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Hepatology Research 26 (2003) 1–9
Hepatology Research
www.elsevier.com/locate/ihepcom

Connective tissue growth factor (CTGF/CCN2) in hepatic fibrosis

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Received 24 December 2002; received in revised form 16 February 2003; accepted 18 February 2003

Abstract

Connective tissue growth factor (CTGF/CCN2) is a highly profibrogenic molecule which is overexpressed in many fibrotic lesions, including those of the liver. CTGF/CCN2 is transcriptionally activated by transforming growth factor-beta (TGF-β) and appears to mediate some of the extracellular matrix (ECM)-inducing properties that have been previously attributed to TGF-β. CTGF/CCN2 and TGF-β stimulate connective tissue cell proliferation and ECM synthesis in vitro and exhibit shared fibrogenic and angiogenic properties in vivo. In fibrotic liver, CTGF/CCN2 mRNA and protein are produced by fibroblasts, myofibroblasts, hepatic stellate cells (HSCs), endothelial cells, and bile duct epithelial cells. CTGF/CCN2 is also produced at high levels in hepatocytes during cytochrome P-4502E1-mediated ethanol oxidation. CTGF/CCN2 expression in cultured HSCs is enhanced following their activation or stimulation by TGF-β while exogenous CTGF/CCN2 is able to promote HSC adhesion, proliferation, locomotion, and collagen production. Collectively, these data suggest that during initiating or downstream fibrogenic events in the liver, production of CTGF/CCN2 is regulated primarily by TGF-β in one or more cell types and that CTGF/CCN2 plays important roles in HSC activation and progression of fibrosis. This article reviews the data that support the importance of CTGF/CCN2 in hepatic fibrosis and highlights the concept that CTGF/CCN2 may represent a new therapeutic target in this disease.

Keywords: CTGF/CCN2; TGF-β; Hepatic stellate cell; Extracellular matrix; Matricellular; CCN

1. Introduction

Connective tissue growth factor (CTGF/CCN2) is a multi-functional matricellular protein that is produced by a variety of cell types and acts via autocrine and paracrine circuits to regulate many cellular functions including growth, proliferation, apoptosis, adhesion, migration, extracellular matrix (ECM) production, and differentiation [1–4]. CTGF/CCN2 appears to play a role in diverse biological processes including angiogenesis, chondrogenesis, embryogenesis, implantation, development, ovarian function, tumorigenesis and fibrosis. CTGF/CCN2 belongs to the CTGF/CYR61/NOV (CCN) family that currently comprises six members that are 30–40 kDa cysteine-rich mosaic proteins [1–4]. CCN proteins likely exert many of their biological effects by direct binding interactions with components of the ECM and the cell surface.

Of the many functions ascribed to CTGF/CCN2, that of its ability to promote fibrosis has attracted considerable attention and has been the major driving force behind many of the studies published to date [5,6]. This area of focus arose following the demonstration that CTGF/CCN2 was induced by TGF-β (itself highly fibrogenic) and that fibroblasts were targets for CTGF/CCN2 action [7–10]. CTGF/CCN2 regulates proliferation, migration, adhesion and production of ECM molecules in cultured fibroblasts [10–14], while subcutaneous injection of CTGF/CCN2 into neonatal mice causes a rapid increase in the amount of granulation tissue comprising connective tissue cells and abundant ECM [13,15]. Additionally, CTGF/CCN2 was shown to be present and frequently over-expressed and

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1386-6346/03/$ - see front matter
doi:10.1016/S1386-6346(03)00115-3

co-expressed with TGF-β in fibrotic skin disorders such as systemic sclerosis, localized skin sclerosis, keloids, scar tissue, eosinic fasciitis, nodular fasciitis, and Dupuytren's contracture [16–21]. CTGF/CCN2 mRNA and/or protein are over-expressed in fibrotic lesions of major organs and tissues including the liver [22–28], kidney [29–37], lung [18,38–40], cardiovascular system [41–43], pancreas [44–46], bowel [47], eye [48–51], and gingiva [52,53]. CTGF/CCN2 is also over-expressed in the stromal compartment of melanoma as well as mammary, pancreatic and fibrohistiocytic tumors that are characterized as having significant connective tissue involvement [54–57]. These studies have led to the notion that CTGF/CCN2 may be a new molecular target for therapeutic intervention in fibrotic diseases [5,6,58,59].

