The control of *ccn2*^{*} (ctgf) gene expression in normal and scleroderma fibroblasts

A Leask, S Sa, A Holmes, X Shiwen, C M Black, D J Abraham

Abstract

Although the role of transforming growth factor β (TGF β) in initiating fibrosis is well established, the role that TGF β plays in maintaining fibrosis is unclear. The gene encoding connective tissue growth factor (*ccn2*; ctgf), which promotes fibrosis, is not normally expressed in dermal fibroblasts unless TGF β is present. However, in dermal fibroblasts cultured from lesional areas of scleroderma, *ccn2* (ctgf) is expressed constitutively. The contribution of several elements in the *ccn2* (ctgf) promoter to basal and TGF β induced *ccn2* (ctgf) expression in normal and scleroderma fibroblasts has been investigated. A functional SMAD binding site in the *ccn2* (ctgf) promoter that is necessary for the TGF β mediated induction of this gene has been identified. The previously termed TGF β responsive enhancer (TGF β RE) in the *ccn2* (ctgf) promoter has been found to be necessary for basal promoter activity in normal fibroblasts. The SMAD element is not necessary for the high *ccn2* (ctgf) promoter activity seen in scleroderma fibroblasts. However, mutation of the previously termed TGF β RE reduces *ccn2* (ctgf) promoter activity in scleroderma fibroblasts to that seen in normal fibroblasts. Thus, the maintenance of the scleroderma phenotype, as assessed by a high degree of *ccn2* (ctgf) promoter activity, appears to be relatively independent of SMAD action and seems to reflect increased basal promoter activity.

(*J Clin Pathol: Mol Pathol* 2001;54:180–183)

Keywords: connective tissue growth factor; scleroderma; transforming growth factor β

Scleroderma (systemic sclerosis) is characterised by the progressive scarring of skin and certain internal organs.¹ There is currently no effective treatment for scleroderma, and the cause of this disorder is unclear, but probably involves an autoimmune component.² Because of the ability of transforming growth factor β (TGF β) to promote fibroblast proliferation and matrix synthesis, attention has been focused on its potential role in initiating and maintaining the fibrotic phenotype (for review, see Blobe and colleagues³), including scleroderma.⁴ In acute drug or surgery induced animal models of fibrosis the neutralisation of TGF β reduces the deposition of collagen and tissue fibrosis (for review, see Blobe and colleagues³).

In scleroderma, the importance of TGF β in the maintenance of fibrosis is less clear—data

supporting a role for TGF β are circumstantial and somewhat contradictory, mainly depending on studies that have examined the histological distribution of TGF β mRNA and protein. Mononuclear cells taken from bronchial lavage fluids of patients with scleroderma have raised TGF β values.⁵ However, in skin lesions, TGF β mRNA is localised to the leading edge of the scleroderma lesion—that is, the region of enhanced inflammation involved in the initiation of the fibrotic response.^{6–8} Furthermore, fibroblasts taken from scleroderma lesions show increased amounts of collagen relative to their normal counterparts, yet show little difference in their ability to produce TGF β or in their ability to bind TGF β , and neither do they show enhanced sensitivity to TGF β treatment.^{9–10} In addition, differential display analysis between normal and scleroderma fibroblasts failed to identify genes known to be involved in the TGF β signalling pathway, such as TGF β receptors.¹¹ Thus, although some data support the role of TGF β in the onset of the fibrotic phenotype in scleroderma, its precise role in initiating or maintaining the scleroderma phenotype is unclear.

ccn2 (ctcf) is constitutively expressed in scleroderma fibroblasts

We have focused on identifying those genes playing a role in scleroderma. Using differential display analysis, we showed that connective tissue growth factor (*ccn2*; ctgf) was upregulated in dermal fibroblasts cultured from patients with scleroderma.¹¹ We estimated that scleroderma fibroblasts in culture synthesise *ccn2* (ctgf) mRNA at approximately 10–20 times the concentration seen in fibroblasts cultured from normal skin.¹¹ *ccn2* (ctgf) mRNA was also greatly increased in fibroblasts derived from unaffected skin from these patients.¹¹ CCN2 (CTGF), encoded by a member of the CCN gene family,^{12–13} is a heparin binding, 38 kDa, cysteine rich peptide that induces proliferation, collagen synthesis, and chemotaxis in mesenchymal cells,^{15–18} and has been shown to potentiate sustained fibrosis when injected together with TGF β in an acute animal model.¹⁹ *ccn2* (ctgf) mRNA and protein are expressed constitutively in numerous fibrotic disorders, both in skin and in internal organs, such as atherosclerosis and pulmonary and renal fibrosis.^{12–20–24} In contrast to TGF β , the expression of *ccn2* (ctgf) correlates well with the severity of fibrosis and collagen synthesis in scleroderma.^{25–26} Hence, in adult tissues constitutive *ccn2* (ctgf) expression is perhaps a more

