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Loss of TGF-β dependent growth control during HSC transdifferentiation

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Abstract

Liver injury induces activation of hepatic stellate cells (HSCs) comprising expression of receptors, proliferation, and extracellular matrix synthesis triggered by a network of cytokines provided by damaged hepatocytes, activated Kupffer cells and HSCs. While 6 days after bile duct ligation in rats TGF-β inhibited DNA synthesis in HSCs, it was enhanced after 14 days, indicating a switch from suppression to DNA synthesis stimulation during fibrogenesis. To delineate mechanisms modulating TGF-β function, we analyzed crosstalk with signaling pathways initiated by cytokines in damaged liver. Lipopolysaccharide and tumor necrosis factor-α enhanced proliferation inhibition of TGF-β, whereas interleukin-6, oncostatin M, interleukin-1α, and interleukin-1β did not. Hepatocyte growth factor (HGF) counteracted TGF-β dependent inhibition of DNA synthesis in quiescent HSCs. Since expression of c-met is induced during activation of HSCs and HGF is overrepresented in damaged liver, crosstalk of HGF and TGF-β contributes to loss of TGF-β dependent inhibition of DNA synthesis in HSCs.

Keywords: Fibrosis; Fibrogenesis; Hepatic stellate cells; Hepatocyte growth factor; Hepatocytes; Proliferation; Cytokine; Inhibition; DNA synthesis

Liver fibrosis results from chronic damage in conjunction with accumulation of ECM proteins [1]. Following chronic injury, HSCs transdifferentiate into myofibroblast-like cells (MFBs), acquiring contractile, proinflammatory, and fibrogenic properties [2,3]. Activated HSCs migrate and accumulate at sites of tissue repair secrete excess amounts of ECM and regulate its degradation. Further, HSCs undergo excessive cellular proliferation in hepatic fibrosis. PDGF is the predominant mitogen for activated HSCs and cytokines regulating the inflammatory response to injury modulate hepatic fibrogenesis [4]. Transforming growth factor (TGF-β) is a key mediator in fibrogenesis [5], favoring activation of HSCs and stimulating ECM production. Disruption of TGF-β synthesis and signaling markedly decreased fibrosis in experimental models [6–8]. TGF-β inhibits [^{3}H]thymidine incorporation in HSCs and pre-treatment of HSCs with TGF-β blocked PDGF-induced cell proliferation [9]. Further, TGF-β response of rat HSCs is modulated during culture activation [10]. HSCs in culture days 1–4 are responsive to TGF-β and display ligand-dependent signal transduction involving Smad proteins, resulting in a dose-dependent control of DNA synthesis. Fully activated HSCs (culture day 7) do not display TGF-β dependent Smad activation and DNA synthesis inhibition.

In the present study, we performed a more detailed study for TGF-β effects on DNA synthesis in HSCs. Using a rat bile duct ligation fibrogenesis model we show that

Abbreviations: BDL, bile duct ligation; ECM, extracellular matrix; HGF, hepatocyte growth factor; HSCs, hepatic stellate cells; Il, interleukin; LPS, lipopolysaccharide; MFBs, myofibroblasts; OSM, oncostatin M; PDGF, platelet-derived growth factor; TGF, transforming growth factor; TNF, tumor necrosis factor.

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in vivo transdifferentiated MFBs similarly loose the DNA synthesis control response to TGF-β. Furthermore, we identify LPS and TNF-α as enhancer and HGF as antagonist for the DNA synthesis inhibitory effect of TGF-β, whereas other cytokines, e.g., Il-6 and OSM have no effect. In summary, loss of TGF-β dependent DNA synthesis control is a prerequisite for HSC activation, and HGF, which is upregulated in fibrogenesis, modulates TGF-β signaling.

Materials and methods

Cytokines. Recombinant human TGF-β1,2,3 and HGF, recombinant rat Il-1α, Il-1β, Il-6, and PDGF-bb were from R&D Systems, Wiesbaden, Germany. Human OSM was from Roche, Mannheim, Germany. LPS was from Sigma, Steinheim, Germany and recombinant rat TNF-α was from Innogenetics, Gent, Belgium.