2. The link between TGF-β and CTGF/CCN2

Early studies using fibroblasts established that CTGF/CCN2 is a TGF-β1-induced immediate early gene [9,10]. Expression of CTGF/CCN2 following TGF-β stimulation occurs within minutes in the absence of de novo protein synthesis, an effect that was initially attributed to a TGF-β response element in the CTGF/CCN2 promoter [60]. However, more recent studies have demonstrated that while this element may be important for basal CTGF/CCN2 expression, TGF-β-induced CTGF/CCN2 expression requires Smad-binding sequences and a novel promoter sequence which is preferentially activated in fibroblasts as compared with epithelial cells [61–63].

The intimate relationship between TGF-β action and CTGF/CCN2 expression has been the basis for examining the role played by CTGF/CCN2 in mediating the biological activities of TGF-β [64]. While in the long run, this may prove to be a somewhat narrow view of the role of CTGF/CCN2, the outcome of these studies has been the demonstration that TGF-β and CTGF/CCN2 share pro-fibrogenic properties whereas anti-inflammatory and immunosuppressive properties are unique to TGF-β. TGF-β1 and CTGF/CCN2 are both over-expressed in many fibrotic diseases and are fibrogenic in vitro and in vivo [5]. TGF-β-induced collagen production is antagonized by CTGF/CCN2 antibodies or antisense oligonucleotides in normal rat kidney cells (NRK) and human fibroblasts [65]. TGF-β is able to support anchorage-independent growth of NRK cells, a process that is antagonized by CTGF/CCN2 antibodies or antisense oligonucleotides [66]. Additionally, subcutaneous injection of TGF-β into neonatal mice, which causes a rapid increase in the amount of granulation tissue comprising connective tissue cells and abundant ECM, results in enhanced levels of CTGF/CCN2 mRNA in connective tissue fibroblasts but not in epithelial cells or endothelial cells [13,67]. Finally, injection of CTGF/CCN2 causes a very similar fibrotic reaction as TGF-β and is not mimicked by other growth factors [13,15,67,68]. Collectively, these data support a role for CTGF/CCN2 as a downstream mediator of some of the fibrogenic actions of TGF-β1, particularly the promotion of fibroblast proliferation and ECM production.

3. Hepatic stellate cells in liver fibrosis

A central event in liver fibrosis is the activation of hepatic stellate cells (HSCs) which represents a transition from quiescent vitamin A-rich cells to vitamin A-deficient, proliferative, fibrogenic and contractile myofibroblasts [69–73]. Thereafter, activated HSCs demonstrate perpetuation which involves changes in cell behavior, proliferation, chemotaxis, fibrogenesis, contractility, matrix degradation, retinoid loss, leukocyte chemotaxis, and cytokine release [71,72]. Collectively, these changes increase ECM accumulation such that HSCs become the principal effector cells of the fibrotic response. HSC activation is also concurrent with a shift from normal low-density basement membrane-like matrix containing non-fibril-forming collagens (types IV and VI) to fibril-forming collagens (types I and III) [71]. Data from several laboratories have demonstrated that CTGF/CCN2 is overexpressed in fibrotic human liver and in animal models of the disease. Importantly, CTGF/CCN2 is produced by HSCs as a function of their activation status and exogenous CTGF/CCN2 is able to modulate HSC function. These aspects are discussed in detail below.

4. TGF-β1 and hepatic fibrosis

Cytokines play an important role in the development of liver fibrosis. One of the major cytokines associated with hepatic fibrosis and involved in HSC activation and deposition of ECM is TGF-β1 [74,75]. The evidence for TGF-β1 having a central role in liver fibrosis is sizable. First, its overexpression has been correlated with the degree of fibrosis in both animal models as well as in human disease [76–82]. Second, TGF-β1 inhibits matrix degradation by upregulation of TIMP-1 and -2, while stimulating the deposition of ECM by HSCs [81,83,84]. Third, TGF-β1 modulates the expression of integrin matrix receptors [85]. Fourth, transgenic mice that over-express TGF-β1 develop acute hepatic fibrosis [86,87]. Finally, blocking TGF-β1 action with neutralizing antibodies, inhibitors or receptor agonists inhibits animal models of renal or liver fibrosis [88–90].