^{*}According to the recommendation issued by the nomenclature committee of the CCN Society (*J Clin Pathol: Mol Pathol* 2001;54:M108), the ctgf gene is referred to as *ccn2*.

**Fibrogen Inc, 225
Gateway Blvd, South
San Francisco CA
94080, USA**
A Leask
S Sa

**Center for
Rheumatology, Royal
Free and University
College Medical
School, Rowland Hill
Street, London
NW3 2PF, UK**
A Holmes
X Shiwen
C M Black
D J Abraham

Correspondence to:
Dr Leask
aleask@fibrogen.com

Accepted for publication
8 March 2001

Figure 1 A transforming growth factor β (TGF β) responsive enhancer (TGF β RE) lies in the *ccn2* (ctgf) promoter, but the SMAD binding site and the TGF β RE are insufficient to confer TGF β responsiveness. Various *ccn2* (ctgf) promoter fragments were cloned upstream of a minimal thymidine kinase (TK) promoter–SEAP reporter construct (Clontech, Palo Alto, California, USA). Constructs were transfected into NIH3T3 cells along with a cytomegalovirus (CMV) β galactosidase (Clontech) plasmid to control for transfection efficiencies, and cells were treated for 24 hours with or without TGF β 2.^{20 28} Three *ccn2* (ctgf) promoter fragments were used: a full length *ccn2* (ctgf) promoter fragment (FLTK, containing a fragment between –805 and –50), a 23 bp DNA fragment containing the TGF β RE (TGF β RETK²⁷), and a 55 bp DNA fragment containing the SMAD element and the TGF β RE element (SMADTGF β RETK). Values are expressed relative to the plasmid without TGF β treatment ($N = 6$, SEM was less than 25% for all samples). Although the FLTK responds to TGF β , neither the TGF β RETK nor the SMADTGF β RETK constructs respond to TGF β , suggesting that there are additional motifs in the *ccn2* (ctgf) promoter involved in the TGF β mediated induction of *ccn2* (ctgf).

clinically relevant molecular marker of fibrosis and is thought to be a mediator of the fibrotic phenotype.^{12–24}

A SMAD binding element in the *ccn2* (ctgf) promoter is necessary for TGF β mediated induction but not for the increased expression seen in scleroderma fibroblasts

In contrast to the situation in fibrotic disorders, *ccn2* (ctgf) is not expressed in fibroblasts unless cells are treated with TGF β .^{14 15 20 27} Induction by TGF β is cell-type specific, because it occurs in connective tissue cells, but not in epithelial cells or lymphocytes.^{14 16} The regulation of *ccn2* (ctgf) expression by TGF β appears to be controlled primarily at the level of gene transcription.^{20 27} Grotendorst's laboratory identified an element in the *ccn2* (ctgf) promoter necessary for the response to TGF β —the TGF β responsive enhancer (TGF β RE).²⁷ However, this sequence by itself is insufficient to confer TGF β responsiveness to a heterologous promoter (fig 1).

For this reason, we assessed the *ccn2* (ctgf) promoter for additional sequences necessary for the response to TGF β . TGF β induction of gene expression has been studied extensively and generally involves the action of members of the SMAD family of proteins.²⁹ Briefly, activation of TGF β mediated gene expression is frequently mediated through SMAD2, SMAD3, and SMAD4. SMAD2 and SMAD3 are normally present in the cytosol. Once activated by TGF β , SMAD2 and SMAD3 interact transiently with type I TGF β receptor kinase and become phosphorylated at the C-terminus. SMAD2 and SMAD3 then form a heteromeric complex with SMAD4, which translocates to the nucleus and activates the expression of target genes. This pathway is negatively regulated by the inhibitory SMADs, SMAD6 and SMAD7. Recent studies have identified a consensus DNA motif, GTCTAGAC, which is a

