Insights Into the Molecular Mechanism of Chronic Fibrosis: The Role of Connective Tissue Growth Factor in Scleroderma

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Connective tissue growth factor (CCN2), a member of the CCN family of proteins, is a cysteine-rich matricellular protein. Connective tissue growth factor is not normally expressed in dermal fibroblasts unless induced. The most potent inducer of connective tissue growth factor thus far identified is transforming growth factor β. Connective tissue growth factor, however, is constitutively overexpressed by fibroblasts present in skin fibrotic lesions, including scleroderma. The overexpression of connective tissue growth factor present in fibrotic lesions contributes to the phenotype of scleroderma in that connective tissue growth factor promotes matrix deposition, and fibroblast adhesion and proliferation. In animal models, whereas either transforming growth factor β or connective tissue growth factor alone produce only a transient fibrotic response, connective tissue growth factor and transforming growth factor β act together to promote sustained fibrosis. Thus the constitutive overexpression of connective tissue growth factor by fibroblasts present in fibrotic lesions would be expected to contribute directly to chronic, persistent fibrosis. This review discusses recent information regarding insights into connective tissue growth factor biology and, using scleroderma as a model system, the part connective tissue growth factor might play in fibrotic disease.

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The extracellular matrix (ECM) is essential in establishing and maintaining the structural and mechanical integrity of skin (Nimni, 1974; Kiely and Shuttleworth, 1997). Virtually every person at some point in their life suffers skin damage, and tissue repair occurs effectively. If the tissue repair process is not appropriately terminated, however, excessive deposition of ECM occurs resulting in the deposition of excessive scar tissue (Silver and Glasgold, 1995). These scars are generally limited in their nature; however, although relatively rare, progressive, pathologic, fatal scarring (fibrotic) disorders can occur. One example of such a connective tissue disease is systemic sclerosis (scleroderma; SSc), which affects the skin as well as internal organs (Simms and Korn, 2002; Valentini and Black, 2002). There is no effective treatment for this disorder, in part because the etiology of this disease is unknown. Thus, in order to develop effective anti-scarring therapies it is necessary to understand the molecular players in the normal wound healing process and how these proteins normally interact to coordinate the proper repair of the epidermis and dermis, and to understand how this process goes awry, leading to scarring and fibrosis.

One of the major cytokines involved with tissue repair and fibrosis in general is transforming growth factor (TGF)-β. TGF-β promotes deposition of the ECM by inducing expression of matrix genes (Ignotz and Massague, 1986; Quaglino et al., 1991) and decreasing the expression of matrix metalloproteinases while increasing that of tissue inhibitors of metalloproteinases (Overall et al., 1989, 1991; Yuan and Varga, 2001). Anti-TGF-β strategies, such as an anti-TGF-β antibody and an adenovirus overexpressing Smad7, are effective at blocking fibrosis in animal models (McCormick et al., 1999; Nakao et al., 1999; Yamamoto et al., 1999; Terada et al., 2002; Lan et al., 2003). In addition, mice knocked out for TGF-β or Smad 3 show alterations in wound healing, such as enhanced re-epithelialization and suppressed matrix deposition; however, these mice also show an impaired inflammatory response (Letterio and Roberts, 1996; Ashcroft et al., 1999; Crowe et al., 2000; Flanders et al., 2002) consistent with the notion that TGF-β controls a wide range of physiologic functions (McCartney-Francis et al., 1998). Thus using a global anti-TGF-β strategy as a prolonged treatment to combat chronic fibrosis might be expected to have harmful consequences for human health.

