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REVIEW ARTICLE

Connective tissue growth factor (CTGF/CCN2) ELISA: a novel tool for monitoring fibrosis

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Abstract

Background: Connective tissue growth factor (CTGF) has been identified as a key factor in the pathogenesis of diseases with significant fibrosis-related complications such as hepatitis, diabetes and renal transplantation. Increasing evidence shows that CTGF levels in plasma, serum and urine have promising biomarker applicability in these disorders.

Objective: To present an overview of current knowledge on CTGF in various patient populations and the technical aspects of CTGF measurement by enzyme-linked immunosorbent assay (ELISA).

Method: We performed a comprehensive literature search by using electronic bibliographic databases.

Conclusion: CTGF is associated with disease severity parameters and outcome in fibrotic disease and may have diagnostic and prognostic values. However, CTGF ELISA needs standardization.

Keywords: Connective *tissue growth factor, fibrosis, biomarker, sandwich ELISA*

Introduction

Fibrosis represents the common final pathway of chronic disease processes and may be triggered by a variety of inflammatory, ischemic and metabolic disorders. The onset is often insidious and ongoing disease leads to distortion of functional architecture, compromising adequate cell signalling and homeostasis (Wynn, 2008). Transforming growth factor-β1 (TGF-β1) is generally regarded as one of the key growth factors involved in fibrosis. However, many of the downstream effects of TGF-β1 leading to deposition of extracellular matrix (ECM) are mediated by connective tissue growth factor (CTGF), a 36–38-kDa polypeptide, which is member of the CCN (CTGF/Cyr6/Nov) family (Leask and Abraham, 2006). CTGF has been shown to be up-regulated in various chronic diseases including liver fibrosis, systemic sclerosis, diabetic nephropathy (DN) and non-diabetic chronic kidney disease, idiopathic interstitial pneumonias, cardiomyopathy, atherosclerotic plaques, nephrogenic systemic fibrosis, peritoneal fibrosis in peritoneal dialysis patients and urethral stricture (Igarashi et al., 1996; Ito et al., 1998; Allen et al., 1999; Paradis et al., 1999; Sato et al., 2000; Yoshisue et al., 2002; Cicha et al., 2005; Zhang et al., 2008; Kono et al., 2010; Mizutani et al., 2010; Schieren et al., 2010).

As the clinical course of chronic fibrotic disorders varies between individual patients, there is great need for non-invasive biomarkers that can help predict progression. CTGF seems an ideal candidate biomarker for monitoring ongoing fibrosis as it is a key mediator of fibrosis and is readily quantifiable in body fluids. Experimental studies indicate that CTGF expression increases over time (Mori et al., 1999; Yokoi et al., 2002; Roestenberg et al., 2006), suggesting that CTGF levels run parallel with

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Abbreviations

ACE	angiotensin-converting enzyme
ACE-I	angiotensin-converting enzyme inhibitor
BA	biliary atresia
BCE-1	basal control element-1
BMP	bone morphogenetic protein
BNP	brain natriuretic peptide
CAN	chronic allograft nephropathy
CI	confidence interval
CLD	chronic liver disease
COPD	chronic obstructive pulmonary disease
CTGF	connective tissue growth factor
DM	diabetes mellitus
DN	diabetic nephropathy
ECM	extracellular matrix
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
GFR	glomerular filtration rate
HBV	hepatitis B virus
HCV	hepatitis C virus
HIF	hypoxia-inducible factor
IGF	insulin-like growth factor
IQR	interquartile range
LPS	lipopolysaccharide
LRP	low-density lipoprotein receptor-related protein
NYHA	New York Heart Association, abbreviation is also used for the NYHA classification for heart failure
Smad	Signalling mother against decapentaplegic peptide
Sp1	signal protein 1
TA/IF	tubular atrophy/interstitial fibrosis
TGF-β	transforming growth factor-β
TrkA	tyrosine kinase protein A
UAE	urinary albumin excretion
VEGF	vascular endothelial growth factor

the process of fibrosis. Here, we review published studies on CTGF in different patient populations. In addition, we evaluate technical aspects of measuring CTGF levels, which is currently performed by a wide variety of enzyme-linked immunosorbent assay (ELISA) formats. For this purpose, a comprehensive literature search was performed with PubMed and Google Scholar using combinations of the following keywords: CTGF, CCN2, ELISA, fibrosis, biomarker. The search was narrowed by eliminating non-human and non-English articles. Bibliographies of selected articles and reviews were searched for other relevant articles.

General aspects of CTGF regulation and function

CTGF displays a variety of biological functions, strongly dependent on cell type and condition. CTGF is known to be induced by TGF-β1 but also by other pathways, involving a variety of factors, including hypoxia-inducible factor (HIF), angiotensin II, signalling mother against decapentaplegic peptide (Smad), endothelin-1 (Kemp et al., 2004), signal protein 1 (Sp1) and basal control element-1 (BCE-1) (Iwanciw et al., 2003). In systemic sclerosis, CTGF induction is more likely to depend on endothelin-1 than on TGF-β1 (Higgins et al., 2004; Leask and Abraham, 2006). A molecule-specific receptor for CTGF has not been published yet. However, interactions of CTGF with insulin-like growth factor (IGF), TGF-β1, bone morphogenetic protein (BMP)-2, BMP-4, BMP-7, vascular endothelial growth factor (VEGF), tyrosine kinase protein A (TrkA), p75 NTR, low-density lipoprotein receptor-related protein (LRP)-1 and LRP-6 were reported in cell cultures and animal models. Additionally, CTGF binds to integrins, aggrecan, heparan sulphate-containing proteoglycans and fibronectin (Kim et al., 1997; Segarini et al., 2001; Abreu et al., 2002; Inoki et al., 2002; Gao and Brigstock, 2004; Mercurio et al., 2004; Wahab et al., 2005; Heng et al., 2006; Hoshijima et al., 2006; Aoyama et al., 2009; Maeda et al., 2009) (Figure 1). Intracellularly, Smad and ERK1/2 MAPK pathways are the main signal transduction pathways known to be influenced by CTGF (Phanish et al., 2010). Resulting biological functions of CTGF include differentiation, proliferation, migration, hypertrophy, angiogenesis, ECM deposition and remodelling, fibronectin production, stimulation or inhibition of angiogenesis and cell–matrix interactions (Perbal, 2004). One of the most important functions of CTGF is the induction of ECM production, both TGF-β1-dependent and -independent. Intracellularly, Smad and ERK1/2 MAPK pathways are the main signal-transducing pathways known to be influenced by CTGF (Nguyen and Goldschmeding, 2008). For a detailed overview on the functions and mechanisms of CTGF regulation, we refer to some of the excellent reviews that have been published recently (Cicha and Goppelt-Struebe, 2009; Phanish et al., 2010).