SMAD responsive promoter element to which SMAD3 and SMAD4 bind.³⁰ A sequence similar to the consensus SMAD site is located in the region of the *ccn2* (ctgf) promoter immediately upstream of the TGF β RE.²⁸ We found that mutation of the TGF β RE in the context of the full length *ccn2* (ctgf) promoter greatly diminishes basal promoter activity but still permits a response to TGF β .²⁸ Conversely, mutating the putative SMAD binding sequence in the context of an otherwise wild-type, full length CTGF promoter–reporter construct resulted in complete abolition of TGF β induced gene expression.²⁸ In addition, gel shift analysis showed that SMAD3 and SMAD4 bound to this sequence.²⁸ Cotransfection of SMAD3 and SMAD4 enhanced *ccn2* (ctgf) expression significantly, and TGF β induction of *ccn2* (ctgf) did not occur in SMAD3 knock-out fibroblasts,²⁸ suggesting that TGF β induction of *ccn2* (ctgf) occurred in a SMAD2 independent fashion. Transfection of the inhibitory SMAD (SMAD7) greatly attenuated the ability of TGF β , SMAD3, and SMAD4 to increase *ccn2* (ctgf) promoter activity, but had little effect on basal expression.²⁸ Conversely, transfecting the inhibitory SMAD (SMAD6) had no effect on the ability of TGF β to induce *ccn2* (ctgf) promoter activity.²⁸ SMAD2/3/4 activity often requires p300 as a transcriptional cofactor.^{31 32} However, we found that transfecting either wild-type or dominant negative p300 had no impact on the TGF β induction of *ccn2* (ctgf).²⁸ Thus, *ccn2* (ctgf) gene induction requires p300 independent SMAD activity. Intriguingly, the SMAD binding site and the TGF β RE are themselves insufficient to confer TGF β responsiveness to a heterologous promoter, suggesting that additional factors are required for the TGF β induction of *ccn2* (ctgf) (fig 1).

Because *ccn2* (ctgf) gene expression is SMAD dependent and CCN2 (CTGF) is produced spontaneously by scleroderma fibroblasts in culture, we wanted to assess the contribution of SMAD dependent TGF β signalling to the high degree of CTGF gene expression seen in scleroderma fibroblasts.¹¹ In these studies, we transfected the full length *ccn2* (ctgf) promoter fused to a SEAP reporter gene into normal or scleroderma dermal fibroblasts and compared its expression to that of a *ccn2* (ctgf) promoter–SEAP reporter construct lacking either a SMAD binding site or a TGF β RE. We found that mutation of the SMAD site had little effect on the increased *ccn2* (ctgf) promoter activity seen in scleroderma (fig 2). However, mutation of the TGF β RE site greatly reduced promoter activity both in scleroderma fibroblasts and in normal fibroblasts²⁸ (fig 2). In fact, mutation of the TGF β RE reduced promoter activity in scleroderma fibroblasts to approximately that seen in normal fibroblasts. Thus, SMAD dependent TGF β signalling seems to have little to do with the maintenance of the scleroderma phenotype, as assessed by constitutive *ccn2* (ctgf) gene expression.

Figure 2 The transforming growth factor β (TGF β) responsive enhancer (TGF β RE) of the *ccn2* (ctgf) promoter is required for the increased basal *ccn2* (ctgf) promoter activity seen in scleroderma fibroblasts. The figure is adapted from data in Holmes et al.³⁰ Three constructs were transfected into normal dermal and scleroderma dermal fibroblasts: a wild-type (nucleotides -805 to +17) *ccn2* (ctgf) promoter-SEAP reporter construct (FL), and otherwise identical constructs that contained a mutated TGF β RE element (TGF β RE) or SMAD element (SMAD).³⁰ Values shown are relative to FL construct expression in normal fibroblasts. The standard error in all cases was less than 25%. Mutating the TGF β RE reduces basal *ccn2* (ctgf) promoter activity in normal and scleroderma fibroblasts, whereas mutating the SMAD element has no effect on basal expression.

