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Regulation of hepatic stellate cells by connective tissue growth factor

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TABLE OF CONTENTS

1. ABSTRACT

Connective tissue growth factor (CTGF/CCN2) regulates cell proliferation, differentiation, adhesion, chemotaxis, migration, apoptosis and extracellular matrix production. Through these diverse actions, CTGF/CCN2 plays a major role in important physiological and pathophysiological processes such as embryogenesis, implantation, angiogenesis, chondrogenesis, tumorigenesis, differentiation, wound healing and fibrosis. Whereas hepatic levels of CTGF/CCN2 are usually low, elevated levels of hepatic CTGF/CCN2 occur in patients with liver fibrosis and in experimental animal models of liver fibrosis. In fibrotic liver, CTGF/CCN2 is produced by multiple cell types but its sustained expression by and action on hepatic stellate cells is particularly important because these cells assume an activated phenotype during fibrosing injury and are principally responsible for the excessive production of fibrillar collagens, a process that is driven by CTGF/CCN2. Through its direct actions and interactions with other molecules such as fibronectin or transforming growth factor beta-1, CTGF/CCN2 promotes proliferation, survival, migration, adhesion, and extracellular matrix production in activated hepatic stellate cells, thereby promoting hepatic fibrogenic pathways. This review focuses on the regulation of hepatic stellate cell function by CTGF/CCN2.

2. INTRODUCTION

Liver fibrosis is a common result of chronic liver injury of different etiologies such as that caused by excessive alcohol consumption, chronic infection with hepatitis virus B or C (HBV, HCV), non-alcoholic fatty liver disease, non-alcoholic steatohepatitis (NASH), or biliary atresia (1-4). Liver fibrosis represents the wound-healing response of the liver to chronic damage and it can eventually advance to liver cirrhosis, leading to liver failure, portal hypertension and even hepatocellular carcinoma (3). One of the most striking features of liver fibrosis and cirrhosis is the overt deposition of scar tissue due to increased expression/synthesis or impaired degradation of extracellular matrix (ECM) components such as collagen type I and III (3, 5, 6). These changes arise due to acquisition of a highly differentiated phenotype by hepatic stellate cells (HSC) which ordinarily reside in non-injured tissue as quiescent cells in the Space of Disse (3). Transcriptionally activated HSC become highly proliferative and responsive to growth factors and chemokines released by injured hepatocytes or infiltrating macrophages, and they are the major source of hepatic ECM components in fibrotic liver (3, 5, 7, 8). Transforming growth factor beta-1plays a critical role in HSC activation and hepatic ECM deposition, inducing the synthesis and release of ECM components and inhibition of ECM

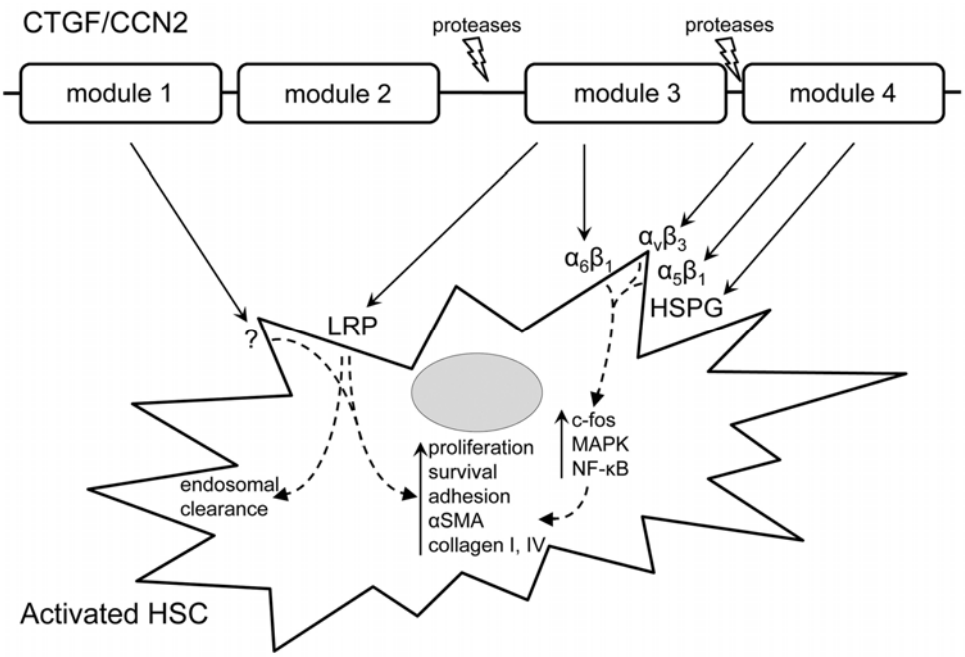

Figure 1. Structural organization of the CTGF/CCN2 protein and its role in driving the activated phenotype in HSC. CTGF/CCN2 comprises four structural modules that show evolutionary conservation with an IGF-binding domain (module1), a von Willebrand factor Type C repeat (module 2), a thrombospondin type 1 repeat (module 3) and a cysteine knot motif (module 4) (18, 22-25). These modules contain a high proportion of cysteine residues that are proposed maintain overall structural conformation via disulfide bridging within each module. Regions between the modules are cysteine-free and are prone to cleavage by serine proteases or matrix metalloproteases, resulting in the production of low mass CTGF/CCN2 proteins that retain biological activity (30, 35, 85, 92, 104-106). Activated HSC express several cell surface receptors or accessory molecules, including integrins, HSPG or LRP, that directly bind to specific motifs in modules 3 or 4 of CTGF/CCN2 (85, 86, 94, 95). Module 1 also binds to HSC via an as yet undetermined mechanism (97). Upon binding to HSC, CTGF/CCN2 triggers stimulation of c-fos, MAPK or NF-kappa B which drives the activated phenotype by increasing cell proliferation, survival, or adhesion, as well as stimulating production of markers of cell activation (alpha-SMA) or fibrogenesis (collagen I, IV) (35, 86, 87, 92, 94, 95, 97, 102). LRP can act as a CTGF/CCN2 scavenger, resulting in its internalization and subsequent degradation in endosomes (101).

