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

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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[10]. Furthermore, expression of CysC mRNA in astrocyte precursor cells is directly linked to the activity of TGF-β[11]. 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)[12].

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), 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 CO2. Prior to treatment with recombinant human CysC (rhCysC), TGF-β1, and PDGF-BB depending on the trans-differentiation status of the cells. However, CysC had no direct effect on TGF-β signaling itself.

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 °C 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 AGC 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 1 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 H2O 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 electrophoretic 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[19] 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), mouse anti-human CysC rat/human CysC and its precursor (1:500) (Upstate Biotechnology, Lake Placid, USA), rabbit anti-phospho-Smad2 (Ser465/467) (1:100) (New England Biolabs GmbH, Frankfurt/Main, Germany), rabbit anti-Smad2/3 (1:500) (Upstate Biotechnology, Lake Placid, USA), mouse anti-Smad2/3 (1:500) (Upstate Biotechnology, Lake Placid, USA), goat anti-Smad2/3 (N-19), (Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse anti-Smad1 and showed cross-reactivity with phosphorylated Smad3 (1:100 000), 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-Smad2/3 (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) (Cymbus). The antiserum PS1 was raised against a phosphorylated C-terminal peptide of Smad1 and showed cross-reactivity with phosphorylated Smad3 (1:1 000). 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[23].

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 cm²) 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)-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 [125I]-TGF-β1 (Amersham Pharmacia Biotech, Freiburg, Germany) and cross-linking were performed as described previously[24]. Briefly, confluent monolayers of HSCs (5×10⁴ cells/10 cm²) 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 MgSO₄, 13 mmol/L CaCl₂, 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 [125I]-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 [125I]-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.

CystC 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

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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, 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. 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. Therefore, cultured HSCs were first infected with 50 MOI Ad-(CAGA)-MLP-luciferase, 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, we performed cross-linking experiments to test if CysC had the same
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...
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 loading control.

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)MLP-luciferase followed by 1 h incubation with the indicated doses of rhCysC prior to exposure to 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 [125I]-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).

of CysC within different liver 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[29]. 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[30]. 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-β1 and PDGF-BB suppressed 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 cells[31], as 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 loading control.

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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. 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 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, 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-β 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.

REFERENCES

1. Barrett AJ, Davies ME, Grubb A. The place of human gamma-trace (cystatin C) amongst the cysteine proteinase inhibitors. Biochem Biophys Res Commun 1984; 120: 651-656
2. Newman DJ, Cystatin C. Ann Clin Biochem 2002; 39: 89-104
3. Koenig W, Twardella D, Brenner H, Rothenbacher D. Plasma concentrations of cystatin C in patients with coronary heart disease and risk for secondary cardiovascular events: more than simply a marker of glomerular filtration rate. Clin Chem 2005; 51: 321-327
4. Brzin J, Popovic T, Turk V, Borchart U, Machleidt W. Human cystatin, a new protein inhibitor of cysteine proteinases. Biochem Biophys Res Commun 1984; 118: 103-109
5. Ghiso J, Pons-Estel B, Frangione B. Hereditary cerebral amyloid angiopathy: the amyloid fibrils contain a protein which is a variant of cystatin C, an inhibitor of lysosomal cysteine proteases. Biochem Biophys Res Commun 1986; 136: 548-554
6. Bollengier F. Cystatin C, alias post-gamma-globulin: a marker for multiple sclerosis? J Clin Chem Clin Biochem 1987; 25: 589-593
7. Palsdottir A, Abrahamson M, Thorsteinsson L, Arnason A, Olafsson I, Grubb A, Jansson O. Mutation in cystatin C gene causes hereditary brain haemorrhage. Lancet 1988; 2: 603-604
8. Shi G, Sukhova GK, Grubb A, Ducharme A, Rhode LH, Lee RT, Ridker PM, Libby P, Chapman HA. Cystatin C deficiency in human atherosclerosis and aortic aneurysms. J Clin Invest 1999; 104: 1191-1197
9. Sokol JP, Schiemann WP. Cystatin C antagonizes transforming growth factor beta signaling in normal and cancer cells. Mol Cancer Res 2004; 2: 183-195
Sole M, Rawson C, Lindburg K, Barnes D. Transforming growth factor beta regulates cystatin C in serum-free mouse embryo (SFME) cells. Biochem Biophys Res Commun 1990; 172: 945-951

Gressner AM, Weisskirchen R, Breitkopf K, Dooley S. Roles of TGF-beta in hepatic fibrosis. Front Biosci 2002; 7: d793-d807

Takeuchi M, Fukuda Y, Nakano I, Kato Y, Hayakawa T. Elevation of serum cystatin C concentrations in patients with chronic liver disease. Eur J Gastroenterol Hepatol 2001; 13: 951-955

Chu SC, Wang CP, Chang YH, Hsieh YS, Yang SF, Su JM, Yang CC, Chiou HL. Increased cystatin C serum concentrations in patients with hepatic diseases of various severities. Clin Chim Acta 2004; 341: 133-138