Other regulators of *ccn2* (ctgf) expression in fibroblasts

It is obviously important to identify why *ccn2* (ctgf) is overexpressed in scleroderma fibroblasts to identify possible targets for drug treatment. Examining the control of *ccn2* (ctgf) expression in normal fibroblasts can provide clues to how overexpression can occur in fibrosis. Several cytokines are known to affect *ccn2* (ctgf) gene expression in fibroblasts, and the signalling pathways through which these factors act on *ccn2* (ctgf) are beginning to be elucidated.

Recently, we found that the proinflammatory cytokine tumour necrosis factor α (TNF α) blocks the TGF β induction of CCN2 (CTGF) in normal and scleroderma fibroblasts.²⁰ However, TNF α had little or no impact on basal *ccn2* (ctgf) expression in scleroderma, suggesting that the increased concentrations of CCN2 (CTGF) found in this disorder may result, in part, from an insensitivity to this cytokine.²⁰ Other regulators of *ccn2* (ctgf) gene expression also exist. For example, *ccn2* (ctgf) is induced in fibroblasts by the coagulation protease thrombin³³ and by dexamethasone,³⁴ and is suppressed by cAMP¹⁷ and prostaglandin E2.³⁵ In addition, *ccn2* (ctgf) is induced by vascular endothelial growth factor in endothelial cells,³⁶ cortisol in osteoblasts,³⁷ and WT1 (Wilms's tumour suppressor) in kidney mesangial cells.³⁸ Whether these agents are active in fibroblasts or contribute to fibrosis in these tissues is unknown.

Conclusion

The maintenance of the scleroderma phenotype, as assessed by increased CCN2 (CTGF) concentrations, does not appear to result from SMAD dependent TGF β signalling.²⁸ The TGF β RE²⁷ is required for basal *ccn2* (ctgf) promoter activity in normal fibroblasts and also contributes to the increased activity seen in scleroderma fibroblasts.^{27, 28} The factor(s) binding to this element are currently under investigation, but appear to be independent of AP-1, cAMP responsive element binding protein, or Sp1, factors known to contribute to TGF β responses in other contexts, because oligonucleotides containing consensus binding sites for these factors do not compete for protein

binding to the TGF β RE in gel shift studies (A Leask and S Sa, 1998, unpublished data). Efforts are under way to identify the factors that bind to the TGF β RE. In addition to the promoter motifs, an element in the 3' untranslated region of *ccn2* (ctgf) mRNA has been shown to act as a transcriptional repressor of *ccn2* (ctgf) in NIH3T3 fibroblasts.^{39, 40} The identity of the protein binding to this sequence is unknown, as is its contribution to fibrosis.

Examining the available evidence in the literature, it appears that in scleroderma the initial expansion of the sclerotic lesion may require the activity of TGF β , although the maintenance of the sclerotic lesion does not result from TGF β action per se.^{6-10, 28} Instead, the maintenance of the scleroderma phenotype might result from the failure to suppress downstream responses to TGF β , such as the induction of CCN2 (CTGF) and collagen synthesis. Thus, although anti-TGF β /anti-SMAD strategies might be effective in blocking the onset or progression of fibrosis,³ they may not be effective at reversing fibrosis once it has occurred. Given the known profibrotic activity of CCN2 (CTGF), the high degree of constitutive, TGF β independent *ccn2* (ctgf) expression present in the scleroderma lesion may be a cause of the excessive scarring that is seen in patients with scleroderma. Thus, the identification of the molecular mechanism underlying the overexpression of *ccn2* (ctgf) in scleroderma might yield effective methods of reversing the fibrotic process in this disorder.

This work was supported in part by NIH grant AR45879, Arthritis Research Campaign (UK), the Raynaud's and Scleroderma Association Trust, and the Nightingale Charitable Trust.

