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REVIEW

Connective tissue growth factor (CTGF, CCN2) gene regulation: a potent clinical bio-marker of fibroproliferative disease?

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Abstract The CCN (*c*yr61, *c*tgf, *n*ov) family of modular proteins regulate diverse biological affects including cell adhesion, matrix production, tissue remodelling, proliferation and differentiation. Recent targeted gene disruption studies have demonstrated the CCN family to be developmentally essential for chondrogenesis, osteogenesis and angiogenesis. CCN2 is induced by agents such as angiotensin II, endothelin-1, glucocorticoids, HGF, TGFβ, and VEGF, and by hypoxia and biomechanical and shear stress. Dysregulated expression of CCN2 has also been widely documented in many fibroproliferative diseases. This mini-review will focus on CCN2, and the recent progress in understanding CCN2 gene regulation in health and disease. That CCN2 should be considered a novel and informative surrogate clinical bio-marker for fibroproliferative disease is discussed.

Keywords CCN2 · CTGF · Fibrosis · Biomarker

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Introduction

The CCN (cyr61, ctgf, nov) family comprises six members (CCN1–6). These proteins act through integrin- and heparan sulfate proteoglycan-mediated adhesive signaling to directly modulate adhesion and indirectly modulate the functional activities of other extracellular ligands such as cytokines, growth factors, morphogens and matrix components (Leask and Abraham 2006). Of these, connective tissue growth factor (CTGF, CCN2) is perhaps the best studied family member.

CCN2 was initially identified as a protein secreted by cultured human endothelial cells (Bradham et al. 1991). In adult mammals, CCN2 exhibits a restricted expression, produced only by hepatic stellate cells (Paradis et al. 1999; Chen et al. 2001) and kidney mesangial cells (Ito et al. 1998; Chen et al. 2002). Although CCN2 is not normally expressed by other mesenchymal cells, it is rapidly induced during the tissue repair process, for example upon injury (Igarashi et al. 1993; Kapoor et al. 2008). The most potent inducer of CCN2 expression thus far identified is TGFβ. The induction of CCN2 by TGFβ is restricted to the mesenchymal cell lineages (e.g. tissue fibroblasts, myofibroblasts, pericytes) and generally not in epithelial cells (Leask et al. 2001, 2003; Kantarci et al. 2006). However, CCN2 is expressed in response to TGFβ in renal proximal tubule epithelial cells (Phanish et al. 2005). These results indicate that CCN2 gene regulation in response to TGFβ can differ based on the cell type examined.

CCN2 is over-expressed in connective tissue pathologies such as in excessive scarring and fibrosis and in stroma surrounding tumors (Blom et al. 2002). Data examining the location of CCN2 expression and the use of genetically modified cells has led to a hypothesis that CCN2 selectively mediates or sustains the specific actions of

TGFβ in mesenchymal cells (Grotendorst 1997; Leask et al. 2004). CCN2 may exert its stimulating effect on TGFβ signaling by decreasing Smad7 availability and increasing Smad2 (Qi et al. 2007). However, CCN2-deficient fibroblasts show only a partial impairment of TGFβ responses and no defect in Smad3-dependent responses (Shi-wen et al. 2006). In this review, we summarize recent key observations concerning the regulation of the expression of the CCN2 gene, insights derived from these observations into the potential contribution that CCN2 may make to cell biology, and evaluate the potential use of CCN2 as a fibrogenic bio-marker.

CCN2 gene expression in health and disease

CCN2 is principally regulated at the level of transcription (Grotendorst et al. 1996). The CCN2 proximal promoter is induced by a number of specific factors such as endothelin-1 (ET-1) and TGFβ in addition to changes such as hypoxia (Holmes et al. 2001; Leask et al. 2001, 2003; Shi-wen et al. 2004; Higgins et al. 2004). TGFβ induction of CCN2 mRNA in fibroblasts is immediate-early, occurring within 30 min of TGFβ treatment, in a fashion that does not involve *de novo* protein synthesis (Grotendorst et al. 1996). This induction is severely impaired in the fibroblasts deficient in Smad3 (Holmes et al. 2001). Consistent with this observation, a functional Smad element resides within the CCN2 promoter (Holmes et al. 2001; Fig. 1). However, the ability of TGFβ to fully induce the CCN2 promoter and protein also requires protein kinase C and the ras/MEK/ERK MAP kinase cascade (Chen et al. 2002; Leask et al. 2003; Fig. 1).