Cell culture reagents. DMEM and L-glutamine were from BioWhittaker Europe, Verviers, Belgium. Penicillin/Streptomycin, phosphate-buffered saline and trypsin/EDTA were from BioChromKG, Berlin, Germany. Fetal calf serum was from Gibco BRL, Karlsruhe, Germany. Bovine serum albumin, calf thymus DNA, and dimethylsulfoxide were from Sigma, Steinheim, Germany. [^{3}H]thymidine was from NET, Boston, MA. Hank's balanced salt solution w/o Ca^{2+} and Mg^{2+} was from PAA, Linz, Austria. SYBR Green was from Biozyme, San Diego, CA.

Ligation of the bile duct in rat. Male Sprague–Dawley rats (body weight of approx. 300 g) were sedated in isoflurane (Abbott, Ludwigshafen, Germany) and anaesthesized by i.v. injection of 50 μL of 2% Rompun (Bayer, Leverkusen, Germany) and 100 mg/kg bodyweight ketamine (Ceva Sante Animale, Düsseldorf, Germany). Anaesthesized animals were treated as described [11,12].

Isolation and culture of HSCs and MFBs. Isolation of HSCs was performed with male Sprague–Dawley rats (body weight approx. 500 g) as described [13]. HSCs were identified by light microscopical autofluorescence. Contamination by Kupffer Cells and endothelial cells was assessed by Dil-Ac-LDL (Harbor Bioproducts, Norwood, MA) and Fluoresbrite 1 mm latex beads (Polyscience Inc., Warrington, PA). Mean purity of isolated HSCs was between 85% and 95%. Yield varied from 10×10^6 to 6×10^7 per animal. Per definitionem, MFBs were generated by secondary culture of trypsinized primary 7-day-old HSCs on plastic dishes [10].

Cytokine treatment and proliferation assay. HSCs were cultured at 37 °C in 5% CO_2-enriched atmosphere humidified with saturated H_2O. Cells were seeded with a density of 90,000 cells per 3.8 cm^2 (12-well), 200,000 cells per 10 cm^2 (6-well) and 2.5×10^6 cells per 63 cm^2 (10 cm Petri dish) in 1, 2 or 10 mL DMEM including 10% FCS, 4 mmol/L L-glutamine and penicillin (100 IU/mL)/streptomycin (100 μg/mL) (day 0). Eighteen hours later, the medium was changed. Cells (day 1) were cultured overnight with serum starvation (0.5% FCS), then (day 2) medium was changed to serum free, followed by stimulation with the selected cytokines for 1 h. Then TGF-β was administered at the indicated concentrations and incubated serum free for 2 h. Finally, FCS was added to a concentration of 0.5% and cells were incubated for 21 h. At day 3, 2 μCi/mL [^{3}H]thymidine was added and cells were incubated for 24 h before the assay was stopped. DNA content was measured fluorometrically with SYBR green and [^{3}H]thymidine incorporation was assessed by scintillation measurement [14,15]. MFBs were treated identically to freshly isolated HSCs.

RNA purification and real time RT-PCR. Total RNA was extracted from HSCs 2 and 7 days after plating according to the RNA isolation kit instructions (Roche, Mannheim Germany). Quantity and purity were assessed by spectrophotometry and agarose gel electrophoresis. Total RNA (1 μg) was reversely transcribed following the instructions of First Strand cDNA Synthesis Kit (Roche). PCR program: 30 s at 95 °C, 30 s at 60 °C, and 60 s at 72 °C (number of cycles: 25 for GAPDH, 33 for c-met), 7 min at 72 °C. Primers: rat-c-met-for, 5′-ATTCTCTACGGGGTGTT TGC-3′; rat-c-met-rev, 5′-GGGTCCATAAAAATGCTGGA-3. Templates and primer sets were mixed with 2× QuantiTect SYBR Green PCR Master Mix (Qiagen, Hilden, Germany) and PCR was performed in ABI-Prism 7700 PCR machine.

