Connective tissue growth factor in renal development and injury
Ito, Y.

Citation for published version (APA):
General Discussion
General Discussion

Connective tissue growth factor (CTGF; CCN2) is a cysteine-rich polypeptide that stimulates a broad repertoire of cellular responses including proliferation, chemotaxis, adhesion, migration and extracellular matrix production, and has been implicated in regulating diverse processes, including angiogenesis, embryogenesis, differentiation, wound healing and fibrosis (1, 2).

The aim of our study was to gain insight into the role of CTGF in renal disease. When we started these projects in 1996, there was no evidence of a role for CTGF in renal diseases or in peritoneal injury in peritoneal dialysis (PD) patients. We established a technique for in situ hybridization of CTGF mRNA in order to investigate the precise localization of CTGF mRNA in the normal kidney and in human glomerulopathies, and established models of proliferative glomerulonephritis induced by anti-Thy-1.1 antibody in rats.

CTGF in the glomerulus

Low glomerular CTGF mRNA and protein expression is only detected in podocytes from normal human and rodent kidneys (3-5). In proliferative glomerulonephritis, CTGF expression is upregulated in crescents and mesangial proliferative lesions leading to sclerosis in most human glomerulopathies, including diabetic and hemodynamic disorders such as diabetic nephropathy and hypertensive nephrosclerosis (3, 4, 6-8). In contrast, in non-proliferative glomerulonephritis with heavy proteinuria, such as minimal change nephritic syndrome and membranous nephropathy, CTGF expression is not elevated (3, 4, 6-10). Numerous factors, including high glucose, advanced glycation end-products (AGE), mechanical cyclic stretching, angiotensin II and transforming growth factor (TGF)-β, induce CTGF in mesangial cells, visceral epithelial cells and tubular epithelial cells (4, 11-17). We confirmed that all TGF-β1, -β2, and -β3 isoforms induce CTGF mRNA in mesangial cells and visceral epithelial cells (4). In vivo, we demonstrated the coexpression of CTGF and TGF-β isoforms in several types of human glomerular injury (6). On precise analysis of the kinetics of Thy-1.1 nephropathy, we observed early upregulation of TGF-β2 and -β3, followed by that of TGF-β1, in association with elevated CTGF expression (4). In human glomerulopathies, sustained overexpression of CTGF is associated with expression of TGF-β isoforms, which indicates a causal relationship in the progression of glomerulosclerosis. In the glomerulus of uninephrectomized spontaneous hypertensive rats, a model of advanced nephrosclerosis, no association could be found between expression of TGF-β and CTGF in the glomeruli. CTGF may be induced by other factors, such as angiotensin II and stretch forces resulting from glomerular hypertension (18). We explored the CTGF and TGF-β isoforms in glomerulogenesis in human and rodents, as glomerular injury and repair may recapitulate development of glomerulonephrosis. CTGF mRNA was present among the immediate precursors of glomerular visceral and parietal epithelial cells in the comma- and S-shaped stages, but not in the earlier stages.
of nephron development (6). During the capillary loop and maturing stages, CTGF protein and mRNA were detected concomitantly with TGF-β isoforms. CTGF protein and mRNA was present among precursors of mesangium and glomerular endothelium. CTGF mRNA and protein expression was restricted to podocytes in normal adult glomeruli co-expressing TGF-β2 and -β3, but not TGF-β1. However, CTGF and TGF-β1 were again coexpressed, often with TGF-β2 and -β3, in particular in podocytes in proliferative glomerulonephritis and in mesangial cells in diabetic nephropathy and IgA nephropathy. Coordinated expression of TGF-β isoforms and CTGF may be involved in normal glomerulogenesis, as well as maintenance of glomerular structure and function in adults (6).

