

Urinary Connective Tissue Growth Factor Excretion in Patients With Type 1 Diabetes and Nephropathy

RICHARD E. GILBERT, MD, PHD¹

AYSEL AKDENIZ, BSC²

STEPHEN WEITZ, MD³

WILLIAM R. USINGER, MD³

CHRISTOPHER MOLINEAUX, MD³

SUSAN E. JONES, MD¹

ROBYN G. LANGHAM, MD, PHD¹

GEORGE JERUMS, MD²

OBJECTIVE — Excretion of growth factors in the urine has been implicated in the pathogenesis of tubulointerstitial disease that characterizes proteinuric renal disease. In this cross-sectional study, we sought to examine the urinary excretion of the profibrotic cytokine connective tissue growth factor (CTGF) in type 1 diabetic patients with incipient and overt diabetic nephropathy.

RESEARCH DESIGN AND METHODS — We recruited 31 subjects with type 1 diabetes from a hospital diabetes outpatient clinic. Of these, 10 subjects were normoalbuminuric, 8 were microalbuminuric and not receiving ACE inhibitor treatment, and 13 were macroalbuminuric, 8 of whom were receiving ACE inhibitor treatment. Urinary CTGF NH₂-terminal fragment (CTGF-N) was determined by enzyme-linked immunosorbent assay and expressed relative to urinary creatinine.

RESULTS — Urinary CTGF-N was closely correlated with the degree of albuminuria ($r = 0.76$, $P < 0.001$). In comparison with normoalbuminuric subjects, urinary CTGF-N was increased 10- and 100-fold in micro- and untreated macroalbuminuric subjects, respectively (CTGF-N-to-creatinine ratio: normoalbuminuria $0.23 \times / \div 1.3$ ng/mg, microalbuminuria $2.1 \times / \div 1.7$ ng/mg, untreated macroalbuminuria $203 \times / \div 3.8$ ng/mg, and geometric mean \times / \div tolerance factor; $P < 0.05$ for normoalbuminuria versus microalbuminuria, $P < 0.001$ for microalbuminuria versus macroalbuminuria). Urinary CTGF-N was lower (<30 -fold) in macroalbuminuric subjects treated with ACE inhibitors ($6.5 \times / \div 1.7$ ng/mg; $P < 0.01$ vs. untreated macroalbuminuria) compared with their untreated counterparts.

CONCLUSIONS — In this cross-sectional study, the magnitude of urinary CTGF-N excretion was related to the severity of diabetic nephropathy. In the context of its known profibrotic actions, these findings suggest that CTGF may contribute to the chronic tubulointerstitial fibrosis that accompanies proteinuric renal disease. Prospective and interventional studies will be needed to determine whether urinary CTGF-N may provide a reliable surrogate marker of renal injury and a meaningful indicator of response to therapy.

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From the ¹Department of Medicine, St. Vincent's Hospital, University of Melbourne, Fitzroy, Australia; ²Fibrogen Inc., San Francisco, California; and the ³Department of Medicine, Austin and Repatriation Medical Centre, University of Melbourne, Heidelberg, Australia.

Address correspondence and reprint requests to Richard E. Gilbert, University of Melbourne, Department of Medicine, St. Vincent's Hospital, 41 Victoria Parade, Fitzroy, Victoria, 3065, Australia. E-mail: gilbert@medstv.unimelb.edu.au.

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Abbreviations: AER, albumin excretion rate; CTGF, connective tissue growth factor; CTGF-N, CTGF NH₂-terminal fragment; ELISA, enzyme-linked immunosorbent assay; TGF- β , transforming growth factor- β .

A table elsewhere in this issue shows conventional and Système International (SI) units and conversion factors for many substances.

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Proteinuria is not only a major correlate of declining renal function but may also directly lead to disease progression by contributing to tubulointerstitial injury (1). The pathogenetic effects of proteinuria are not well understood but may be the result of excessive tubular protein resorption leading to the release of inflammatory and vasoactive substances into the interstitium (1). However, additional mechanisms may also contribute. For instance, the enhanced ultrafiltration of growth factors that occurs in proteinuric states has also been implicated as pathogenetically linked to the development of tubulointerstitial disease (2).

