



Connective tissue growth factor hammerhead ribozyme attenuates human hepatic stellate cell function

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Abstract

AIM: To determine the effect of hammerhead ribozyme targeting connective tissue growth factor (CCN2) on human hepatic stellate cell (HSC) function.

METHODS: CCN2 hammerhead ribozyme cDNA plus two self-cleaving sequences were inserted into pTriEx2 to produce pTriCCN2-Rz. Each vector was individually transfected into cultured LX-2 human HSCs, which were then stimulated by addition of transforming growth factor (TGF)- β 1 to the culture medium. Semi-quantitative RT-PCR was used to determine mRNA levels for CCN2 or collagen I, while protein levels of each molecule in cell lysates and conditioned medium were measured by ELISA. Cell-cycle progression of the transfected cells was assessed by flow cytometry.

RESULTS: In pTriEx2-transfected LX-2 cells, TGF- β 1 treatment caused an increase in the mRNA level for CCN2 or collagen I, and an increase in produced and secreted CCN2 or extracellular collagen I protein levels. pTriCCN2-Rz-transfected LX-2 cells showed decreased basal CCN2 or collagen mRNA levels, as well as produced and secreted CCN2 or collagen I protein. Furthermore, the TGF- β 1-induced increase in mRNA or protein for CCN2 or collagen I was inhibited partially in pTriCCN2-Rz-transfected LX-2 cells. Inhibition of

CCN2 using hammerhead ribozyme cDNA resulted in fewer of the cells transitioning into S phase.

CONCLUSION: Endogenous CCN2 is a mediator of basal or TGF- β 1-induced collagen I production in human HSCs and regulates entry of the cells into S phase.

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Key words: Connective tissue growth factor; Fibrosis; Hepatic stellate cell; Transforming growth factor- β 1

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INTRODUCTION

Activation of hepatic stellate cells (HSCs) is a central event in the pathobiology of hepatic fibrosis^[1,2]. In response to chronic liver injury, quiescent HSCs undergo gradual phenotypic changes that result in their trans-differentiation into α -smooth muscle actin (SMA)-positive, proliferative, myofibroblast-like cells. In the injured liver, activated HSCs are responsible for producing much of the excess extracellular matrix, including fibrillar collagen^[1,2]. At the molecular level, this process is driven by a variety of growth factors, cytokines and matricellular proteins. While transforming growth factor (TGF)- β has received special attention because of its potent fibrogenic effects *in vivo* and its ability to stimulate fibrogenic pathways in HSCs^[3], targeting of this molecule for therapeutic intervention is problematic because of its other important actions in the liver, including tumor suppression and immune modulation. However, a potentially more attractive target is connective tissue growth factor (CCN2), which appears to mediate many of the fibrogenic properties of TGF- β ^[4].

CCN2 was discovered as a TGF- β -immediate early gene in 1989^[5] and numerous studies since then have described an intimate relationship between these molecules in many fibrotic diseases, including those of the liver^[6]. CCN2 is recognized increasingly as a central player in hepatic fibrosis and may offer new options for prognosis, diagnosis and therapy^[7]. Previously, we have shown that exposure of HSCs to CCN2 induces cell adhesion, migration and proliferation, the latter of which is associated with transient induction of c-fos and activation of the extracellular signal-regulated kinase 1/2 signaling pathway^[8-10]. In addition, CCN2 induces expression of α -SMA and type I collagen in HSCs, consistent with a role in activation and fibrogenesis^[8]. We have shown that adhesive signaling by CCN2 in HSCs increases expression of collagen type I, fibronectin, and tissue inhibitor of metalloproteinase (TIMP)-1, and decreases expression of caspase 8 and hepatocyte growth factor^[11]. CCN2 also stimulates survival pathways in activated HSCs^[12]. These data suggest that CCN2 drives both fibrogenic and anti-apoptotic pathways in activated HSCs and reinforce the notion that CCN2 is a potential novel therapeutic target in liver fibrosis.

