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BASIC RESEARCH

Variable expression of cystatin C in cultured trans-differentiating rat hepatic stellate cells

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Abstract

AIM: To study the expression of cystatin C (CysC), its regulation by transforming growth factor-β1 (TGF-β1) and platelet-derived growth factor (PDGF) and the potential interference of CysC with TGF-β1 signaling in this special cell type.

METHODS: We evaluated the CysC expression in cultured, profibrogenic hepatic stellate cells and trans-differentiated myofibroblasts by Northern and Western blotting and confocal laser scanning microscopy.

RESULTS: CysC was increased significantly in the course of trans-differentiation. Both TGF-β1 and PDGF-BB suppressed CysC expression. Furthermore, CysC secretion was induced by the treatment with TGF-β1. Although CysC induced an increased binding affinity of TGF-β receptor type III (beta-glycan) as assessed by chemical cross-linking with [^{125}I]-TGF-β1, it did not modulate TGF-β1 signal transduction as shown by evaluating the Smad2/3 phosphorylation status and [CAGA]-MLP-luciferase reporter gene assay. Interestingly, the shedding of type III TGF-β receptor beta-glycan was reduced in CysC-treated cells. Our data indicated that CysC expression was upregulated during trans-differentiation.

CONCLUSION: Increased CysC levels in the serum of patients suffering from liver diseases are at least partially due to a higher expression in activated hepatic stellate cells. Furthermore, TGF-β1 influences the secretion of CysC, highlighting a potentially important role of cysteine proteases in the progression of hepatic fibrogenesis.

Key words: Cystatin C; TGF-β; Hepatic stellate cells; Trans-differentiation; Beta-glycan

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INTRODUCTION

Cystatin C (CysC), belonging to the type II cystatin gene superfamily, is the most abundant extracellular inhibitor of cysteine proteinases[1]. Mature CysC is a 13-kD, positively charged, secreted protein composed of 120-122 amino acids expressed by many cell types. It is present in large quantities in cerebrospinal fluid, seminal plasma, serum and other body fluids. One of the most prominent functions of CysC is related to the inactivation of the cathepsin family members of cysteine proteinases, which are involved in the cleavage of membrane and extracellular matrix proteins among others and thus in disease-related tissue remodeling. The diagnostic value and prognostic significance of CysC determination have been reported for several diseases[2,3], and correlations have been found between CysC expression, mutations, and clinical status of patients with autoimmune disease, cerebral amyloid angiopathy, hereditary brain hemorrhage, atherosclerosis, aortic aneurysms, and multiple sclerosis[4-8].

A further highly relevant function of CysC has been recently reported, which is concentrated on the inhibitory effect on transforming growth factor-β (TGF-β)-signal transduction in normal and malignant cells[9]. The TGF-β antagonizing mechanism is at least partially due to the interference of ligand binding to the type II TGF-β receptor. *In vitro*, TGF-β1 is a potent inducer of CysC secretion in vascular smooth muscle cells[8]. Furthermore, expression of CysC mRNA in astrocyte precursor cells is directly linked to the activity of TGF-β[10]. TGF-β is known to be the fibrogenic master cytokine in human and experimental liver fibrosis due to its ability to stimulate the expression and inhibition of degradation of extracellular matrix (ECM) proteins and to promote the trans-differentiation of profibrogenic hepatic stellate cells (HSCs) to ECM-producing hepatic myofibroblasts (MFBs)[11]. The phenotypic and functional trans-differentiation of

HSCs is initiated in acute and chronic inflammatory liver diseases, ultimately leading to organ fibrosis and cirrhosis. Significantly elevated serum concentrations of CysC have been recently described in chronic liver diseases showing a strong correlation between the degree of elevation and the severity of disease[12,13]. Based on these new findings, we tried to figure out the potential functional relevance of CysC in the profibrogenic liver cell subtype, HSCs. Therefore, we studied in cultured rat HSCs, the expression of CysC, its regulation by TGF-β1 and platelet-derived growth factor (PDGF) and the potential interference of CysC with TGF-β signaling in this special cell type. The results showed that CysC expression in HSCs was modulated by TGF-β and platelet-derived growth factor-BB (PDGF-BB) depending on the trans-differentiation status of the cells. However, CysC had no direct effect on TGF-β signaling itself.

MATERIALS AND METHODS

Cell isolation and culture

HSCs, Kupffer cells (KCs), and sinusoidal endothelial cells (SECs) were isolated from male Sprague-Dawley rats by the pronase-collagenase method[14]. HSCs were further purified by a single-step density gradient centrifugation as previously described[15]. KCs and SECs were enriched by centrifugal elutriation[16] and RNA was directly isolated without cell cultivation. HSCs were seeded in Dulbecco's modified Eagle's medium (DMEM) (BioWhittaker Europe, Verviers, Belgium) supplemented with 100 mL/L fetal calf serum (FCS) (Hyclone Fetal Bovine Serum, Perbio), 4 mL glutamine (PAA Laboratories GmbH, Linz, Austria), 100 IU/mL penicillin and 100 μg/mL streptomycin (PAA Laboratories). Hepatocytes (PCs) were isolated following the collagenase method of Seglen[17]. The medium was renewed one day after initial plating and then every day. Cultures were maintained at 37 °C in a humidified atmosphere containing 50 mL/L CO_2. Prior to treatment with recombinant human CysC (rhCysC), TGF-β1, and PDGF-BB (R&D Systems, Wiesbaden, Germany), the cells were starved for 16 h in 0.2% FCS.