A number of studies have documented increased urinary excretion of transforming growth factor- β (TGF- β) in association with diabetic kidney disease and have suggested that this profibrotic and immunomodulatory cytokine may be both a marker and pathogenetic factor in the development of progressive renal disease (3–5). A more recent addition to the list of profibrotic growth factors is connective tissue growth factor (CTGF), a 349-amino acid cysteine-rich peptide, belonging to the CCN (CTGF, *cyr 16/cef 10, nov*) family of peptides. Like TGF- β , CTGF has also been implicated in the pathogenesis of renal diseases, including diabetic nephropathy (6).

In this cross-sectional study, we first sought to examine the association between urinary excretion of NH₂-terminal fragment of CTGF (CTGF-N) and albuminuria in type 1 diabetic patients with various stages of nephropathy. Second, we also sought to explore the relationship between CTGF-N and TGF- β excretion in these subjects.

RESEARCH DESIGN AND METHODS

Subjects

We recruited 31 subjects with type 1 diabetes from the diabetes outpatient clinic of the Austin and Repatriation Medical Center, a university teaching hospital in

Table 1—Characteristics of study patients

	Normoalbuminuria	Microalbuminuria	Macroalbuminuria no ACEI	Macroalbuminuria with ACEI
n	10	8	5	8
Sex (M:F)	7:3	6:2	4:1	4:4
Age (years)	33 ± 4	39 ± 6	57 ± 8*†	39 ± 4
Duration (years)	14 ± 3	20 ± 7	35 ± 8*	23 ± 3
SBP (mmHg)	130 ± 4	130 ± 7	154 ± 9*†	136 ± 8
DBP (mmHg)	84 ± 2	83 ± 5	83 ± 5	83 ± 6
HbA _{1c} (%)	8.3 ± 0.6	9.4 ± 0.8	9.1 ± 1.4	9.5 ± 0.7
Se creat (μmol/l)	0.14 ± 0.06	0.09 ± 0.02	0.39 ± 0.13*†‡	0.16 ± 0.02
Creat cl (ml/s)	2.2 ± 0.2	1.8 ± 0.4	0.8 ± 0.4*†‡	1.3 ± 0.3

Data are means ± SE. * $P < 0.05$ vs. normoalbuminuria; † $P < 0.05$ vs. microalbuminuria; ‡ $P < 0.05$ vs. macroalbuminuria with ACE inhibitor treatment. Creat cl, creatinine clearance; DBP, diastolic blood pressure; SBP, systolic blood pressure; Se creat, serum creatinine.

Melbourne, Australia. Patients with various stages of diabetic nephropathy were recruited according to established criteria (7). Patients were defined as having incipient nephropathy (microalbuminuria) if in at least two of three consecutive timed urine collections the albumin excretion rate (AER) was between 20 and 200 μg/min. Overt nephropathy (macroalbuminuria) was defined by an AER >200 μg/min. In addition to micro- and macroalbuminuric patients, a group of patients who had remained persistently normoalbuminuric despite having type 1 diabetes for >10 years were also included. We recruited 10 subjects with normoalbuminuria, 8 with microalbuminuria not receiving ACE inhibitor treatment, and 13 who were macroalbuminuric, 8 of whom were receiving ACE inhibitor treatment. Only macroalbuminuric patients were receiving treatment with antihypertensive agents, including β-blockers (4), calcium channel blockers (3), and diuretics (3). Among the eight patients receiving ACE inhibitor therapy, agents used included perindopril (4–8 mg/day), enalapril (5–20 mg/day), and fosinopril (10 mg/day). In four of the five patients with macroalbuminuria not receiving ACE inhibitor therapy, such treatment was deemed inappropriate because of a propensity to significant hyperkalemia. The remaining patient was a new referral to our medical center, and the reason for not using ACE inhibitor therapy was uncertain.

No patients were receiving treatment with an angiotensin receptor blocker. A 24-h urine collection was performed in all subjects. Both albuminuria and urinary CTGF-N were expressed as a ratio relative to creatinine, measured by autoanalyzer.

In addition, blood was drawn for HbA_{1c} measurement and blood pressure was measured after 5 min of quiet recumbency. An aliquot from each urine collection was stored at –20°C. Before assay, specimens were thawed and assayed for albumin, CTGF, and TGF-β. Urinary CTGF-N, creatinine, and albumin were measured in all 31 study subjects. Urinary TGF-β was assayed in a subset of 24 patients in whom sufficient urine was available (7 with normoalbuminuria, 6 with microalbuminuria, 7 with macroalbuminuria receiving ACE inhibitor therapy, and 4 with macroalbuminuria not receiving ACE inhibitor therapy).