We and others have shown that CCN2 mRNA and protein are expressed increasingly during progressive activation of cultured primary rat HSCs, or in response of the cells to stimulation by TGF- β , vascular endothelial growth factor, lipid peroxidation products, acetaldehyde or platelet-derived growth factor-BB^[8,13-16]. We have shown further that CCN2 promoter activity is enhanced in a Smad7-dependent fashion by TGF- β in primary rat HSCs transfected with the CCN2 promoter, luciferase reporter construct^[10], and that CCN2 production in the HSCs is stimulated by TGF- β ^[6]. We recently showed that TGF- β -induced CCN2 promoter activity in activated mouse HSCs requires Smad and Ets-1 elements in the CCN2 promoter^[16], as described for normal fibroblasts or mesangial cells^[17-20]. However, CCN2 promoter activity in activated HSCs is uniquely antagonized by ALK4/5/7 inhibition^[16]. CCN2 gene regulation in activated HSCs is distinct from that of scleroderma, which is independent of Smad/ALK5-mediated TGF- β signaling, but is dependent on endothelin-1^[17,21,22], or pancreatic cancer, which depends on activated ras/MEK/ERK rather than TGF- β or endothelin-1^[23].

Surprisingly, there is no information regarding the role of basal or TGF- β -induced CCN2 in the function of human HSCs. In the present study, we investigated the effect of a hammerhead ribozyme that targets CCN2 on TGF- β 1-induced collagen I synthesis or cell-cycle progression in the LX-2 human HSC line, which was generated originally by spontaneous immortalization of primary human HSCs in low-serum conditions^[24].

MATERIALS AND METHODS

Reagents

Dulbecco's Modified Eagle's Medium (DMEM),

Lipofectamine™ 2000 and TRIzol were from Life Technologies (Carlsbad, CA, USA). Fetal bovine serum (FBS) was obtained from Hyclone (Logan, UT, USA). Human TGF- β 1 was purchased from PeproTech (Rocky Hill, NJ, USA). The human HSC line, LX-2, was a kind gift of Dr. Scott Friedman (Mount Sinai Hospital, New York). pTriEx2 vector was obtained from Novagen (Gibbstown, NJ, USA). *Xho* I and *Nco* I were from Toyobo (Osaka, Japan). Avian myeloblastosis virus and Taq DNA polymerase were from Promega (Madison, WI, USA). ELISA kits for human CCN2 or collagen I were from, respectively, USCN Life Science and Technology Co. (TX, USA) or Cosmo Bio (Tokyo, Japan).

Ribozyme design and recombinant plasmid construction

The mRNA sequence of human CCN2 (NCBI, gi: 98986335) was scanned for potential hammerhead ribozyme cleavage sites using proprietary design software developed by the Shanghai Institute of Biological Chemistry of the Chinese Academy of Sciences. Of the five potential hammerhead ribozyme cleavage sites, one site located at 896 of CCN2 mRNA was identified with optimal secondary folding. A 34-mer hammerhead ribozyme cDNA that targeted at the C-U-A at position 896 of CCN2 mRNA (sense: 5'-CTTCTCCTGATGAGTCCGTGAGGACGAAAGCCTG-3'; antisense: 5'-CAGGCTTTCGTCTCACGGACTCATCAGGAGAAG-3'), plus two self-cleaving sequences, were synthesized chemically and inserted into pBluescript II SK9(-) cloning vector. Both pTriEx2 and pBluescript II SK9(-) were linearized with *Xho* I and *Nco* I individually, and the digested products were ligated using T4 DNA ligase. The resulting vector, termed pTriCCN2-Rz, (in which synthesis of the ribozyme was driven by the chicken β -actin promoter and the cytomegalovirus enhancer) was confirmed by DNA sequencing.

Cell culture and transfection

LX-2 cells were allowed to grow at 37°C for 2 d in DMEM that contained 10% FBS, 100 U/mL penicillin and 100 μ g/mL streptomycin. The cells were digested with 0.25% trypsin, and washed with Hank's Balanced Salt Solution (without Ca²⁺ or Mg²⁺). The cells were then cultured in either 20 \times 100-mm cell culture dishes for RNA isolation and determination of cell-cycle progression, or 12-well plates for measurement of production and secretion of CCN2 or collagen I. Prior to transfection, the cells were grown in DMEM that contained 2.5% FBS for 24 h, followed by DMEM that contained 0.1% FBS for another 24 h. Cells were transfected with vectors using Lipofectomine™ 2000 reagent under serum-free conditions for 4 h. The transfected cells were incubated for another 24 h in the presence or absence of 20 ng/mL TGF- β 1.