CTGF has numerous biological activities in mesangial cells. Recombinant CTGF induced its own expression in mesangial cells (12). CTGF stimulates production of extracellular matrix (ECM) components, including fibronectin, type IV collagen and plasminogen activator inhibitor-1 (14). CTGF upregulates the expression of integrins on the cell surface, possibly facilitating deposition and assembly of ECM proteins (19, 20). CTGF stimulates actin disassembly, which may facilitate increased mesangial migration (21-23). CTGF also induces mesangial cell arrest in G1 phase due to induction of cyclin-dependent kinase inhibitors, which are related to mesangial hypertrophy (24). CTGF has been shown to have several ligands, which include other growth factors (TGF-β, Bone morphogenetic proteins (BMPs) (25), Insulin like growth factor-1 (IGF-1) (26) and Vascular endothelial growth factor (VEGF) (27) whose function is then modified, cell surface proteins (integrins (28), low density lipoprotein receptor–related protein (LRP) (29) and tyrosine kinase receptor TrkA (30) through which intracellular signaling may be initiated, and extracellular matrix proteins (fibronectin (20) and heparan sulfate proteoglycans (28)) (1). These functions may be involved in glomerulogenesis as well as in development of glomerulosclerosis.

CTGF expression in the tubulo-interstitial area
The underlying mechanisms of renal interstitial fibrosis have similarities in different kidney diseases. In our studies, CTGF was observed at the sites of chronic tubulointerstitial damage, which correlated with the extent of damage (3). In the tubulo-interstitial area, the majority of CTGF-positive cells were α-smooth muscle actin (α-SMA) positive fibroblasts (3, 7). In human glomerulopathies and in kidneys of uninephrectomized rats with spontaneous hypertension, CTGF was detected in the flattened spindle-shaped tubular epithelial cells lining atrophic tubules, which may have been undergoing epithelial-mesenchymal transition (3, 7). In contrast, CTGF mRNA was detected in the proximal and distal tubules with a relatively preserved structure in the diabetic kidney (8). CTGF protein levels are reportedly elevated in proximal and distal tubular epithelial cells in STZ-induced diabetic animals (31, 32), which is consistent with our observations (8). These findings suggest that diabetic conditions may be unusual for CTGF induction in tubular epithelial cells, as high glucose and/or AGE levels directly induce CTGF.
Proximal tubular epithelial cell (PTEC) in culture show increased fibronectin mRNA expression after incubation with rCTGF (31). CTGF induces upregulation of collagen IIIα1 mRNA in synergistic action with insulin-like growth factor I (26, 31). In tubular epithelial cells and fibroblasts, CTGF induced fibronectin and collagen IV secretion. The combination of TGF-β and CTGF was additive in their effects on fibronectin and collagen IV secretion in both PTEC and cortical fibroblasts (33). Overall, CTGF produced by myofibroblasts plays an important role in the development of chronic tubulo-interstitial fibrosis, possibly by both autocrine and paracrine stimulation. The precise role of CTGF in the transition of several cell types including tubular epithelial cells, pericytes and endothelial cells to myofibroblasts remains poorly defined. Nevertheless, CTGF-antisense oligonucleotide markedly reduced myofibroblast levels in unilateral ureter obstruction models (34). CTGF siRNA reduced epithelial to mesenchymal transition (EMT) induced by AGE or TGF-β in the NRK-52E renal epithelial cell line (15). These findings suggest that CTGF is involved in the development of EMT and tubulo-interstitial fibrosis.

**CTGF as a biomarker for renal diseases**

Biomarkers have various functions that indicate different stages in the development of disease. Biomarkers can assist in the care of patients who have no apparent disease (screening biomarkers), those who are suspected to have disease (diagnostic biomarkers) and those who present with overt disease (prognostic biomarkers) (35, 36).

CTGF is strongly expressed during the development of various fibrotic disorders and has been acknowledged as one of the key growth factors in extracellular matrix production and other profibrotic activities mediated by TGF-β. Therefore, CTGF has been proposed as a possible biomarker for fibrotic disorders. Serum levels of CTGF were significantly correlated with the progression of hepatic fibrosis in biliary atresia (37) and chronic liver diseases (38). Serum CTGF levels were elevated in patients with systemic sclerosis, and were correlated with skin sclerosis and severity of pulmonary fibrosis (39). It was shown that N-terminal CTGF is a marker of fibrotic phenotypes in systemic sclerosis (40). N-terminal CTGF fragment content is increased in the vitreous fluid of patients with active proliferative diabetic retinopathy (41). In human renal transplant recipients, serum and urine CTGF levels are significantly elevated when compared with normal individuals. In addition, urinary levels of CTGF are correlated with the histological presence of chronic allograft nephropathy (42). In patients with type 1 diabetic nephropathy, we observed that plasma CTGF is an independent predictor of end-stage renal disease and mortality (43) and that urinary CTGF excretion is correlated with clinical markers of renal disease (44).