- Black CM. The aetiopathogenesis of systemic sclerosis. *J Intern Med* 1993;234:3-8.
- Feghali CA, Wright TM. Identification of multiple, differentially expressed messenger RNAs in dermal fibroblasts from patients with systemic sclerosis. *Arthritis Rheum* 1999;42:1451-7.
- Blobe GC, Schiemann WP, Lodish HF. Role of transforming growth factor beta in human disease. *N Engl J Med* 2000;342:1350-8.
- Smith E, Leroy EC. A possible role for transforming growth factor-beta in systemic sclerosis. *J Invest Dermatol* 1990;95s:125s-7s.
- Deguchi Y. Spontaneous increase of transforming growth factor beta production by bronchoalveolar mononuclear cells of patients with systemic autoimmune diseases affecting the lung. *Ann Rheum Dis* 1992;51:362-5.
- Querfeld C, Eckes B, Huerkamp C, et al. Expression of TGF-beta 1, -beta 2 and -beta 3 in localized and systemic scleroderma. *J Dermatol Sci* 1999;21:13-22.
- Withdrawn
- Higley H, Persichitte K, Chu S, et al. Immunocytochemical localization and serologic detection of transforming growth factor beta 1. Association with type I procollagen and inflammatory cell markers in diffuse and limited systemic sclerosis, morphea, and Raynaud's phenomenon. *Arthritis Rheum* 1994;37:278-88.
- Moreland LW, Goldsmith KT, Russell WJ, et al. Transforming growth factor beta within fibrotic scleroderma lungs. *Am J Med* 1992;93:628-36.
- Needleman BW, Choi J, Burrows-Mezu A, et al. Secretion and binding of transforming growth factor beta by scleroderma and normal dermal fibroblasts. *Arthritis Rheum* 1990;33:650-6.
- Shi-wen X, Pennington D, Holmes A, et al. Autocrine overexpression of CTGF maintains fibrosis: RDA analysis of fibrosis genes in systemic sclerosis. *Exp Cell Res* 2000;259:213-24.
- Grotendorst GR, Lau L, Perbal B. CCN proteins are distinct from and should not be considered members of the insulin-like growth factor-binding protein superfamily. *Endocrinology* 2000;141:2254-6.
- Moussad EEA, Brigstock DA. Connective tissue growth factor: what's in a name? *Mol Genet Metab* 2000;71:276-92.
- Bradham DM, Igarishi A, Potter RL, et al. Connective tissue growth factor: a cysteine-rich mitogen secreted by human vascular endothelial cells is related to the SRC-induced