Semi-RT-PCR

Total RNA was extracted from cultured LX-2 cells using TRIzol reagent. An optimal RNA template (0.8 μ g)

was generated by semi-quantitative RT-PCR using a UNOII thermocycler (Biometra, Germany). The expression of mRNA for human CCN2, collagen $\alpha 1$ (I), or β -actin was examined by RT-PCR using the following forward and reverse primers: CCN2 forward, 5'-CCTGGTCCAGACCACAGAGT-3'; CCN2 reverse, 5'-ATGTCTTCATGCTGGTGCAG-3'; collagen $\alpha 1$ (I) forward, 5'-CCTCAAGGGCTCCAACGAG-3'; collagen $\alpha 1$ (I) reverse, 5'-TCAATCACTGTCTTGCCCCA-3'; β -actin forward, 5'-GTCCTCTCCCAAGTCCACAC-3'; β -actin reverse, 5'-GGGAGACCAAAAGCCTTCAT-3'.

Briefly, 0.8 μ g RNA was reverse transcribed using Moloney murine leukemia virus reverse transcriptase in the presence of oligo(dT) and dNTP, followed by amplification of cDNA (1 μ L), using Taq DNA polymerase in a final volume of 50 μ L that contained 1 mmol/L $MgCl_2$, 0.2 μ mol/L of each dNTP, and 0.5 μ mol/L of each primer. Amplification conditions were 94°C for 30 s, 58°C for 30 s, and 72°C for 1 min, over 32 cycles. Amplification products were visualized on ethidium-bromide-stained agarose gels. Image analysis of the individual transcripts was carried out using Gel-Pro 3.2 software (Media Cybernetics, Bethesda, MD, USA). Data were expressed as the gray-scale ratio of either CCN2 mRNA or collagen $\alpha 1$ (I) mRNA relative to that of β -actin mRNA.

CCN2 or collagen I ELISA

CCN2 or collagen I levels were measured in cell lysates or conditioned medium of serum-starved, cultured LX-2 cells in the absence or presence of 20 ng/mL TGF- $\beta 1$ for 24 h using a sandwich ELISA following the manufacturer's instructions for each kit. Briefly, microtiter wells were pre-coated for 2 h (CCN2) or 30 min (collagen I) at 37°C, with 100 μ L of each standard, or 1:20 dilutions of LX-2 cell lysates or conditioned medium supernatant. The plates were then developed by addition of biotinylated anti-CCN2 antibody or anti-collagen I antibody, followed by avidin-conjugated horseradish peroxidase. The color reaction was developed using tetramethylbenzidine substrate solution and measured by its absorbance at 450 nm using a Model 550 plate reader (BioRad, Hercules, CA, USA).

Flow cytometry

LX-2 cell-cycle progression was determined by resuspending the cells at 1×10^6 cells/mL in PBS, fixing the cells with 75% ethanol overnight, and then staining the cells with 0.1 μ g/mL propidium iodide in a 0.1% sodium citrate/0.1% Triton X-100 solution for 30 min at room temperature in the dark, in the presence of 0.2 mg/mL Rnase. Analysis of cellular DNA content after cell staining with propidium iodide was performed by flow cytometry at an excitation wavelength of 488 nm. The distribution of cells in three major phases of the cycle (G0/G1, S, G2/M) was analyzed using CellQuest software (BD Biosciences, San Jose, CA, USA).

Statistical analysis

The values reported represent the mean \pm SD of the

measurements of at least four different experiments. Photographs of RT-PCR products are representative of at least three independent experiments. Statistical analysis of the data was performed using SPSS for Windows version 11 (SPSS, Chicago, IL, USA). The Student *t* test was used for paired data that were normally distributed, and *P* < 0.05 was considered significant.

RESULTS

Effects of hammerhead ribozyme on CCN2 mRNA and protein expression in cultured LX-2 cells

Previous studies have demonstrated that the LX-2 cell line contains CCN2 mRNA^[25], a feature that is characteristic of the activated HSC phenotype^[6]. Since many TGF- β pro-fibrogenic activities are mediated by CCN2^[26] and TGF- β stimulates collagen production in LX-2 cells^[24], we examined the functional relationship between TGF- β and CCN2, utilizing hammerhead ribozymes designed to cleave CCN2 mRNA in the cells. Initial experiments were performed in which the LacZ reporter gene was inserted downstream of CCN2 hammerhead ribozyme cDNA in pTriCCN2-Rz, and into the downstream multiple cloning site of pTriEx2, to investigate the transcriptional activity of both vectors. When histochemical X-gal staining was performed on cell cultures transfected with either pTriEx2 or pTriCCN2-Rz individually, about 30% of the cells were positive for each vector (data not shown), and this transfection efficiency was sufficient to assess subsequently the functional effect of the hammerhead ribozyme that targeted CCN2.