Most previous published studies have been cross-sectional studies that predicted disease outcomes. Accurate markers that indicate the effectiveness and optimal dose of therapy are important when patients are medically treated. In our analysis, urinary CTGF was significantly higher in patients with diabetic nephropathy (DN) renal
insufficiency, DN renal failure and nephrosclerosis with slight proteinuria. Urinary CTGF was correlated with urinary-protein to creatinine ratios in diabetic nephropathy and nephrosclerosis. In contrast, urinary CTGF was not elevated in the patients with minimal change nephrotic syndrome and membranous nephropathy. The ratios were much higher in nephrosclerosis than in diabetic nephropathy. In serial studies, urinary CTGF reflects the development of renal dysfunction and response to therapy (8). These findings suggest that urinary CTGF is a useful monitoring marker in renal diseases. In recent studies, filtered CTGF is normally reabsorbed almost completely in proximal tubules via megalin, and elevated urinary CTGF may reflect proximal tubular dysfunction (45). Serum levels of CTGF are also reported to be an indicator of bone diseases in multiple myeloma (46).

**CTGF in PD patients**

In peritoneal dialysis, there are several biomarkers which reflect a high peritoneal transport rate. IL-6, VEGF and TGF-β were reported to be elevated in the peritoneal effluent and were correlated with peritoneal transport D/P Cr (47-50). BMP-7 has been known to reverse EMT in kidney. In human mesothelial cells, BMP-7 was shown to also reverse EMT morphology and to normalize Ezrin and α-SMA expression (51). In dialysate the level of BMP-7 was positively correlated with small solute transport (52). Notwithstanding these interesting findings however, so far routine biomarkers in the clinical settings have not been established. CTGF has also been detected in peritoneal fluid of patients undergoing PD (53). However, as these studies used small numbers of patients, the power was insufficient to draw conclusions on the potential use of CTGF as a biomarker in PD patients and on the possible patho-physiological role of CTGF in peritoneal transport. In our studies, CTGF content in PD effluent from 178 PD patients and local peritoneal production of CTGF estimated from the peritoneal transport line of each patient was correlated with the dialysate to plasma ratio for creatinine (D/P Cr), a marker of peritoneal permeability. Peritoneal tissue CTGF expression was significantly higher in ultrafiltration failure, as assessed by real-time PCR and immunohistochemistry, and was correlated with the peritoneal thickness. In cultured human peritoneal mesothelial cells from spent peritoneal dialysate, TGF-β1-induced expression of CTGF mRNA was elevated at 12 and 24 h, and was correlated with D/P Cr. These findings suggest that a high peritoneal transport state is associated with fibrosis and increased CTGF expression, as well as production by mesothelial cells, which can be stimulated with TGF-β1. Dialysate CTGF concentration could be a biomarker for both peritoneal fibrosis and membrane function, and may thus be useful to monitor the peritoneal damage in PD patients (54).

Recent progress in understanding roles of CTGF in fibrosis based on Transgenic (Tg) and Knockout (KO) mice (Tables 1 and 2)

The direct roles of CTGF in the pathogenesis of tissue fibrosis have been controversial. CTGF has been reported to act on certain cells in concert with one or more other factors,
for example TGF-β, and has no effect if such factors are absent. For instance, TGF-β-induced proliferation in NRK-49F cells and myofibroblasts was greatly enhanced by CTGF. In contrast, CTGF alone had no effect on these cells (55). Serial injections of CTGF after TGF-β resulted in a sustained elevation of COL1A2 mRNA expression compared with consecutive injection of TGF-β, while collagen induction by CTGF alone was weak (56). However, in another study, collagen and fibronectin induction was demonstrated in response to CTGF alone (57).

In order to address these issues, several systems have recently been reported, in which CCN2/CTGF was individually expressed in podocytes (34), hepatocytes (58), cardiomyocytes (59, 60), respiratory epithelial cells (61, 62), fibroblasts (63) (64) and osteoblasts (65) to achieve overexpression in the kidney, liver, lung, heart, skin and bone (Table 1).