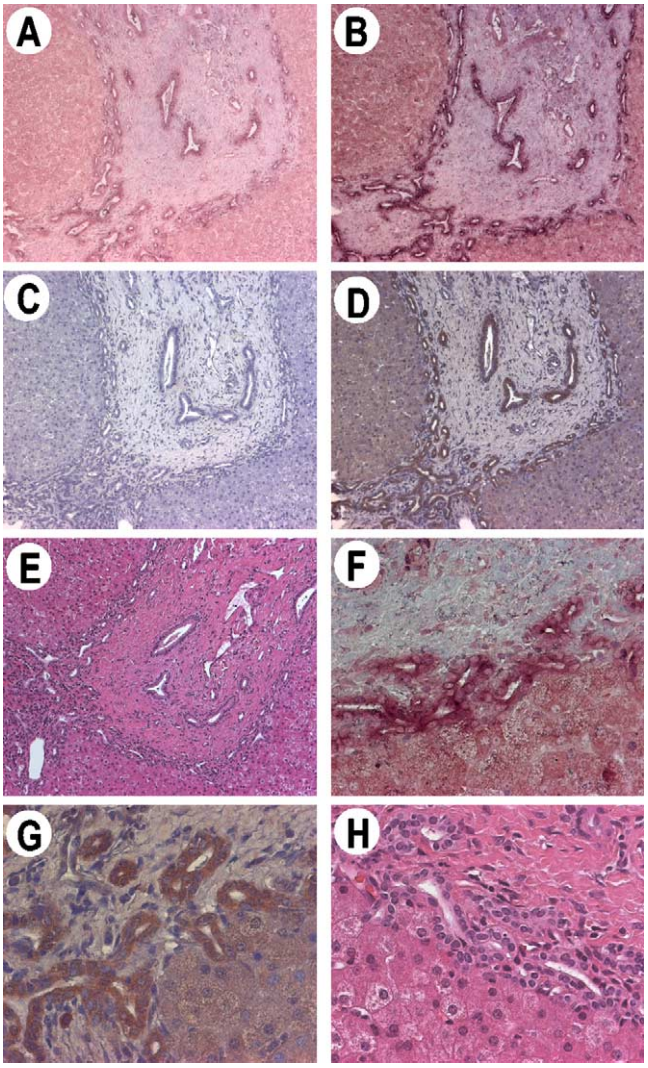

Fig. 1. CTGF/CCN2 expression in human congenital hepatic fibrosis. Sections of liver from a 9-year-old patient with congenital hepatic fibrosis were treated with (A) sense CTGF/CCN2 mRNA, (B, F) antisense CTGF/CCN2 mRNA, (C) normal rabbit IgG, (D, G) affinity-purified rabbit anti-CTGF/CCN2 (81–94) peptide or (E, H) hemotoxylin and eosin. CTGF/CCN2 mRNA (purple staining) and protein (brown staining) was localized primarily to bile duct epithelial cells; this staining pattern was observed in 3/3 cases of the disease. A–E, 10 × ; F–H, 40 × .