Assays

Radioimmunoassay for albumin was performed by a double antibody method with intra- and interassay coefficients of variation (CVs) of 1.8 and 4.8%, respectively, for a concentration of 27 mg/l (8). Urinary CTGF-N was assayed by sandwich enzyme-linked immunosorbent assay (ELISA) using two noncross blocking monoclonal antibodies reacting to distinct NH₂-terminal epitopes (Fibrogen, San Francisco, CA). Levels of CTGF-N were expressed as nanograms CTGF relative to creatinine. In brief, ELISA plates (Immulon 2) were coated overnight with a monoclonal antibody directed to the amino terminus of CTGF. Wells were washed and blocked with buffer containing BSA and then rinsed. Another amino-terminal anti-CTGF monoclonal antibody solution (50 μl) conjugated to biotin was added. Then 50 μl (in duplicate wells) of each standard, control, or urine sample, prediluted in assay buffer, was added to the plate. The plate was covered and incubated at 4°C on a plate

shaker for 1.5 h. The plate was washed three times with wash buffer, and 50 μl of a solution of streptavidin conjugated to alkaline phosphatase was added to each well. The plate was covered and incubated at 4°C on a plate shaker for 1.5 h. The plate was again washed three times with wash buffer. Then 100 μl of substrate buffer containing para nitrophenyl phosphate was added to each well. After proper color development, the enzyme activity was stopped by the addition of 50 μl of 4 N NaOH. The plate was read at 405 nm, and data were fitted using a quadratic fit option. Standards were made from purified full-length CTGF and expressed in nanograms per milliliter. The described CTGF ELISA is capable of detecting both full-length CTGF as well as an amino-terminal fragment of CTGF. CTGF-N is ~17 kDa and has been found in both plasma and urine of patients and normal volunteers, whereas full-length CTGF has not been detected in urine (in >200 samples examined to date, including those with proteinuria, data not shown). Thus far, no biological activity has been demonstrated to the CTGF-N. The intra- and interassay CVs were 5 and 15%, respectively.

For TGF-β measurements, urine samples were placed in a filter unit (Centricon-10 filter; Amicon, Danver, MA) and concentrated 20-fold by centrifugation for 60 min at 6,500 rpm, as previously described (4,9). In brief, urinary TGF-β1 was assayed by solid-phase ELISA (Quantikine; R & D Systems, Abingdon, U.K.) according to the manufacturer's instructions. The intra- and interassay CVs were 7.5 and 12.2%, respectively.

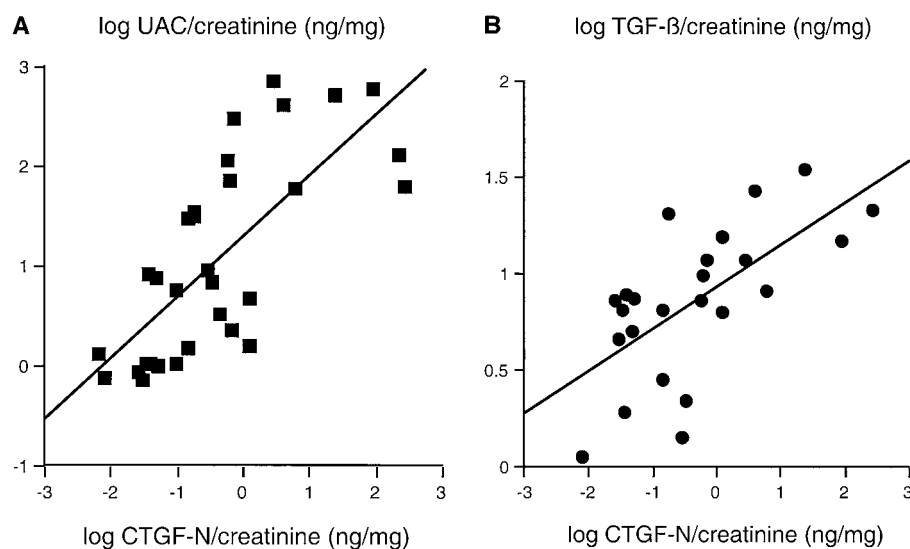


Figure 1—A: Correlation between urinary albumin and CTGF-N in patients with type 1 diabetes and nephropathy. $n = 31$, $R = 0.76$, $P < 0.001$. B: Correlation between TGF- β albumin and CTGF-N. $n = 24$, $R = 0.65$, $P < 0.001$.