As for all Smad-responsive promoters, additional basal transcription factors (co-activators/co-repressors) are required for complete coordinated regulation of CCN2 expression by TGFβ. The Smad element of the CCN2 promoter is not sufficient to confer TGFβ-responsiveness to a heterologous promoter (Leask et al. 2001), but rather acts in concert with a tandem repeat of an element similar to an Ets/TEF recognition motif (Leask et al. 2003). The protein(s) which bind to this element are enriched in fibroblasts relative to keratinocytes, suggesting that this protein directly contributes to the specific induction of CCN2 in different cell types (Leask et al. 2003). Recently, we have shown this motif to be a functional ETS binding element; Ets-1 is required for the ability of TGFβ to induce CCN2 expression. siRNA recognizing Ets-1 ablates the ability of TGFβ to induce CCN2 (van Beek et al. 2006; Fig. 1). Moreover, the oncogenic transcriptional co-activator YAP also operates through this site (14). Additional elements, including a BCE-1 (basal control element-1) site and a Sp1 site (Holmes et al. 2001, 2003), have been shown to play a role in basal activity

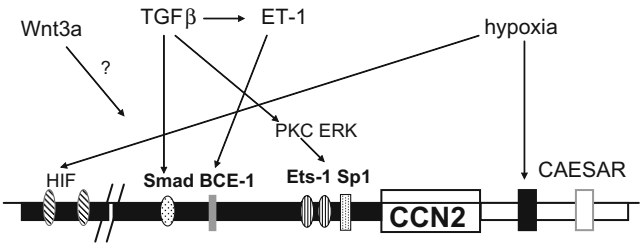

Fig. 1 Elements in the CCN2 promoter involved with its expression in normal mesenchymal cells. HIF-1=hypoxia inducible factor-1; BCE-1=basal control element-1; TEF/ets=transcription enhancer factor; CAESAR=cis-acting element of structure-anchored repression

of the CCN2 promoter (Fig. 1) and hence are indirectly required for TGFβ induced CCN2 gene expression. Sp1, similar to factors binding to a TATA box, though required for formation of a functional transcriptional complex is not directly required for TGFβ induced CCN2 expression (Holmes et al. 2003). The BCE-1 site was originally thought to be involved with the TGFβ-induction of CCN2, but has subsequently been shown to be involved with basal promoter activity (Grotendorst et al. 1996; Leask et al. 2001; Holmes et al. 2001) and in response of the CCN2 promoter to ET-1, through ras/MEK/ERK signal transduction cascade (Shi-wen et al. 2004). Since TGFβ induces ET-1 and ET-1 is essential for the ability of TGFβ to induce CCN2 (Shi-wen et al. 2007b), this element appears to be, albeit indirectly, a TGFβ response element (Grotendorst et al. 1996). Finally, Wnt3a induces CCN2 through a β-catenin-dependent mechanism, although the promoter sequences mediating this action are unknown, they reside within in the first 805 base pairs of the CCN2 proximal promoter (Luo et al. 2004; Chen et al. 2007).

It is now appreciated that post-transcriptional controls also contribute to CCN2 gene regulation. The chicken *ccn2* gene is regulated not only at the transcriptional level, but also by the interaction between a post-transcriptional element in the 3′-untranslated region (3′-UTR) and a nucleophosmin (NPM/B23) cofactor (Mukudai et al. 2008). The 3′-untranslated region (3′-UTR) has a suppressive effect on CCN2 gene expression via a minimal RNA element in the 3′-UTR, which acts as a cis-acting element of structure-anchored repression (CAESAR) (Kubota et al. 2000). Moreover, hypoxia increases stability of CCN2 mRNA in chondrosarcoma cells (Kondo et al. 2006). Finally, the unusually long 5′UTRs of CCNs 1, 2, and 4 harbor cryptic promoters that show varying degrees of activity in different cell types (Huang et al. 2007).

In fibrotic fibroblasts isolated from the involved skin area of scleroderma patients, CCN2 is over-expressed independent of TGFβ and Smads but is dependent on BCE-1, Sp1 and endothelin-1 (Holmes et al. 2001, 2003; Chen et al. 2006; Shi-wen et al. 2007a). Finally, a functional polymorphism has been found in some sclero-

derma patients which results in increased Sp1-dependent transcription (Fonseca et al. 2007). However, increased expression of CCN2 in cell-culture activated hepatic stellate cells is dependent on TGFβ (Leask et al. 2008). Intriguingly, elective disruption of TβRII in mouse skin fibroblasts increases constitutive expression CTGF/CCN2 (Denton et al. 2009). It remains to be established whether the rise in CCN2 expression in patients correlates with the increased expression of ET-1 or TGFβ. Overall, these data reveal that the regulation of CCN2 expression in fibrotic conditions is complex and is perhaps disease/organ specific.