degradation (3, 5-11). Indeed, TGF-beta 1 has been considered a particularly important factor that drives liver fibrosis (9, 11-13), and suppression of TGF-beta 1 expression or its downstream signaling pathway can ameliorate or even prevent liver fibrosis (10, 14). However, suppression of TGF-beta 1 is a challenging approach to liver fibrosis therapy because of its other important functions such as its anti-inflammatory, anti-proliferative, anti-angiogenic or anti-tumorigenic actions (9, 15). For example, TGF-beta 1 gene knockout mice showed an excessive level of inflammation with tissue necrosis in multiple organs, eventually leading to multiple system dysfunction and death (16).

A new lead in the field arose with the observation that connective tissue growth factor (CTGF; also known as CCN2; "CTGF/CCN2") is a TGF-beta 1 immediate early gene that acts downstream of TGF-beta leading to the notion that CTGF/CCN2 is a mediator of the pro-fibrogenic properties of TGF-beta 1 but not its inhibitory action on epithelial cells or modulation of immune or inflammatory cells (14, 17-19). Since other fibrotic factors such as endothelin or angiotensin drive CTGF/CCN2 production as well (20, 21), CTGF/CCN2 may be a common downstream mediator of the fibrotic action of diverse pro-fibrotic signaling pathways and therefore a broad-spectrum anti-fibrotic target. Moreover, emerging evidence has unequivocally established that activated HSC are an important source of CTGF/CCN2 in fibrosing liver injury and that the differentiated functions of these cells are dependent on paracrine or autocrine pathways of CTGF/CCN2 action. In this article, we summarize the evidence that CTGF/CCN2 circuitry plays a central role in activated HSC function.

3. CTGF/CCN2 AND FIBROSIS

CTGF/CCN2 is a multifunctional heparin-binding glycoprotein that is normally expressed at low levels but is dramatically enriched in virtually all fibrotic conditions, and it is well known for its roles in tissue remodeling and ECM production (22). Structurally, the CTGF/CCN2 molecule is very complex, comprising 349 residues that are organized into four discrete domains (modules 1-4; Figure 1) which, in addition to binding to cell surface receptors, interact with and regulate the activity

or bioavailability of other signaling molecules in the extracellular environment (18, 22-26). CTGF/CCN2 over-production has been proposed to play a major role in pathways that lead to fibrosis in many organs (14, 18, 22, 23). Cell-based studies have shown that CTGF/CCN2 regulates multiple processes that contribute to fibrogenesis, including cell proliferation, migration, adhesion, survival and ECM production; that it does so as both a downstream and cooperative mediator of TGF-beta signaling; and that it affects a variety of cell types that broadly participate in fibrogenic processes including, for example, mesenchymal stem cells, HSC, renal podocytes, mesangial cells, parietal and tubular epithelial cells, pulmonary type II alveolar cells, mesothelial cells, vascular smooth muscle cells, endothelial cells, cardiomyocytes, pericytes and fibroblasts (27). Moreover, correlative studies in diseased human tissues indicate important links between CTGF/CCN2 and TGF-beta in many fibrotic disease states such as diabetic nephropathy, idiopathic and non-idiopathic pulmonary fibrosis, liver fibrosis, skin fibrosis (including keloids and scleroderma), systemic sclerosis, atherosclerosis, congestive heart failure, pancreatitis and various forms of malignant disease (14, 28).

4. CTGF/CCN2 PRODUCTION IN FIBROTIC LIVER

CTGF production in normal healthy liver is usually very low whereas enhanced hepatic CTGF/CCN2 expression or production is commonly observed in fibrotic liver. Increased hepatic CTGF/CCN2 expression occurs in patients with chronic fibrosing pathologies of the liver including those due to HBV, HCV, excessive alcohol consumption, NASH, biliary atresia, or that are of congenital etiology (29-40). Likewise, enhanced hepatic CTGF/CCN2 expression occurs in rodent models of hepatic steatosis or fibrosis such as those in response to toxins or carcinogens (e.g. carbon tetrachloride, dimethylnitrosamine, thioacetamide), methionine/choline-deficient diets, or bile duct ligation (29, 30, 32, 41-63). Hepatic CTGF/CCN2 is also over-expressed in genetic models of liver fibrosis or chronic cholangitis in mice (64, 65). Many of these studies have documented an intimate temporal and spatial association between CTGF/CCN2 and TGF-beta, as well as other markers of fibrosis such as pro-collagen alpha 1(I) or tissue inhibitor of matrix metalloprotease-1 (TIMP-1). Hepatic or circulating CTGF/CCN2 levels have been correlated with a higher liver fibrosis score leading to the suggestion that such measurements may be useful diagnostic or prognostic indicators (29, 36, 38, 66-70), although the utility of CTGF/CCN2 as a non-invasive marker for certain fibrosing liver diseases (e.g. biliary atresia) is controversial (71) and may be confounded by the presence of diseases such as hepatocarcinoma (72) or Type II diabetes (70).