Statistics

Data are expressed as means \pm SE unless otherwise stated. Correlation was determined by linear regression analysis, and between-group differences were determined by ANOVA with a Fisher's post hoc comparison. Because of their skewed distribution, albuminuria, urinary CTGF-N, and TGF- β were logarithmically transformed before statistical analysis and expressed as the geometric mean \times/\div tolerance factor. To adjust for potential confounding effects, multivariate analysis was performed using ANCOVA to examine the associations between CTGF-N and clinical variables after adjustment for either urinary TGF- β or albuminuria. Clinical variables examined included age, sex, duration, HbA_{1c}, and blood pressure (systolic and diastolic). Analyses were performed using StatView (SAS Institute, Cary, CA) on an Apple Macintosh G4 computer (Apple Computer, Cupertino, CA). A P value <0.05 was regarded as statistically significant.

RESULTS

Clinical parameters

While not reaching predefined levels of statistical significance, patients with nephropathy (both micro- and macroalbuminuric) trended toward worse glycemic control and longer diabetes duration. These differences reached significance in patients with overt nephropathy who

were not receiving ACE inhibitor therapy, were older, had longer duration of diabetes, and had higher systolic blood pressures than normoalbuminuric subjects (Table 1). In addition, macroalbuminuric subjects not treated with an ACE inhibitor had worse renal function (higher serum creatinine and reduced creatinine clearance) than the other study groups (Table 1).

Urinary CTGF and TGF- β excretion

A close correlation was noted between urinary CTGF-N and albuminuria (Fig. 1) and between TGF- β and CTGF-N (Fig. 1). In comparison with normoalbuminuric subjects, urinary CTGF-N-to-creatinine ratio was increased 10- and 100-fold in micro- and untreated macroalbuminuric subjects, respectively (Table 2). Urinary CTGF-N was lower (<30 -fold) in macroalbuminuric subjects treated with ACE inhibitors compared with their untreated counterparts (Table 2). Urinary TGF- β excretion was also increased in macroalbuminuric compared with normo- and microalbuminuric subjects, albeit to a lesser extent than CTGF-N (Table 2).

For the association between albuminuria and CTGF-N, multivariate analysis (ANCOVA) revealed no significant association with age, sex, diabetes duration, HbA_{1c}, or blood pressure after adjustment for albuminuria. For the association between TGF- β and CTGF-N, multivariate

analysis similarly revealed no significant association between CTGF-N and clinical variables after adjustment for urinary TGF- β .

CONCLUSIONS— In the present study we examined the excretion of two major profibrotic growth factors that have been implicated in the pathogenesis of diabetic nephropathy. Both urinary CTGF-N and TGF- β were increased in patients with diabetic nephropathy, although only CTGF-N excretion was elevated in patients at the microalbuminuric stage. Moreover, the magnitude of the incremental change between normo-, micro-, and macroalbuminuric stages of the disease was greater for CTGF-N than for TGF- β .

A number of clinical studies have shown a relationship between the extent of proteinuria and the rate of progression of renal disease (10–12). Indeed, experimental studies have also suggested that proteinuria, rather than being simply a manifestation of renal disease, may also be involved in its pathogenesis. These studies suggest that proteinuria directly contributes to tubulointerstitial injury, itself a major factor in the progression of a range of chronic nephropathies, including diabetes (1,13). While tubular proteinuria may be detrimental in a general sense, recent studies suggest that qualitative changes such as the increased ultrafiltration of high molecular weight growth factors (2,15) also contribute (14). Like TGF- β , CTGF in tubular fluid may act directly on tubular epithelial cells to induce the expression of extracellular matrix proteins, as demonstrated in cultured proximal tubular epithelial cells (16). However, it is also possible that the elevated urinary CTGF-N with worsening albuminuria found in the present study may be a consequence of renal disease as well as a potential contributor to it.

