Downregulation of syndecan-1 expression induces activation of hepatic stellate cells via the TGF-β1/Smad3 signaling pathway

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Abstract. The activation of hepatic stellate cells (HSCs) is considered associated with liver fibrosis. However, the exact role of syndecan-1 (SDC1), a protein that regulates the interaction between cells and the microenvironment, in the activation of HSCs resulting in liver fibrosis remains elusive. The objective of the present study was to explore the effects and mechanism of action of SDC1 in the activation of HSCs. HSCs were isolated from mouse liver and cultured to detect the expression of SDC1, transforming growth factor (TGF)-β1, Smad3 and α-smooth muscle actin (α-SMA; a marker of HSC activation) by western blotting and reverse transcription-quantitative PCR. The expression of SDC1 was found to be downregulated, while the expression of TGF-β1, Smad3 and α-SMA was upregulated in HSCs during cell culture. In addition, following stimulation of HSCs with recombinant SDC1, the expression of TGF-β1, Smad3 and α-SMA in HSCs was downregulated, whereas small interfering RNA targeting Smad3 antagonized the effects of recombinant SDC1 on α-SMA. Taken together, these data suggest that SDC1 plays a key role in the development of liver fibrosis.

Introduction

Liver fibrosis is an inevitable pathological event of cirrhosis that develops in a background of chronic liver disease induced by various pathogenic factors, including viral infection, poisoning, parasite infection, alcoholic hepatitis and fatty liver. The continuous and repeated hepatocyte inflammation or necrosis leads to a specific repair response in the body, frequently resulting in fibrous tissue hyperplasia, insufficient fiber degradation, extensive deposition of extracellular matrix (ECM) in the liver and development of liver fibrosis. Hepatic stellate cells (HSCs) are the major cells responsible for ECM synthesis in the liver, and their activation can create an imbalance between synthesis and degradation of ECM, with ensuing liver fibrosis (1). In chronic liver injury, the proliferation and phenotype of HSCs are altered. A switch occurs from the static phenotype, which is rich in vitamin A, to the activated myofibroblast-like phenotype, which is a hallmark of HSC activation (2). Various clinical investigations have indicated that liver fibrosis may be reduced or reversed by fiber degradation. In addition, early cirrhosis may also be reversed. The prognosis of chronic patients with liver disease is markedly improved by inhibition, alleviation or reversal of liver fibrosis. Thus, early diagnosis of liver fibrosis is crucial for arresting its development and improving the clinical outcome of patients with chronic liver disease (3).

The name syndecan-1 (SDC1) originates from the Greek word ‘syndein’, which means the binding of cell microenvironment components with the cytoskeleton. This protein regulates the interaction between cells and the microenvironment, and acts as a cell-surface co-receptor that participates in the regulation of a series of physiological processes, such as differentiation and development of tissues and organs, vascularization and tissue regeneration (4). The SDC proteins are removed from the cell surface by trypsin in cultured epithelial cells. This causes the cells to lose their adhesion characteristics and to exhibit pleiomorphic-like anchoring non-dependent growth. The full-length cDNA sequence of SDC1 can be integrated into the aforementioned cells in order to construct a stably expressing SDC1 cell line. The epithelial cell shape and growth characteristics can recover in the presence of constitutive SDC1 expression (5,6). However, whether SDC1 has an important role in the activation of HSCs has not been previously reported. The aim of the present study was to investigate the functional expression of the SDC1 protein in mouse HSCs.

A number of previous studies have indicated that the transforming growth factor-β1 (TGF-β1)/Smad3 signaling pathway participates in the development of liver fibrosis (7,8). It was recently demonstrated that the SDC family of proteins is an important regulatory factor in the TGF-β1/Smad3 signaling pathway (9). It remains unclear whether this signaling pathway...
is also regulated by SDC1 during liver fibrosis. The effects and possible mechanism underlying the role of SDC1 in the development of liver fibrosis were investigated at the cellular and molecular levels in the current study, hoping to provide a new target for the prevention and treatment of this disease.

The phenotypic transformation of myofibroblasts through HSC activation is considered as the hallmark of liver fibrosis. In the present study, mouse HSCs were isolated and cultured in vitro. Freshly isolated HSCs are spontaneously activated during in vitro culture. This model mimics the process of liver injury. Thus, in the present study, the changes in the mRNA and protein levels of α-smooth muscle actin (α-SMA), a marker of HSC activation, SDC1 and TGF-β1/Smad3 signaling pathway-related proteins were investigated during the spontaneous activation of freshly isolated mouse HSCs that were cultured in vitro. At specific time points (days 1, 3 and 7), reverse transcription-quantitative PCR (RT-qPCR) analysis was used to quantitatively measure the mRNA levels of the aforementioned markers, whereas western blotting was used to quantitatively analyze their corresponding protein levels. To further analyze the effect of SDC1 on the activation of HSCs, SDC1 recombinant protein was used to stimulate HSCs. Smad3-siRNA transfection resulted in the activation of mouse HSCs, whereas SDC1 recombinant protein was also used to stimulate HSCs and observe their activated status.