5. CTGF/CCN2 PRODUCTION BY HSC

In steatosing or fibrosing liver, CTGF/CCN2 frequently localizes to regions of excessive ECM deposition (29, 32, 34, 57), consistent, first, with its production by cells that are intimately involved in ECM production (e.g. myofibroblasts, HSC) and, second, with the well-characterized physical association and functional interaction between CTGF/CCN2 and ECM components such as heparan sulfate proteoglycans (HSPGs; see below). However, diverse cellular sources of hepatic CTGF/CCN2 in fibrosing injury have been reported that include hepatocytes (36, 40), fibroblasts (31), myofibroblasts (31, 33, 57), HSC (29, 31, 33, 36, 38, 41, 50, 54, 57, 73) , endothelial cells (31, 38, 40), mononuclear cells (34), inflammatory cells (40), or bile duct epithelial cells (31, 35, 38, 40, 41). Nonetheless, with few exceptions, the specific biological role(s) of CTGF/CCN2 produced by most of these cell types is unclear and its actual relationship to the underlying process of fibrotic pathogenesis remains largely unexplored. A paracrine action of CTGF/CCN2, in which it acts on HSC after its production by other hepatic cell populations is plausible, but will require more definitive experimental approaches, especially *in vivo*. To date, credence for this notion is supported by the findings that: (i) production of alpha smooth muscle actin (alpha-SMA; a marker of HSC activation) or collagen deposition is increased after liver injury in transgenic mice overexpressing hepatocyte CTGF/CCN2 (74); (ii) HepG2 hepatocytes stably expressing HCV core protein produce enhanced levels of TGF-beta 1 and CTGF/CCN2 themselves and elicit enhanced production of CTGF/CCN2, TGF-beta 1, alpha-SMA, collagen type I or TGF-beta 1 receptor type II (TβRII) when co-cultured with HSC (75); (iii) hepatocytes expressing HBV X protein (HBx) induce paracrine activation of HSC, including stimulation of CTGF/CCN2 expression, via enhanced TGF-beta secretion (76); and (iv) HSC cultured with conditioned medium from leptin-treated Kupffer cells (resident hepatic macrophages) produce enhanced levels of CTGF/CCN2, TGF-beta, alpha-SMA, TIMP-1 or collagen I (77). However, it is equally important to recognize that the production of CTGF/CCN2 by epithelial, endothelial or immune cells, especially during the early phases of liver injury, may reflect alternative functions relating to wound healing, angiogenesis, regeneration, cell survival, or immune modulation rather than fibrosis (78-84) and this is an area requiring much more careful investigation of the dynamics of CTGF/CCN2 production and its broader biological effects during acute and chronic injury.

Much evidence has emerged that activated HSC themselves are a biologically significant source of CTGF/CCN2 which directly contributes to the role of this cell type in fibrosing liver injury. Evidence for the production of CTGF/CCN2 by activated HSC is supported by its cellular localization in fibrotic livers. For example, in chronic hepatitis in humans, CTGF/CCN2 was immunohistochemically localized to portal tracts and fibrous speta in fibrotic regions and to adjacent sinusoidal cells and there was a significant association between alpha-SMA-positive cells and CTGF/CCN2 staining intensity (29). *In situ* hybridization showed that CTGF/CCN2 messenger ribonucleic acid (mRNA) was present in sinusoidal cells but not in hepatocytes (29). In cirrhotic livers from patients with chronic hepatitis, primary biliary cholangitis, primary sclerosing cholangitis, alcoholic liver disease (ALD), or cryptogenic, CTGF/CCN2 mRNA was

principally localized to connective tissue spindle cells (myofibroblasts / fibroblasts) in fibrous portal tracts and in fibrous septa and these cells were presumptive activated HSC based on co-localization of alpha-SMA (31, 33). Similarly, HSC in the fibrotic regions of livers from patients with biliary atresia or ALD were positive for CTGF/CCN2 (36, 73), while livers from pediatric patients diagnosed with autoimmune hepatitis, chronic HCV, Alagille syndrome or Budd-Chiari syndrome demonstrated CTGF/CCN2 expression in presumptive HSC in the portal and centrolobular areas (38). In rats treated with carbon tetrachloride, CTGF/CCN2 staining was restricted to cells that were desmin-positive, a characteristic of HSC (41). Importantly, studies of clinical specimens and animal models showed that while CTGF/CCN2 is detectable in the activated HSC population in fibrotic livers, there is no such signal in the quiescent HSC population of normal healthy liver (31, 33, 36, 38, 41, 50, 73) suggesting that CTGF/CCN2 is induced in HSC as a specific response to injury.