RNA isolation and Northern blot analysis

Total RNA was purified using the RNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. After resuspension in water, the concentration was determined by UV absorbance. Northern blot analysis was carried out as previously described[18].

cDNA preparation, RT-PCR, real time PCR

To generate a probe for Northern blot analysis of CysC expression, total RNA (2 μg) from rat HSCs was reverse transcribed using the Omniscript transcriptase (Qiagen) and random hexamer primers (Invitrogen, Karlsruhe, Germany). The reaction was allowed to proceed at 37℃ for 60 min and cDNA products were stored at -20 °C until use. For amplification of rat CysC, aliquots of first strand cDNAs were subjected to PCR with primers 5'-AGT ACA ACA AGG GCA GCA ACG ATG-3' and 5'-AGG AGA AGA GAA CCA GGG GAC AGC-3', dNTPs (each 10 mmol/L dATP, dCTP, dGTP, dTTP) in 1× PCR reaction buffer and 2.5 U *Taq* DNA polymerase (Roche Molecular Biochemicals, Mannheim, Germany) resulting in the amplification of a 454-bp CysC fragment[19]. PCR conditions were at 94 °C for 5 min (initial denaturation), at 94 °C for 60 s, at 60 °C for 60 s, at 72 °C for 3 min (40 cycles), and at 72 °C for 10 min (final extension). Aliquots of the reaction products were separated and visualized on 1% (w/v) agarose gels and the identity of the fragment was demonstrated by sequencing. Quantitative analysis of rat CysC mRNA was performed with a LightCycler (LC) System in 20 μL reaction volume using the LC-FastStart DNA Master SYBR Green I kit (Roche). Thermocycling was performed with 2 μL of cDNA products and 0.75 μmol/L of each primer specific for CysC. No-template controls were prepared by adding 2 μL PCR grade, sterile H_2O to 18 μL of master mix. Cycling conditions were one cycle of denaturation at 95 °C for 10 min, followed by 45 amplification cycles at 95 °C for 10 s, at 60 °C for 5 s, at 72 °C for 22 s. The PCR products were melted in a temperature transition procedure from 65 °C to 95 °C in steps of 0.1 °C/s and fluorescence was measured and plotted online against the temperature to obtain the fragment-specific melting points. Differences between crossing points were taken to estimate the relative concentrations.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting

For immunoblotting, proteins from rat liver cells were separated under reducing conditions on 4-12% Bis-Tris gradient gels using MES running buffer (Invitrogen). The electroblotting onto 0.2-μm protran NC membranes (Schleicher & Schuell, Dassel, Germany) was carried out according to standard procedures. Blocking and incubation of the membranes with individual antibodies were performed as described previously[18] with slight modifications. Briefly, membranes were blocked with 5% (w/v) non-fat milk powder in Tris-buffered saline-Tween 20 (TBST). For Western blot analysis, the following primary antibodies were diluted in 2.5% (w/v) non-fat milk powder in TBST: clone asm-1 (Cymbus Biotech., Chandlers Ford, UK) against α-smooth muscle actin (α-SMA) (1:10 000), rabbit anti-human CysC rat/human CysC and its precursor (1:500) (Upstate Biotechnology, Lake Placid, USA), rabbit anti-phospho-Smad2 (Ser465/467) (1:1000) (New England Biolabs GmbH, Frankfurt/Main, Germany), rabbit anti-Smad2 (1:200) (Zymed Laboratories, San Francisco, CA, USA), goat anti-Smad2/3 (N-19), (Santa Cruz Biotechnology, Santa Cruz, CA, USA) (1:100), mouse anti-β-actin (1:10 000) (Cymbos). The antiserum PS1 was raised against a phosphorylated C-terminal peptide of Smad1 and showed cross-reactivity with phosphorylated Smad3 (1:1 000)[20]. Primary antibodies were visualized using horseradish peroxidase (HRP)-conjugated anti-mouse, -rabbit or -goat IgG (Santa Cruz Biotechnology) or alkaline phosphatase (AP)-conjugated anti-rabbit or mouse IgG (Santa Cruz Biotechnology). Alternatively, the primary antibodies against CysC and Smad2/3 (N-19) were detected with swine anti-rabbit and rabbit anti-goat IgG biotin antibody followed by streptavidin-HRP conjugate (DAKO Diagnostics, Hamburg, Germany). Staining was

performed with the SuperSignal West Dura Extended chemiluminescent substrate (Pierce, Rockford, IL, USA) for HRP exposed in a Lumi-Imager™ (Roche) or a color staining using NBT/BCIP substrate (Perbio Science, Bonn, Germany).

Sequence analysis

All primers were obtained from MWG-Biotech (Ebersberg, Germany) and sequencing was done with the ABI PRISM BigDye® termination reaction kit (PE Applied Biosystems, Weiterstadt, Germany) as described[21].

