Effects of Antiproteinuric Intervention on Elevated Connective Tissue Growth Factor (CTGF/CCN-2) Plasma and Urine Levels in Nondiabetic Nephropathy

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Summary
Background and objectives Connective Tissue Growth Factor (CTGF/CCN-2) is a key player in fibrosis. Plasma CTGF levels predict end-stage renal disease and mortality in diabetic chronic kidney disease (CKD), supporting roles in intra- and extrarenal fibrosis. Few data are available on CTGF in nondiabetic CKD. We investigated CTGF levels and effects of antiproteinuric interventions in nondiabetic proteinuric CKD.

Design, setting, participants, & measurements In a crossover randomized controlled trial, 33 nondiabetic CKD patients (3.2 [2.5 to 4.0] g/24 h proteinuria) were treated during 6-week periods with placebo, ARB (100 mg/d losartan), and ARB plus diuretics (100 mg/d losartan plus 25 mg/d hydrochlorothiazide) combined with consecutively regular and low sodium diets (193 ± 62 versus 93 ± 52 mmol Na+/d).

Results CTGF was elevated in plasma (464 [387 to 556] pmol/L) and urine (205 [135 to 311] pmol/24 h) of patients compared with healthy controls (n = 21; 96 [86 to 108] pmol/L and 73 [55 to 98] pmol/24 h). Urinary CTGF was lowered by antiproteinuric intervention, in proportion to the reduction of proteinuria, with normalization during triple therapy (CTGF 99 [67 to 146] in CKD versus 73 [55 to 98] pmol/24 h in controls). In contrast, plasma CTGF was not affected.

Conclusions Urinary and plasma CTGF are elevated in nondiabetic CKD. Only urinary CTGF is normalized by antiproteinuric intervention, consistent with amelioration of tubular dysfunction. The lack of effect on plasma CTGF suggests that its driving force might be independent of proteinuria and that short-term antiproteinuric interventions are not sufficient to correct the systemic profibrotic state in CKD.

Introduction
Connective-tissue growth factor (CTGF/CCN-2) is a main mediator of fibrogenesis both downstream and independent of transforming growth factor–β1 (1–3). CTGF was shown to be a key player in the development and progression of diabetic renal fibrosis. In experimental diabetic nephropathy, glomerular and tubulointerstitial CTGF overexpression induces glomerulosclerosis, tubulointerstitial fibrosis, and albuminuria (4–6). Likewise, in human diabetic nephropathy, CTGF overexpression in renal biopsies is associated with tubulointerstitial fibrosis, proteinuria, and renal function impairment (7,8), and urinary CTGF levels correlate with albuminuria and renal function impairment (9,10). Plasma CTGF levels independently predict end-stage renal disease, intima-media thickness, and mortality in diabetic nephropathy (11,12), supporting a role in intrarenal as well as extrarenal fibrotic processes (13,14). This is underscored by efficacy of CTGF inhibition in experimental models (15). Few data are available, however, on the role of CTGF in nondiabetic chronic kidney disease (CKD), although intra- and extrarenal fibrosis are of well recognized importance in this disease condition (16–19). We therefore investigated plasma and urinary levels of CTGF and the effects of antiproteinuric intervention in nondiabetic proteinuric CKD.

Materials and Methods
Patients and Protocol
This is a post hoc analysis of a randomized, double-blind, placebo-controlled crossover trial. The protocol was described in detail elsewhere (20). In short, all patients (n = 33) had stable proteinuria (>2 and <10 g/24 h) because of nondiabetic CKD, were middle aged (18 to 70 years), and had stable creatinine clearance (>30 ml/min, <6 ml/min/yr decline). Renal diagnoses were membranous nephropathy (n = 7), focal segmental glomerulosclerosis (n = 7), IgA nephropathy (n = 5), hypertensive nephropathy (n = 5), membranoproliferative glomerulonephritis (n = 2), minimal-change disease with secondary glomerulo-
sclerosis (n = 2), Alport syndrome (n = 1), and nonconclusive diagnosis (n = 4).