The concept that CTGF/CCN2 is produced principally by HSC as a function of their activation or as a response to fibrosing stimuli has gained considerable traction from *in vitro* studies using either primary HSC isolated from normal livers or HSC cell lines. In the case of primary cell cultures, quiescent HSC isolated from normal livers become autonomously activated *in vitro* and show a progressive increase in CTGF/CCN2 expression that is correlated with the expression of other key markers of activation or fibrogenesis such as TGF-beta 1, alpha-SMA or collagen I (30, 50, 85, 86). Both primary HSC and HSC lines show enhanced CTGF/CCN2 production or promoter activity after (i) exposure to agents that are strongly implicated in fibrosing liver injury and/or HSC activation including TGF-beta, leptin, HBx, acetaldehyde, ethanol or CTGF/CCN2 itself (30, 35, 73, 77, 85, 87-89); or (ii) incubation with conditioned medium from or co-culture with other hepatic cell types which produce known fibrotic mediators such as HCV core protein- or HBx- producing hepatocytes (see above; (75, 76)) or leptin-treated Kupffer cells (77).

6. REGULATION OF CTGF/CCN2 mRNA EXPRESSION IN HSC

Considerable interest lies in understanding the mechanisms by which CTGF/CCN2 expression is regulated in HSC, especially by TGF-beta. Blockage of *de novo* transcription in HSC using actinomycin D prevents activation-dependent CTGF/CCN2 production as well as that in response to added TGF-beta (88). These phenomena appears to be attributable in large part to the induction of CTGF/CCN2 by TGF-beta acting through Smad 3/4 and Ets-1 (Figure 2) since the high basal CTGF/CCN2 promoter activity in activated HSC or that in response to TGF-beta was reduced by mutation of the Smad and Ets elements in the CTGF/CCN2 promoter (88). CTGF/CCN2 production and promoter activity were also blocked by inhibiting TGF-beta receptor Type 1 showing that in activated HSC, CTGF/CCN2 mRNA expression either during activation or in response to exogenous TGF-beta relies on an intact TGF-beta signaling pathway (88). These findings are supported by other studies showing that transfection of HSC with inhibitory Smad 7 decreases basal or TGF-beta-stimulated CTGF/CCN2 promoter activity in HSC (87), or that TGF-beta small interfering RNA (siRNA) blocks ethanol-induced TGF-beta or CTGF/CCN2 expression in HSC(73). In the latter study, the TGF-beta-dependent CTGF/CCN2 response involved the Smad promoter element mentioned above as well as a basal control element that is indirectly responsive to TGF-beta since it is a response element for endothelin 1 which is induced by TGF-beta and is essential for TGF-beta to induce CTGF/CCN2 (21) (Figure 2). ALK/Smad signaling also accounts for the stimulation of CTGF/CCN2 production by interleukin 13, although in this case it is driven by Stat6-independent activation of the ERk-MAPK pathway rather than via TGF-beta directly (90). Finally, the nuclear translocation of Smad 2 is blocked by adiponectin, resulting in reduced expression of TGF-beta 1 or CTGF/CCN2 (42).

Several studies have shown that CTGF/CCN2 production is regulated by growth factors and other growth-modulating molecules in cultured HSC. CTGF/CCN2 production or promoter activity in HSC is stimulated by amphiregulin (a member of the epidermal growth factor family) (91) or platelet derived growth factor (87, 92), the latter of which was TGF-beta-dependent as shown by its inhibition by Smad 7 overexpression (87) or TGF-beta 1 immuno-neutralization (92) (Figure 2). CTGF/CCN2 production in HSC is phosphatylinositol-3-kinase-dependent (93) and is stimulated by glucose or insulin (29), thus supporting a role for CTGF/CCN2 in mediating the pro-fibrotic effects of hyperglycemia or insulin in patients with NASH.

7. RESPONSES OF HSC TO CTGF/CCN2

Several notable biological responses of HSC have been described after their exposure to exogenous recombinant CTGF/CCN2. Culture-activated, but not freshly isolated, HSC are able to utilize CTGF/CCN2 as an adhesive substrate via the engagement of cell surface molecules such as integrins, low density lipoprotein-related protein (LRP), or HSPG (Figure 1) (85, 94). Indeed the apparent absence of a functional repertoire of CTGF/CCN2 receptors in HSC in normal liver is likely an important element of their quiescent phenotype and helps to explain why mere overexpression of hepatic CTGF/CCN2 in liver parenchymal cells is not sufficient in itself to promote HSC activation or fibrosis (74). Adhesion of activated HSC to CTGF/CCN2 is sufficient to promote a pattern of gene expression that is pro-fibrogenic and pro-survival (19). Discrete peptide domains in module 3 are involved in binding of CTGF/CCN2 to LRP or integrin alpha 6 beta 1 on the surface of HSC (85, 95) while module 4 binds to integrin alpha v beta 3, integrin alpha 5 beta 1, or HSPG (86, 94), the latter of which function as critical co-receptors for integrin binding by CTGF/CCN2 (Figure 1). Although specific downstream events in HSC have not been attributed to binding of one or more of these CTGF/CCN2 receptors, studies of other cell types have shown that