Enzyme linked fluorescence cytological labeling assay (ELF 97 assay)

Ten thousand cells per well were cultured in black 96-well plates for different times under standard conditions and fixed with 4% (w/v) paraformaldehyde in phosphate buffered saline (PBS) (pH 7.4). For generation of myofibroblasts (MFBs), HSCs cultured for seven days were trypsinized and subcultured for further 3 days. The cells were permeabilized on ice in 0.1% (w/v) Triton X-100/0.1% (w/v) sodium citrate, blocked in 1% (w/v) BSA/PBS pH 7.4 and incubated with anti-α-smooth muscle actin (1:500) or anti-CysC antibody (1:200). Non-immune IgGs were used as negative controls. The ELF-97 assay (Molecular Probes Europe; Leiden, NL) was performed according to an established procedure[22]. The fluorescence intensity was measured in a Multilabel counter (Victor, Wallac ADL GmbH, Freiburg, Germany) for the quantitative evaluation of α-SMA and CysC (excitation 365 nm, emission 515 nm). The staining intensity of negative controls was subtracted from the determined values and in parallel the fluorescence was also recorded in an inverted fluorescence microscope (Axiovert M135, Zeiss, Frankfurt, Germany). Subsequently, to normalize the fluorescence values (FU) of α-SMA or CysC, the DNA content of parallel cultures was measured fluorometrically (excitation 485 nm, emission 525 nm) and quantified against calf thymus DNA as standard using the SYBR Green I dye (Molecular Probes Europe).

Immunofluorescence staining and confocal microscopy

Forty thousand cells per well (1.8 cm^2) were cultured in 4 chamber polystyrene vessels (Becton, Dickinson, Franklin Lakes, NJ, USA) for different times under standard conditions and fixed with 4% (w/v) paraformaldehyde in PBS (pH 7.4). Permeabilization, blocking and CysC antibody (Upstate Biotechnology, 1:200) incubation were carried out as described above. After washing, a biotin-linked swine anti-rabbit IgG (DAKO) (1:300) was used as the secondary antibody followed by incubation with streptavidin-FITC (DAKO) (1:150). For the detection of α-SMA, a direct CY3-conjugated antibody (clone AC-40; Sigma, Taufkirchen, Germany) was used (1:200). Non-immune IgGs (Santa Cruz) served as negative controls. After washing and mounting with antifade, the cells were subjected to high-resolution confocal laser scanning microscopy in a LS 410 inverse microscope (Zeiss) using a standard objective (40×1.3 oil).

Adenoviral infection of HSCs and luciferase measurement

Adenoviral stocks of Ad $(CAGA)_9$-MLP-luciferase were prepared as previously described[23]. Two thousand cells per well were cultured in black 96 well plates. After infection at a multiplicity of infection (MOI) of 50, cells were serum starved at 0.2% FCS for 16 h, pre-incubated with or without rhCysC for 1.5 h, respectively, followed by cytokine stimulation as indicated. The luciferase activity was measured with the Steady-Glo luciferase assay substrate (Promega, Madison, WI, USA) according to the manufacturer's protocol.

Affinity labeling and cross-linking of TGF-β receptors

Affinity labeling with [^{125}I]-TGF-β1 (Amersham Pharmacia Biotech, Freiburg, Germany) and cross-linking were performed as described previously[24]. Briefly, confluent monolayers of HSCs (5×10^5 cells/10 cm^2) were cultured for 4 days in medium containing 10% FCS. After aspiration of medium, monolayers were washed with Krebs-Ringer-HEPES binding buffer (128 mmol/L NaCl, 5 mmol/L KCl, 5 mmol/L $MgSO_4$, 13 mmol/L $CaCl_2$, 50 mmol/L HEPES) containing 0.5% (w/v) BSA (radioimmunoassay grade) and pre-incubated with or without CysC for 1 h at 37 °C. Cells were washed with ice-cold buffer and receptor binding was performed in the presence or absence of CysC. After equilibration for 10 min on ice, cells were incubated with 2.8 ng/mL [^{125}I]-TGF-β1 (1 621 Ci/mmol) with or without a 200-fold excess of unlabeled TGF-β1 (R&D Systems, Minneapolis, USA) for 3 h at 4 °C. As a further control, an antibody directed against the extracellular domain of betaglycan was used to suppress ligand binding. Bound ligand was cross-linked to its receptor in Krebs-Ringer-HEPES buffer lacking the BSA with disuccinimidyl suberate (Perbio Science) for 15 min at 4 °C. The reaction was stopped by aspiration of the supernatant followed by washing with STE buffer [10 mmol/L Tris-HCl pH 7.4, 250 mmol/L sucrose, 1 mmol/L ethylenediaminetetraacetic acid (EDTA)] including 1 mmol/L phenyl methyl sulfonyl fluoride (PMSF). Cross-linked [^{125}I]-TGF-β1 was extracted with TTE buffer (10 mmol/L Tris-HCl, pH 7.4, 10 mL/L (v/v) Triton X-100, 1 mmol/L EDTA) including proteinase inhibitor cocktail complete (Roche). The detergent soluble, cell debris-free fraction was precipitated by ice-cold 10% TCA (w/v) in the presence of 0.1 mg/mL sodium deoxycholate (DOC). The protein precipitates were subsequently washed with acetone, air dried, and dissolved in a small volume of LDS electrophoresis sample buffer (Invitrogen). Sample volumes corresponding to the normalized DNA contents were subjected to NuPAGE SDS-PAGE. For autoradiography, dried gels were exposed to KODAK X-OMAT AR films (Eastman Kodak Company, Rochester, NY, USA) using intensifying screens.