Inhibitors of CCN2 expression

It has long been suggested that agents that inhibit CCN2 expression may be used as anti-fibrotic therapies (Blom et al. 2002). As discussed above, inhibitors of TGFβ or endothelin receptors may be used to reduce CCN2 expression in activated hepatic stellate cells or scleroderma fibroblasts, respectively (Leask et al. 2008; Shi-wen et al. 2007a). Moreover, prostaglandins [PGE(2)] and prostacyclins (such as the synthetic prostacyclin Iloprost) can antagonize CCN2 expression, including in scleroderma fibroblasts (Ricupero et al. 1999; Stratton et al. 2001, 2002). It has been shown that 9-cis-retinoic acid reduced CCN2 expression in scleroderma fibroblasts, possibly through its ability to produce PGE(2) production (Xiao et al. 2008). Iloprost works, at least in part, by antagonizing MEK/ERK signaling (Stratton et al. 2002) and, indeed, the MEK inhibitor U0126 reduces CCN2 expression in response to TGFβ and ET-1 in fibroblasts as well as constitutive over-expression of CCN2 in the pancreatic cancer cell line PANC-1 (Pickles and Leask 2007). TNFα suppresses TGFβ-induced gene expression in fibroblasts but has no appreciable effect on the constitutive CCN2 expression in scleroderma fibroblasts (Abraham et al. 2000). Caffeine also reduces TGFβ-induced CCN2 expression in hepatocytes by blocking Smad activation (Gressner et al. 2008; Leask 2008). Finally, it has been shown recently that the addition of the lipid second messenger C2-ceramide can also reduce TGFβ-induced CCN2 expression in human foreskin fibroblasts (Kennedy et al. 2008).

CCN2 as a surrogate marker of fibroproliferative disease

As discussed above, CCN2 is over-expressed in fibrotic disorders. Early studies examining the kinetics of CCN2 induction showed that, in the anti-Thy-1.1 antibody model of rat kidney fibrosis, CCN2 induction paralleled the progression of fibrogenesis and repair (Ito et al. 2001; Table 1). Subsequent studies have established that CCN2 levels in biological fluids correlate with the levels of fibrosis in patient samples (Table 1). For example, urinary CCN2 levels appear at both stage nephropathy and appear to predict those patients who are destined for progressive glomerulosclerosis and end-stage renal disease (Riser et al. 2003). In addition, glomerular basement membrane thickness correlates with tubular and total CCN2 levels (Thomson et al. 2008)

In particular, studies using an enzyme-linked immunosorbent assay (ELISA) to examining the appearance of amino-terminal fragments of CCN2 appear especially promising as potential diagnostic tools to show the severity of fibrosis. An ELISA detecting amino-terminal CCN2 appears to be superior to ELISAs detecting full-length of carboxy-terminal CCN2 as the latter moieties of CCN2 bind the cell surface and are internalized through the heparin- and integrin-binding carboxy-terminal domain of CCN2. The advantage of these ELISAs is that CCN2 can be readily detected in body fluids (e.g., urine, blood, blister fluid). Amino-terminal CCN2 ELISAs may be especially useful in diagnosing fibrosis associated with diabetes or, alternatively, in clinical trials testing the efficacy of anti-fibrotic compounds. For example, in type 1 diabetic patients with incipient and overt diabetic nephropathy, the magnitude of urinary amino-terminal excretion relates to the severity of diabetic nephropathy (Gilbert et al. 2003). Similarly, NH2-terminal CCN2 is also increased in the vitreous of patients with active progressive diabetic proliferative vitreoretinopathy, suggesting that it represents a surrogate marker of fibrosis in the disorder (Hinton et al. 2002). Indeed, plasma CCN2 levels contribute significantly to prediction of end-stage renal disease and mortality in patients with type 1 diabetic nephropathy (Nguyen et al. 2008). Such a diagnostic tool may not merely be limited to diabetes. N-terminal CCN2 levels in plasma and dermal interstitial fluid correlated with severity of skin disease of

Table 1 CCN2 levels as a surrogate marker of fibrosis: Diseases in which CCN2 levels, detected by ELISA, correlate with severity of fibrosis

Hepatic fibrosis in biliary atresia	(Tamatani et al. 1998)
Pulmonary fibrosis in scleroderma	(Sato et al. 2000)
diabetic nephropathy	(Gilbert et al. 2003)
proliferative vitreoretinopathy	(Hinton et al. 2002).
skin fibrosis in scleroderma	(Dziadzio et al. 2005)
Kidney allograft fibrosis	(Cheng et al 2006)
liver fibrosis	(Gressner et al. 2006)
chronic viral hepatitis	(Gressner et al. 2006)
Fibrotic carcinoid tumors	(Kidd et al 2007)
diabetic retinopathy	(Kuiper et al 2008)
End-stage renal disease	(Nguyen et al. 2006)
myocardial fibrosis	(Koitabashi et al. 2008)

scleroderma and (negatively) with disease duration (Dziadzio et al. 2005). Moreover in liver fibrosis, the mean concentration of total CCN2 is highest in the fibrosis group (5.2-fold) and in the chronic viral hepatitis group (4.3-fold) but lower in those patients with fully developed cirrhosis (Gressner et al. 2006). Similarly, CCN2 concentration appears to predict myocardial fibrosis in chronic heart failure patients (Koitabashi et al. 2008).

Conclusion

CCN2 was identified over 15 years ago; however, the actual physiological relevance of CCN2 is only just beginning to emerge. However, it remains clear that studies on CCN2 expression have established that CCN2 is an effective marker of fibroproliferative disease. Thus ELISAs examining CCN2 levels in patients are warranted as diagnostic tools for fibrosis as well as in investigating the efficacy of drugs in clinical trials.

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