The CTGF molecule

The CTGF molecule consists of four distinct cysteine-rich interaction domains and a signal peptide. Between domains 2 and 3, the protein contains a cysteine-free hinge region, which contains multiple cleavage sites susceptible to proteolysis by a number of different proteases generating N- and C-terminal fragments of similar molecular weight. The C-terminal half can be further cleaved yielding a fragment of 9–12 kDa containing domain 4 (Brigstock et al., 1997; Grotendorst and Duncan, 2005) (Figure 1). In cell culture supernatants and in body fluids, both full-length CTGF and its fragments have been detected. Full-length CTGF and N-fragment were found in plasma, serum, urine, vitreous fluid (Hinton et al., 2004; Kuiper et al., 2006), dermal interstitial fluid (Dziadzio et al., 2005) and peritoneal dialysate (Zarrinkalam et al., 2003). Importantly, in plasma and urine the amount of N-fragment is much

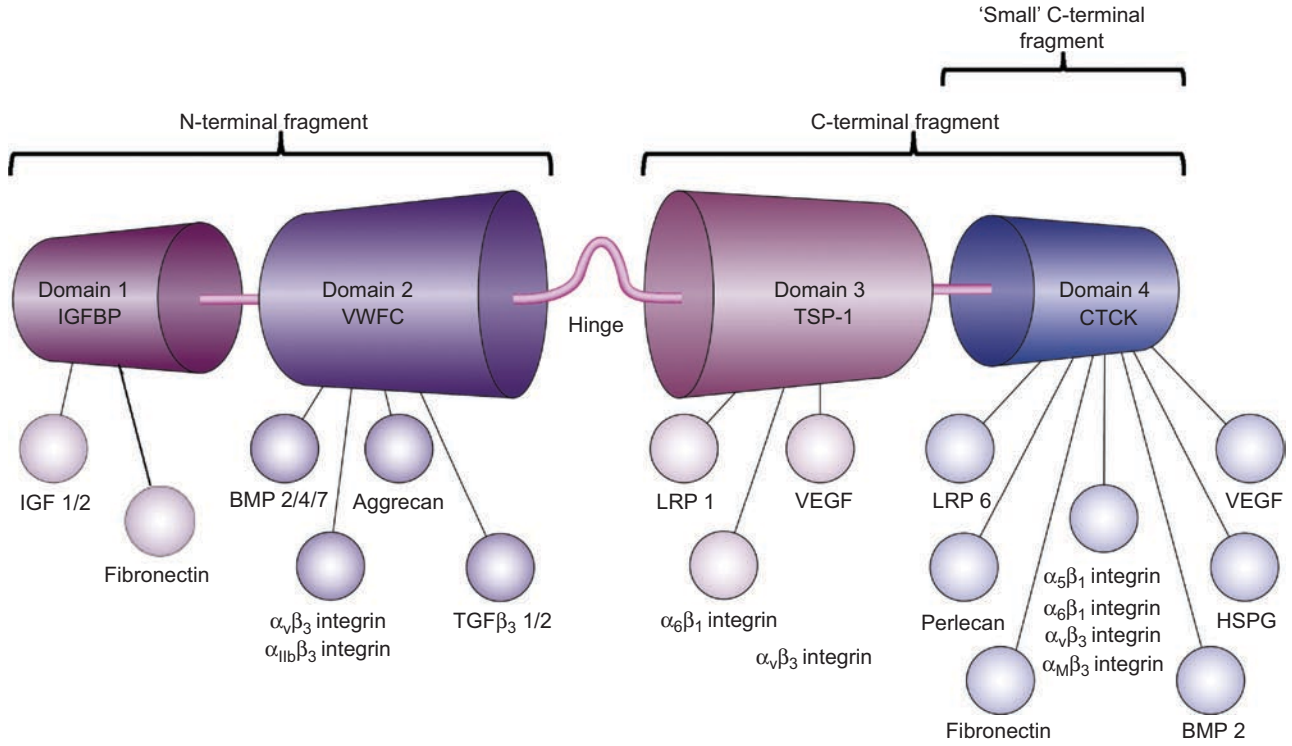

Figure 1. Connective tissue growth factor (CTGF) structure and its binding partners. The CTGF molecule consists of four domains and hinge region, which can be cleaved by proteases generating an N- and C-terminal fragment. The C-terminal half can be further cleaved yielding a fragment containing domain 4. The individual domains interact with different ligands, as depicted below each domain. BMP, bone morphogenetic protein; CTCK, C-terminal cysteine knot-like domain; HSPG, heparan sulphate proteoglycan; IGF, insulin-like growth factor; IGFBP, insulin-like growth factor-binding protein domain; LRP, lipoprotein receptor-related protein; TGF-β, transforming growth factor-β; TSP-1, thrombospondin repeat type 1 domain; VEGF, vascular endothelial growth factor; VWFC, von Willebrand factor type C repeat domain.

more abundant than full-length protein, which is hardly detectable by available techniques (Cicha et al., 2004; Kubota et al., 2004; Roestenberg et al., 2004; Miyazaki et al., 2010). C-fragment was recovered from serum and urine, and western blotting of peritoneal dialysate also yielded a 23-kDa heparin-binding fragment probably representing C-terminal fragment (Zarrinkalam et al., 2003). A 12-kDa fragment was detected in urine of diabetic patients by western blotting (Riser et al., 2003).