CysC secretion in HSCs

Serum-starved HSCs were treated with rhTGF-β1 (10 ng/mL) for 24 or 48 h and the cell-free supernatants were subjected to standard immunoprecipitation using goat antibody (sc-16989) directed against CysC (Santa Cruz). The resulting precipitates were washed and analyzed for CysC expression by Western blotting using a primary rabbit antibody directed against CysC (Upstate) and

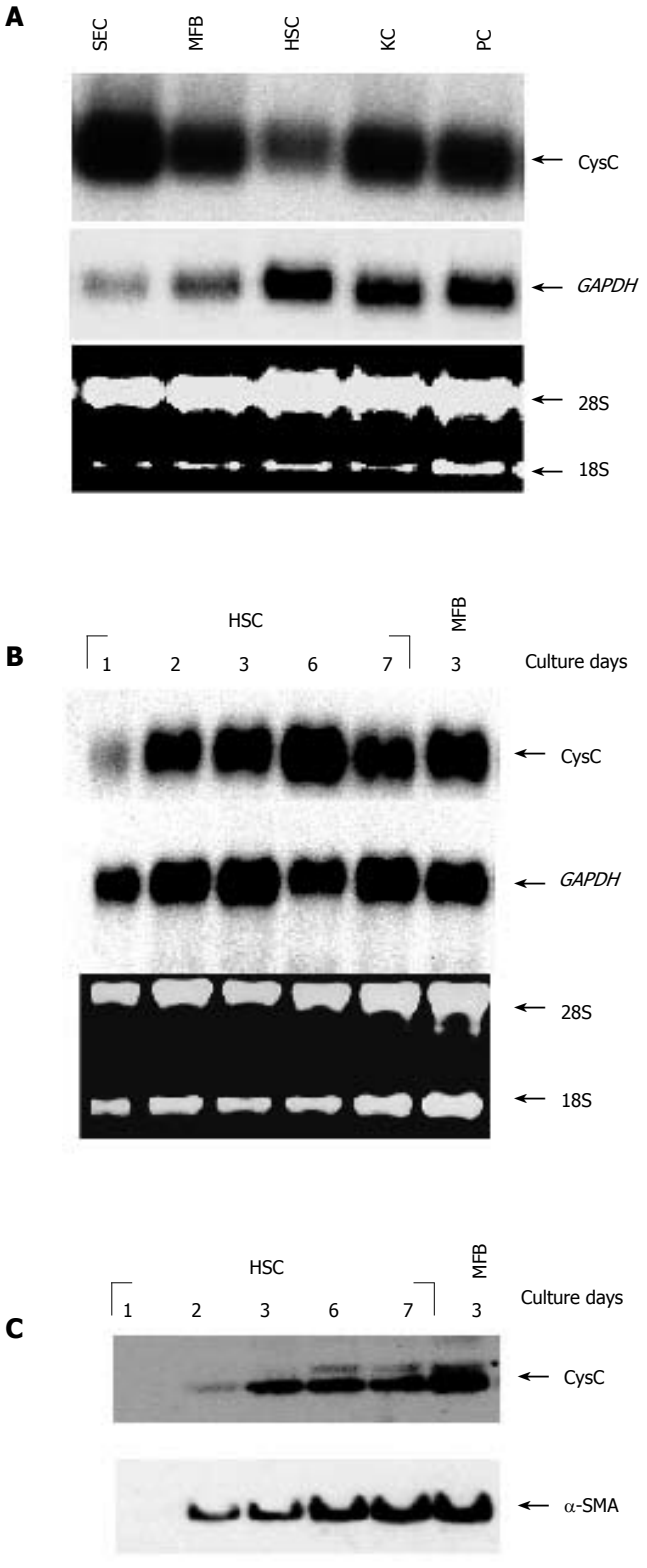

Figure 1 CysC mRNA expression in different rat liver cell types. **A:** Equal amounts of total RNA samples (10 μg) isolated from cultured SECs, MFBs, HSCs, KCs, and PCs were analyzed by Northern blot. The blot was hybridized with a ^{32}P-labeled probe specific for rat CysC; **B:** total RNA samples (5 μg/lane) of trans-differentiating HSCs/MFBs at indicated time intervals were analyzed for CysC expression by Northern blot analysis. As internal loading controls **(A and B)**, the 18S and 28S rRNA as well as signals obtained after hybridization with a *GAPDH*-specific probe are shown. The blots were exposed for 4 (GAPDH) or 24 h (CysC), respectively; **C:** protein extracts (20 μg/lane) from primary rat HSCs cultured for 1 to 7 days or rat MFBs cultured for 3 days were analyzed for CysC expression by Western blotting under reducing conditions (50 mmol/L DTT) using antibody P-14 (sc-16989). To verify cellular trans-differentiation, the blot was stripped, and tested for α-SMA expression using a mouse monoclonal antibody (clone asm-1).

visualized with a goat anti-rabbit IgG-HRP conjugate.