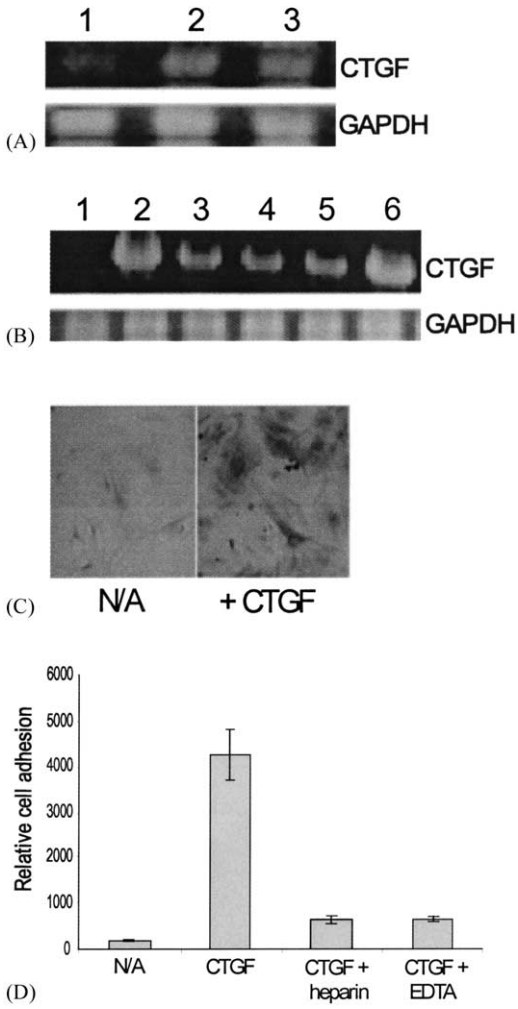

Fig. 2. CTGF/CCN2 production and action in HSC cells. (A) Myofibroblastic HSCs (HSC-T6; Scott Friedman, Mount Sinai Hospital, New York, NY) were grown for 3 days in DMEM containing 0.2% fetal bovine serum until they reached >90% confluence. Cells were left untreated (lane 1) or stimulated for 4 h with 2 ng/ml TGF-β1 (lane 2) or 1 μg/ml recombinant CTGF/CCN2 (lane 3). Total RNA from the cells was isolated and subjected to RT-PCR to amplify mRNA for CTGF/CCN2 or GAPDH. (B) Balb/c 3T3 cells were grown to confluence and quiescence in DMEM containing 10% calf serum and then left untreated (lane 1) or stimulated for 4 h with 20 ng/ml TGF-β1 (lane 2), 200 μM acetaldehyde (lane 3), 2 mM acetaldehyde (lane 4), 12 mM ethanol (lane 5) or 120 mM ethanol (lane 6). RNA from the cells was isolated and subjected to RT-PCR to amplify mRNA for CTGF/CCN2 or GAPDH. (C) Day 7 primary activated rat HSCs were left untreated or stimulated with 100 ng/ml CTGF/CCN2(247–349) [68,97] for 24 h. αSMA was detected by immunocytochemical staining. (D) Non-tissue culture plastic ELISA plates were coated with 1 μg/ml human recombinant CTGF/CCN2 and then blocked with 3% BSA. Wells were incubated for 1 h at 37 °C with 100 μl PBS containing 5×10^4 HSC-T6 cells in the presence or absence of 5 μg/ml heparin or 10 mM EDTA. Adherent cells were fixed and quantified as described [68].

5. CTGF/CCN2 and hepatic fibrosis

5.1. Human disease

Data reported in the last few years have provided compelling evidence that CTGF/CCN2 mRNA and protein levels are correlated with the degree of hepatic fibrosis, irrespective of the type of disease. Ribonuclease protection assay analysis of cirrhotic livers from patients with primary biliary cirrhosis (PBC), primary sclerosing cholangitis (PBC) or biliary atresia showed that CTGF/CCN2 mRNA in fibrotic livers was elevated 3–5-fold as compared with their normal counterparts [25]. Similarly, Northern blot analysis showed that, as compared with normal livers, CTGF/CCN2 and TGF-β1 mRNA expression was 6- to 8-fold higher in cirrhotic livers from patients with chronic viral hepatitis, PBC, PSC, crypto-

genic and alcoholic liver disease (ALD) [24]. Elevated levels of CTGF/CCN2 mRNA were also present in livers from patients with non-alcoholic steatohepatitis (NASH) and the degree of fibrosis was correlated with CTGF/CCN2 protein levels in the ECM [91]. Immunohistochemical staining of normal and hepatitis C livers showed that increased CTGF/CCN2 protein levels were associated with a higher score of fibrosis [26] .