Little is known about the biological significance of CTGF fragments. *In vitro* studies suggest that the various CTGF proteins differ in their biological actions. In normal rat kidney fibroblasts, the N-terminal fragment induced myofibroblast differentiation and collagen synthesis in the presence of IGF, whereas the C-terminal fragment domain stimulated proliferation in the presence of epidermal growth factor (EGF). Activity of the fragments was approximately one-tenth of that of full-length CTGF (Grotendorst and Duncan, 2005). In isolated cardiomyocytes, overexpression of full-length CTGF induced hypertrophic growth, whereas deletion of the C-terminal domain antagonized the hypertrophic effect (Yoon et al., 2010). In hepatic stellate cells, module 3 of CTGF exhibited integrin binding, signalling and fibrogenic properties (Tong and Brigstock, 2006). Interestingly, the LRP-binding site is also located on domain 3 of the C-terminal fragment (Gao et al., 2003) and might be involved in clearance of the full-length molecule and the C-fragment. The N-terminal fragment might therefore have a longer half-life, which might explain the relative abundance of the N-fragment in body fluids. *In vivo* data on CTGF fragments are even scarcer. Recently, Sánchez-López et al. (2009) reported that intravenous injection of a recombinant 11.2-kDa human CTGF peptide representing domain 4 of the protein induced renal inflammatory responses by activating the NF-κB pathway.

Technical aspects of CTGF measurement

In general, sandwich ELISA technology is most suitable for determination of small proteins and peptides present in limited amounts. In this technique, the antibody attached to the plate provides immune-specific antigen capture, whereas the second antibody linked to an enzyme provides immune-specific detection and amplification. This approach enables accurate and sensitive detection of the antigen. An additional advantage of ELISA is the fact that the results are highly quantitative and generally reproducible. A weakness of the method is dependency on epitope stability.

For CTGF detection, different ELISA formats have been applied with antibodies directed against different CTGF domains, thus detecting different CTGF isoforms

(Table 1). The most commonly applied assays detect either the full-length protein and the N-fragment or the full-length protein and the C-fragment. The clinical significance of the individual fragments is not known, but the various formats might have different clinical value. Regarding the specificity of the ELISA assay, some studies report that they checked for cross-reactivity with other members of the CCN family, including Cyr61, NOV, Wisp1–3. This might be important because of significant structural and sequence homology, although most assays have employed antibodies raised against peptides representing CTGF-specific amino acid sequences (Chen and Lau, 2009).

Most of the clinical studies have used plasma for determination of CTGF in blood, whereas others have used serum (Table 1). However, platelets contain a large amount of full-length CTGF, which were shown to be released into serum during the coagulation of blood. This might theoretically interfere with reliable CTGF measurement in serum and we therefore advocate the use of plasma instead of serum. Also, shear stress and storage at room temperature are shown to induce release of full-length CTGF from platelets and careful handling of the samples and storage on ice seems warranted (Cicha et al., 2004; Miyazaki et al., 2010). N-Fragment levels are not confounded by platelet activation (Miyazaki et al., 2010).

For plasma, the type of anticoagulant might also matter since the CTGF molecule contains a heparin-binding domain. Theoretically, when using heparin plasma for CTGF measurement, heparin binding may prevent binding of the CTGF molecule to the antibodies of the sandwich ELISA by steric hindrance, obscuring the actual plasma levels in the patient. Since the heparin-binding domain is located at the C-terminal fragment, this especially concerns measurement of full-length CTGF and the C-fragment. For this reason, we discourage the use of heparin plasma for the CTGF ELISA.

The stability of CTGF differs between molecules and matrices. The full-length molecule is known to be less stable than the N-terminal fragment. This is, at least in part, due to degradation of the full-length protein by proteases in urine and possibly also in plasma. The N-terminal fragment is resistant to protease degradation and is stable in urine during storage at 4°C for at least 24 h (Weitz and Usinger, 2003). In plasma, the N-fragment was found to be stable for at least four cycles of freezing (–80°C) and thawing (own observations) and during storage at 25°C for at least 24 h (Miyazaki et al., 2010). The stability of the C-fragment is not known.

Recently, a Chinese group developed an alternative ELISA method for detection of urinary CTGF. It concerns a competitive indirect ELISA method using rabbit anti-CTGF polyclonal (H-55, Santa Cruz Biotechnology, Santa Cruz, CA). The advantages of competitive ELISA tests include faster performance, consisting of one-step addition of reagents, and reducing the total test time to <2 h. However, this test has not yet been validated on human samples (Bao et al., 2008).

Besides pre-analytical, methodological and technical considerations, it is not known to what extent CTGF levels vary over time in the individual patient.

In conclusion, validation of the different testing methods, inter-test comparisons and longitudinal studies of CTGF levels within the same patient are highly warranted to allow clinical implementation and comparison between studies.

Clinical studies

Table 1 gives an overview of the clinical studies evaluating CTGF levels in various patient populations and summarizes the associations of CTGF levels with clinical parameters.

CTGF in fibrotic liver disease

There is a great need for non-invasive testing to estimate the degree of fibrosis in chronic liver disease (CLD), mainly because liver biopsy is associated with a substantial risk of bleeding in CLD. CTGF levels are strongly elevated in liver fibrosis. Hepatic stellate cells are a major source of CTGF in liver fibrosis, but increased CTGF expression is also reported in hepatocytes, bile duct epithelial cells, sinusoidal and vascular endothelial cells, depending on the pathological condition (Paradis et al., 1999; Narkewicz et al., 2005).