RESULTS

Expression of CysC in isolated liver cells

To analyze the expression of CysC in different liver cell subpopulations, we performed Northern blot analysis (Figure 1A). In agreement with the general assumption that CysC is a constitutive active gene expressed in all nucleated cells at a constant rate[25], we found a single CysC transcript of 0.8-0.9 kb in size in SECs, KCs, PCs, HSCs and their trans-differentiated myofibroblastic phenotype. However, compared to HSCs, we found a significant increase of CysC transcripts in their trans-differentiated phenotype. Subsequent analysis revealed that the activation of HSCs was accompanied with an upregulation of CysC expression during the trans-differentiation process (Figure 1B). In these analyses, the levels of mRNA encoding the enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as well as the 28S and 18S rRNAs were used as internal quality and loading controls. To demonstrate CysC expression at the protein level, we further performed Western blot analysis using cellular extracts isolated from cells at different time points of trans-differentiation (Figure 1C). We found that the amount of CysC protein correlated with the amounts of transcripts. To illustrate and confirm the increase of CysC protein more quantitatively, we further performed confocal immunocytochemistry (Figure 2A) and ELF-assay (Figure 2B), respectively. Again, both visualizations of cellular CysC expression by confocal laser scanning microscopy and quantitative ELF-assay indicated that CysC expression was strongly increased during cellular activation and trans-differentiation.

CysC did not modulate TGF-β1 signal transduction in HSCs

A recent report demonstrated that CysC is able to antagonize TGF-β binding to its cell surface receptors thereby inhibiting TGF-β signaling[9]. To test if this antagonizing effect on TGF-β signaling was also present in HSCs, we monitored the changes in the TGF-β sensitive CAGA-MLP-luciferase reporter assay system[26]. Therefore, cultured HSCs were first infected with 50 MOI Ad-(CAGA)$_9$-MLP-luciferase[22], preincubated with different concentrations of rhCysC and then exposed to TGF-β1. The TGF-β-stimulated luciferase activity was compared to unstimulated control cells, which revealed that preincubation with CysC had no effect on the reporter assay (Figure 3A). Similarly, Western blot analysis further revealed that TGF-β-induced phosphorylation of Smad2 and Smad3 did not change in the presence of CysC, again demonstrating that this cysteine protease inhibitor could not antagonize TGF-β signaling in cultured HSCs (Figure 3B).

CysC promoted TGF-β binding to TGF-β type III receptor betaglycan

Based on a recent report demonstrating that CysC could dose-dependently inhibit the physical interaction of TGF-β to murine TGF-β type II receptor[9], we performed cross-linking experiments to test if CysC had the same