In situ hybridization of cirrhotic livers resulting from chronic viral hepatitis, PBC, PSC, cryptogenic, and ALD contained marked CTGF/CCN2 staining in fibroblast and myofibroblast-like cells within the fibrotic portal tracts and fibrous septa [22,24,92]. CTGF/CCN2 mRNA was also observed in HSCs, myofibroblast-like spindle cells around proliferating ductules, a few duct and ductular epithelial cells, inflammatory cells, and sinusoidal and vascular endothelial cells [24,26]. CTGF/CCN2 protein staining was present in portal tracts, the ECM of the fibrous septa, sinusoidal lining and in the proliferating bile ducts in hepatitis C diseased livers [22,26]. Additionally, many cells in fibrotic liver that stained for α-smooth muscle actin (αSMA) were also shown to contain CTGF/CCN2 protein [26]. In congenital hepatic fibrosis, which is marked by profound hyperplasia of the bile ducts, CTGF/CCN2 mRNA and protein are strongly expressed by bile duct epithelial cells (Fig. 1). In ALD, hepatocytes may be an important source of CTGF/CCN2 since it is produced in cultured HepG2 cells downstream of cytochrome P-4502E1-mediated ethanol oxidation [92,93]. In livers from patients suffering from idiopathic portal hypertension, periductal mononuclear cells demonstrate enhanced CTGF/CCN2 expression and this was correlated with collagen and elastin deposition and the presence of activated HSCs and pericellular fibrosis [94]. Thus, while its timing of production and mode of action may vary according to the initial hepatic insult and type of damage, CTGF/CCN2 has the potential of being produced by virtually all major cell types in the liver. Moreover, high intra-hepatic levels of CTGF/CCN2 are associated with its entry into the circulation since, as compared with control subjects, serum CTGF/CCN2 levels were higher in patients with biliary atresia and were correlated with the progression of liver fibrosis [28].

5.2. Animal models of hepatic fibrosis

Analysis of mRNA by real-time PCR or reverse transcriptase PCR showed that hepatic CTGF/CCN2 and collagen expression was increased with the degree of fibrosis following CCl_4 treatment or bile duct ligation (BDL) in rats [25,26]. Both CTGF/CCN2 and TGF-β mRNA increased 6 h post CCl_4 administration and levels peaked at 72 h [23]. In the BDL model, TGF-β and CTGF/CCN2 mRNA were increased 4- and 7-fold, respectively, as compared with normal liver [23]. CTGF/CCN2 mRNA and protein was also upregulated in the obesity and type II diabetes Zucker (fa/fa) rat as compared with the wild type counterpart [91]. In the CCl_4 model, CTGF/CCN2 mRNA was expressed at high levels in HSCs and the protein was localized to fibrous septa and centrilobular regions [23,26]. In contrast, the main site of CTGF/CCN2 mRNA in BDL livers was proliferating bile duct epithelial cells, though the protein was present in the fibrous septa as well as in areas of ductular proliferation [23,26]. TGF-β was only expressed at minimal levels in these cells but was highly expressed in the surrounding HSCs [23].

5.3. CTGF/CCN2 biology in cultured HSCs

In a cultured HSC line, CTGF/CCN2 was localized exclusively to the Golgi apparatus and found to be quantitatively secreted into the medium [95]. Cultured activated primary HSCs were found to contain CTGF/CCN2 by Western blotting [26] and both Northern and Western blots demonstrated CTGF/CCN2 was increasingly expressed during progressive activation of cultured primary rat HSCs [25]. Additionally, both intermediate and activated cultured HSCs were induced by TGF-β1 to express CTGF/CCN2 mRNA and protein [25]. CTGF/CCN2 mRNA expression was also induced in HSCs treated with VEGF, lipid peroxidation products, acetaldehyde or PDGF-BB [27]. Similarly, CTGF/CCN2 mRNA expression is stimulated by both TGF-β1 and CTGF/CCN2 in HSC-T6 cells and by TGF-β1, ethanol, or acetaldehyde in mouse fibroblasts (Fig. 2A, B). Finally, HSCs isolated from fa/fa rats incubated with glucose or insulin showed a TGF-β-independent increase in CTGF/CCN2 mRNA and protein [91]. While these data show that CTGF/CCN2 is produced as a function of HSC activation or under conditions that favor activation, the relative contribution to the activation process of CTGF/CCN2 derived from HSCs as compared with the other cells in the liver remains uncertain. Nonetheless, both autocrine and paracrine pathways of action of CTGF/CCN2 on HSCs seem likely since the cells are responsive to exogenous CTGF/CCN2. For example treatment of rat HSCs with CTGF/CCN2 induces migration and proliferation [27], as well as stimulated expression of type I collagen mRNA [27] and αSMA (Fig. 2C). Moreover when added to CTGF/CCN2-coated ELISA plates, HSC-T6 cells demonstrate heparin-dependent and divalent-cation-dependent adhesion (Fig. 2D), consistent with previously reported data on the interaction of CTGF/CCN2 with heparin-like molecules and integrins on fibroblasts [12,96]. These binding interactions appear to occur principally with the C-terminal 103 residues of CTGF/CCN2 [97] which can stimulate αSMA production in the absence of the rest of the molecule (Fig. 2C and [68]). However, residues 199–