Knook DL, Blansjaar N, Sleyvester EC. Isolation and characterization of Kupffer and endothelial cells from the rat liver. Exp Cell Res 1977; 109: 317-329

Schäfer S, Zerbe O, Gressner AM. The synthesis of proteoglycans in fat-storing cells of rat liver. Hepatology 1987; 7: 680-687

Gressner AM, Lotfi S, Gressner G, Hältner E, Kropf J. Synergism between hepatocytes and Kupffer cells in the activation of fat-storing cells (perisinusoidal lipocytes). J Hepatol 1993; 19: 117-132

Seglen PO. Preparation of isolated rat liver cells. Methods Cell Biol 1976; 13: 29-83

Fehrenbach H, Weisskirchen R, Kasper M, Gressner AM. Up-regulated expression of the receptor for advanced glycation end products in cultured rat hepatic stellate cells during transdifferentiation to myofibroblasts. Hepatology 2001; 34: 943-952

Cole T, Dickson PW, Esnard F, Averill S, Risbridger GP, Gauthier F, Schreiber G. The cDNA structure and expression analysis of the genes for the cysteine proteinase inhibitor cystatin C and for beta 2-microglobulin in rat brain. Eur J Biochem 1989; 186: 35-42

Brodin G, ten Dijke P, Funa K, Heldin CH, Landström M. Increased smad expression and activation are associated with apoptosis in normal and malignant prostate after castration. Cancer Res 1999; 59: 2731-2738

Weisskirchen R, Moser M, Weisskirchen S, Erdel M, Dahmen S, Buettner R, Gressner AM. LIM-domain protein cysteine- and glycine-rich protein 2 (CRP2) is a novel marker of hepatic stellate cells and binding partner of the protein inhibitor of activated STAT1. Biochem J 2001; 359: 485-496

Wolff B, Zsak M, Rabeck C. Immunofluorescence assay for the quantitative and qualitative evaluation of intracellular interleukin-8 in microtiter plates. Anal Biochem 1997; 244: 53-59

Dooley S, Hamzavi J, Breitkopf K, Wiercinska E, Said HM, Lorenzen J, Ten Dijke P, Gressner AM. Smad7 prevents activation of hepatic stellate cells and liver fibrosis in rats. Gastroenterology 2003; 125: 178-191

Dooley S, Delvoy B, Lahme B, Mangasser-Stephan K, Gressner AM. Modulation of transforming growth factor beta response and signaling during transdifferentiation of rat hepatic stellate cells to myofibroblasts. Hepatology 2000; 31: 1094-1106

Abrahamson M, Olafsson I, Palsdottir A, Ulvsbäck M, Lundwall A, Jnsson O, Grubb A. Structure and expression of the human cystatin C gene. Biochem J 1990; 268: 287-294

Dennler S, Ioth S, Vivien D, ten Dijke P, Huet S, Gauthier JM. Direct binding of Smad3 and Smad4 to critical TGF-beta-inducible elements in the promoter of human plasminogen activator inhibitor-type 1 gene. EMBO J 1998; 17: 3091-3100

Afonso S, Tovar C, Romagnano L, Babiarz B. Control and expression of cystatin C by mouse decidual cultures. Mol Reprod Dev 2002; 61: 155-163

Esnard F, Esnard A, Faucher D, Capony JP, Derancourt J, Brillard M, Gauthier F. Rat cystatin C: the complete amino acid sequence reveals a site for N-glycosylation. Biol Chem Hoppe Seyler 1990; 371 Suppl: 161-166

Murawaki Y, Yamada S, Koda M, Hirayama C. Collagenase and collagenolytic cathepsin in normal and fibrotic rat liver. J Biochem 1990; 108: 241-244

Leyo G, Tumminello FM, Pizzolanti G, Montalto G, Soresi M, Gebbia N. Lysosomal cathepsins B and L and Stefin A blood levels in patients with hepatocellular carcinoma and/or liver cirrhosis: potential clinical implications. Oncology 1997; 54: 79-83

Ray S, Lukyanov P, Ochieng J. Members of the cystatin superfamily interact with MMP-9 and protect it from autolytic degradation without affecting its gelatinolytic activities. Biochim Biophys Acta 2003; 1652: 91-102

Olafsson I, Gudmundsson G, Abrahamson M, Jnsson O, Grubb A. The amino terminal portion of cerebrospinal fluid cystatin C in hereditary cystatin C amyloid angiopathy is not truncated: direct sequence analysis from agarose gel electropherograms. Scand J Clin Lab Invest 1990; 50: 85-93

Velasco-Loyden G, Arribas J, López-Casillas F. The shedding of betaglycan is regulated by pervanadate and mediated by membrane type matrix metalloprotease-1. J Biol Chem 2004; 279: 7721-7733

Rotter D, Roth M, Lutz M, Lindemann D, Sebald W, Knaus P. Type III TGF-beta receptor-indpendent signalling of TGF-beta2 via TbetaRII-B, an alternatively spliced TGF-beta type II receptor. EMBO J 2001; 20: 480-490