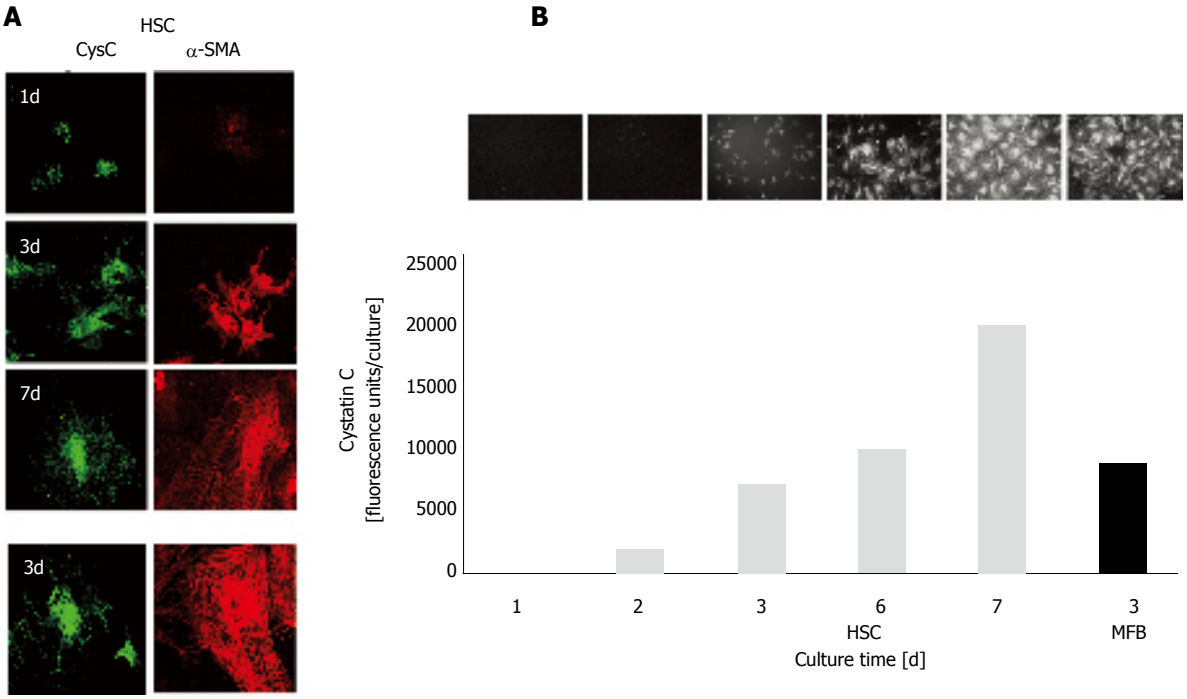

Figure 2 Confocal laser scanning microscopy and ELF assay for quantitative determination of CysC expression in HSCs and MFBs. **A:** HSCs/MFBs were seeded on glass cover slides for indicated time intervals. After fixation, cells were permeabilized and incubated with primary antibodies directed against CysC or α-SMA. Primary antibodies were visualized with a FITC-labeled secondary antibody (CysC, green fluorescence) or a Cy3-labeled secondary antibody (α-SMA, red fluorescence). Original magnification was ×400. Negative controls, using normal IgGs instead of primary antibodies showed no staining (data not shown); **B:** HSCs/MFBs cultured in 96-well plates for indicated time intervals were fixed and stained for CysC. The staining was visualized by fluorescence microscopy (original magnification ×200). Fluorescence intensities were measured quantitatively using a 96-well fluorescence reader (excitation 365 nm, emission ≥515 nm).

effects in the rat system. Interestingly, we found that the binding of TGF-β to the type II receptor was not affected in the presence of physiological concentration of CysC (data not shown). Since the transmembrane signaling by TGF-β was critically modulated in the presence of the type III receptor (betaglycan), which was expressed at high level in HSCs and in turn associated with the type II receptor in a second step, we next addressed the question if CysC was able to modify the binding of TGF-β to this receptor. Binding of TGF-β1 to betaglycan was analyzed by cross-linking experiments using iodinated TGF-β1. HSCs cultured for 4 days were first incubated with rhCysC. Thereafter, the cells were exposed to [^{125}I]-TGF-β1 and the iodinated ligand was chemically cross-linked to the receptors. Subsequently, betaglycan was separated by SDS-PAGE, and the amount of ligand bound to this receptor was visualized by autoradiography (Figure 4, lanes 1 and 2). Interestingly, we found that the binding to betaglycan was elevated after preincubation with CysC. The specificity for the betaglycan labeling was demonstrated by preincubation with a betaglycan-specific antibody (Figure 4, lanes 3 and 4).

TGF-β and PDGF-BB suppressed CysC expression in HSCs

Several reports demonstrated that CysC transcript expression could be stimulated by TGF-β1[9,10,27]. To test if CysC expression was induced in HSCs by TGF-β1, we performed Northern blot analysis with RNA isolated from HSCs that were treated with TGF-β1 (5 ng/mL) for 18 or 38 h, respectively (Figure 5A, lanes 2 and 5). Surprisingly, the contents of CysC transcripts were decreased in TGF-β-stimulated HSCs. Furthermore, the same effect was observed when PDGF-BB was applied causing a significant decrease of CysC transcript expression (Figure 5A, lanes 3 and 6). To confirm that CysC expression in HSCs was indeed suppressed by TGF-β and PDGF-BB, we repeated our experiments at the protein level. As shown in Figure 5B, both TGF-β1 and PDGF-BB significantly suppressed the synthesis of CysC in HSCs.

TGF-β increased secretion of CysC in HSCs

To further explore the impact of TGF-β on CysC regulation, we tested if TGF-β1 could modify CysC secretion. Therefore, we treated serum-starved HSCs with TGF-β1 (10 ng/mL) for 24 or 48 h and determined the amount of CysC in the cell-free supernatants. Compared to control cells, the amount of secreted CysC was markedly increased in cultures stimulated with TGF-β1 (Figure 6). Furthermore, we detected two different CysC protein bands with an approximate molecular mass of 13 and 18 ku most likely representing proteins with a different N-glycosylation status[28].

DISCUSSION

The response to chronic liver injury is wound healing and subsequently fibrosis representing the enhanced ECM production and deposition ending in cirrhosis. In the last decades a growing body of evidence indicates that HSCs and their trans-differentiated phenotype, the MFBs, as