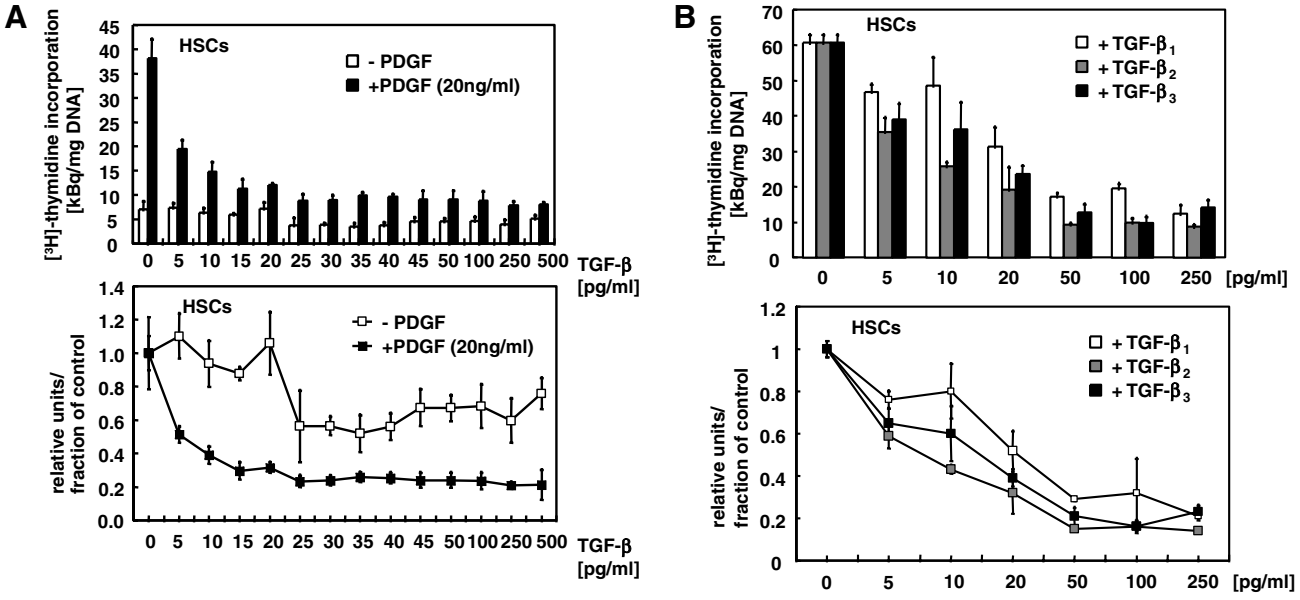

Fig. 1. TGF-β isoforms TGF-β1, TGF-β2, and TGF-β3 provide similar potential to inhibit DNA synthesis in HSCs dose dependently. (A) HSCs were treated with different concentrations of TGF-β1 with or without PDGF stimulation (20 ng/mL), as indicated at culture day 2. DNA synthesis measured by [^{3}H]-thymidine incorporation is presented as absolute values of radioactivity incorporated per DNA (upper diagram) or relative to not TGF-β treated controls (lower diagram). Black bars indicate continuous stimulation with PDGF (20 ng/mL) 1 h prior to TGF-β stimulation. Presented is one representative experiment (mean values ± SD of $n = 3$). (B) Quiescent HSCs were treated with different concentrations of TGF-β isoforms as indicated at culture day 2. DNA synthesis measured by [^{3}H]-thymidine incorporation is presented as absolute values of radioactivity incorporated per DNA (upper diagram) or relative to the untreated controls (lower diagram). All cells were treated with PDGF (20 ng/mL) 1 h prior to TGF-β stimulation. Presented is one out of three independent experiments, which all show similar results (mean values ± SD of $n = 4$).