Biliary atresia (BA) is a congenital disorder of unknown aetiology leading to neonatal jaundice, liver fibrosis and eventually end-stage liver disease necessitating transplantation in most patients. The hallmark of the pathogenic process is fibrosis of biliary ducts, in which TGF-β1 and CTGF are presumed to play a role (Kobayashi et al., 2005; Roach and Bruny, 2008). In a cross-sectional study, serum levels of 36 BA patients and 10 healthy controls were analysed for CTGF (Tamatani et al., 1998). Patients were subdivided according to liver function (normal liver function, moderate liver dysfunction and severe liver dysfunction) by laboratory parameters. CTGF levels were especially raised in moderate liver dysfunction (20-fold), with lower levels in patients with severe liver dysfunction.

In addition, in adult liver fibrosis CTGF levels are elevated in all types of CLD (e.g. alcoholic, viral or cryptogenic) (Gressner et al., 2006; Kovalenko et al., 2009; Zhang et al., 2009) (Table 1). Most of the evidence for an association between CTGF levels and liver fibrosis stage come from studies looking at chronic hepatitis B virus (HBV) or hepatitis C virus (HCV) infection (Kovalenko et al., 2009; Zhang et al., 2009; Guo-Qiu et al., 2010). The diagnostic value of serum CTGF in HCV-related liver fibrosis might be explained by the known interaction between the HCV core protein and the TGF-β1 promotor, upstream from CTGF regulation (Kovalenko et al., 2009). However, Morikawa et al. (2007) found no relationship between CTGF level and fibrosis stages in

Table 1. Overview of clinical studies reporting ELISA-measured CTGF levels in various chronic diseases.

Authors	Disease	Material	Form of CTGF detected	Antibodies	Study groups	CTGF values	Conclusion
Tamatani et al. (1998)	Biliary atresia	Serum	Full-length CTGF, fragments not specified	Mouse monoclonal MHCT1 and MHCT2 (from hybridomas)	BA, normal liver function ($N=17$) BA, moderate dysfunction ($N=12$) BA, severe dysfunction ($N=7$) Healthy controls ($N=10$)	4.3 ng/mL (<0.5–28.0) 82.9 ng/mL (0.7–22.1) 2.7 ng/mL (<0.5–14.0) Undetectable	Serum CTGF is elevated in BA patients with normal and moderate liver dysfunction ($P<0.01$), with marked increase in moderate liver dysfunction. Cross-sectional study design
Gressner et al. (2006)	Chronic liver disease of diverse aetiology	Serum	Full-length CTGF	Polyclonal goat L-20, polyclonal rabbit H-55 (Santa Cruz Biotechnology)	Chronic HBV/HCV, suspected for fibrosis or cirrhosis, no biopsy ($N=17$) Fibrosis ($N=16$) Cirrhosis ($N=50$) Healthy controls ($N=24$)	975 ng/mL (IQR 801–2388) 1805 ng/mL (IQR 1264–2778) 895 ng/mL (IQR 504–1607) 199 ng/mL (IQR 120–294)	Serum CTGF is elevated in all patient groups ($P<0.001$) with higher levels in fibrosis than in cirrhosis ($P=0.003$). Cross-sectional study design
Morikawa et al. (2007)	HCV-related liver fibrosis Idiopathic portal hypertension	Serum	Not specified	Monoclonals M295 and 8-86-2 (Japan Tobacco Inc., Tokyo, Japan)	HCV Fibrosis stage 1 ($N=24$) HCV Fibrosis stage 2 ($N=24$) HCV Fibrosis stage 3 ($N=24$) HCV Fibrosis stage 4 ($N=12$) Idiopathic portal hypertension ($N=76$) Healthy controls ($N=38$)	36.1 ± 11.7 ng/mL 31.7 ± 8.5 ng/mL 32.1 ± 6.2 ng/mL 34.0 ± 9.8 ng/mL 28.8 ± 32.8 ng/mL 19.1 ± 12.5 ng/mL	Serum CTGF is elevated in HCV-related fibrosis ($P<0.05$) and in idiopathic portal hypertension ($P<0.05$). No difference between stages of fibrosis. Cross-sectional study design
Kovalenko et al. (2009)	Chronic liver disease, including HCV	Serum	Not specified	Monoclonal mouse and polyclonal goat antibody (Santa Cruz Biotechnology)	HCV patients ($N=138$) CLD patients, diverse aetiology ($N=129$) Healthy controls ($N=36$)	46.0 ng/mL (11–189) 21.7 ng/mL (0–400) 15.4 ng/mL (0–33.2)	Serum CTGF is elevated in hepatitis C ($P<0.001$) and in CLD of diverse aetiology ($P=0.003$). Highest CTGF is found in HCV patients with cirrhosis (fibrosis stage F4). Cross-sectional study design
Zhang et al. (2009)	Liver fibrosis (67% HBV-related)	Serum	Full-length CTGF, N-CTGF	Monoclonal and polyclonal antibody, details not specified (RapidBio Lab, Columbia, CA)	Liver fibrosis patients ($N=125$) Healthy controls ($N=58$)	11.5 ng/mL (CI 5.4–29.8) 4.5 ng/mL (CI 2.1–5.6)	Serum CTGF is elevated in liver fibrosis with a clear correlation with fibrosis stage ($r=0.69$, $P<0.001$). Cross-sectional study design.
Guo-Qiu et al. (2010)	Chronic hepatitis B liver disease	Serum	Full-length CTGF, C-CTGF	Monoclonal mouse (from hybridoma), polyclonal rabbit (not further specified)	HBV patients ($N=83$) Healthy controls ($N=18$)	162 ± 108 ng/mL 52 ± 5.8 ng/mL	Serum CTGF is elevated in hepatitis B ($P<0.001$) and correlates strongly with fibrosis stage ($r=0.89$, $P<0.005$). Cross-sectional study design.
Sato et al. (2000)	Systemic sclerosis	Serum	Full-length CTGF, fragments not specified	Mouse monoclonal MHCT1 and MHCT2 (from hybridomas)	Diffuse scleroderma ($N=28$) Limited scleroderma ($N=32$) Healthy controls ($N=30$)	Exact values not shown	Serum CTGF is elevated in both diffuse scleroderma and limited scleroderma with higher levels in diffuse scleroderma. Cross-sectional study design

Table 1. continued on next page

Table 1. Continued.