Materials and methods

Animals and HSC isolation. Male Kunming mice (8-10 weeks old, 22±2 g) were provided by CHI Scientific, Inc. The mice were all raised under standard laboratory conditions with free access to food and water, and were allowed to acclimatize for 1 week. The experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals of China Jiaxing University, and approved by the Ethics Committee of Animal Experimentation. All the experimental procedures were performed with the permission and under the surveillance of the institutional ethics committee. Pronase E (0.05%) and type IV collagenase (0.1%) were used to perfuse mouse livers; the HSCs were isolated by Nycodenz density gradient centrifugation at 4°C for 20 min (1,400 x g) and cultured. The cell yield was measured by cell counting method, and the survival rate of the cells was measured by Trypan blue staining (1 g/l) for 3 min at 25°C. The isolated HSCs were seeded in DMEM containing 10% FBS at 1x10^5/cm^2. At specific time points (days 1, 3 and 7), reverse transcription-quantitative PCR (RT-qPCR) was used to analyze the expression of the genes of interest. The amplification was performed using 40 cycles of 95°C for 30 sec, 95°C for 5 sec and 60°C for 34 sec. The 2^-ΔΔCq method was used for normalization of raw data using the housekeeping gene GAPDH. The results were repeated at least three times. The sequences of the primers were as follows: GAPDH, forward 5'-TGG GAA GAT GAG ATG GTT-3' and reverse 5'-CCCTCCTGGGACGATGAT-3'; α-SMA, forward 5'-GCTGCTCCAGTATGTTGA-3' and reverse 5'-GTTCTTCCATGTGCTGACAGT-3'; and SDC1, forward 5'-CAGCGACAAACCGAGAC-3', SDC1, forward 5'-CCTGGGCAAGTTTCCAGAG-3' and reverse 5'-CCA TCGCTGGCATCTTGTG-3'.

Western blot analysis. RIPA lysis buffer (Zhaoji Boster) was used to extract total protein from HSCs, and the protein concentration was measured using a UV spectrophotometer following the bichinchoninic acid method. Proteins were separated on a 10% SDS-PAGE gel and transferred to a PVDF membrane (EMD Millipore). Subsequently, the membrane was blocked with 5% BSA (Zhaoji Boster) in TBS with Tween-20 for 1 h at room temperature and incubated overnight at 4°C with the primary antibodies, including anti-SDC1 (1:1,000; cat. no. BM3896; Wuhan Boster Biological Technology, Ltd.) and anti-GAPDH (1:1,000; cat. no. BM3896; Wuhan Boster Biological Technology, Ltd.) was used as an internal reference, and the densities of the western blot bands were analyzed using Image Lab Software 5.2.1 (Bio-Rad Laboratories, Inc.)

Exogenous SDC1 stimulation. After 6 days of incubation, the isolated HSCs were seeded in a 96-well plate at a density of 1x10^5 cells/ml. Following cell adherence, serum-free culture...
medium was used to replace the medium after 24 h. The cells were divided into three groups and incubated with recombinant SDC1 protein solution at concentrations of 10 and 20 ng/ml in order to stimulate the cells for 24 h. Subsequently, the cells were collected for α-SMA, SMAD3 and TGF-β1 detection.

Small interfering RNA (siRNA) targeting of mouse Smad3. siRNA targeting was performed for the cDNA sequence of mouse Smad3. Guangzhou RiboBio Co., Ltd synthesized the oligonucleotides. The sequences were as follows: 5'-CAG UCU ACCUCUCCAGUGU UdTd T-3' (siRNA-1), 5'-CCA UGACGUAUAGGC UdTd T-3' (siRNA-2) and 5'-CGC AGA CUGU AACCAC AdTd T-3' (siRNA-3). A negative control siRNA (siRNanc) was used as control (5'-UAUCAC UGCGAUACAU GC UdTd T-3'). To measure the knockdown efficacy of different siRNA oligonucleotides, primary cultured mouse HSCs (2x10^5 cells/ml) were transfected with 16 μg/ml siRNAs (siRNanc, siRNA1, siRNA2 and siRNA3) using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). Following transfection, the cells were cultured for 48 h and the inhibitory effects of the different siRNA oligonucleotides were determined by measuring Smad3 mRNA using RT-qPCR. The siRNA molecule with the highest inhibitory rate was selected for subsequent experiments.