The protein connective tissue growth factor (CTGF) was initially discovered in 1991 as a secreted protein in the conditioned media of cultured human umbilical vascular endothelial cells (Bradham et al., 1991). In adult skin, however, CTGF is not normally expressed unless induced, for example during the normal wound repair process (Igarashi et al., 1993). One of the most exciting early observations concerning CTGF was that TGF-β induced CTGF expression in dermal fibroblasts, but not in epidermal keratinocytes (Igarashi et al., 1993; Frazier et al., 1996; Grotendorst et al., 1996). In contrast to the situation in normal fibroblasts, CTGF is constitutively overexpressed in dermal fibrotic lesions, such as in scleroderma (Igarashi et al., 1996; Abraham et al., 2000; Shi-wen et al., 2000; Holmes et al., 2001). These observations suggest that CTGF...
may act as a downstream effector of at least some of the pro-fibrotic effects of TGF-β and therefore that blockading CTGF might be expected to alleviate the symptoms of fibrosis, but TGF-β-dependent, CTGF-independent events unaltered (Grotendorst, 1997). This review focuses on the role that connective tissue growth factor (CTGF, CCN2) may play as marker and a mediator of the fibrotic phenotype of the disease scleroderma.

**CTGF functions**

CTGF, a member of the CCN family of matricellular proteins (Bork, 1993; Lau and Lam, 1999; Moussad and Brigstock, 2000; Perbal, 2001), promotes fibroblast proliferation, matrix production, and granulation tissue formation (Bradham et al, 1991; Frazier et al, 1996; Brigstock et al, 1997). In addition, CTGF promotes cell adhesion and migration in a wide variety of cell types (Kireeva et al, 1997; Babic et al, 1999; Shimo et al, 1999; Crean et al, 2002). During development, CTGF is strongly expressed in mesenchyme such as in hypertrophic chondrocytes in the growth plate of cartilage, the developing glomerulus and connective tissue surrounding developing muscle (Yamamoto et al, 2002; Friedrichsen et al, 2003). In addition, CTGF is expressed in the placenta during embryo implantation (Surveyor et al, 1998) and during ovarian folliculogenesis (Slee et al, 2001). Recently, CTGF knockout mice were generated. The phenotype of these mice is consistent with a role for CTGF in matrix synthesis and remodeling and cell proliferation and migration. Mice homozygous for a deletion of the CTGF gene die soon after birth, displaying an inability of the rib cage to ossify properly, including a failure of the embryo to induce expression of bone-specific matrix, such as aggrecan, and a failure of chondrocyte proliferation (Ivkovic et al, 2003). CTGF knockout mice also display a reduction in the number of capillaries invading cartilage (Ivkovic et al, 2003). CTGF knockout animals also show a decreased expression of the pro-angiogenic factor vascular endothelial growth factor (Ivkovic et al, 2003). These activities are consistent with the notion that recombinant CTGF induces chondrocyte differentiation and proliferation in vitro (Nakanishi et al, 2000) through p38 and mitogen-activated protein kinase, respectively (Yosimichi et al, 2001), and angiogenesis in vivo and in vitro (Babic et al, 1999; Shimo et al, 1999, 2001).

**CTGF receptors**

In spite of substantial efforts expended by several laboratories, a specific CTGF receptor has not yet been identified. Rather, CTGF promotes many of its activities by binding to integrins (Lau and Lam, 1999). For example, CTGF promotes angiogenesis endothelial cells through integrin αvβ3 (Babic et al, 1999), and adhesion to human foreskin fibroblasts through integrin α6β1, to human platelets through integrin αIIβ3, to endothelial cells through αvβ3 and to blood monocytes through integrin αMβ2 (Babic et al, 1999; Jedsadayamata et al, 1999; Chen CC et al, 2001; Schober et al, 2002). CTGF directly binds integrin α6β1 through a motif in the carboxyl-terminal half of CTGF (Leu et al, 2003), in the so-called thrombospondin motif (for review, see Perbal, 2001). Functional studies, however, have emphasized the essential nature of the heparin-binding domain of CTGF, which is carboxy-terminal of the thrombospondin motif, in CTGF-mediated fibroblast proliferation and attachment (Brigstock et al, 1997; Ball et al, 2003a). These results suggest that the ability of CTGF to bind cell surface heparin sulfate proteoglycans is essential for CTGF activity. Indeed, CTGF-mediated adhesive signaling possesses both integrin- and heparin sulfate proteoglycan-mediated components (Chen CC et al, 2001). Although the specific heparin sulfate proteoglycans and heparin side chains that bind CTGF have not been fully elucidated, a recent report found that the ability of CTGF to induce chondrocyte proliferation and migration required the heparin sulfate proteoglycan perlecian (Nishida et al, 2003). Thus, the available evidence suggests that CTGF mediate its effects through integrin- and heparin sulfate proteoglycan-dependent mechanisms. Iodinated CTGF has also been shown to interact with the low-density lipoprotein receptor (Segarini et al, 2001). This interaction has not yet been shown to result in a CTGF-mediated signaling event, but is involved with CTGF internalization and degradation in the endosome (Chen Y et al, 2001; Segarini et al, 2001).