In a recent report by Sonnylal et al., transgenic mice over-expressing CTGF in fibroblasts under the control of the fibroblast-specific collagen α2(I) promoter enhancer exhibited tissue fibrosis in the skin, lung, kidney and vasculature, without additional manipulation (63). CTGF-dependent activation of several key signaling pathways was evidenced by increased levels of phosphorylated p38, ERK-1/2, JUNK and Akt in CTGF-transgenic mouse embryonic fibroblasts, but not by p-Smad3 (63). In contrast, hepatic and glomerular overexpression in CTGF mice did not result in fibrosis. However, the effect of the CTGF transgene was to exacerbate some aspects of the fibrotic phenotype in diabetic nephropathy (34) and liver injury induced by carbon tetrachloride (CCl4) administration (58). Homozygous transgenic mice exhibit more severe hepatic fibrosis than heterozygous transgenic mice (58). In homozygous transgenic mice, lung fibrosis due to disruption of alveologenesis and capillary formation during the critical period of alveolar development was also observed (62). Overexpression of CTGF decreased alveolarization and vascular development leading to pulmonary hypertension in association with macrophage and neutrophil infiltration in alveolar spaces and perivascular regions in neonatal mice (61). However, there is no information regarding whether adult mice develop pulmonary hypertension. The profibrotic effects of CTGF may be dependent on the developmental stage of the target organ and environment, and susceptibility may be important to CTGF activity.

Interestingly, CTGF-tg mice showed reductions in myocardial infarct size in a myocardia ischemia-reperfusion injury model via enhanced phosphorylation and activity of the Akt/p70 S6 kinase/GSK-3β salvage kinase pathway induction of several genes with reported cardioprotective functions (60).

Another strategy to investigate the functional role of CTGF in vivo has been the generation of CTGF KO mice. While CTGF-/- mice die soon after birth from respiratory failure due to skeletal malformation, heterozygous CTGF+/- mice have been used to analyze the roles of CTGF in diabetic nephropathy (66, 67). CTGF+/- mice with diabetes induced by intraperitoneal injection of streptozotocin had approximately 50% lower CTGF mRNA and protein, less albuminuria, no thickening of glomerular basement membrane, and preserved matrix metalloproteinase activity when compared with diabetic CTGF+/-
mice (66). pSmad1/5/8 was relatively preserved in podocytes from diabetic CTGF +/- mice (67). This indicates that CTGF expression was elevated, and that it inhibits BMP6 or -7 signaling in diabetic nephropathy. Recent studies showed that CTGF-/- mice had hypoplastic lung disorders due to both disruption of the basic lung developmental process with reduced cell proliferation and increased apoptosis at embryonic day 18.5 (68). In addition, embryonic fibroblasts showed reduced expression of pro-adhesive, pro-inflammatory and pro-angiogenic genes such as interleukin-6, ceruloplasmin, thrombospondin-1, lipocalin-2 and syndecan 4 (69). These findings suggest that CTGF plays an important role in organogenesis, differentiation, tissue repair and fibrosis.