CCN2 mRNA and protein in pTriEX-2-transfected LX-2 cells were present under basal growth conditions and there was an approximately 2.5- and 3.5-fold increase, respectively, in CCN2 transcript and protein levels (*P* < 0.01, paired *t* test) after stimulation of the cells with TGF- β for 24 h (Figures 1A and 2A). In contrast, after transfection with the pTriCCN2-Rz vector, the LX-2 cells demonstrated reduced constitutive expression of CCN2 mRNA and protein (*P* < 0.05) under baseline conditions and moreover, the stimulation of CCN2 mRNA and protein (*P* < 0.05) in response to TGF- β was attenuated substantially (*P* < 0.05). These data confirmed the ability of the hammerhead ribozymes to target CCN2 mRNA and to block CCN2 production and secretion, thus allowing for its function in LX-2 cells to be further explored.

Effects of hammerhead ribozyme on TGF- $\beta 1$ -induced collagen I mRNA and protein expression in cultured LX-2 cells

Under basal conditions, pTriEx-2-transfected LX-2 cells produced collagen $\alpha 1$ (I) mRNA and the amount of this transcript was enhanced two-fold by TGF- β treatment (*P* < 0.01; Figure 1B). On the other hand, under basal conditions, pTriCCN2-Rz-transfected LX-2 cells produced approximately 60% of the level of collagen $\alpha 1$ (I) mRNA as control cells (*P* < 0.05). Whereas TGF- β treatment of pTriCCN2-Rz-transfected

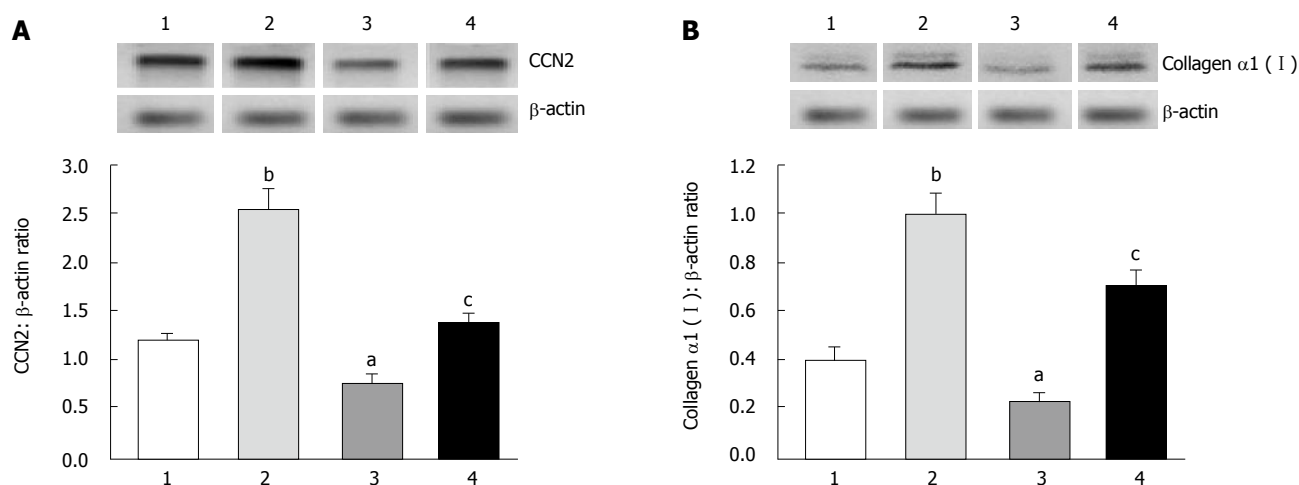


Figure 1 Effect of CCN2 hammerhead ribozyme on basal or TGF- β -induced mRNA expression of CCN2 or collagen $\alpha 1$ (I). The figure shows the RT-PCR reaction products (upper panel) and the densitometric scans (lower panel) for (A) CCN2 mRNA or (B) collagen $\alpha 1$ (I) after transfection of LX-2 cells with pTriEx2 (lanes 1 and 2) or pTriCCN2-Rz (lanes 3 and 4) under basal conditions (lanes 1 and 3), or after stimulation with 20 ng/mL TGF- $\beta 1$ for 24 h (lanes 2 and 4). ^a $P < 0.05$ vs lane 1; ^b $P < 0.01$ vs lane 1; ^c $P < 0.05$ vs lane 2.