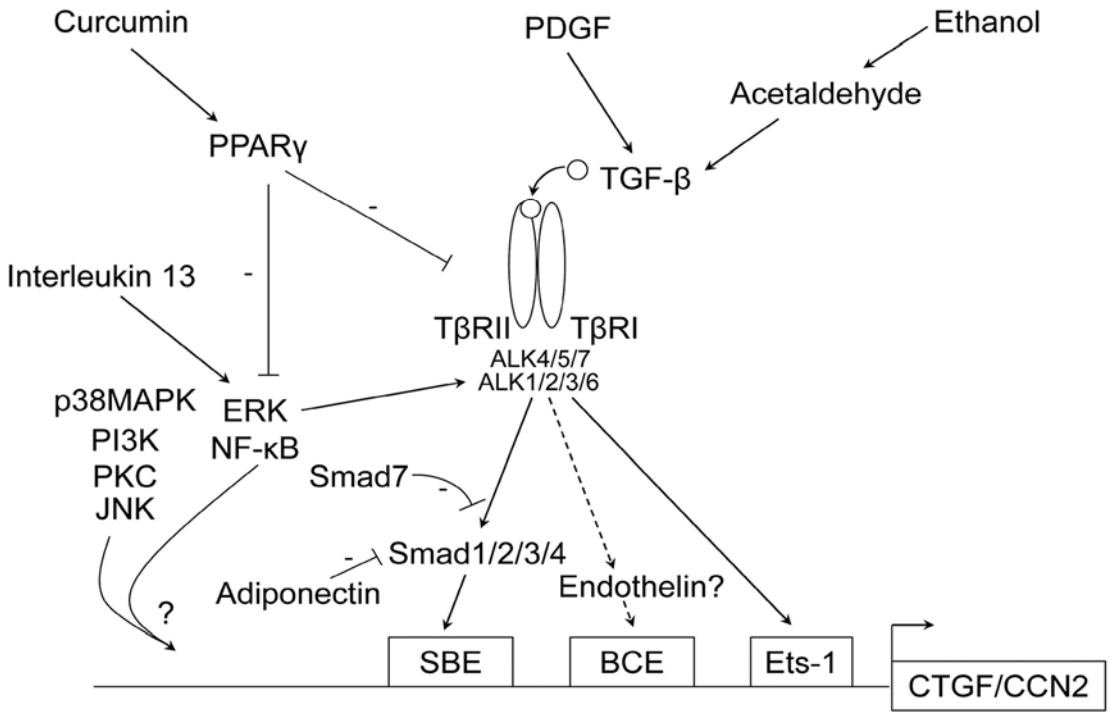

Figure 2. Regulation of CTGF/CCN2 transcription in activated HSC. CTGF/CCN2 is a TGF-beta immediate early gene that mediates many of the pro-fibrotic actions of TGF-beta. CTGF/CCN2 or TGF-beta mRNA are concomitantly increased during culture-induced activation of primary HSC (88). Basal CTGF/CCN2 promoter activity is stimulated during the activation process as well as in response to TGF-beta; this is TGF-beta type I receptor/ALK4/5/7-dependent and requires transcriptional activation of the Smad and Ets-1 elements in the CTGF/CCN2 promoter (73, 88). Ethanol-stimulated stimulated CTGF/CCN2 production is dependent on the metabolism of ethanol into active metabolites such acetaldehyde which act in a TGF-beta-dependent manner to drive the CTGF/CCN2 promoter via a Smad-binding element (SBE) or basal control element (BCE) (73), the latter of which responds to endothelin 1 produced downstream of TGF-beta 1 in fibrotic fibroblasts (21). The BCE element is also activated by ethanol in HSC (73) but the specific metabolic and siganling pathways utilized have not been reported. PDGF-induced CTGF/CCN2 promoter activity is dependent on TGF-beta 1 up-regulation (92) and is blocked by the presence of inhibitory Smad7 (87). CTGF/CCN2 gene expression is inhibited by curcumin which acts via PPAR-gamma to suppress the TGF-beta type II receptor (117) and which inhibits NF-kappa B or ERK signaling (119). Interleukin 13 stimulates TGF-beta-independent activation CTGF gene transcription via ERK which appears to co-operatively activate Alk4/5/7 and Alk1/2/3/6 resulting in activation of Smad 1/2/3 (90). Adiponectin attenuates expression of TGF-beta 1 or CTGF/CCN2 by inhibiting nuclear translocation of Smad2 (42). Although inhibition of ERK, p38 MAPK, PKC, ALK4/5/7 or PI3K reduces CTGF/CCN2 mRNA expression in activated HSC (88, 93), the underlying mechanisms have yet to be clarified and may involve some or all of the signaling pathways shown elsewhere in the figure.

specific responses (e.g. migration, adhesion, proliferation) to the closely-related cysteine-rich-61 (CYR61/CCN1) protein are selectively determined through its ability to engage one specific integrin subunit versus another (96). Expression of the integrin alpha v, alpha 5, beta 1, or beta 3 subunits by HSC is regulated during activation or in response to exogenous CTGF/CCN2, though this latter effect is complex and dependent on the activation status of the cells (86). In addition, fibronectin (FN), which is also a ligand for integrin alpha 5 beta 1, interacts co-operatively with CTGF/CCN2 to promote HSC adhesion (86), a phenomenon that has also been reported for hepatic oval cells (97) as well as fibroblasts (98) and chondrocytes (99). This interaction is likely important in the context of fibrotic liver disease since the provisional matrix contains enriched levels of each molecule, and FN regulates the cellular response to CTGF/CCN2 (100). Finally, incubation of HSC with iodinated CTGF/CCN2 has identified a LRP-coupled endosomal clearance pathway in HSC (Figure 1) (101), but it remains unclear how this is related, if at all, to the binding of CTGF/CCN2 to its integrin receptors.