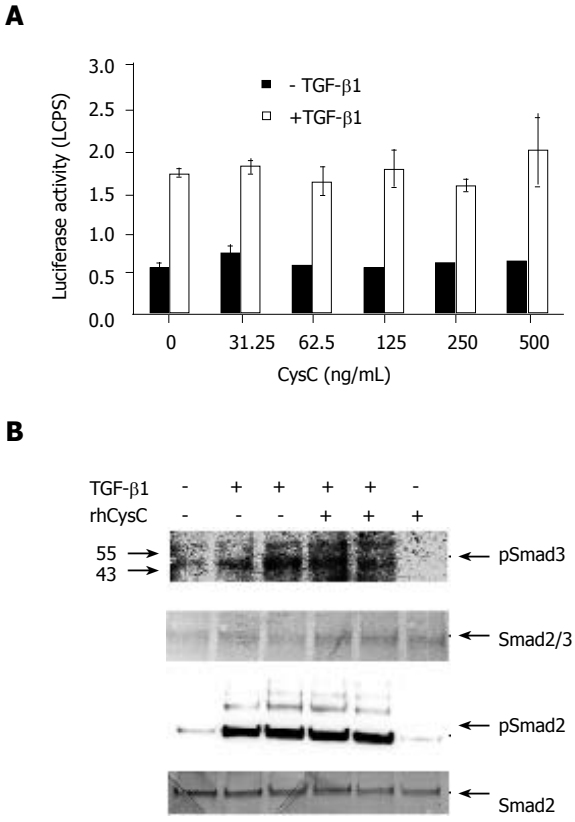

Figure 3 Effect of rhCysC on TGF-β1-induced reporter gene activity and Smad2/3 phosphorylation. **A:** Cultured HSCs were infected with 50 MOI Ad-$(CAGA)_9$-MLP-luciferase followed by 1 h incubation with the indicated doses of rhCysC prior to exposure with TGF-β1 (1 ng/mL) for 4 h or left untreated. The mean ± SD of the measured luciferase activities (n = 4) was given; **B:** phosphorylated Smad2 (pSmad2) and Smad3 (pSmad3) were detected by immunoblotting of whole cell protein lysates prepared after treatment of HSCs with or without 500 ng/mL rhCysC for 1 h followed by incubation with 1 or 5 ng/mL TGF-β1, respectively for an additional hour. The amount of total Smad2/3 or Smad3 was used as internal loading control.

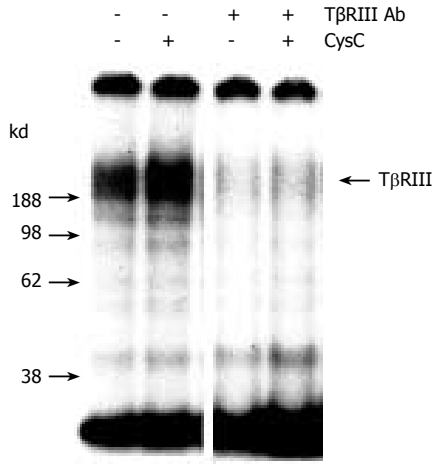

Figure 4 Effect of rhCysC on TGF-β1 binding to beta-glycan. Cultured HSCs were incubated for 1 h with or without rhCysC or an antibody specific for the extracellular domain of beta-glycan followed by the application of [^{125}I]-TGF-β1. The ligand was chemically cross-linked to the receptor and subsequently TβRIII was immunoprecipitated, separated by SDS-PAGE, and visualized by autoradiography. Control experiments including a 200-fold excess of unlabeled TGF-β1 showed no receptor labeling (data not shown).

well as TGF-β1 and PDGF-BB are central cell types and mediators in this wound healing process. In healthy livers, the balance between synthesis and degradation of ECM is tightly controlled. During injury and fibrosis progression, there is increased expression of matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs) resulting in an imbalance in the turnover of ECM and a conversion of the low-density subendothelial matrix into matrix-rich interstitial collagens. Additionally, the activity of collagenolytic cathepsins is elevated in experimental and clinical hepatic fibrogenesis[29,30] and elevated serum concentrations of CysC are found in patients suffering from chronic liver diseases[12,13]. Therefore, it is possible that an imbalance between cathepsins and CysC contributes to liver fibrosis. The demonstration that members of the cystatin superfamily can physically interact with metalloproteinases thereby protecting them from autolytic activation, further suggesting that there is a direct linkage between the MMP/TIMP and the cathepsin/cystatin enzyme systems[31]. Although all these studies have clearly shown that elevated serum CysC concentrations are clinically important for the progression of liver fibrogenesis, the exact cellular source and the regulation of CysC within different liver cell subpopulations have not been explored.

In the present study, all liver cell types tested were able to synthesize CysC, CysC expression was up-regulated in activated HSCs and trans-differentiated MFBs, TGF-β1 and PDGF-BB suppressed CysC expression at the RNA and protein level, CysC did not modulate TGF-β1 signal transduction in HSCs but induced affinity of betaglycan for TGF-β1, TGF-β1 induced secretion of this inhibitor of cysteine proteinases.

The finding that all liver cells tested express CysC (Figure 1A) is not surprising since it has been well established that CysC behaves like a "housekeeping gene" expressed in all nucleated cells, in all tissues and cell types, although mRNA levels vary several-fold between and among the tissues[25]. However, the finding that CysC expression is strongly increased in activated HSCs and trans-differentiated MFBs is somewhat unexpected since previous studies have demonstrated that the CysC promoter is of constitutive nature[32]. This upregulation is consistent with the findings that patients with liver cirrhosis have an average three-fold greater serum CysC concentration which closely matches to the degrees of fibrosis and is elevated even in patients with mild fibrosis. Thus, increased CysC levels in serum of these patients are at least partially due to a higher expression of this protein inhibitor of cysteine proteinases. The observation that both TGF-β and PDGF-BB could suppress the expression of CysC is at first somewhat striking and unexpected since TGF-β1 and PDGF-BB are the main effectors during hepatic fibrogenesis and, moreover, TGF-β1 can stimulate CysC expression in murine embryo cells, uterine decidual and 3T3-L1 cells[9,10,27]. The conflict might be explained by the finding that only HSCs respond to TGF-β1, whereas fully trans-differentiated MFBs are insensitive to the treatment with TGF-β1[24]. Thus, in ongoing hepatic fibrogenesis, when HSCs become refractory against