**CTGF and TGF-β synergize to promote a sustained fibrotic response**

CTGF has been directly implicated in the excessive matrix deposition characteristic of scleroderma lesions. Experiments using recombinant CTGF and neutralizing antibodies targeting CTGF have suggested that CTGF mediates at least some of the effects of TGF-β on fibroblast proliferation, adhesion, and ECM production (Grotendorst, 1997; Duncan et al, 1999; Lau and Lam, 1999). One of the most compelling pieces of evidence showing CTGF can independently induce matrix is that an expression vector encoding CTGF transfected into fibroblasts can activate a cotransfected reporter construct driven by the type I collagen promoter (Shi-wen et al, 2000). These results suggest that a CTGF response element exists in the type I collagen promoter. Studies are currently underway to identify the cis-acting sequences, trans-acting factors and signal transduction mechanisms involved with this response.

Physiologically, the effect of CTGF induced by TGF-β might result in a prolonged induction of expression of collagen mRNA. In fact, CTGF seems act with TGF-β to promote a sustained fibrotic response. Mori et al (1999) showed that subcutaneous injection of TGF-β1 into neonatal mice caused a transient fibrotic response, which dissipated when ligand was no longer injected. Conversely, although injection of CTGF alone had little effect, coinjection of CTGF and TGF-β resulted in sustained fibrosis that persisted for at least a week after cessation of injection (Mori et al, 1999). Other experiments have shown that CTGF alone causes an increase in matrix deposition when injected subcutaneously (Frazier et al, 1996; Ball et al, 2003b). Recently it was shown, using an adenoviral delivery system, that overexpression of CTGF in lung tissue caused a modest,
transient fibrosis, suggesting that another stimulus might be required for sustained fibrosis (Bonniaud et al., 2003). Alternatively, it was recently suggested that CTGF may bind to TGF-β and potentiate TGF-β binding to the TGF-β type II receptors and thus contribute to the effects of TGF-β on cells, for example, by promoting activation of Smad, at least in Xenopus (Abreu et al., 2002). The relevance of these latter observations to mammalian systems is currently under investigation. The available data, however, suggest that, whereas TGF-β or CTGF alone may only result in transient fibrosis, TGF-β in the presence of high levels of CTGF may result in a sustained fibrotic response.

**Elevated levels CTGF expression in scleroderma fibroblasts are independent of the TGF-β response element of the CTGF promoter but dependent on Sp1**

It has long been known that TGF-β induction of collagen occurs directly in an immediate early fashion, requiring action of the general TGF-β signaling mediators the Smad (Chen et al., 1999). The Smad family of proteins has been extensively reviewed (for example, Roberts, 1999; Attisano and Wrana, 2002; Verrecchia and Mauviel, 2002). Briefly, in the presence of TGF-β ligand, the receptor-activated Smad, Smad2 and 3, are phosphorylated directly by the TGF-β receptor I kinase and then translocate to the nucleus complexed to the common mediator, Smad4. Maximal affinity of recombinant Smad3 and Smad4 to DNA is observed with the 5 bp sequence, CAGAC (Zawel et al., 1998). Smad2, on the other hand, does not bind DNA directly, requiring a nuclear DNA-binding protein of the Fast family to bind DNA, in association with Smad4, and activate transcription in response to TGF-β and activin (Liu et al., 1999). A third group of Smad proteins, the inhibitory Smad (I-Smad), such as Smad6 or Smad7 prevent R-Smad phosphorylation and subsequent nuclear translocation of R-Smad/Smad4 heterocomplexes (Nakao et al., 1997).

