Urinary Connective Tissue Growth Factor Excretion Correlates With Clinical Markers of Renal Disease in a Large Population of Type 1 Diabetic Patients With Diabetic Nephropathy

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OBJECTIVE — Levels of connective tissue growth factor (CTGF; CCN-2) in plasma are increased in various fibrotic disorders, including diabetic nephropathy. Recently, several articles have reported a strong increase of urinary CTGF excretion (U-CTGF) in patients with diabetic nephropathy. However, these studies addressed too small a number of patients to allow general conclusions to be drawn. Therefore, we evaluated U-CTGF in a large cross-sectional study of patients with type 1 diabetes.

RESEARCH DESIGN AND METHODS — Subjects were 318 type 1 diabetic patients and 29 normoglycemic control subjects. U-CTGF was measured by sandwich enzyme-linked immunosorbent assay. Groups were compared by Kruskal-Wallis and Mann-Whitney analysis. The relation between U-CTGF and markers of diabetic nephropathy was determined by regression analysis.

RESULTS — U-CTGF in patients with diabetic nephropathy ($n = 89$, median 155 pmol/24 h [interquartile range 96–258]) was significantly higher than in microalbuminuric ($n = 79$, 100 [65–78]) and normoalbuminuric ($n = 150$, 85 [48–127]) patients and control subjects ($n = 29$, 100 [78–114]). U-CTGF correlated with urinary albumin excretion (UAE) ($R = 0.31$) and glomerular filtration rate ($R = -0.38$) in patients with diabetic nephropathy. A standardized increase in U-CTGF was associated with diabetic nephropathy (odds ratio 2.3 [95% CI 1.7–3.1]), which was comparable with the odds ratios for diabetic nephropathy of increased HbA_{1c} (2.0 [1.5–2.7]), and blood pressure (2.0 [1.5–2.6]).

CONCLUSIONS — This is the first large cross-sectional study addressing U-CTGF in human type 1 diabetes. The observed association of U-CTGF with UAE and glomerular filtration rate might reflect a role of CTGF as progression promoter in diabetic nephropathy.

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Connective tissue growth factor (CTGF; CCN-2) is a 349–amino acid cysteine-rich polypeptide belonging to the CCN (CTGF/CYR61/NOV) family (1). The CCN family consists of six matricellular regulatory proteins, which participate in diverse biological processes like angiogenesis and wound healing and are involved in the control of cell proliferation and differentiation (2). In vitro studies have shown that CTGF is mainly involved in extracellular matrix synthesis and fibrosis. Upregulation of CTGF mRNA and increase of CTGF protein levels have been observed in various diseases, including diabetic nephropathy and cardiomyopathy, fibrotic skin disorders, systemic sclerosis, biliary atresia, liver fibrosis and idiopathic pulmonary fibrosis, and nondiabetic acute and progressive glomerular and tubulointerstitial lesions of the kidney (3–8). Furthermore, renal CTGF gene expression is strongly upregulated in experimental diabetic nephropathy (9,10).

CTGF is considered to be of particular interest to diabetic nephropathy because its expression is induced by high glucose and because it mediates transforming growth factor-β–dependent and –independent fibrogenesis (11,12). Recently, we have reported that plasma CTGF is increased in type 1 diabetic patients with diabetic nephropathy and that plasma CTGF levels in diabetic patients correlate with urinary albumin excretion (UAE) and glycemic control (13). Furthermore, urinary CTGF excretion (U-CTGF) is also increased in patients with diabetic nephropathy, and this is reduced by treatment with ACE inhibitors or angiotensin II receptor blockers (ARBs) (14–16). However, the latter studies were performed in relatively small groups of patients. Therefore, we set out to analyze in a large population of type 1 diabetic patients how U-CTGF levels relate to clinical parameters associated with (severity of) diabetic nephropathy.