Treatment of partially activated HSC with CTGF/CCN2 causes an acceleration of pathways that are associated with activation or fibrogenesis. These include stimulation of c-fos gene activation and expression, proliferation through activation of the mitogen-activated protein kinase (MAPK) pathway, migration, and stimulation of alpha-SMA or collagen type I gene expression (Figure 1) (35, 87, 92). Also, CTGF/CCN2 confers a survival advantage on activated HSC which is attributable, at least in part, to its ability to activate nuclear factor-kappa B (NF-kappa B) signaling pathways (Figure 1) (102) and which has previously been associated with

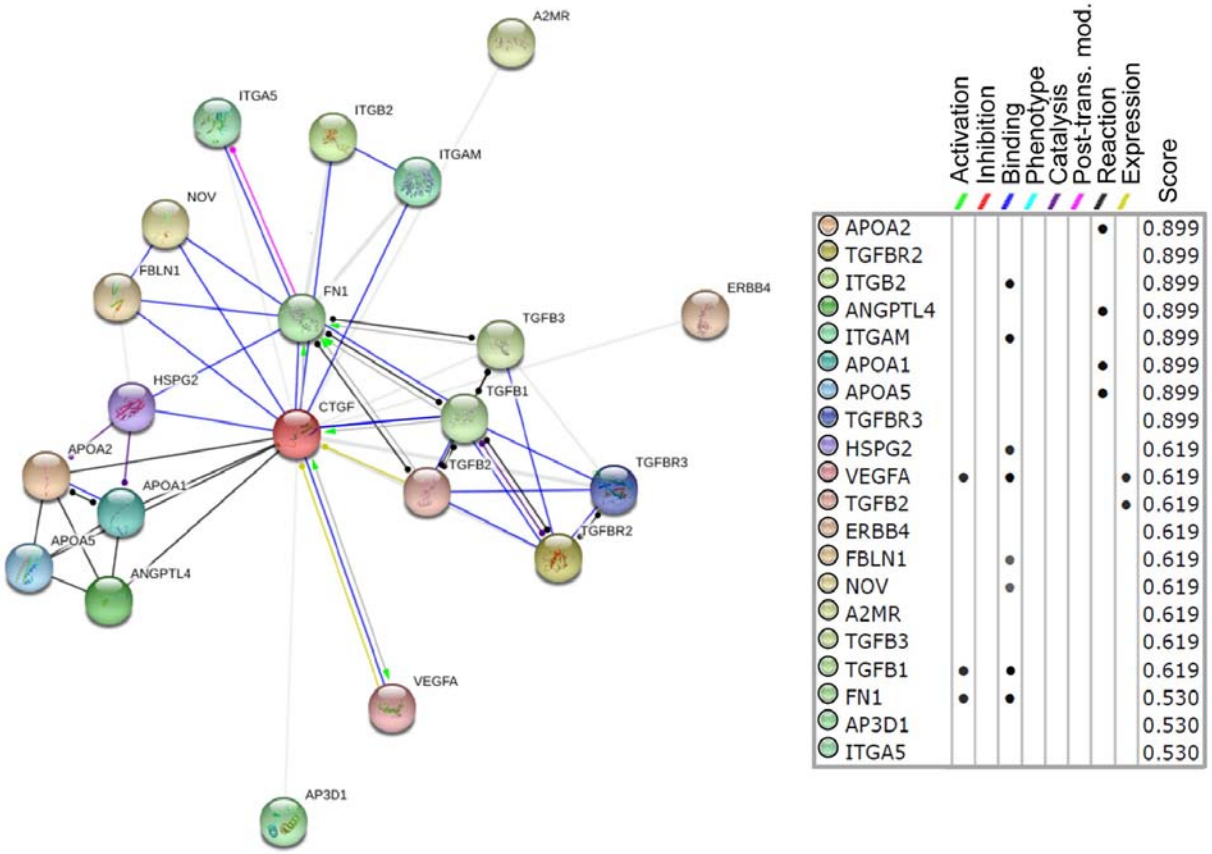

Figure 3. Action of CTGF/CCN2 as a molecular hub. The figure shows an action view for human CTGF/CCN2 and its predicted functional partners determined using String 9.0^{TM} predictive software (http://string-db.org). Predictions were based on experimental data and database information (confidence >0.4) and the string network was modified by the authors to take account of additional information in the literature. The different modes of action are shown in different colors. Nodes are colored if they are directly linked CTGF/CCN2 or light grey if they require higher iteration/depth. A dark blue node indicates a direct binding interaction that in some cases (e.g. CTGF/CCN2 with FN) causes the activity of the binding partner becomes modified. Studies of activated HSC to date have documented direct binding interactions between CTGF/CCN2 and FN, integrins, HSPG, or LRP (85, 86, 94, 95, 101). Abbreviations (not used elsewhere in the text): APO, apolipoprotein; TGFB: TGF-beta; TGFBR, TGFB receptor; ITG, integrin; ANGPTL4, angiopoeitin-like 4; VEGF, vascular endothelial growth factor; ERB4, v-erb-a erythroblastic leukemia viral oncogene protein homolog 4; FBLN, fibulin 1; NOV, nephroblastoma overexpressed gene (also termed CCN3); A2MR; Pro- LRP-1; AP3D1, adapter-related protein complex3, delta 1 subunit.