Statistical analysis. The statistical analysis of the data was performed using SPSS 15.0 statistical software for Windows (SPSS, Inc.). The results are presented as mean ± SEM. The data were analyzed by the one-way ANOVA or repeated-measures ANOVA tests followed by Tukey's post-hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

Spontaneous activation of mouse-isolated HSCs during culture. The results of the western blot analysis indicated that the relative expression levels of α-SMA on days 3 and 7 were increased compared with those of the cultured HSCs on day 1 (P<0.05; n=6; Fig. 1A). The relative expression levels of α-SMA on day 7 were significantly increased compared with those on day 3 (P<0.05; n=6; Fig. 1A). These data demonstrated that the expression of α-SMA increased in a time-dependent manner. The results of the RT-qPCR analysis were consistent with the aforementioned data on the protein expression of α-SMA.
α-SMA. The α-SMA mRNA levels of the aforementioned groups exhibited significant differences (P<0.05; n=6; Fig. 1B). Immunofluorescence staining indicated that, after 7 days of HSC culture, α-SMA protein was highly expressed in myocytes that were transformed into myofibroblasts (Fig. 1D). Double immunofluorescence staining indicated cellular co-localization of α-SMA and muscle markers (desmin) in HSCs cultured for 1 and 7 days (Fig. 1C and D, respectively). The aforementioned experimental results highlighted that the isolated HSCs had undergone transformation from static to activated cells during *in vitro* culture.

**Expression of SDC1 is downregulated during HSC culture.** The results of the western blot analysis indicated that the protein expression of the SDC1 was significantly downregulated following culture of HSCs for 3 and 7 days compared with that of the HSCs on day 1 (P<0.05; n=6; Fig. 2A). The relative expression levels of the SDC1 protein on day 7 were also significantly decreased compared with those of the cultured HSCs on day 3 (P<0.05; n=6; Fig. 2A). The results of the SDC1 mRNA levels as detected by RT-qPCR were consistent with those of the western blot assay (P<0.05; n=6; Fig. 2B). Upon activation of HSCs, the expression levels of SDC1 exhibited a time-dependent decrease.

**TGF-β1/Smad3 signaling pathway is activated during HSC culture.** The results of the western blot analysis indicated that HSCs isolated and cultured on day 1 exhibited upregulation of the TGF-β1 and Smad3 proteins on days 3 and 7 (P<0.05; n=6; Fig. 3). The relative expression levels of the TGF-β1 and Smad3 proteins on day 7 were further upregulated compared with those observed on day 3 (P<0.05; n=6; Fig. 3). In the present study, the TGF-β1/Smad3 signaling pathway was also activated during the HSC culture.

**SDC1 negatively regulates the activation of HSCs and the TGF-β1/Smad3 signaling pathway during HSC culture.** Having demonstrated that the protein expression levels of α-SMA, TGF-β1 and Smad3 in HSCs cultured for 7 days were significantly increased, recombinant SDC1 protein (10 and 20 ng/ml) was added to stimulate HSCs for 24 h on day 6. The expression levels of α-SMA, TGF-β1 and Smad3 were significantly downregulated compared with those of the control group (P<0.05; n=6; Fig. 4). Thus, it was deduced that SDC1 negatively affects the activation of HSCs via the TGF-β1/Smad signaling pathway.
Smad3 expression is successfully silenced by Smad3-siRNA. In order to select a siRNA oligonucleotide for efficient knockdown of Smad3, three siRNA oligonucleotides targeting mouse Smad3 were co-transfected to HSCs isolated from mice. The transfection was performed after 7 days of initial HSC culture, and the Smad3 mRNA expression levels were evaluated by RT-qPCR. siRNA1 and siRNA3 achieved effective knockdown of the Smad3 protein, with siRNA1 exhibiting the highest efficiency (P<0.05; n=6; Fig. 5). Therefore, siRNA1 was selected to knock down Smad3 in subsequent experiment.

SDC1 negatively regulates the levels of α-SMA upon HSC activation via the TGF-β1/Smad3 signaling pathway. As shown in Fig. 6, the expression levels of α-SMA in HSCs cultured for 7 days were measured by RT-qPCR and western blot assays. SDC1 stimulation significantly decreased the expression levels of α-SMA in HSCs compared with those of the control groups (P<0.01; n=6; Fig. 6). Pretreatment with Smad3-siRNA reduced the effects of SDC1, which increased the expression levels of α-SMA (P<0.01; n=6; Fig. 6). These data suggested that SDC1 markedly inhibits HSC activation, which is likely mediated by activation of the TGF-β1/Smad3 signaling pathway.