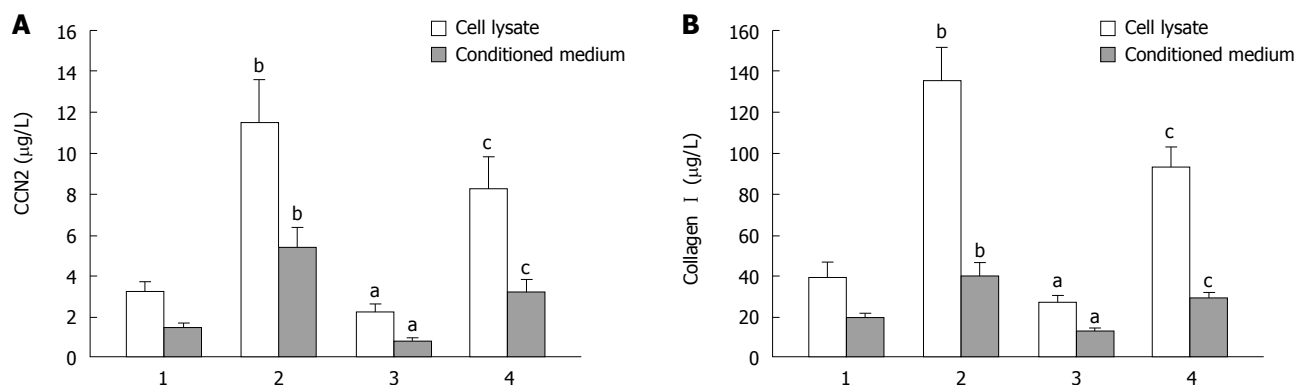


Figure 2 Effect of CCN2 hammerhead ribozyme on basal or TGF- β -induced protein production or secretion of CCN2 or collagen I. The figure shows the protein levels of CCN2 (A) or collagen I (B) in cell lysates or conditioned medium measured by ELISA after transfection of the cells with pTriEx2 (lanes 1 and 2) or pTriCCN2-Rz (lanes 3 and 4) under basal conditions (lanes 1 and 3), or after stimulation with 20 ng/mL TGF- $\beta 1$ for 24 h (lanes 2 and 4). ^a $P < 0.05$ vs lane 1; ^b $P < 0.01$ vs lane 1; ^c $P < 0.05$ vs lane 2.

LX-2 cells resulted in stimulation of the amount of collagen $\alpha 1$ (I) mRNA, the level attained was only 70% of that seen in TGF- β -stimulated control cells ($P < 0.05$; Figure 1B). Essentially identical results were obtained when cell lysates or conditioned medium from the cells were tested by ELISA, in that basal and TGF- β -stimulated collagen I protein production and secretion was attenuated significantly in pTriCCN2-Rz-transfected LX-2 cells as compared to those transfected with pTriEx-2 ($P < 0.05$; Figure 2).

Effects of hammerhead ribozyme on LX-2 cell-cycle progression

We next used flow cytometry to assess the effect of basal CCN2 expression on LX-2 cell-cycle progression. As shown in Table 1, after pTriCCN2-Rz transfection, a relatively higher proportion of cells were in the G0/G1 phase ($P < 0.05$) and a lower proportion were in S phase ($P < 0.05$), as compared to pTriEx2-transfected cells. This showed that endogenous CCN2 played a role in the G0/G1 \rightarrow S transition in LX-2 cells. To verify that

this difference was not caused by greater cytotoxicity of pTriCCN2-Rz than pTriEx2, the viability of transfected LX-2 cells was verified by trypan blue exclusion assay. Less than 3% of the pTriEx2- or pTriCCN2-Rz-transfected cells stained blue, and there was no difference in the frequency of trypan-blue-positive cells between pTriEx2 and pTriCCN2-Rz transfection (data not shown). Moreover, comparable amounts of β -actin mRNA were amplified from pTriEx2- or pTriCCN2-Rz-transfected cells (Figure 1).