Smad3 is essential for the induction of matrix (Verrecchia et al., 2001b). Indeed, a functional Smad element resides within the CTGF promoter; the TGF-β induction of CTGF is grossly impaired in embryonic fibroblasts taken from Smad3 knockout mice (Holmes et al., 2001; Fig 1). In addition to the Smad binding element, the TGF-β-induction of CTGF also requires a consensus TEF binding element (Leask et al., 2003; Fig 1).

Smad3 is essential for the initiation of fibrosis; radiation-induced fibrosis does not develop in Smad3 knockout animals (Flanders et al., 2002). Consistent with this idea, elevated levels of Smad3 and phosphorylated Smad3 are observed during bleomycin-induced skin fibrosis for 3 wk after injection of bleomycin (Takagawa et al., 2003). Another study from the same group examined the level of unphosphorylated and phosphorylated Smad3 present in SSc fibroblasts cultured from the leading edge of the fibrotic lesion (Mori et al., 2003); that is, in the area of the fibrotic lesion involved with the initiation and expansion of the fibrotic response. In this latter study, elevated levels of phosphorylated Smad3 and expression of a generic Smad3-responsive promoter were observed in leading edge SSc fibroblasts relative to normal fibroblasts (Mori et al., 2003). Collectively, these results suggest that an elevated level of Smad3 activation is involved with the initiation of the fibrotic response in SSc. When we examined Smad expression in SSc fibroblasts cultured from established fibrotic lesions, however, we were not able to show a consistent pattern of Smad3 overexpression (Holmes et al., 2001). Thus, it is not clear whether a generally activated Smad3-dependent signaling pathway is sufficient to result in prolonged pathologic fibrosis. For example, it remains to be seen if every transcript that is Smad3 responsive is overexpressed in lesional SSc fibroblasts, or if every gene overexpressed in lesional SSc fibroblasts is Smad3 responsive in normal fibroblasts. In addition, it remains to be seen if activity of a generic Smad3-responsive promoter is upregulated in SSc fibroblasts cultured from established lesions.

In contrast to the situation in normal dermal fibroblasts, CTGF is constitutively overexpressed in dermal fibrotic lesions, such as in scleroderma (Igarashi et al., 1996; Abraham et al., 2000; Shi-wen et al., 2000; Holmes et al., 2001). As CTGF is thus an effective marker of an activated, sclerotic fibroblast, examining the mechanism underlying the overexpression of CTGF in fibroblasts cultured from fibrotic lesions is likely to provide insights into understanding the mechanism underlying persistent fibrosis.

Thus to probe the molecular mechanism underlying the maintenance of the fibrotic phenotype and to investigate the role of Smad-dependent and Smad-independent signaling pathways in the maintenance of the fibrotic phenotype in established SSc lesions, we have identified elements in the CTGF promoter required for its overexpression in lesional SSc fibroblasts. We found that the elevated expression of the CTGF promoter in SSc fibroblasts seems to be independent of its Smad response element (Holmes et al., 2001) and TGF-β response element (Leask et al., 2003). Instead, the elevated expression of the CTGF promoter in SSc fibroblasts was dependent, at least in part, on an Sp1

![Figure 1](image.png)

**Figure 1**

The regulation of CTGF in normal and scleroderma fibroblasts. A diagram of the CTGF promoter is shown, with the elements that are required for the expression of CTGF in normal and scleroderma fibroblasts. The expression of CTGF in normal fibroblasts requires induction by TGF-β and its TGF-β response element, namely Smad and TEF recognition sequences (circles with stippled and vertical lines, respectively; Holmes et al., 2001; Leask et al., 2003). The expression of CTGF in scleroderma fibroblasts requires an Sp1-binding element (open box; Holmes et al., 2003) and is independent of the TGF-β response element of the CTGF promoter.
binding element in the CTGF promoter and an elevated level of Sp1 DNA binding present in nuclear extracts prepared from scleroderma fibroblasts (Holmes et al., 2003; Fig 1). The Sp1 element of the CTGF promoter was not required for the ability of the CTGF promoter to respond to TGF-β (Holmes et al., 2003). Previously, it was shown that Sp1 regulates a wide variety of matrix genes (Verrecchia et al., 2001c). Intriguingly, the transcription factor fil-1, which suppresses collagen promoter activity through an Sp1-dependent mechanism (Czuwara-Ladykowska et al., 2001), was recently found to be markedly downregulated in SSC lesions (Kubo et al., 2003). Collectively, these results suggest that elevation of matrix genes in lesional SSC fibroblasts may involve elevated Sp1 activity.

