two data points per concentration) with correlations (r^2 values) >0.955 for each immunoassay. For the TGF-β1 immunoassay, there is less than or equal to 3% cross-reactivity with TGF-β2 or -β3, and for the TGF-β2 immunoassay there is less than or equal to 3% cross-reactivity with TGF-β1 or -β3.

3. Results

3.1. PF express all three TGF-β receptors

TGF-β signals through the sequential action of two serine-threonine kinase cell surface receptors, TβRI and TβRII. Signaling by the TGF-β isoform TGF-β2, however, is greatly enhanced in some cells by the presence of betaglycan, a proteoglycan accessory receptor that acts by enhancing the affinity of TβRI and TβRII for the ligand [19]. Although TGF-β1 is the major TGF-β isoform expressed in the liver, TGF-β2 is produced at high levels in the proliferating bile ducts seen in biliary fibrosis [15]. We therefore sought to determine whether PF, which are adjacent to the biliary epithelial cells, express betaglycan and can potentially respond to TGF-β2. Cells were affinity labeled with [^{125}I]TGF-β1 and cell lysates were immunoprecipitated with antibodies against all three TGF-β receptors. As seen in Fig. 1 (left panel), all three receptors are expressed on the surface of PF. This is in contrast to HSC, which express betaglycan in vitro only when quiescent or in an intermediate state, but not when activated (M.D.A. Gaça and R.G. Wells, unpublished results) and passaged (Fig. 1, right panel).

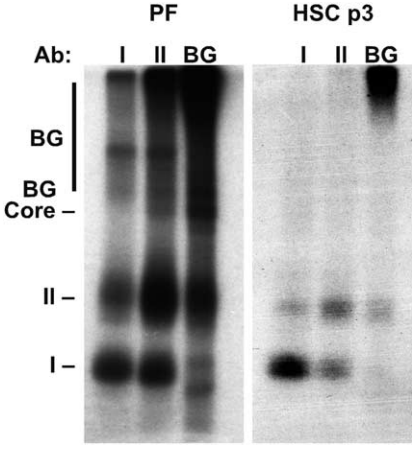

Fig. 1. PF express all three TGF-β receptors. Left: PF in culture were affinity labeled with [^{125}I]TGF-β1. Cells were lysed, and lysates immunoprecipitated with antibodies against the type I (I) and type II (II) receptors and betaglycan (BG). All three cell surface receptors are present on the surface of these cells. Right: In vitro activated HSC at the same passage number were similarly treated, but demonstrate labeling primarily of the type II receptor.

Table 1
TGF-β isoform production (pg/10^5 cells)

Isoform	PF	HepG2
TGF-β1	52.6 ± 4.1 (n = 24)	237.1 ± 26.2 (n = 16)
TGF-β2	3341 ± 1340 (n = 17)	328.2 ± 80.9 (n = 15)

3.2. PF produce minimal matrix in response to TGF-β

In order to determine whether PF respond to TGF-β, and in particular whether they produce matrix in response to TGF-β, we measured the expression of fibronectin and PAI-1 with and without TGF-β treatment. Cells show a modest (less than 2-fold) but significant increase in expression of both matrix-active components when treated with TGF-β1 or -β2 (Fig. 2), similar to the response we have observed with activated HSC in vitro (Fig. 2 and [17]). Additionally, cells were treated with a TβRI kinase inhibitor to determine whether baseline matrix production resulted from autocrine TGF-β production. Although there is a small but significant decrease in matrix production after inhibitor treatment, suggesting that autocrine TGF-β is responsible for some of the baseline matrix production, the lack of complete inhibition suggests that multiple factors are responsible for PF matrix production. As a control, Rat1 fibroblasts were treated with TGF-β1 and TGF-β2 in parallel, and demonstrated a 1.6–1.8-fold increase in fibronectin secretion and a 13–14-fold increase in PAI-1 deposition (data not shown). Interestingly, these cells showed minimal background production of either matrix component, unlike PF and activated HSC. Northern blotting for collagen α1(I) showed no change in mRNA levels with TGF-β or inhibitor treatment (data not shown).