To date, studies that have examined urinary growth factor excretion in diabetic nephropathy have mostly focused on TGF- β (3–5). However, a number of experimental studies have indicated that CTGF is an important downstream mediator of the profibrotic, as distinct from the immunomodulatory effects of TGF- β (6,17,18). CTGF is overexpressed in a range of fibrotic diseases including scleroderma (19), hepatic cirrhosis (20), and pulmonary fibrosis (21), as well as in the kidney in both human (22) and experi-

Table 2—Urinary excretion of albumin, CTGF-N, and TGF- β in patients with type 1 diabetes

	Normoalbuminuria	Microalbuminuria	Macroalbuminuria no ACEI	Macroalbuminuria with ACEI
n	10	8	5	8
Albuminuria (mg/mg)	1.1 \times/\div 1.1	6.0 \times/\div 1.4*	177.8 \times/\div 2.1†	124 \times/\div 1.1†‡
CTGF-N (ng/mg)	0.23 \times/\div 1.3	2.1 \times/\div 1.7*	203 \times/\div 3.8†	6.5 \times/\div 1.7‡
TGF- β (ng/mg)	0.77 \times/\div 0.07	0.61 \times/\div 0.24	1.09 \times/\div 0.14†	1.11 \times/\div 0.11†

* $P < 0.05$ vs. normoalbuminuria; † $P < 0.001$ vs. microalbuminuria; ‡ $P < 0.01$ vs. macroalbuminuria without ACEI. ACEI, ACE inhibitor.

mental diabetic nephropathy (23,24). Furthermore, in addition to glucose (25), in vitro studies have also demonstrated that a range of other factors implicated in the pathogenesis of diabetic nephropathy also induce CTGF expression, including advanced glycation end products (26) and static pressure (27). Indeed, the ability of static pressure to induce CTGF expression in mesangial cells is TGF- β independent (27), indicating that while TGF- β is a potent inducer of CTGF, other factors involved in the pathogenesis of renal disease may also contribute to the regulation of its expression. In the present study, there was a close correlation between urinary TGF- β and CTGF excretion, consistent with the concept that CTGF acts downstream from TGF- β . Indeed the greater incremental change in CTGF compared with TGF- β is also consistent with the view that TGF- β leads to the induction of CTGF in sensitive cells (28).

In diabetic nephropathy, as in many other forms of progressive renal disease, the magnitude of proteinuria, the extent of tubulointerstitial disease, and the progression of renal dysfunction are interrelated (13). In the present study, the excretion of urinary CTGF-N increased progressively from normo- to micro- through to the macroalbuminuric phases of diabetic nephropathy. As in experimental animals (2), the present study suggests that growth factor ultrafiltration is also a component of diabetic nephropathy in humans.

Blockade of the renin-angiotensin system is an important therapeutic strategy in patients with type 1 diabetes and nephropathy (29). In this study, urinary CTGF-N was significantly lower in macroalbuminuric patients treated with an ACE inhibitor compared with those who were not. However, the latter also had worse renal function, were older, and had higher blood pressure. Thus, whether the described findings reflect a specific ther-

apeutic effect of ACE inhibitor therapy on CTGF production or whether they are a consequence of the better renal function, blood pressure, and age in these patients cannot be determined from this cross-sectional study.

The tissue source (local or systemic) and locus of degradation of urinary CTGF to CTGF-N have not been identified. While it has been shown that CTGF increases in the kidney parenchyma during the development of diabetic nephropathy (22), the present study cannot determine the proportion of urinary CTGF that reflects local intrarenal production versus that accompanying the increased transglomerular passage of proteins. Indeed, catheterization studies examining the renal handling of TGF- β have highlighted that the kidney can be both a net extractor and a producer of growth factors and that the extent of each can be modified by disease states (30). Whether similar physiologic and pathophysiologic mechanisms apply to CTGF is uncertain.

In summary, the findings of the present study indicate that the magnitude of urinary CTGF-N excretion is related to the severity of diabetic nephropathy, and in the context of its known profibrotic actions, these findings suggest that CTGF may contribute to the chronic tubulointerstitial fibrosis that accompanies proteinuric renal disease. However, as participants in the present study were examined cross-sectionally, we are not able to draw firm conclusions about the influence of ACE inhibition on any of the urinary parameters measured. Prospective and interventional studies will be needed to determine whether urinary CTGF-N fragment may provide a reliable surrogate marker of renal injury and a meaningful indicator of response to therapy.

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