RESEARCH DESIGN AND METHODS

— For the present study, CTGF levels were determined in 24-h urine collections from 318 well-characterized adult type 1 diabetic patients and 29 healthy normoglycemic control subjects. Patients were selected from the outpatient clinic at Steno Diabetes Center (Copenhagen, Denmark) for a

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Abbreviations: ARB, angiotensin II receptor blocker; CTGF, connective tissue growth factor; GFR, glomerular filtration rate; UAE, urinary albumin excretion; U-CTGF, urinary CTGF excretion.

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

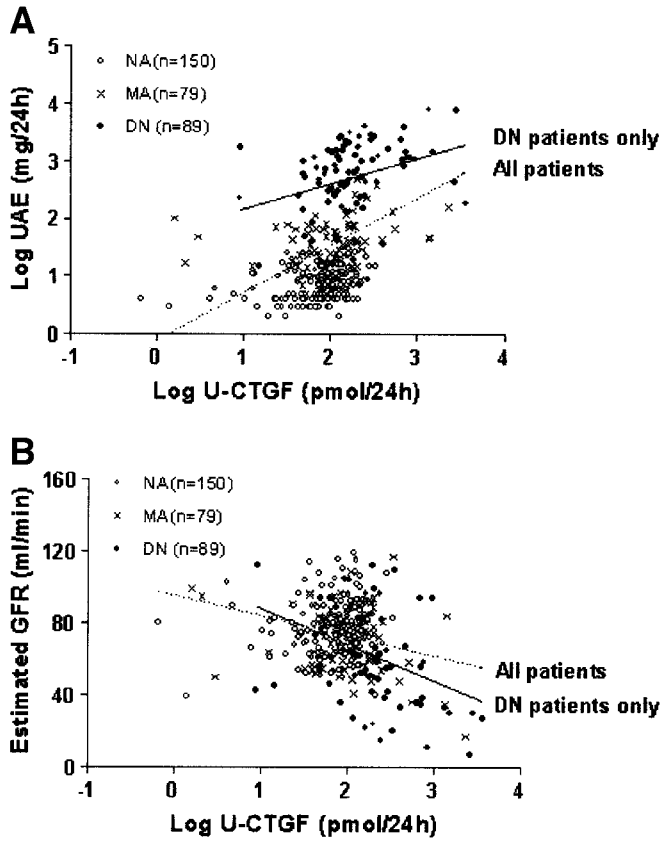

Figure 1—*Correlations between U-CTGF and clinical markers of renal disease. A: Log-transformed U-CTGF correlates with log-transformed UAE in all patients (dotted line, R = 0.43, P < 0.01) and in patients with diabetic nephropathy (solid line, R = 0.31, P < 0.01). B: Log U-CTGF inversely correlates with estimated GFR in all patients (dotted line, R = −0.26, P < 0.001), and this correlation is the strongest in diabetic nephropathy patients (continuous line, R = −0.37, P < 0.001). DN, diabetic nephropathy; MA, microalbuminuria; NA, normoalbuminuria.*

study of diabetic nephropathy and proliferative retinopathy. Forty-three of 89 patients with diabetic nephropathy had been previously analyzed in a longitudinal study examining the impact of ARBs on U-CTGF (16). Diabetic patients were categorized as having normoalbuminuria when UAE was persistently <30 mg/24 h, and type 1 diabetes was diagnosed at least 10 years ago. Patients were categorized as having microalbuminuria when UAE was between 30 and 300 mg/24 h in urine collections of at least two of three consecutive visits to the outpatient clinic. Patients were categorized as having diabetic nephropathy if they had persistent albuminuria (>300 mg/24 h) and diabetic retinopathy and no other kidney or renal tract disease. Demographic and clinical data were recorded, including age, sex, duration of diabetes, weight, height, and medication. Blood pressure values were measured twice with a Hawksley sphygmomanometer after 10 min of supine rest. Presence of retinopathy was scored as nil, simplex, or proliferative by fundus photography.

UAE was determined in 24-h urine collections by turbidimetry. In venous blood samples, plasma creatinine was determined (Cobas Mira Plus; Roche), and HbA_{1c} (A1C) was measured by high-performance liquid chromatography (normal range 4.1–6.4%) (Variant; Bio-rad Laboratories). Estimated glomerular filtration rate (GFR) was calculated by the Modification of Diet in Renal Disease study equation (17).