Authors	Disease	Material	Form of CTGF detected	Antibodies	Study groups	CTGF values	Conclusion
Dziadzio et al. (2005)	Systemic sclerosis	Plasma	Full-length CTGF, N-CTGF	Monoclonal human FG-3019, monoclonal human FG-3001 (FibroGen Inc.)	Diffuse scleroderma ($N=26$) Limited scleroderma ($N=21$) Healthy controls ($N=18$)	N-CTGF: 83 ng/mL (IQR 60–116) 42 ng/mL (IQR 25–50) 25 ng/mL (IQR 17–30)	Plasma N-CTGF is elevated in scleroderma ($P<0.0001$) and correlates with severity of skin involvement ($r=0.43$, $P=0.0015$). Cross-sectional study design.
Gilbert et al. (2003)	Diabetic nephropathy in type 1 diabetes	Urine	Full-length CTGF, N-CTGF	Monoclonal human FG-3019, monoclonal human FG-3001 (FibroGen Inc.)	Macroalbuminuria, no ACE-I ($N=5$) Macroalbuminuria with ACE-I ($N=8$) Microalbuminuria ($N=8$) Normoalbuminuria ($N=10$)	203 ± 3.8 ng/mL 6.5 ± 1.7 ng/mL 2.1 ± 1.7 ng/mL 0.23 ± 1.3 ng/mL	Urinary CTGF is elevated in macroalbuminuric ($P<0.001$) and microalbuminuric ($P<0.05$) patients as compared with normoalbuminuric patients but attenuated by ACE inhibition. Urinary CTGF excretion correlates with UAE ($r=0.76$, $P<0.001$). Cross-sectional study design
Roestenberg et al. (2004)	Diabetic nephropathy in type 1 diabetes	Plasma	Full-length CTGF, N-CTGF	Monoclonal human FG-3019, monoclonal human FG-3001 (FibroGen Inc.)	Macroalbuminuria ($N=10$) Microalbuminuria ($N=12$) Normoalbuminuria ($N=40$) Healthy controls ($N=21$)	290 ± 101 pmol/L 203 ± 203 pmol/L 80 ± 66 pmol/L 103 ± 51 pmol/L	Plasma CTGF is elevated in DN and correlates with UAE ($r=0.57$, $P<0.001$) and (inversely) with GFR. Cross-sectional study design
Andersen et al. (2005)	Diabetic nephropathy in type 1 diabetes	Urine	Full-length CTGF, N-CTGF	Monoclonal human FG-3019, monoclonal human FG-3001 (FibroGen Inc.)	Diabetic nephropathy, baseline Diabetic nephropathy, after 36 months of losartan treatment	7.1 μg/24 h (CI 5.7–8.8) 4.7 μg/24 h (CI 3.6–5.9)	Losartan treatment reduces urinary CTGF excretion by 22% (CI 12–32) (mean follow-up of 36 months). Reduction in urinary CTGF excretion correlates with reduction in UAE ($r=0.54$, $P<0.01$) and inversely with rate of decline in GFR ($r=0.23$, $P=0.05$). Prospective study design.
Nguyen et al. (2006)	Diabetic nephropathy in type 1 diabetes	Urine	Full-length CTGF, N-CTGF	Monoclonal human FG-3019, monoclonal human FG-3001 (FibroGen Inc.)	Macroalbuminuria ($N=89$) Microalbuminuria ($N=79$) Normoalbuminuria ($N=150$) Healthy controls ($N=29$)	155 pmol/24 h (IQR 96–258) 100 pmol/24 h (IQR 65–78) 85 pmol/24 h (IQR 48–127) 100 pmol/24 h (IQR 78–114)	Urinary CTGF is increased in macroalbuminuric DN and correlates with UAE ($r=0.43$, $P<0.01$) and GFR ($r=-0.26$, $P<0.01$). Cross-sectional study design.
Nguyen et al. (2008)	Diabetic nephropathy in type 1 diabetes	Plasma	Full-length CTGF, N-CTGF	Monoclonal human FG-3019, monoclonal human FG-3001 (FibroGen Inc.)	Macroalbuminuria ($N=198$) Normoalbuminuria ($N=188$)	381 pmol/L (IQR 270–630) 235 pmol/L (IQR 168–353)	Plasma CTGF is higher in macroalbuminuric DN ($P<0.001$). Baseline plasma CTGF correlates with rate of decline in GFR. Elevated baseline plasma CTGF is an independent predictor of end-stage renal disease and mortality in DN (HR 1.6 ($P=0.03$) and 1.4 ($P=0.005$), respectively; HR 4.5 ($P<0.001$) and 3 ($P=0.01$), respectively, in nephrotic range albuminuric patients). Prospective study design.

Table 1. continued on next page

Table 1. Continued.