sustained expression of integrin alpha v beta 3 (103), a CTGF/CCN2 receptor in HSC (see above). Some of these events are mimicked by fragments of the CTGF/CCN2 protein which have been helpful in mapping functional domains in the CTGF/CCN2 protein. For example, recombinant CTGF/CCN2 module 3, a ligand of integrin alpha 6 beta 1, stimulates adhesion, activation of focal adhesion kinase or MAPK, or enhances production of FN or collagen IV in HSC (Figure 1) (95). Similarly recombinant CTGF/CCN2 module 4 engages integrin alpha v beta 3, integrin alpha 5 beta 1, or HSPG on HSC and promotes HSC adhesion or alpha-SMA production (Figure 1) (35, 86, 94). CTGF/CCN2 modules 1 or 4 were also reported to be selectively adhesive to HSC (97). The ability of HSC to respond to various CTGF/CCN2 fragments is likely physiologically significant because these domains appear to be liberated post-translationally from the full length molecule by the action of serine proteinases or matrix metalloproteases (104-106). Western blot analysis of cell lysates or conditioned medium have provided support for the presence of both full-length and lower mass CTGF/CCN2 proteins in HSC cultures (30, 35, 85, 92).

While these *in vitro* studies have helped to clarify the nature of HSC responses to CTGF/CCN2, they may actually be giving an incomplete picture. An emerging concept in the field is the recognition of CTGF/CCN2 and other CCN proteins as matricellular factors that regulate cell function as non-structural components of the ECM (23, 26, 96, 107); in this manner CTGF/CCN2 acts as "molecular hub" that is capable of controlling diverse cellular responses though its ability to bind to and regulate the activity of a variety of signaling molecules in the extracellular environment (Figure 3). Thus the biological effects of CTGF/CCN2 on HSC are likely influenced by the ability of the CTGF/CCN2 to participate in complex binding interactions with other proteins, causing modulation of their biological actions. For example, CTGF/CCN2 can regulate FN-mediated cell adhesion, an

effect that may be attributable to the presence of specific FN-binding domains with the CTGF/CCN2 protein (86, 97-99). In the context of fibrosis, it may be particularly relevant that post-translational co-operation between CTGF/CCN2 and TGF-beta has been reported in *in vivo* models of dermal, pulmonary, kidney or multi-organ fibrosis (108, 109). These findings are consistent with evidence that module 2 of CTGF/CCN2 participates in direct protein-protein interaction with TGF-beta which causes the binding of TGF-beta to its receptors to be enhanced (110). While this mode of action has yet to be proven definitely for either liver fibrosis or HSC activation, it is clear that HSC responses (alpha-SMA production, collagen deposition) to over-expressed hepatocyte CTGF/CCN2 *in vivo* are exacerbated in the presence of injury-induced signaling in the liver (74, 107).

8. EXPERIMENTAL ANTAGONISM OF CTGF/CCN2 PRODUCTION IN HSC

The ability of exogenous CTGF/CCN2 to elicit biologically important responses in activated HSC supports the notion that a variety of hepatic cell types may contribute to CTGF/CCN2 paracrine pathways that regulate HSC function during fibrosing injury. However the fact that activated HSC themselves produce CTGF/CCN2 has prompted investigations of the autocrine role of CTGF/CCN2 in HSC themselves. An ever-increasing number of studies conducted in experimental fibrosis models *in vivo* or in HSC cultures *in vitro* have examined the ability of anti-fibrotic agents to down-regulate elevated CTGF/CCN2 levels in activated HSC. Frequently, these investigations have tended to focus on the efficacy of these compounds in modulating the expression of a variety or fibrosis-related molecules, including CTGF/CCN2 - which has emerged as a *de facto* marker of fibrosis or of the activated HSC phenotype. To date, this approach has been somewhat "survey-oriented" but the general mode of action of these drugs may provide clues as the signaling pathways that regulate CTGF/CCN2 production. Certain anti-fibrotic molecules such as rapamycin (46), rosiglitazone (54), canderstan or olmasartan (angiotensin II type I receptor antagonists) (49, 51), angiotensin-(1-7) (an angiotensin II antagonist) (55), rofecoxib (a cyclo-oyygenase-2 inhibitor) (52), perindopril (59), eplerenone (a selective mineralocorticoid receptor antagonist) (111), SMND-309 (a potent metabolite of salvianolic acid) (62), or Amomum xanthoides (an anti-oxidant) (63) have been shown to attenuate hepatic CTGF/CCN2 levels in models of liver fibrosis *in vivo* but this has not been specifically proven to occur in HSC. On the other hand, CTGF/CCN2 production in activated HSC *in vitro* is blocked by anti-fibrotic compounds that include olmasartan (an antagonist of the angiotensin II type I receptor) (112), adiponectin (42), Salvia Miltioohiza (47), epigallocatechin-3-gallate (an anti-oxidant) (113), Ginko biloba extract (114), rosemarinic acid (58), Cinnamomum cassia Blume (61), or CIP-A5 (a N1-acetyl substituted pyrrolidine derivative) (115) and these effects are invariably accompanied by reduced proliferation, migration, or production of alpha-SMA, TGF-beta 1, collagen, and/or TIMP-1 in the cells as well. The most comprehensive and mechanistic data along these lines has come from studies of HSC in which the activity or expression of peroxisome proliferator-activated receptor-gamma (PPAR-gamma), a nuclear receptor and ligand-activated transcription factor, was experimentally modulated (116-119). In activated HSC, stimulation of PPAR-gamma expression or activity resulted in a reversion to quiescence as evidenced by decreased cell proliferation, increased apoptosis, and suppression of alpha-SMA, collagen alpha 1(I) or FN expression, with the latter changes in gene expression due to inhibition by PPAR-gamma of TGF-beta-induced CTGF/CCN2 expression (116-118). Curcumin, which activates PPAR-gamma, was shown to exert its inhibitory effect on CTGF/CCN2 in activated HSC *in vitro* by blocking NF-kappa B or MAPK pathways (119).