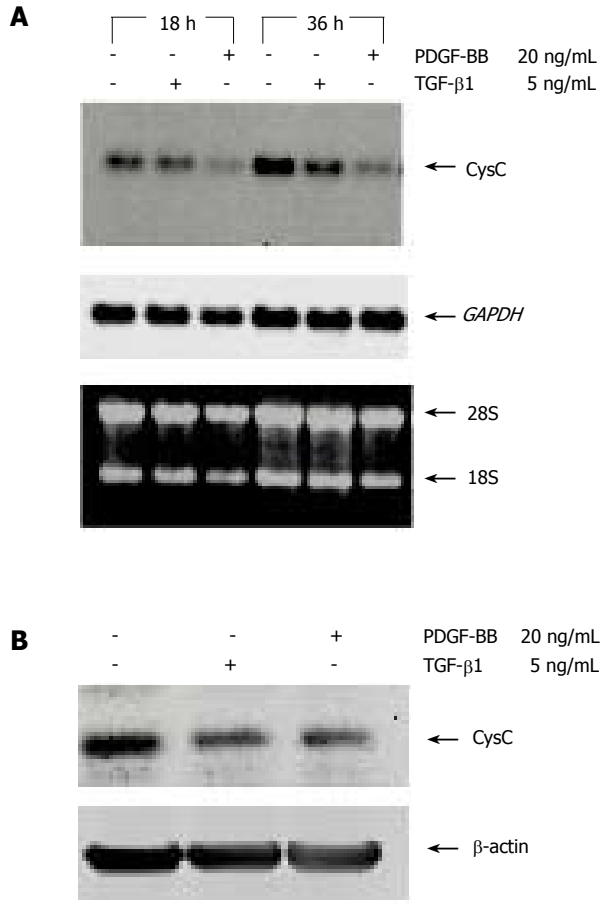

Figure 5 Effect of PDGF-BB and TGF-β1 on CysC expression in HSCs. **A:** Cultured HSCs were serum starved to 2 mL/L FCS followed by treatment with or without PDGF-BB or TGF-β1 for 18 and 36 h, respectively. Total RNA (5 μg/lane) was isolated and analyzed by Northern blot for CysC expression. As internal loading controls, the 18S and 28S rRNA as well as signals obtained after hybridization with a GAPDH-specific probe were shown; **B:** for CysC protein expression, cells were treated with or without PDGF-BB or TGF-β1 for 72 h and protein lysates (20 μg/lane) were subjected to Western blot analysis using an antibody specific for CysC. The expression of β-actin was used as internal protein loading control.

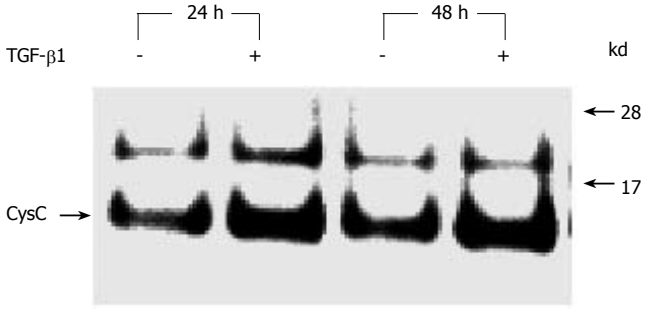

Figure 6 TGF-β1-induced CysC secretion in HSCs. Serum-starved HSCs were treated with TGF-β1 (10 ng/mL) for 24 or 48 h and the cell-free supernatants were subjected to immunoprecipitation using goat antibody P-14 directed against CysC. Subsequently, the precipitates were analyzed for CysC expression by Western blotting using an independent primary rabbit antibody directed against CysC and visualized with a goat anti-rabbit IgG-HRP conjugate.

TGF-β, they start to increase CysC expression potentially leading to the observed higher serum concentrations.

In HSCs, TGF-β signaling is not influenced by the presence of CysC. Although CysC has been found to increase the ligand binding to betaglycan, we observed that it had no effects on TGF-β receptor type II binding (data not shown) and TGF-β reporter gene assay or Smad2/3 phosphorylation. Possibly, CysC inhibits a specific proteinase required for shedding of betaglycan. In line with such a hypothesis, the shedding of betaglycan is mediated by membrane type MMP-1[33] and members of the cystatin superfamily can interact with MMPs and protect them from autolytic degradation[31]. The missing impact of CysC on TGF-β-binding to the type II receptor might be due to the absence of specialized spliced forms of the type II receptor, i.e. type IIb present in mice[34] but not in rat HSCs/MFBs (Meurer, Gressner, Weiskirchen, unpublished observation).

Finally, we found that TGF-β induced secretion of CysC in HSCs. This ability of TGF-β is also described in vascular smooth muscle cells[8], suggesting that it might be a general hallmark of smooth muscle cells. It is also conceivable that the observed decrease of cellular CysC content upon stimulation with TGF-β1 is a consequence of CysC release from intracellular storage sites. However, this effect might only in part contribute to the intracellular decrease of CysC since we observed a clear decrease of CysC mRNA in cells stimulated with TGF-β1.

In conclusion, the concentration of serum CysC is correlated with the severity of liver dysfunction. CysC expression is upregulated in the course of HSC activation and trans-differentiation into MFB, which gives a plausible explanation for the correlation of CysC concentrations with the progression of liver fibrosis.

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