Authors	Disease	Material	Form of CTGF detected	Antibodies	Study groups	CTGF values	Conclusion
Tam et al. (2009)	Diabetic nephropathy, type not specified	Urine	Full-length CTGF, C-CTGF	Polyclonal goat L-20 (Santa Cruz) and polyclonal rabbit pAb839	Macroalbuminuria ($N=17$) Microalbuminuria ($N=14$) Normoalbuminuria ($N=12$)	5.9 µg/mmol Cr (IQR 4.3–10.8) 10.0 µg/mmol Cr (IQR 1.3–13.9) 0.08 µg/mmol Cr (IQR 0–5.1)	Urinary CTGF is elevated in microalbuminuria ($P<0.01$) but declines in macroalbuminuria. Urinary CTGF correlates with rise in microalbuminuria over 1 year ($r=0.49$, $P<0.05$). Prospective study design.
Cheng et al. (2006)	Kidney transplant patients	Serum Urine	Full-length CTGF, N-CTGF	Monoclonal human FG-3019, monoclonal human FG-3001 (FibroGen Inc.)	Transplant recipients ($N=29$) Healthy controls ($N=8$) Transplant recipients ($N=29$) Healthy controls ($N=20$)	Serum: 50.1 ± 3.9 ng/mL 15.4 ± 3.6 ng/mL Urine: 32.2 ± 6.0 ng/mg Cr 0.8 ± 0.2 ng/mg Cr	Serum and urinary CTGF are elevated in transplant patients ($P<0.001$ and $P<0.0001$, respectively). Urinary CTGF is lowest in patients without rejection (22.2 ± 4.0 ng/mg Cr), intermediate in acute rejection (39.0 ± 11.6 ng/mg Cr) and highest in CAN (57.4 ± 16.0 ng/mg Cr) ($P=0.005$). Urinary CTGF is higher in CAN than in acute rejection. Urinary CTGF correlates with proteinuria ($r=0.49$, $P=0.007$). Cross-sectional study design.
Ito et al. (2010)	Hypertensive nephro-sclerosis	Plasma Urine	Full-length CTGF, N-CTGF	Monoclonal human FG-3019, monoclonal human FG-3001 (FibroGen Inc.)	Hypertensive nephrosclerosis ($N=21$) Hypertension ($N=19$) Healthy controls ($N=28$) Hypertensive nephrosclerosis ($N=21$) Hypertension ($N=19$) Healthy controls ($N=28$)	Plasma: 543 ± 336 pmol/L 286 ± 260 pmol/L 182 ± 123 pmol/L Urine: 1113 ± 1620 pmol/mg Cr 92 ± 48 pmol/mg Cr 73 ± 37 pmol/mg Cr	Plasma and urinary CTGF are elevated in patients with hypertensive nephrosclerosis (plasma: $P<0.05$ versus hypertension and control group; urine: $P<0.005$ versus hypertension group, $P<0.001$ versus control group). Cross-sectional study design.
Koitabashi et al. (2008)	Chronic heart failure	Serum	Full-length CTGF, fragments not specified	Mouse monoclonal MHCT1 and MHCT2 (from hybridomas)	NYHA class III ($N=4$) NYHA class II ($N=24$) NYHA class I ($N=24$)	14.3 ng/mL (IQR 8.9–24.0) 9.2 ng/mL (IQR 6.6–12.3) 2.5 ng/mL (IQR 0–8.9)	Plasma CTGF is elevated in chronic heart failure patients in proportion to their NYHA class ($P<0.001$) and correlates with plasma BNP ($r=0.34$, $P<0.01$) and echocardiographic E/E' value ($r=0.59$, $P<0.05$). Cross-sectional study design.
Bergestuen et al. (2010)	Carcinoid heart disease	Plasma	Full-length CTGF, C-CTGF	Monoclonal MAB660 and goat polyclonal BAF 660 (R&D Systems, Abingdon, UK)	Severe valvular involvement ($N=3$) No/minimal valvular involvement ($N=29$) Controls ($N=18$)	166 ng/mL (IQR 14–4000) 60 ng/mL (IQR 32–112) 78 ng/mL (IQR 59–347)	Plasma CTGF correlates with right ventricular function ($r=-0.47$, $P<0.001$). CTGF ≥ 77 ng/mL is an independent predictor of reduced right ventricular function (OR 15.36, $P<0.001$) and CTGF ≥ 86 ng/mL an independent predictor of right-sided valvular regurgitation (OR 7.8, $P=0.008$). Prospective study design.

Data presented as mean ± SD or median (range) unless indicated otherwise. Abbreviations are explained in the abbreviation list.

HCV-related liver fibrosis. Interestingly, in idiopathic portal hypertension, a condition without parenchymatous fibrosis, CTGF levels were also significantly higher than in controls.

CTGF in systemic sclerosis

In scleroderma, studies have shown that TGF-β1 is mainly elevated at the start of the fibrotic response, at the active site of early skin lesions (Higley et al., 1994). CTGF, on the contrary, seems to be overexpressed at every step of the pathogenic process, in early lesions as well as in established fibrotic skin (Igarashi et al., 1996). Promoter polymorphisms in the CTGF gene are able to influence its transcription and expression, leading to more severe disease (Fonseca et al., 2007; Kawaguchi et al., 2008; Granel et al., 2010; Kovalenko et al., 2009). These data emphasize the important pathophysiological role of CTGF in systemic sclerosis. The first report of elevated systemic CTGF levels in systemic sclerosis was made by Sato et al (Sato et al., 2000). Serum CTGF was higher in diffuse scleroderma associated with pulmonary complications than in limited scleroderma and healthy controls. Moreover, CTGF levels were significantly higher in scleroderma than in patients affected by systemic lupus erythematosus or dermatomyositis/polymyositis. When a cutoff value of more than mean + 2 SD was used to differentiate scleroderma from these other connective tissue disorders, only 40% of scleroderma patients had a CTGF concentration above this level. Seven percent of lupus patients also showed a high CTGF. As such, the predictive value of elevated CTGF levels for the diagnosis of scleroderma is rather low when used at one time point. The study, however, stresses the role of CTGF as a marker of ongoing fibrosis, as highest CTGF levels were found in patients suffering between 1 and 3 years of scleroderma.