- immediate early gene product CEF-10. *J Cell Biol* 1991;114:1285-94.
- 15 Brigstock DR, Steffen CL, Kim GY, *et al.* Purification and characterization of novel heparin-binding growth factors in uterine secretory fluids. Identification as heparin-regulated Mr 10,000 forms of connective tissue growth factor. *J Biol Chem* 1997;272:20275-82.
 - 16 Grotendorst GR. Connective tissue growth factor: a mediator of TGF-beta action on fibroblasts. *Cytokine Growth Factor Rev* 1997;8:171-9.
 - 17 Duncan MR, Frazier KS, Abramson S, *et al.* Connective tissue growth factor mediates transforming growth factor beta-induced collagen synthesis: down-regulation by cAMP. *FASEB J* 1999;13:1774-86.
 - 18 Shimo T, Nakanishi T, Kimura Y, *et al.* Inhibition of endogenous expression of connective tissue growth factor by its antisense oligonucleotide and antisense RNA suppresses proliferation and migration of vascular endothelial cells. *J Biochem* 1998;124:130-40.
 - 19 Mori T, Kawara M, Shinozaki N, *et al.* Role and interaction of connective tissue growth factor with transforming growth factor-beta in persistent fibrosis: a mouse fibrosis model. *J Cell Physiol* 1999;181:153-9.
 - 20 Abraham DJ, Shiwen X, Black CM, *et al.* Tumor necrosis factor alpha suppresses the induction of connective tissue growth factor by transforming growth factor-beta in normal and scleroderma fibroblasts. *J Biol Chem* 2000;275:1522-5.
 - 21 Dammeier J, Brauchle M, Falk W, *et al.* Connective tissue growth factor: a novel regulator of mucosal repair and fibrosis in inflammatory bowel disease? *Int J Biochem Cell Biol* 1998;30:909-22.
 - 22 di Mola FF, Friess H, Martignoni ME, *et al.* Connective tissue growth factor is a regulator for fibrosis in human chronic pancreatitis. *Ann Surg* 1999;230:63-71.
 - 23 Igarashi A, Nashiro K, Kikuchi K, *et al.* Connective tissue growth factor gene expression in tissue sections from localized scleroderma, keloid, and other fibrotic skin disorders. *J Invest Dermatol* 1996;106:729-33.
 - 24 Ito Y, Aten J, Bende RKJ, *et al.* Expression of connective tissue growth factor in human renal fibrosis. *Kidney Int* 1998;53:853-61.
 - 25 Igarashi A, Nashiro K, Kikuchi K, *et al.* Significant correlation between connective tissue growth factor gene expression and skin sclerosis in tissue sections from patients with systemic sclerosis. *J Invest Dermatol* 1995;105:280-4.
 - 26 Sato S, Nagaoka T, Hasegawa M, *et al.* Levels of interleukin 12, a cytokine of type 1 helper T cells, are elevated in sera from patients with systemic sclerosis. *J Rheumatol* 2000;27:149-54.
 - 27 Grotendorst GR, Okochi H, Hayashi N. A novel transforming growth factor beta response element controls the expression of the connective tissue growth factor gene. *Cell Growth Differ* 1996;7:469-80.
 - 28 Holmes A, Abraham DJ, Sa S, *et al.* CTGF and SMADs: maintenance of scleroderma phenotype is independent of SMAD signalling. *J Biol Chem* 2001;276:10594-601.
 - 29 Kretzschmar M, Massague J. SMADs: mediators and regulators of TGF-beta signaling. *Curr Opin Genet Dev* 1998;8:103-11.
 - 30 Zawel L, Dai JL, Buckhaults P, *et al.* Human Smad3 and Smad4 are sequence-specific transcription activators. *Molecular Cell* 1998;1:11-17.
 - 31 Janknecht R, Wells NJ, Hunter T. TGF-beta-stimulated cooperation of smad proteins with the coactivators CBP/p300. *Genes Dev* 1998;12:3084-95.
 - 32 Shen X, Hu PP, Liberati NT, *et al.* TGF-beta-induced phosphorylation of Smad3 regulates its interaction with coactivator p300/CREB-binding protein. *Mol Cell Biol* 1998;9:3309-19.
 - 33 Pendurthi UR, Allen KE, Ezban M, *et al.* Factor VIIa and thrombin induce the expression of Cyr61 and connective tissue growth factor, extracellular matrix signaling proteins that could act as possible downstream mediators in factor VIIa x tissue factor-induced signal. *J Biol Chem* 2000;275:14362-41.
 - 34 Dammeier J, Beer HD, Werner S. Dexamethasone is a novel potent inducer of connective tissue growth factor expression. Implications for glucocorticoid therapy. *J Biol Chem* 1998;273:18185-90.
 - 35 Ricupero DA, Rishikof DC, Kuang PP, *et al.* Regulation of connective tissue growth factor expression by prostaglandin E(2). *Am J Physiol* 1999;277:L1165-71.
 - 36 Suzuma K, Naruse K, Suzuma I, *et al.* Vascular endothelial growth factor induces expression of connective tissue growth factor via KDR, flt1, and phosphatidylinositol 3-kinase-akt-dependent pathways in retinal vascular cells. *J Biol Chem* 2000;275:40725-31.
 - 37 Pereira RC, Durant D, Canalis E. Transcriptional regulation of connective tissue growth factor by cortisol in osteoblasts. *Am J Physiol Endocrinol Metab* 2000;279:E570-6.
 - 38 Stanhope-Baker P, Williams BR. Identification of connective tissue growth factor as a target of WT1 transcriptional regulation. *J Biol Chem* 2000;275:38139-50.
 - 39 Kubota S, Kondo S, Eguchi T, *et al.* Identification of an RNA element that confers post-transcriptional repression of connective tissue growth factor/hypertrophic chondrocyte specific 24 (ctgf/hcs24) gene: similarities to retroviral RNA-protein. *Oncogene* 2000;19:4773-86.
 - 40 Kondo S, Kubota S, Eguchi T, *et al.* Characterization of a mouse ctgf 3'-UTR segment that mediates repressive regulation of gene expression. *Biochem Biophys Res Commun* 2000;278:119-24.

Find out what's in the latest issue
the moment it's published

Email Alerts

Sign up to receive the table of contents by email every month. You can select from three alerts:
Table of Contents (full), TOC Awareness (notice only); *Journal of Clinical Pathology* related announcements.

www.jclinpath.com