Discussion
Liver fibrosis is an irreversible process caused by progression of chronic liver disease of varying etiologies. Prevention and early intervention to target fibrotic tissues is an effective measure for stabilizing the disease and preventing progression from liver fibrosis to cirrhosis, which can ultimately prevent the development of liver cancer (11). Thus, the exploration of the development of liver fibrosis is crucial for the prevention and treatment of chronic liver diseases (12). HSCs belong to the family of hepatic stromal cells, accounting for 5-8% of total liver cells. Static HSCs are in a non-proliferative state and do not express α-SMA. Upon liver injury, the phenotype of static HSCs is altered, resulting in the formation of myofibroblasts. These cells have a high proliferative rate and express α-SMA. α-SMA promotes the transformation of HSCs into myofibroblasts and may be used as a clinical diagnostic index for the initial stages of liver fibrosis (13).

Freshly isolated HSCs can be spontaneously activated during in vitro culture. The magnitude of activation is parallel to the induction of liver injury. Thus, mouse HSCs were used as a model in the present study. Western blot analysis and immunofluorescence were used to detect the relevant mechanism underlying HSC activation. The aim of these experiments was to obtain crucial information on HSC activation that may prove useful in the prevention and treatment of liver fibrosis. The results indicated that the expression of α-SMA was increased in a time-dependent manner in an HSC culture. Immunofluorescent staining demonstrated that the expression levels of α-SMA were high on day 7, indicating that HSCs had differentiated to...
myofibroblasts, transforming from a static to an activated state.

The development of liver fibrosis is regulated by multiple growth factors, such as TGF, platelet-derived growth factor and epidermal growth factor, which are essential regulatory factors for HSC activation and proliferation (14,15). The TGF-β1/Smad signaling pathway plays an important role in the development of liver fibrosis, although its exact mechanism of action remains unclear. Several studies to date have demonstrated that the TGF-β1/Smad signaling pathway participates in the activation of HSCs (16). The results of the present study are in accordance with this conclusion. The inhibition of the classic or the non-classic TGF-β1/Smad signaling pathway may inhibit the proliferation of HSCs and induce apoptosis. However, the exact mechanism through which this process affects liver function is unclear. Furthermore, the direct inhibition of this pathway may have several adverse effects. Recent studies demonstrated that the SDC family of proteins regulates the TGF-β1/Smad signaling pathway, thus playing an important role in tissue differentiation, organ formation and liver disease progression (17,18).

SDC1 is a surface transmembrane proteoglycan that acts as a membrane adhesion receptor. SDC1 is an important component of the intercellular region and of the plasma membrane (19). SDC1 regulates the interaction between cells and the microenvironment by covalent binding to ligands, and participates in physiological processes, such as vascularization and tissue regeneration. Its expression is highly regulated and is cell type- and developmental stage-specific (20). The cells employ integrin and SDC1 receptors in order to mediate cell-ECM adhesion, cell proliferation and differentiation, and tumor growth inhibition (21). The present study demonstrated that, during HSC culture in vitro, the SDC1 mRNA and protein expression levels decreased in a time-dependent manner. Although it is reported that SDC1 is upregulated in patients with liver cirrhosis (22), a recent study demonstrated that SDC1 inhibits the early stages of liver fibrogenesis by interfering with the action of TGF-β1 and upregulating matrix metalloproteinase 14 (23). There is a change in SDC1 over time; its expression during the earlier stages of fibrogenesis is higher in liver cells, whereas it starts to decrease during the later stages (23). These results indicated that SDC1 may also participate in the regulation of HSC activation.

In the current study, there was a negative association of SDC1 with TGF-β1, Smad-3 and SMA. The activation of HSCs was reduced following treatment with SDC1 recombinant protein. Concomitantly, the levels of the TGF-β1/Smad3 pathway protein expression were reduced, in accordance with a recent study demonstrating that the overexpression of SDC1 decreases TGF-β1 signaling (23). Treatment with SDC1 inhibited the Smad3-siRNA-induced decrease in the levels of α-SMA compared with that in the control group. These results indicated that SDC1 is involved in the regulation of HSC activation, which is mediated by the TGF-β1/Smad pathway.

In summary, the present study demonstrated that reduced SDC1 expression induces activation of HSCs via the TGF-β1/Smad3 signaling pathway. These findings provide evidence supporting a regulatory role of SDC1 in the activation of HSCs via TGF-β1/Smad3 signaling. The identification of this novel mechanism may prove to be useful in the prevention and treatment of liver fibrosis.
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Availability of materials and data

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

MD, XS and MY performed the experiments and analyzed the data; LX, XX and ZZ interpreted the experimental results; MD and LX drafted the manuscript; MD and MY edited and revised the manuscript; MY was responsible for the conception and design of the research. All authors have read and approved the final version of this manuscript.

Ethics approval and consent to participate

Experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals of China Jiaxing University, and approved by the Ethical Committee of Animal Experimentation. All the laboratory procedures were performed with the permission and under the surveillance of the institutional ethics committee.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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