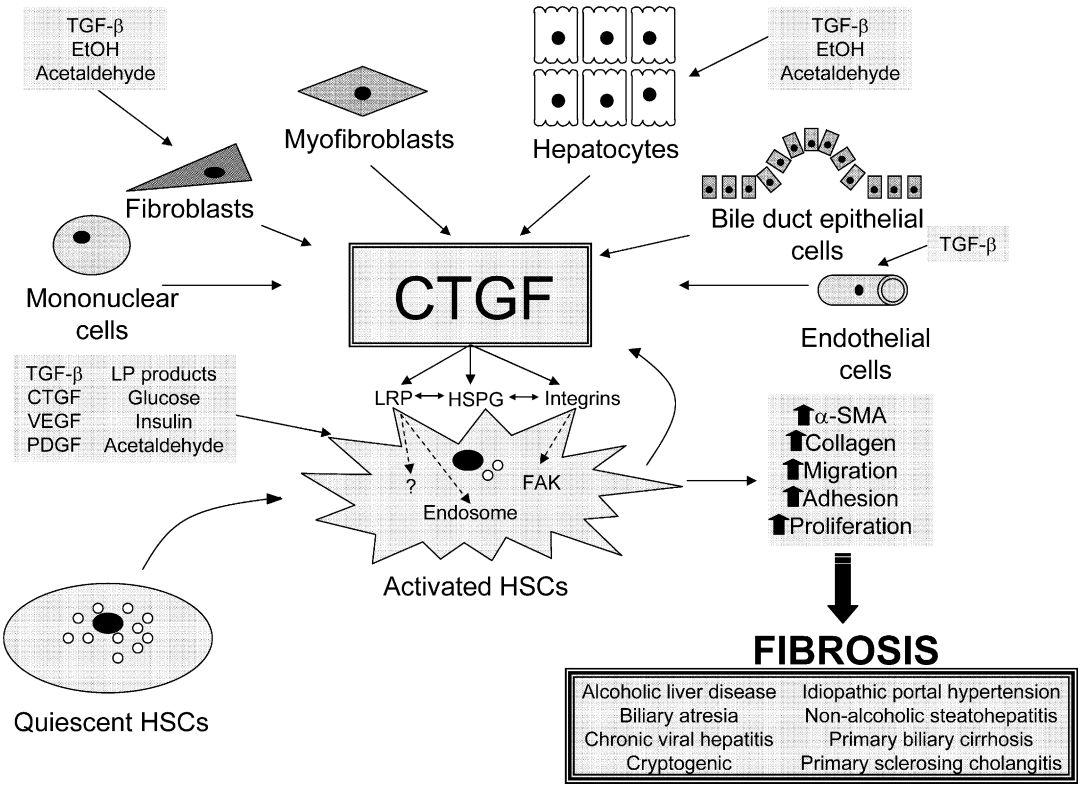

Fig. 3. Pathways of CTGF/CCN2 production and action in hepatic fibrosis. CTGF/CCN2 is produced by most major cell types in the liver in response to diverse stimuli, including those associated with fibrosis and/or HSC activation. As the principal effector cells of the fibrotic response, HSCs appear to be particularly important as autocrine or paracrine targets for CTGF/CCN2, especially in their activated state. CTGF/CCN2 binds to both LRP and integrins on the HSC surface via mechanisms that involve HSPGs. Binding to LRP may facilitate CTGF/CCN2 clearance via endosomal degradation, though additional pathways may also be activated. By analogy to data obtained with fibroblasts, binding of integrins to CTGF/CCN2 likely results in the activation of intracellular signaling cascades that promote proliferation, adhesion, motility and collagen synthesis. As a mosaic protein, CTGF/CCN2 may also be involved in direct synergistic binding interactions with other fibrogenic molecules such as TGF-β or PDGF.