Patients were randomly assigned to one of four treatment sequences, namely: (1) RS + PLA > RS + ARB > RS + ARB + diuretics > LS + ARB + diuretics > LS + PLA; (2) RS + PLA > RS + ARB + diuretics > RS + ARB > LS + ARB > LS + ARB + diuretics > LS-PLA; (3) LS + PLA > LS + ARB > LS + ARB + diuretics > RS + ARB + diuretics > RS + ARB > RS + PLA; and (4) LS + PLA > LS + ARB + diuretics > LS + ARB > RS + ARB + diuretics > RS + PLA, where LS is a low sodium diet (target, 50 mmol Na+/d), RS is a regular sodium diet (target, 200 mmol Na+/d), ARB is angiotensin-receptor blockade (losartan 100 mg/d), and diuretics is 25 mg/d hydrochlorothiazide. Additional antihypertensive drugs were allowed for BP control (except for Renin-Angiotensin Aldosterone System blockers—diuretics is 25 mg/d hydrochlorothiazide. Additional antihypertensive drugs were allowed for BP control (except for Renin-Angiotensin Aldosterone System blockers—diuretics is 25 mg/d hydrochlorothiazide. Additional antihypertensive drugs were allowed for BP control (except for Renin-Angiotensin Aldosterone System blockers—diuretics)

Healthy Controls

Healthy volunteers (n = 21) were derived from the Prevention of Renal and Vascular End-stage Disease (PREVEND) study and served as controls. Healthy volunteers were kept on a regular sodium diet and, by definition, had no diabetes mellitus or renal function impairment.

Measurements and Calculations

Proteinuria was measured by the pyrogallol red-molybdate method in 24-hour urine samples. Dietary sodium intake was assessed from urinary sodium excretion. Peripheral blood was drawn by venipuncture. Aliquots from blood and 24-hour urine were stored at −80°C until CTGF analysis.

CTGF levels were determined by enzyme-linked immunosorbent assay, using monoclonal antibodies against two distinct epitopes on the NH2-terminal part of human CTGF (FibroGen, San Francisco, CA), as described previously (9). This assay detects both CTGF NH2-terminal fragments and full-length CTGF with similar efficiency. Recoveries of full length CTGF and CTGF-N fragment spiked in plasma were identical, but in urine, full-length CTGF rapidly disappeared, whereas detection of CTGF-N fragment remained stable. To avoid confusion caused by differences in molecular mass of full-length CTGF and fragments, CTGF levels are expressed as picomoles (per ml or 24 hour) instead of milligrams.

BP was measured at 1-minute intervals by an automatic device (Dinamap, GE Medical Systems, Milwaukee, WI), with the patient in supine position. After 15 minutes of measurements, the mean of the last four readings was used for further analysis.

Data Analyses

The data were given as means with standard error when normally distributed (i.e., gender, age, proteinuria, BP, creatinine clearance, urinary sodium excretion, and body weight) or geometric mean with 95% confidence interval if skewed (i.e., plasma CTGF and urinary CTGF). Before statistical testing, skewed variables were natural log-transformed to obtain normality. Associations between variables in patients were evaluated with Pearson correlation tests or Spearman rank tests. Drug effects in patients were determined using paired t tests. The variables in patients versus healthy controls were compared using unpaired t tests. P < 0.05 was considered statistically significant. SPSS 16.0 for Windows (SPSS Inc., Chicago, IL) was used for all of the analyses.