The study was performed according to the principles of the Declaration of Helsinki and approved by the ethical committee of Copenhagen County. All patients and control subjects gave their informed consent.

Enzyme-linked immunosorbent assay for U-CTGF

U-CTGF was determined by a sandwich enzyme-linked immunosorbent assay, using monoclonal antibodies against two distinct epitopes on the NH_2-terminal part of human CTGF (FibroGen, South San Francisco, CA), as described earlier (11). Briefly, microtiter plates were coated overnight with capture antibody and blocked with BSA. Urine samples were diluted threefold in assay buffer, and a 50-μl diluted sample was added to each well together with 50-μl CTGF detection antibody conjugated with alkaline phosphatase. Plates were washed and a substrate solution containing *p*-nitrophenyl phosphate was added to each well. Absorbance was read at 405 nm. Purified recombinant human CTGF (FibroGen) was used for calibration. The detection limit of this assay was 4 pmol/l, and intra- and interassay variations were 6 and 20%, respectively. The assay detects both CTGF NH_2-terminal fragments as well as full-length CTGF. To avoid confusion due to differences in molecular mass of full-length CTGF and fragments, U-CTGF is expressed as picomoles per 24 h.

Statistical analysis

Data are expressed as means ± SD, unless otherwise stated. UAE, plasma creatinine, and U-CTGF were logarithmically transformed before analysis because of their skewed distribution. Difference in U-CTGF between groups was determined by Mann-Whitney analysis or Kruskal-Wallis analysis followed by Dunn's method. Pearson or Spearman correlations were calculated between U-CTGF and clinical markers of diabetic nephropathy. Forward stepwise regression analysis was used to compare CTGF levels with relevant patient characteristics and clinical parameters. Logistic regression analysis was used to identify parameters that are associated with diabetic nephropathy. Odds ratios for continuous variables were standardized for 1-SD difference. In all cases, $P < 0.05$ was considered significant (two tailed).

RESULTS

U-CTGF excretion is increased in patients with diabetic nephropathy

General characteristics and clinical parameters of healthy subjects and diabetic patients are summarized in Table 1. A significant difference in U-CTGF was observed between patients with diabetic nephropathy and patients with microalbuminuria, between patients with diabetic nephropathy and patients with normoalbuminuria, and between patients

Table 1—*General and clinical parameters of healthy control subjects and type 1 diabetic patients*

	Control subjects	Type 1 diabetic patients		
		Normoalbuminuria	Microalbuminuria	Diabetic nephropathy
General patient characteristics				
n (% men)	29 (66)	150 (48)	79 (48)	89 (48)
Age (years)	42 ± 9*†	53 ± 13‡§	53 ± 12‡§	45 ± 10*†
Duration of diabetes (years)	—	34 ± 11	37 ± 10§	32 ± 10†
BMI (kg/m^2)	24.2 ± 2.4	24.4 ± 4.7	24.8 ± 3.3	25.2 ± 3.4
Retinopathy (nil/simplex/proliferative)	—	52/1/97	8/2/69	0/10/79
Antihypertensive treatment (ACE inhibitor/ARB/other)	0/0/0	24/4/58	47/9/32	20/15/24#
Glycemic control				
Blood glucose (mmol/l)	4.6 ± 0.6*†§	9.4 ± 4.4‡	9.1 ± 4.5‡	8.9 ± 4.7‡
A1C (%)	5.4 ± 0.3*†§	8.3 ± 1.1†‡§	8.8 ± 0.9*‡§	8.9 ± 1.2*‡
Diabetic nephropathy				
UAE (mg/24 h)	5 (4–7)†§	6 (4–11)†§	36 (17–68)*‡	661 (251–1495)*†‡
Plasma creatinine (μmol/l)	90 (78–102)§	83 (74–92)§	89 (80–100)§	97 (84–135)*†‡
Estimated GFR (ml · min^{-1} · 1.73 m^{-2})	80 ± 13§	79 ± 15§	73 ± 18§	63 ± 25*†‡
Systolic blood pressure (mmHg)	123 ± 10*†§	140 ± 20‡	145 ± 20‡	145 ± 19‡
Diastolic blood pressure (mmHg)	74 ± 6	74 ± 9	74 ± 9	81 ± 12*†‡
CTGF levels				
U-CTGF excretion (pmol/24 h)	100 (78–114)§	85 (48–127)§	100 (65–78)§	155 (96–258)*†‡