3.3. PF are growth inhibited by TGF-β but are stimulated by FGF-2

The effect of TGF-β on growth of PF was determined by measuring incorporation of [^{3}H]thymidine. Both TGF-β1 and -β2 cause a reproducible inhibition in [^{3}H]thymidine incorporation, even when used at 10 pM (Fig. 3). Similar results were seen with TGF-β3 (data not shown). Interestingly, treatment with FGF-2 stimulates growth, although PDGF has no effect. Treatment of cells with the TβRI kinase inhibitor increased growth, suggesting that autocrine TGF-β production causes a tonic growth inhibition. PF behaved differently than HSC at

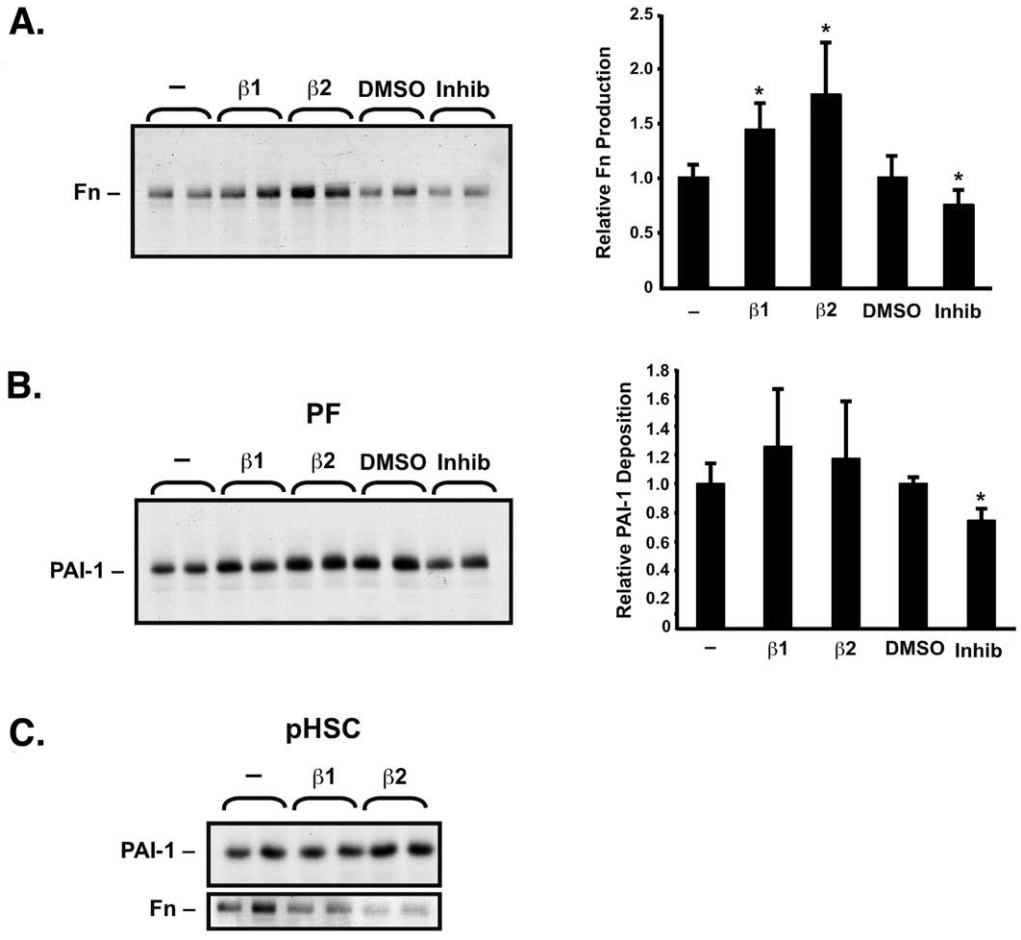

Fig. 2. TGF-β does not induce matrix production by PF. PF were treated with growth factors and a TβRI kinase inhibitor as indicated and assayed for deposition of fibronectin (A) and PAI-1 (B). Duplicates represent independent plates of cells. Autoradiographs of proteins were quantitated and the results shown graphically. Values are mean ± S.D. for two independent experiments with four to five replicates total. $*P < 0.05$ compared with (−) (for TGF-β1 and -β2 treatment), or compared with DMSO alone (for inhibitor treatment). C: In vitro activated HSC at the same passage number were treated similarly.

similar passage number; [^{3}H]thymidine incorporation by HSC was strongly enhanced by PDGF, but the other growth factors and the TβRI kinase inhibitor had no effect.

3.4. Autocrine production of TGF-β1 and TGF-β2 by PF

Production of biologically active TGF-β1 and TGF-β2 was measured using a commercially available immunoassay (Table 1). PF secreted significant amounts of TGF-β1 and, surprisingly, even larger amounts (64 times as much) TGF-β2. HepG2 human hepatoblastoma cells, used as a control, produced similar amounts of TGF-β1 and TGF-β2, as has been reported in the literature [20]; our HepG2 data are also quantitatively similar to previously reported values [20,21].

4. Discussion

PF are one of several potentially fibrogenic cell types identified in the liver. PF are of special interest because they are restricted to the portal region, particularly around intrahepatic bile ducts, and thus may be important in the biliary fibrosis that occurs with such diseases as primary biliary cirrhosis, primary sclerosing cholangitis, and cystic fibrosis [9].