DISCUSSION

In the present study, a recombinant vector, pTriCCN2-Rz, bearing hammerhead ribozyme cDNA plus two self-cleaving sequences that targeted the 896 site of CCN2 mRNA, was constructed and transfected into the LX-2 human HSC line. The hammerhead ribozyme blocked CCN2 mRNA transcription and protein production in LX-2 cells and inhibited basal or TGF- $\beta 1$ -induced transcription and production of collagen I in LX-2 cells.

Table 1 Distribution of cell-cycle phases in pTriEx2- or pTriCCN2-Rz-transfected LX-2 cells

Vector	G0G1 (%)	S (%)	G2M (%)
pTriEx2	60.64 ± 2.26	27.53 ± 1.76	11.83 ± 1.36
pTriCCN2-Rz	80.45 ± 3.12 ^a	10.83 ± 1.02 ^b	8.27 ± 0.68

^a*P* < 0.05, ^b*P* < 0.01 compared with pTriEx2.

That the knockdown achieved in these experiments was only partial was likely attributable to the transfection efficiency of 30%, but even so, the data clearly showed a CCN2-dependency of basal or TGF- β -stimulated collagen I production in activated human HSCs. This finding, coupled with the ability of hammerhead ribozyme targeting of CCN2 to attenuate the rate of HSC proliferation, suggest that it is a useful therapeutic strategy in fibrosis of the liver and possibly, of other organ systems.

In recent years, the relationship between CCN2 and TGF- β in fibrogenic pathways has been explored using blocking strategies that exploit the targeting of CCN2 mRNA with antisense oligonucleotides or small interfering RNA (siRNA)^[27]. The use of CCN2 antisense oligonucleotides has helped to establish the importance of CCN2 for TGF- β -induced collagen production in a variety of cell types including kidney mesangial cells, NRK cells, corneal fibroblasts, and conjunctival fibroblasts^[28-33]. In *in vivo* animal models, CCN2 antisense oligonucleotides have proven effective in reducing matrix expansion or fibrosis in the skin or kidney^[34-36], and scarring around breast implants^[37]. However, while CCN2 antisense therapy in CCl₄-induced liver fibrosis is associated with reduced expression of mRNA for CCN2 or collagen, fibrous deposits are not reduced, an effect that has been attributed to high expression of TIMP-1^[38].

Insight into fibrogenic mechanisms has also been obtained from experiments that employ CCN2 siRNA technology that relies on short dsRNA for gene silencing, and shows improved potency and specificity as compared to antisense oligonucleotides^[27]. CCN2 siRNA was effective in blocking collagen synthesis in scleroderma fibroblasts^[39], glucose-induced matrix production in vascular smooth muscle cells^[40], or TGF- β -stimulated collagen production in dermal fibroblasts, osteoblasts or rat HSCs^[41-43]. Plasmids expressing short hairpin RNA, which are easier to produce and more effective than some traditional siRNA approaches, have been shown to disrupt CCN2 gene expression in cultured rat HSCs, and to cause marked attenuation in the production of collagen III and IV, laminin, and hyaluronic acid^[44]. Finally, *in vivo* studies have shown that CCN2 siRNA delivery to rats is an effective anti-fibrotic therapy in renal allografts after transplantation or in livers after exposure to N-nitrosodimethylamine^[45,46]. In mice, an HSC-specific targeting strategy has been employed for CCN2 siRNA therapy in CCl₄-induced hepatic fibrosis^[27].

Additional approaches for antagonizing the

production or action of CCN2 have been described, such as those employing pharmacological inhibitors or neutralizing antibodies^[27]. However, pharmacological inhibitors are not necessarily specific to CCN2, while the use of neutralizing antibodies or antisense oligonucleotides relies on a stoichiometric and relatively inefficient 1:1 complex formation with their respective CCN2 protein or RNA target. Since, in our studies, the CCN2 ribozyme acted catalytically by binding and cleaving its target RNA, whereupon the fragments were released and the cycle was repeated numerous times, this mode of CCN2 antagonism was considerably more efficient than other methods. On the other hand, since the hammerhead ribozyme motif cleaves the phosphodiester bond downstream of a NUY triplet (where N is any base and Y is any base except G), a potential drawback to the use of ribozymes in some applications is the availability of this required NUY cleavage site in the target RNA molecule.