The divergence between the requirement of TGF-β for the induction of CTGF in normal fibroblasts and the constitutive, elevated level of CTGF expression in scleroderma dermal fibroblasts is supported by other observations. Histologic studies examining the distribution of CTGF and TGF-β mRNA in skin sections showed that in diffuse SSC lesions TGF-β expression is only overexpressed in the leading inflammatory edge of the lesion, but not in the lesional area itself (Querfeld et al., 1999). In contrast, CTGF shows an expression pattern that correlates with the severity of fibrosis; that is, CTGF expression is abundant in fibrotic lesions, even in the absence of markedly elevated amounts of TGF-β ligand (Sato et al., 2000). Additional data suggesting that the elevated level of CTGF expression in fibrotic lesions is TGF-β-independent comes from data showing that tumor necrosis factor α, which is believed to contribute to the downregulation of the fibrotic response in vivo in part through the suppression Smad activity in fibroblasts (Verrecchia et al., 2000), blocks the ability of TGF-β to induce CTGF in normal and SSC fibroblasts, but does not affect the basal, constitutive levels of expression of CTGF observed in scleroderma fibroblasts (Abraham et al., 2000). Taken together, in contrast to the situation in normal dermal fibroblasts whereby CTGF is not normally expressed unless induced by TGF-β through the TGF-β response element, the persistent level of CTGF observed in lesional SSC fibroblasts seems to be independent of TGF-β ligand.

**Hypothesis:** The elevated, TGF-β-independent levels of CTGF promotes a sustained fibrotic response in scleroderma

TGF-β and the TGF-β signaling mediators, the Smad, are necessary for the induction of fibrosis in animal models (McCormick et al., 1999; Nakao et al., 1999; Yamamoto et al., 1999; Terada et al., 2002; Lan et al., 2003). In animal models, however, whereas subcutaneous injection of TGF-β or CTGF alone only causes a transient fibrotic response, simultaneous injection of TGF-β and CTGF—that is, when CTGF is present in levels over and above those normally induced by TGF-β—causes a sustained fibrotic response (Mori et al., 1999). We hypothesize that in the normal wound healing response, the upregulation of CTGF is dependent on the TGF-β response element of the CTGF promoter, and is therefore subject to the controls that normally negatively regulate the TGF-β-induced wound healing response, such as tumor necrosis factor α (Abraham et al., 2000; Fig 2). In pathologic fibrosis, however, fibroblasts display an elevated level of CTGF expression that is independent of the TGF-β response element of the CTGF promoter (Holmes et al., 2001, 2003). Thus, the elevated level of CTGF expression observed in the scleroderma fibroblast is therefore not subject to the negative regulatory controls that normally suppress the TGF-β-induced wound healing response. In fact, it is tempting to speculate that, whereas TGF-β is essential for the initiation of fibrosis it is the persistent, TGF-β-independent CTGF expression characteristic of fibrotic lesions perpetuates the wound healing response, resulting in sustained, chronic, pathologic fibrosis (Fig 2).

In conclusion, the observations concerning CTGF expression and function have led to the intriguing notion that CTGF might be a novel, molecular target for therapeutic
intervention in fibrotic disease (Grotendorst, 1997; Perbal, 2001; Leask et al, 2002; Rachal and Brigstock, 2003). As further research is conducted, the precise functional role of CTGF concerning the regulation of cellular function should emerge, and result in the development of novel methods for anti-fibrotic intervention to prevent dermal scarring and alleviate the symptoms of fibrosis.

References

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Perbal B: NOV (nephroblastoma overexpressed) and the CCN family of genes. Mol Cell Biol 26:14064–14071, 1991