Data are means ± SD or median (interquartile range), unless otherwise indicated. #In the framework of participation in another study, 43 of 89 patients with diabetic nephropathy had stopped antihypertensive medication 4 weeks prior to sample collection (16). *$P < 0.05$ vs. normoalbuminuria; †$P < 0.05$ vs. microalbuminuria; ‡$P < 0.05$ vs. control subjects; §$P < 0.05$ vs. diabetic nephropathy.

with diabetic nephropathy and healthy control subjects ($P < 0.01$).

Mean U-CTGF was not significantly different between 20 patients with diabetic nephropathy who received ACE inhibition at the time of sample collection and 69 patients with diabetic nephropathy who were not treated with ACE inhibition (194 in treated vs. 146 pmol/24 h in untreated patients, $P = 0.27$). Of 69 patients without ACE inhibitors, 26 had never used this medication, while 43 had stopped antihypertensive medication 4 weeks before sample collection in the framework of participation in another longitudinal study examining the impact of ARBs on U-CTGF (16).

No significant difference was observed in U-CTGF between diabetic patients with or without simplex or proliferative retinopathy ($P = 0.34$, data not shown). In the subgroup of normoalbuminuric patients, U-CTGF was slightly lower in patients with retinopathy compared with patients without retinopathy (61.6 vs. 85.1 pmol/24 h, respectively, $P < 0.05$).

U-CTGF correlates with albuminuria and with declined renal function

U-CTGF correlated with UAE in all patients ($R = 0.43$, $P < 0.01$) and also in the subgroups of normoalbuminuric ($R = 0.29$, $P < 0.01$) and microalbuminuric ($R = 0.25$, $P < 0.01$) patients and patients with diabetic nephropathy ($R = 0.31$, $P < 0.01$, Fig. 1*A*).

Since GFR was not measured in all patients, we estimated GFR using the Modification of Diet in Renal Disease study method described by Levey et al. (17). U-CTGF inversely correlated with estimated GFR in all patients ($R = -0.26$, $P < 0.01$). When subgroups of patients were examined separately, this correlation was the strongest in patients with diabetic nephropathy ($R = -0.37$, $P < 0.01$, Fig. 1*B*) and also present in microalbuminuric patients ($R = -0.26$, $P < 0.05$) but not in normoalbuminuric patients ($R = 0.14$, $P = 0.08$).

Associations between U-CTGF, diabetic nephropathy, and clinical parameters

Forward stepwise regression analysis was performed to examine associations of U-CTGF with relevant clinical parameters (i.e., sex, age, duration of diabetes, BMI, A1C, UAE, GFR, systolic and diastolic blood pressure, presence of retinopathy, and use of ACE inhibitors or ARBs). Parameters with $P < 0.1$, as determined by Pearson or Spearman correlations, were sex, diastolic blood pressure, UAE, and GFR (Table 2). These parameters were subsequently entered into forward stepwise regression analysis with U-CTGF as a dependent variable. In all patients, U-CTGF correlated most strongly with UAE ($R = 0.43$), whereas UAE and GFR together resulted in a cumulative R of 0.45. Correlation of U-CTGF with UAE, GFR, and sex together yielded a cumulative R of

Table 2—*Correlations of log-transformed U-CTGF with clinical parameters*

Parameter	*R*	*P* value
Sex*	0.178	<0.001
Age	−0.050	0.357
Duration of diabetes	0.038	0.494
BMI	0.035	0.511
A1C	0.064	0.235
UAE	0.419	<0.001
GFR	−0.243	<0.001
Systolic blood pressure	0.061	0.254
Diastolic blood pressure	0.094	0.080
Presence of retinopathy*	0.011	0.847
ACE inhibitors*	0.070	0.224
ARBs*	0.058	0.311

Correlations were determined by calculation of Pearson or *Spearman *R*.