Dziadzio et al. (2005) studied the presence of CTGF in plasma and in dermal interstitial fluid. Three groups were evaluated in a cross-sectional approach: 26 patients with diffuse scleroderma, 21 patients with limited scleroderma and 18 healthy controls. Full-length CTGF, N-CTGF and C-CTGF were measured separately. Plasma and dermal interstitial fluid levels for N-CTGF were strongly and significantly elevated in patients as compared with controls and correlated with severity of skin disease. The highest levels were found in diffuse scleroderma. Remarkably, full-length CTGF and C-CTGF were not markedly elevated in patients (Dziadzio et al., 2005).

CTGF in diabetes mellitus

CTGF is known to play an important role in the pathogenesis of microvascular complications of diabetes mellitus (DM), such as DN and diabetic retinopathy (Kuiper et al., 2008; Nguyen et al., 2008). In DN, CTGF mRNA was shown to be up-regulated in mesangial cells, podocytes and tubulointerstitial fibroblasts (Ito et al., 1998). Emerging evidence also indicates a role in macrovascular complications (Jaffa et al., 2008). Both plasma and urinary CTGF have been investigated as biomarkers in DM, in particular in the context of DN and cardiovascular morbidity.

Plasma CTGF

The presence of CTGF in plasma of patients with type 1 diabetes was first investigated by Roestenberg et al. (2004). An ELISA detecting full-length CTGF and the N-fragment was used, but the authors detected only N-CTGF as full-length CTGF fell below the sensitivity limit of the assay. Plasma N-CTGF was significantly increased in macroalbuminuric patients, but not in microalbuminuric patients although the variation was much larger in micro- than in normoalbuminuric patients. N-CTGF correlated with urinary albumin excretion (UAE) and glomerular filtration rate (GFR) (Roestenberg et al., 2004). In a subsequent prospective observational study with a larger number of patients, N-CTGF levels also correlated with UAE and GFR in macroalbuminuric patients. In a Cox proportional hazard regression model including baseline GFR and UAE, N-CTGF levels predicted end-stage renal disease as well as mortality in macroalbuminuric patients, and in diabetic patients with nephrotic range albuminuria, N-CTGF was the only predictor for renal function loss (Nguyen et al., 2008).

Jaffa et al. studied the relationship between plasma and urinary CTGF levels and vascular disease in 1052 type 1 diabetic patients. Plasma N-CTGF was higher in hypertensive subjects and also urinary N-CTGF showed a strong association with blood pressure. Multiple regression analysis showed an independent association of plasma N-fragment with UAE and estimated GFR (eGFR). Baseline plasma N-CTGF levels were independently associated with common and internal carotid intima media thickness at 6 years follow-up on multivariate analysis including UAE. It must be noted that eGFR was not included in the multivariate models, which may have confounded the relationship since decreased renal clearance causes elevated N-CTGF (unpublished observation).

Urinary CTGF

Several small studies have evaluated urinary CTGF (uCTGF) in diabetic patients. In a cohort of 43 diabetic patients (nine patients with type 1 and 34 with type 2 DM), Tam et al. (2009) measured significantly higher CTGF levels in patients with macro- and microalbuminuria than in those with normoalbuminuria, using an ELISA capable of detecting whole CTGF and the C-terminal fragment. Urinary CTGF had a tendency to be lower in macroalbuminuric than in microalbuminuric patients and authors suggested that uCTGF may be an early marker of DN (Tam et al., 2009). Gilbert et al. (2003) also measured elevated CTGF concentrations in macroalbuminuric and microalbuminuric type 1 diabetic patients, but levels were 10-fold higher in macroalbuminuric than in microalbuminuric patients, although it must be noted that the mean age

of the macroalbuminuric patients was also significantly higher. In this study, an ELISA capable of detecting both full-length CTGF and the N-terminal fragment was used. However, the rise in uCTGF could be fully accounted for by N-CTGF. Urinary N-CTGF excretion showed close correlation with urinary albumin and TGF-β1 excretion. Remarkably, patients treated with angiotensin-converting enzyme inhibitors (ACE-I) showed much lower uCTGF than untreated patients (Gilbert et al., 2003). In a prospective clinical study, treatment with losartan in a dose range from 50 to 150 mg significantly decreased uCTGF levels in hypertensive type 1 diabetic patients with nephropathy. Changes in albuminuria and rate of decline of GFR significantly correlated with changes in uCTGF (Andersen et al., 2005). A large cross-sectional study of 386 type 1 diabetic patients confirmed that urinary N-CTGF was elevated in macroalbuminuric patients. However, no significant difference between normoalbuminuric and microalbuminuric patients was observed. N-CTGF levels did correlate with UAE and GFR, underscoring the potential value of urinary N-fragment as a progression marker (Nguyen et al., 2006).

CTGF in non-diabetic chronic kidney disease

Three small studies reported elevated urinary CTGF in non-diabetic chronic kidney disease. Gerritsen et al. (2010) measured elevated urinary N-CTGF in 108 patients with primary glomerular diseases and observed a tight correlation between urinary N-CTGF and the degree of tubular dysfunction as reflected by urinary β2-microglobulin excretion indicating that decreased tubular reabsorption is a major determinant of uCTGF in glomerular diseases. Ito et al. (2010) reported elevated plasma and urinary CTGF in 21 patients with hypertensive nephrosclerosis. Slagman et al. (2009) reported elevated uCTGF in 33 patients with non-diabetic proteinuric CKD, which could be reduced by sodium restriction, losartan and addition of hydrochlorothiazide to losartan.