243 of CTGF are also involved in promoting HSC adhesion through the ability of this domain to bind to cell surface low density lipoprotein receptor-related protein (LRP) [98], a multifunctional scavenger, signaling, and adhesion receptor [99]. Upon binding to HSCs, soluble ^{125}I-labeled CTGF/CCN2 is internalized and degraded in the endosome [95] through a process that may involve LRP [100].

5.4. CTGF/CCN2 transgene expression in vivo

While several reports have documented the development of fibrotic lesions after repeated subcutaneous injections of CTGF/CCN2 [13,15,68], there is no published evidence that CTGF/CCN2 is able to drive organ fibrosis in vivo. This will likely be obtained from animal models in which CTGF/CCN2 expression or action is ablated or in which the CTGF/CCN2 gene is over-expressed. Short-term (3 days) over-expression of CTGF/CCN2 or TGF-β in mouse livers was recently achieved by infection with replication-deficient recombinant adenovirus [101]. Gene array and Northern blot analysis demonstrated that both CTGF/CCN2 and TGF-β caused enhanced expression of urokinase receptor, laminin receptor, bone morphogenic protein-1 and the serine proteinase inhibitors Spi 2 and Spi 2.4, whereas TGF-β alone also suppressed immune response genes that were induced by adenoviral infection [101]. While the time-course of these experiments was too short to study disease processes per se, these data support an overlapping role for CTGF/CCN2 and TGF-β in stimulating expression of genes in vivo that are involved in tissue repair, fibrosis and angiogenesis.

6. Summary and perspectives

The rapid pace of research into CTGF/CCN2 over the last 5–6 years can be attributed largely to studies focusing attention on its relationship to TGF-β and its role in fibrosis. From the standpoint of specific organs, most studies of CTGF/CCN2 expression and action have focused on the liver, kidney and skin and there much anticipation that CTGF/CCN2 may offer a new lead as a therapeutic target in fibrosis of these and other organ systems [5,6,20,31,34]. It remains to be deter-

mined whether the excitement surrounding the possibility that CTGF/CCN2 is an effective target for TGF-β-mediated fibrosis is well founded, but it should be noted that several TGF-β-independent pathways of CTGF/CCN2 action have already been identified [2]. In addition to its role in fibrosis, additional studies need to be undertaken to more carefully establish the role of CTGF/CCN2 in development, differentiation, and normal function of the liver. Since CTGF/CCN2 has also been implicated in liver regeneration [102] and hepatocarcinoma [103], a better understanding is needed of the pathways by which CTGF/CCN2 is overexpressed and whether it exerts distinct or overlapping biological roles in various disease states. Since many hepatic cell types have the potential for CTGF/CCN2 synthesis, complex paracrine networks of CTGF/CCN2 action within the liver are likely in view of its broad interactions with target cells that express cell surface heparin-like molecules and/or integrins [3]. Nonetheless, it is already fairly clear that HSCs are one of the principal target cells for CTGF/CCN2 in liver fibrosis, as summarized in Fig. 3. Additionally, a major role of CTGF/CCN2 may be to act as a binding protein for other bioactive molecules, thereby regulating the activity and bioavailability of both binding partners [104]. As further knowledge of CTGF/CCN2 protein–protein and protein–cell interactions within the liver is gained, its role and mechanisms of action in normal liver function, injury, tumorigenesis, and fibrosis will be clarified.

Acknowledgements

We thank Dr E. Moussad and Dr M. Rageh for technical assistance. D.R. Brigstock is funded by NIH grant AA12817-02.

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