There is now increasing evidence that there are several populations of myofibroblasts in the diseased liver in addition to those derived from HSC [2,4,5,8,22]. PF may be a population of cells, akin to HSC, that differentiate into myofibroblasts. It has been suggested that PF transdifferentiation occurs early in biliary fibrosis, and that HSC activate and migrate into the periportal area at a later point [8,9]. Although the stimuli for transdifferentiation of either PF or HSC into myofibroblasts are not well understood, they are clearly different. One group has demonstrated that PDGF stimulates α-smooth muscle actin expression in a PF-like fibroblast population isolated from bile duct preparations [5]. HSC do not express the PDGF receptor β-subunit until activated, indicating that PDGF cannot be an early stimulus for HSC transdifferentiation [23].

Our data demonstrate additional differences between PF and culture-activated HSC at a similar passage number. PF are growth inhibited by TGF-β, whereas activated HSC are not, and PF, as demonstrated in Fig. 1, express all three TGF-β receptors on the cell surface, while activated HSC express minimal cell surface betaglycan (Figs. 1 and 3 and M.D.A. Gaça and R.G. Wells, unpublished results). Activated HSC proliferate in response to PDGF, while PF respond to FGF-2 (Fig. 3 and [24]). Both cell types, however, demonstrate a less than 2-fold increased matrix production in response to TGF-β, suggesting that TGF-β is not the major direct fibrogenic

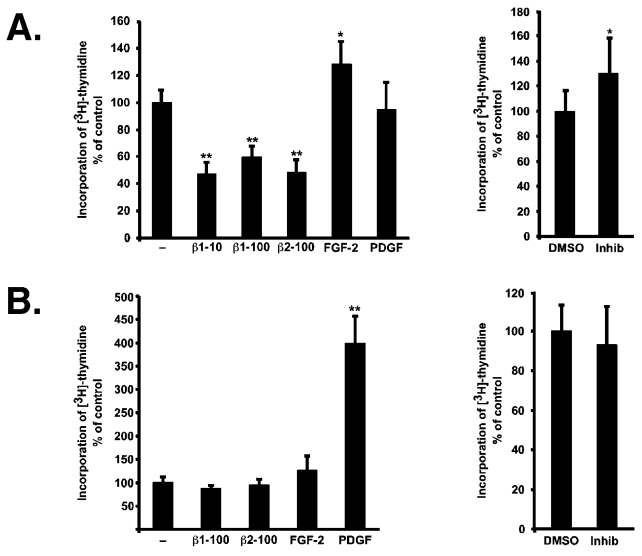

Fig. 3. TGF-β1 and -β2 inhibit proliferation of PF. PF were labeled with [^{3}H]thymidine for 26 h in the presence of growth factors and inhibitors. A, left panel: Cells were treated with TGF-β1 and -β2 as well as FGF-2 and PDGF. A, right panel: Cells were treated with the TβRI kinase inhibitor NPC-34016 (solubilized in DMSO) or with equivalent amounts of DMSO alone. B: HSC at a similar passage number were treated similarly. Note that the *y* axes differ in each graph, and that mitogenic effects of various treatments are lower for PF than for passaged HSC. Values are mean ± S.D. for six replicates, and are representative of findings in three separate experiments. $*P < 0.05$; $**P < 0.001$; compared with (−) or DMSO controls.

stimulus [16,17,25]. Activated HSC and PF both have a high baseline level of matrix production compared with control cells, suggesting that they are fibrogenic even in the absence of exogenous stimuli.

We have shown that PF, unlike activated HSC, express betaglycan and are growth inhibited by TGF-β2. This implies that activated HSC have a growth advantage in biliary fibrosis, particularly in the periportal region, where bile duct epithelial cells in proliferating bile ducts and, as we have shown, PF themselves, produce TGF-β2 [15]. The data suggest that autocrine production of large amounts of TGF-β2 by PF and TGF-β1 by PF and activated HSC is also likely to growth suppress PF preferentially, while production of PDGF by HSC and biliary epithelial cells facilitates HSC but not PF proliferation [26]. Taken collectively, our findings are consistent with the hypothesis that biliary fibrosis results from the sequential transdifferentiation of PF and HSC, and suggest mechanisms whereby HSC become the dominant myofibroblast population.

The interactions between HSC and PF in biliary fibrosis are not well understood. Autocrine production of TGF-β by PF could play a direct role in activation of HSC; alternatively, the production of even small amounts of matrix by PF in response to TGF-β could represent an important change in the liver milieu that leads to HSC activation. The ability to isolate PF now makes it possible to study their interactions with HSC directly, as well as the role of growth factors such as FGF-2, PDGF, and TGF-β2.

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