We conducted these studies with LX-2 cells because they are viable in serum-free media, demonstrate many of the hallmarks of activated HSCs, and because human HSCs have not been studied previously in the context of CCN2 mRNA inhibition. Our data support the potential use of hammerhead ribozyme in targeting CCN2 in fibrotic livers since, in the presence or absence of added TGF- β , it reduced collagen synthesis and secretion by the cells, and reduced the frequency of S-phase cells, even though the cells were constitutively activated and had a high basal proliferative rate. This latter observation is of interest because we are aware of only one other report in which a CCN2 hammerhead ribozyme has been developed, and this has been shown to block TGF- β -mediated proliferation of dermal fibroblasts^[47]. Thus, our data suggest that targeting CCN2 mRNA will likely cause anti-fibrogenic and anti-proliferative effects within the activated HSC population in fibrotic liver.

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COMMENTS

Background

Fibrosis is a debilitating pathology that can occur in many different organs and affects millions of people around the world. It is characterized by excessive collagen deposition that can prevent essential organs from functioning properly, and can ultimately lead to organ failure. In the liver, a cell type called the hepatic stellate cell (HSC) is responsible for producing much of the collagen that contributes to hepatic fibrosis, and recent evidence suggests that connective tissue growth factor (CCN2) is an important molecule that drives collagen production in these cells.

Research frontiers

There is much optimism that antagonism of CCN2 may be a novel and effective means of preventing or reversing fibrosis. Methods for preventing the production or action of CCN2 are at the forefront of research in this area, and it is important to examine the various options available so that rational therapeutic approaches can be developed.

Innovations and breakthroughs

Previously, it has been shown that transforming growth factor (TGF)- β is potent

fibrogenic molecule *in vivo* and can stimulate fibrogenic pathways in HSCs. However, TGF- β plays essential roles in the liver, such as tumor suppression and immune modulation, such that targeting of this molecule for therapeutic intervention is problematic. Since CCN2 acts downstream of TGF- β in fibrogenic cascades and mediates many of the fibrogenic properties of TGF- β , it is becoming accepted that CCN2 is a more appropriate molecule to target than TGF- β . Recently, methods have begun to emerge that achieve antagonism of CCN2 mRNA through the use of blocking cDNA or RNA sequences. In the present study, the authors used this type of blocking approach in a human HSC line, LX-2, to prevent CCN2 mRNA transcription and protein production, to inhibit basal or TGF- β 1-induced transcription and production of collagen I, and to reduce the number of actively dividing cells. The innovative features of these studies involve the use of human HSCs and the delivery of the antisense molecule in the form of a hammerhead ribozyme, which has a more efficient blocking action than some other conventional antisense methods. Their data show that the anti-fibrotic properties of CCN2 hammerhead ribozyme are caused by a reduction of collagen production and cell proliferation.

Applications

The study results suggest that CCN2 hammerhead ribozyme may have utility as a therapeutic agent for treating hepatic fibrosis *in vivo*.

Terminology

HSCs are a minor and quiescent cell type in the liver that usually reside in the space of Disse, but which undergo activation after hepatic injury to produce large quantities of fibrillar collagens, which are deposited as insoluble fibrous matrix. TGF- β is a multifunctional molecule that has immunomodulatory and tumor-suppressive actions in the liver, but which also is fibrogenic through its stimulation of CCN2 transcription.

Peer review

In this study Gao and Brigstock have focused on CCN2 that is considered to mediate many TGF- β -induced fibrotic reactions, to develop potential treatment of liver fibrosis, because TGF- β -targeting therapeutic intervention has been found to be problematic because of side effects in the liver. The authors examined the effects of CCN2 gene-targeting blocking on the TGF- β -dependent activation of LX-2 cells, utilizing the hammerhead ribozyme method to suppress CCN2 gene expression. Recombinant vector pTriCCN2-Rz was prepared by inserting CCN2 hammerhead ribozyme cDNA plus two self-cleaving sequences into pTriEx2 vector, and transfecting LX-2 cells together with a null vector, pTriEx2. The collagen type I synthetic and proliferative activities were assessed at the gene and protein levels, and compared between the cells transfected with pTriCCN2-Rz and pTriEx2 vectors. As a result, the authors were able to demonstrate the expected attenuation of collagen synthesis activity by CCN2-Rz transfection not only in the TGF- β -treated cells, but also in the control cells. Introduction of pTriCCN2-Rz gene also suppressed the entry of the cells into the S phase.

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