CTGF in renal transplant recipients

Chronic allograft nephropathy (CAN) is the leading cause of progressive renal failure and graft loss after kidney transplantation (Fletcher et al., 2009). At the moment, histological analysis appears to be the only sensitive test to determine CAN. There is an urgent need for non-invasive markers for CAN. Serum creatinine is most commonly applied, but is insensitive and nonspecific for predicting CAN (Yilmaz et al., 2007). Experimental studies show a clear correlation between urinary CTGF and the degree of tubular atrophy and interstitial fibrosis (TA/IF) and suggest that increase of uCTGF might even precede pathological changes (Shi et al., 2009; Yue et al., 2010). In 29 transplant patients, Cheng et al. measured significantly elevated serum CTGF as compared with healthy controls, although part of the increase of serum CTGF may be accounted for by decreased renal clearance of N-CTGF since serum creatinine was significantly higher in the CAN group. No difference in serum CTGF level was observed between biopsy diagnoses classified as 'no rejection', 'acute rejection' or 'TA/IF' based on the BANFF 1997 criteria. In urine, only N-fragment was detected, which was significantly elevated in transplant patients and correlated with proteinuria. Urinary N-CTGF was lowest in patients without rejection, intermediate in patients with acute rejection and highest in patients with biopsies demonstrating CAN. High uCTGF in CAN might result from increased CTGF mRNA expression, which was observed in CAN biopsies, or decreased tubular reabsorption by malfunctioning atrophied tubules in CAN (Gerritsen et al., 2010). These studies suggest that urinary N-fragment may be a biomarker for CAN.

CTGF in cardiac fibrosis

Emerging evidence indicates a pathogenic role in myocardial fibrosis and cardiac hypertrophy. CTGF is one of the earliest growth factors transcriptionally induced by hypertrophic stimuli in cardiac myocytes (Ahmed et al., 2004; Kemp et al., 2004). Histological examination on endomyocardial biopsy samples from patients with diastolic heart failure function showed that CTGF-immunopositive cardiac myocytes correlated with the degree of interstitial fibrosis (Koitabashi et al., 2007). In 52 patients with chronic heart failure, plasma CTGF was correlated with brain natriuretic peptide (BNP) and echocardiographic E/E' value as an estimate of left ventricular end diastolic filling pressure reflecting left ventricular diastolic function, but was not correlated with systolic function or left ventricular mass (Koitabashi et al., 2008). Also in carcinoid heart disease, a complication of neuroendocrine tumours, plasma CTGF seems to be a marker for right heart fibrotic lesions. Bergestuen et al. (2010) observed a significant inverse correlation between right ventricular function and plasma CTGF levels. Elevated plasma CTGF was an independent predictor of both reduced right ventricular function and the presence of any right-sided valvular regurgitation.

CTGF in interstitial lung disease

Apart from the prominent involvement of CTGF in systemic sclerosis-associated lung disease (Abraham, 2008), CTGF might also play a role in organ-confined interstitial lung disease. Both in experimental bleomycin-induced lung fibrosis and in human idiopathic pulmonary fibrosis, CTGF was up-regulated, namely in interstitial fibroblasts and type II alveolar epithelial cells (Lasky et al., 1998; Pan et al., 2001). Moreover, cigarette smoke and the inflammatory cytokine lipopolysaccharide (LPS) have the potential to up-regulate CTGF in chronic obstructive pulmonary disease (COPD) patients (Ning et al., 2004; Nishioka et al., 2010). Recently, clinical data by Kono et al. (2010) showed that N-CTGF levels are elevated in idiopathic

interstitial pneumonias and associated with deteriorating functional vital capacity at 6 months in idiopathic pulmonary fibrosis.

Summary and challenges for future developments

In general, CTGF seems to reflect activity of fibrotic processes. Consistently, a significant body of evidence has emerged that CTGF might be a valuable biomarker in chronic fibrotic disorders. However, the heterogeneity of assay formats detecting different CTGF molecules is problematic and standardization is needed. As outlined in 'Technical aspects of CTGF measurement section, technical aspects such as protein stability, platelet activation and type of anticoagulant may have considerable influence on CTGF measurement, especially on readout of full-length CTGF levels. This suggests that the more stable, platelet activation-independent, non-heparin-binding N-fragment is a more accurate reflection of CTGF production and turnover rate. However, it should be kept in mind that the clinical significance of CTGF levels measured by different techniques might depend on the molecular forms of CTGF that are detected. This might also explain the lack of congruence between two recent studies in diabetic kidney disease that applied different assay formats detecting urinary N-CTGF and C-CTGF, respectively (Nguyen et al., 2006; Tam et al., 2009).

In CLD, circulating CTGF is a candidate non-invasive biomarker for use in liver fibrosis prediction models, especially in the setting of viral hepatitis. In DN, urinary N-fragment seems promising as a progression marker. On the contrary, the C-fragment might be an early marker of incipient DN. Plasma N-CTGF might be a potent prognostic marker to identify macroalbuminuric diabetic patients at high risk for loss of renal function or life-threatening events and may also be prognostic for cardiovascular morbidity in DM. In glomerular diseases, urinary N-CTGF reflects proximal tubular dysfunction. In renal transplant patients, urinary N-CTGF, but not serum CTGF, indicates CAN. In heart disease, plasma CTGF may serve as a marker of cardiac fibrosis, both in diastolic dysfunction in the context of left ventricular hypertrophy as well as right ventricular dysfunction in carcinoid heart disease. In pulmonary fibrosis, high CTGF is associated with pulmonary function decline. In systemic sclerosis, circulating CTGF levels do seem to point out the pathogenic role of CTGF, but there is no clear evidence for its applicability in the clinical setting as a prognostic marker, yet.

Thus far, most CTGF biomarker studies have been retrospective although some had baseline CTGF measurements with considerable clinical follow-up. To better assess the value of CTGF for prediction of fibrosis progression and to determine when in the disease process CTGF measurement is useful, more prospective longitudinal studies are needed in well-described patient populations. Validation and comparison of the different assay formats is highly warranted before clinical implementation. In addition, studies on the individual CTGF molecules in the various patient populations are needed to identify the most relevant CTGF molecule for a certain clinical purpose. Intervention studies will be needed to assess whether CTGF levels should have therapeutical consequences or could be used as a predictor and/or indicator of response to therapy.

Declaration of interest

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