In summary, CTGF is involved in organogenesis of several organs including kidney and has been shown to be elevated in progressive renal disease and peritoneal fibrosis and may be a useful biomarker for kidney diseases and to monitor the disease progression in PD. Based on our findings, several studies have examined CTGF and have suggested that CTGF is an alternative to TGF-β as a therapeutic target in renal diseases and peritoneal fibrosis.
<table>
<thead>
<tr>
<th>Article</th>
<th>Promoter</th>
<th>Organ/cells</th>
<th>Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endocrinology (2008 Smerdel-Ramoya)</td>
<td>Osteocalcin</td>
<td>Bone</td>
<td>Osteopenia secondary to decreased bone formation, possibly by antagonizing BMP, Wnt and IGF-I signaling and activity.</td>
</tr>
<tr>
<td>Kidney Int (2008 Yokoio)</td>
<td>Nephrin</td>
<td>Podocytes</td>
<td>No glomerular abnormalities in tg mice. In streptozotocin-induced diabetic tg mice, more severe proteinuria, mesangial expansion was seen than in diabetic wild-type mice.</td>
</tr>
<tr>
<td>Hepatology (2009 Tong)</td>
<td>Albumin enhancer promoter</td>
<td>Hepatocytes</td>
<td>Liver histology and function of testes were unaffected in tg mice. After chronic administration of CCL4, hepatic fibrosis was more severe than in wild-type mice.</td>
</tr>
<tr>
<td>PLoS One (2009 Panek)</td>
<td>Myosin light chain-2</td>
<td>Cardiomyocytes</td>
<td>No increased fibrosis in tg mice. Angiotensin II-treated CTGF-tg mice displayed preserved cardiac function. Collagen Ia mRNA levels were similar between wild-type and tg mice after treatment with angiotensin II.</td>
</tr>
<tr>
<td>Am J Respir Cell Mol Biol (2010 Wu)</td>
<td>Surfactant protein C, tetracycline operator and minimal CMV</td>
<td>Respiratory epithelial cells</td>
<td>Lung fibrosis by disruption of alveologenesis and capillary formation during the critical period of alveolar development. On post-natal days 1-14, thicker alveolar septa and decreased secondary septa formation were observed.</td>
</tr>
<tr>
<td>Arthritis Rheum (2010 Sonnyal)</td>
<td>Colla2 gene</td>
<td>Fibroblasts</td>
<td>4- and 8-week-old tg mice: severe hair loss, fibrosis in skin, lung and kidney (focal glomerulosclerosis and TI fibrosis), increased number of endothelial cells in intima.</td>
</tr>
<tr>
<td>Am J Physiol Heart Circ Physiol (2011 Ahmed)</td>
<td>α-MHC</td>
<td>Cardiomyocytes</td>
<td>Slight increase in myocollagen deposition compared with control, but no evidence of contractile dysfunction. CTGF-tg mice showed reduced myocardial infarct size in myocardia ischemia-reperfusion injury model.</td>
</tr>
<tr>
<td>Am J Physiol Lung Cell Mol Physiol (2011Chen)</td>
<td>Surfactant protein C, tetracycline operator and minimal CMV</td>
<td>Respiratory epithelial cells</td>
<td>Disruption of alveolarization (Post natal days 1-14). CTGF tg mice showed decreased alveolarization and vascular development leading to pulmonary hypertension.</td>
</tr>
</tbody>
</table>

tg: transgenic; TI: tubulo-interstitial
### Table 2  Phenotypes of CTGF homozygous and hemizygous knockout mice

<table>
<thead>
<tr>
<th>Article</th>
<th>Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Development (2003 Ivkovic)</td>
<td><em>ctgf −/−</em> mice: Die at birth from respiratory failure due to skeletal malformation. Skeletal dysmorphism as a result of impaired chondrocyte proliferation and ECM composition within the hypertrophic zone. Endochondral ossification is impaired.</td>
</tr>
<tr>
<td>Exp Cell Res (2007 Kennedy)</td>
<td><em>ctgf −/−</em> mice: Embryonic fibroblasts showed reduced expression of pro-adhesive, pro-inflammatory and pro-angiogenic genes such as interleukin-6, ceruloplasmin, thrombospondin-1, lipocalin-2 and syndecan 4.</td>
</tr>
<tr>
<td>Developmental Dynamics (2008 Baguma-Nibasheka)</td>
<td><em>ctgf −/−</em> mice: Hypoplastic lung due to both disrupted lung developmental processes and restricted thoracic expansion with reduced cell proliferation and increased apoptosis at embryonic day 18.5.</td>
</tr>
<tr>
<td>J Histochem Cytochem (2007 Kuiper)</td>
<td><em>ctgf−/−</em> mice, <em>ctgf+/-</em> mice: Downregulation of CTGF levels does not affect neovascularization.</td>
</tr>
<tr>
<td>J Histochem Cytochem (2009 Turk)</td>
<td><em>ctgf+/-</em> mice: pSmad1/5/8 was decreased in diabetic <em>ctgf+/-</em> mice, which is consistent with CTGF inhibitory effects on BMP signaling.</td>
</tr>
</tbody>
</table>
REFERENCES