Table 3—*Forward stepwise regression analysis of log-transformed U-CTGF with predictors in all patients and the subgroups of diabetic patients*

Parameter	Cumulative *R*	*P* value
All patients ($n = 347$)		
UAE	0.430	<0.001
GFR	0.451	<0.001
Sex	0.481	<0.001
Normoalbuminuria ($n = 150$)		
UAE	0.291	<0.001
Sex	0.334	<0.001
Diastolic blood pressure	0.375	<0.001
Microalbuminuria ($n = 79$)		
GFR	0.262	0.019
Sex	0.371	0.004
Diabetic nephropathy ($n = 89$)		
GFR	0.371	<0.001
UAE	0.468	<0.001

0.48. Addition of other parameters did not significantly contribute to the correlation. Within the subgroup of patients with diabetic nephropathy, U-CTGF correlated most strongly with GFR ($R = 0.37$), whereas GFR and UAE together yielded a cumulative R of 0.47. In normoalbuminuric and microalbuminuric patients, U-CTGF correlated most strongly with UAE ($R = 0.29$) and GFR ($R = 0.26$), respectively (Table 3). When in patients with diabetic nephropathy, instead of U-CTGF, UAE or GFR was taken as a dependent variable in the forward stepwise regression analysis of this dataset, U-CTGF was identified as the strongest independent predictor of these parameters ($R = 0.31$ and 0.36, respectively). It thus appears that in patients with diabetic nephropathy, U-CTGF is correlated with the two most important clinical markers for severity of renal disease.

Logistic regression analysis was performed to further assess the association of U-CTGF and other parameters with diabetic nephropathy. The parameters used in this analysis were U-CTGF, sex, A1C, BMI, and blood pressure. A standardized (1-SD) increase in U-CTGF was associated with diabetic nephropathy (odds ratio 2.3 [95% CI 1.7–3.1]), which was comparable with the odds ratios for diabetic nephropathy of increased A1C (2.0 [1.5–2.7]) and blood pressure (2.0 [1.5–2.6]).

CONCLUSIONS — In the present study, we analyzed U-CTGF excretion in 318 well-characterized patients with type 1 diabetes and 29 healthy control subjects. Thus far, three much smaller studies have been published that addressed elevated CTGF levels in urine of patients with diabetic nephropathy (14–16). Our results, which were obtained in a large population of type 1 diabetic patients, confirm that U-CTGF is significantly increased in diabetic nephropathy. To this, we add that in patients with diabetic nephropathy, U-CTGF is correlated with UAE ($R = 0.31$, $P < 0.01$) and GFR ($R = -0.37$, $P < 0.01$), both important clinical markers for severity of renal disease. Although these R values are relatively small, logistic regression analysis revealed that the association of U-CTGF with diabetic nephropathy was comparable with that of the established risk factors A1C and blood pressure. The low R values of individual parameters most likely relate to the multifactorial pathogenesis of diabetic nephropathy, which, in addition to the above risk factors, also includes genetic susceptibility.

Although individual levels ranged up to 37 times the mean level in normal control subjects, mean U-CTGF in patients with diabetic nephropathy was only 1.6-fold higher than in healthy control subjects, and there was extensive overlap between the patient and control groups. Although statistically significant, the observed differences thus appear less impressive than those reported in previous, much smaller studies.

In a preliminary report, Riser et al. (14) observed an approximate sixfold increase of U-CTGF in seven diabetic patients with renal disease compared with six healthy volunteers. However, because of different methodological approach, comparison with our study is difficult.