The role of CTGF/CCN2 as an autocrine regulator of HSC function has been established unequivocally using CTGF/CCN2 antisense oligonucleotides or siRNA to specifically antagonize CTGF/CCN2 mRNA production. CTGF/CCN2 siRNA has been shown to antagonize mRNA for alpha-SMA or collagens I and III, to reduce secreted levels of hyaluronic acid or type III collagen, and to increase the frequency of cell cycle arrest in rat HSC (120). Similar effects on activation and collagen production have been reported in mouse or human HSC after exposure to CTGF/CCN2 siRNA which blocked TGF-beta-dependent alpha-SMA or collagen alpha 1(I) expression in response to ethanol or acetaldehyde (73) and which also resulted in an alteration in expression of the integrin subunits that function as CTGF/CCN2 receptors (86). Also, rat HSC treated with plasmids expressing short hairpin RNA caused decreased CTGF/CCN2 gene expression as well as decreased production of collagens III and IV, laminin, and hyaluronic acid (121), while hammerhead ribozymes designed to cleave CTGF/CCN2 mRNA blocked basal or TGF-beta-stimulated collagen synthesis and entry into S phase of human HSC (122). Collectively, these findings highlight a central role for CTGF/CCN2 in autocrine regulation of HSC activation and fibrogenesis. Moreover, several anti-CTGF/CCN2 strategies have proven to be effective anti-fibrotic therapies in experimentally-induced liver fibrosis in animal models (43, 48, 50) and strategies that target the activated HSC population in fibrotic livers may be more efficacious in this regard (123).

9. SUMMARY

The production and action of CTGF/CCN2 in activated HSC have emerged as functionally central components of the dynamic response exhibited by these cells during chronic injury in the liver (Table 1). Acting downstream of TGF-beta, CTGF/CCN2 drives pathways of activation and fibrogenesis in HSC, and it has become recognized as a generally reliable marker of these processes. Indeed, the therapeutic value of targeting hepatic CTGF/CCN2 is based largely on the expectation that the differentiated functions of activated HSC will be compromised. While CTGF/CCN2 may ultimately emerge as a viable marker or molecular target for liver fibrosis, this cannot be done in isolation from other important molecular read-outs, nor without considering disease etiology and the

Table 1. Evidence that CTGF/CCN2 contributes to liver fibrosis and that it drives the pro-fibrogenic functions of activated HSC

Experimental Evidence	References
Elevated level of CTGF/CCN2 in patients with chronic liver diseases or fibrosis	(29-40, 66-71)
Elevated level of CTGF/CCN2 in experimental animal models of liver fibrosis	(29, 30, 32, 41-63)
Hepatic CTGF/CCN2 overexpression increases susceptibility to develop liver fibrosis	(74)
Elevated CTGF/CCN2 production by HSC during activation or in response to fibrotic stimuli	(29-31, 33, 35, 36, 38, 41, 50, 73, 75-77, 85-90)
CTGF/CCN2 drives HSC activation, adhesion, proliferation, survival, migration, or ECM production	(35, 86, 87, 92, 94, 95, 97, 102)
Down-regulating CTGF/CCN2 expression attenuates HSC activation or fibrogenesis, or is therapeutic in liver fibrosis in animal models	(43, 48, 50, 73, 86, 120-123)

many other variables that contribute to fibrotic liver disease in humans.

While *in vitro* studies have played an important role in validating the importance of CTGF/CCN2 in HSC function, more refined approaches are needed especially given the limitations of cell culture techniques for studying CCN biology (107). For example, models of cell-specific CTGF/CCN2 knockout or over-expression in the liver would be particularly useful for understanding the contribution of CTGF/CCN2 produced by different hepatic cell types. Finally, it should be realized that the intense focus on the regulation of fibrogenesis by CTGF/CCN2 has, perhaps, resulted in a myopic view of the action of this molecule, which may contribute to a plethora of other important biological processes in the injured liver. Indeed it seems very likely that a much broader investigation of CTGF/CCN2 action in HSC and other hepatic cell types will likely yield unexpected and complex data, as has been shown for its close relative, CYR61/CCN1 (96).

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Abbreviations: ALD: alcoholic liver disease; alpha-SMA: alpha smooth muscle actin; CTGF/CCN2: connective tissue growth factor; CYR61/CCN1: cysteine-rich 61; ECM: extracellular matrix; FN: fibronectin; HBV: hepatitis B virus; HBx: HBV X protein; HCV: hepatitis C virus; HSC: hepatic stellate cells; HSPG: heparan sulfate proteoglycans; LRP: low density lipoprotein-related protein; mRNA: messenger ribonucleic acid; NASH: non-alcoholic steatohepatitis; NF-kappa B: nuclear factor kappa B; PPAR-gamma: peroxisome proliferator-activated receptor-gamma; siRNA: small interfering ribonucleic acid; TIMP-1: tissue inhibitor of matrix metalloprotease-1

Key Words: Connective tissue growth factor, Hepatic stellate cell, Liver fibrosis, Transforming growth factor-beta, Fibrogensis, Matricellular, Review, CCN, CCN2, CTGF, TGF-beta, Review

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