In the study by Gilbert et al. (15), U-CTGF level was determined using a sandwich enzyme-linked immunosorbent assay with the same antibodies and standard as applied in the present study. In the former study, 8 untreated (i.e., without ACE inhibition) diabetic patients with microalbuminuria and 5 patients with macroalbuminuria had U-CTGF levels 10- and 100-fold higher than 10 diabetic patients with normoalbuminuria. However, the mean age of the five untreated diabetic patients with macroalbuminuria in this study was 24 years higher, and mean duration of diabetes in these patients was 21 years longer than that of diabetic patients with normoalbuminuria. It thus appears that within the population of diabetic nephropathy, there are huge differences in U-CTGF between individual patients but that mean U-CTGF is only moderately increased.

As for the effect of antihypertensive treatment on U-CTGF level, the study cited above (15) also reported that U-CTGF of an additional eight patients with diabetic nephropathy who received ACE inhibition was 30-fold lower compared with the U-CTGF of those without ACE inhibition. However, as discussed already in that report, the patients in the untreated group were older and had longer duration of diabetes, as well as worse renal function than the treated patients. In the present study, no effect of ACE inhibition on U-CTGF was observed, but the untreated patients with diabetic nephropathy had slightly better GFR than the treated patients. After adjustment for differences in renal function, U-CTGF levels in the present study are comparable with those of Gilbert et al. (15). Although the direct impact of ACE inhibition on U-CTGF thus might seem to be limited, conclusions regarding the impact of ACE inhibitors and ARBs compared with conventional antihypertensive treatment or no blood pressure–lowering therapy should not be drawn from this cross-sectional study because of confounding by indication (18), i.e., the most severely affected patients are likely to have received treatment that differs from that of less severely affected patients. Irrespective of this, reported induction of CTGF by angiotensin II (19) and reduction of U-CTGF following angiotensin II receptor blockade (16) do clearly imply involvement of the renin-angiotensin-aldosterone system in regulation of U-CTGF.

Diabetic retinopathy is closely associated with diabetic nephropathy. Diabetic retinopathy is much more frequent and often precedes the onset of diabetic nephropathy (20). Patients with diabetic retinopathy have increased CTGF in the vitreous and microvascular pericytes of the retina (21,22). Since diabetic retinopathy might be a reflection of systemic microvascular damage (23), its presence and severity might influence plasma CTGF, which would also be reflected in the (filtered) CTGF level in urine. However, U-CTGF levels in diabetic patients with retinopathy were not increased compared with those in patients without retinopathy. In normoalbuminuric patients, U-

CTGF was even slightly lower in patients with diabetic retinopathy compared with patients without diabetic retinopathy, but this might relate to lower GFR in normoalbuminuric patients with diabetic retinopathy than in those without diabetic retinopathy (77 vs. 83 ml · min^{-1} · 1.73 m^{-2}, $P < 0.05$).

It is still unclear how much plasma CTGF levels contribute to U-CTGF. From their physicochemical properties, CTGF and its fragments are predicted to be cleared from plasma by glomerular filtration. Plasma CTGF levels were not available for the patients in the present study. However, in other studies of human and experimental diabetes, we have observed an approximate two- to threefold increase of plasma CTGF and two- to fourfold upregulation of CTGF mRNA expression in kidney, heart, and liver (13 and R.G., unpublished data). These data suggest that both local production of CTGF in the kidney and renal filtration of (elevated) plasma CTGF might be involved in increased U-CTGF. In addition, tubular dysfunction and/or saturation of tubular reabsorption capacity in proteinuric patients with diabetic nephropathy might also contribute to higher levels of CTGF in voided urine.

In summary, the main finding of this study is that in patients with diabetic nephropathy, U-CTGF is elevated and correlates with severity of renal disease in terms of both UAE and decreased GFR. This suggests that CTGF, probably in conjunction with other factors, might act as a progression promoter in diabetic nephropathy. The possible pathogenic role of CTGF in (progression of) diabetic nephropathy is now the subject of studies in animal models, in which CTGF ligand availability is manipulated on a background of experimental diabetes (24,25). In addition, prognostic clinical studies will be necessary to evaluate U-CTGF as an additional parameter for monitoring of deterioration of renal function in diabetic nephropathy.

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