Results

Baseline Characteristics

The data obtained during placebo combined with the regular sodium diet were taken as baseline values. CKD patients and healthy controls had the same gender (73% versus 76% men, NS; Table 1) and race (all Caucasian), but patients were slightly younger (50 ± 2 versus 58 ± 1 years, P = 0.001). At baseline, the patients had overt proteinuria (3.2 [2.5 to 4.0] g/24 h), hypertension (systolic and diastolic BP, 143 ± 3 and 86 ± 2 mmHg), and a relatively preserved renal function (creatinine clearance, 89 ± 5 ml/min). As expected, healthy controls had no relevant proteinuria (0.2 [0.1 to 0.2] g/24 h, P < 0.001 versus CKD), a lower BP (systolic and diastolic BP 120 ± 3 and 72 ± 2 mmHg, P < 0.001 and P < 0.001 versus CKD), and better renal function (creatinine clearance, 111 ± 6 ml/min, P = 0.006 versus CKD) than patients. Dietary sodium intake, as reflected by urinary sodium excretion, was comparable in patients at baseline and controls (199 ± 10 versus 162 ± 16 mmol Na+/24 h, P = 0.083).

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<th>Table 1. Participant characteristics</th>
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<tr>
<td>Number</td>
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<tr>
<td>Age, years</td>
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<tr>
<td>Male gender, n (%)</td>
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<tr>
<td>Caucasian race, n (%)</td>
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<tr>
<td>Systolic blood pressure, mmHg</td>
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<tr>
<td>Diastolic blood pressure, mmHg</td>
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<tr>
<td>Proteinuria, g/d</td>
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<td>Creatinine clearance, ml/min</td>
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The data are shown as means ± SE or as geometric mean (95% confidence interval).
Response of Proteinuria and BP to ARB, LS, and Diuretics

The average urinary sodium excretion was 196 ± 9 mmol Na+ /24 h during the three periods on a regular sodium diet and 92 ± 8 mmol Na+ /24 h during the three periods on LS (P < 0.001), indicating an adequate dietary compliance (Table 2).

Proteinuria was significantly reduced by monotherapy with either LS (residual proteinuria 2.3 [1.7 to 3.1] g/24 h, P < 0.001 versus baseline; Figure 1A) or ARB (2.1 [1.7 to 2.7] g/24 h, P < 0.001 versus baseline). Proteinuria was further reduced by combination therapy with ARB+LS (1.1 [0.9 to 1.7] g/24 h, P < 0.001 versus ARB) or ARB+diuretics (1.3 [1.0 to 1.6] g/24 h, P < 0.001 versus ARB). The maximal antiproteinuric effect was achieved by triple therapy with ARB+LS+diuretics (0.4 [0.1 to 1.2] g/24 h, P = 0.005 versus ARB+diuretics, P < 0.001 versus ARB+LS). BP decreased accordingly (Table 2). Body weight and creatinine clearance decreased as well, consistent with a negative fluid balance during LS and/or diuretics.

Response of CTGF to ARB, LS, and Diuretics

At baseline, plasma CTGF levels in CKD patients were approximately five-fold higher than in healthy controls (464 [387 to 556] versus 96 [86 to 108] pmol/L, P < 0.001; Figure 1B). Urinary CTGF excretion was approximately three-fold higher than in controls (205 [135 to 311] versus 73 [55 to 98] pmol/24 h, P = 0.001; Figure 1C). Baseline urinary CTGF excretion correlated positively with baseline plasma CTGF levels (r = 0.41, P = 0.027) and inversely with baseline creatinine clearance (r = −0.54, P = 0.002), but not with baseline proteinuria. Plasma CTGF was not correlated with proteinuria or renal function.

Plasma CTGF levels remained completely unaltered by ARB, LS, and/or diuretics. Urinary CTGF excretion was stepwise reduced by the antiproteinuric intervention, paralleling the reduction in proteinuria (Figure 2), resulting in values not significantly different from healthy controls during the treatment regimens with the lowest proteinuria, i.e., during triple therapy with ARB+LS+diuretics (99 [67 to 146]) in CKD versus 73 [55 to 98] pmol/24 h in controls, P = 0.82).

Discussion

Plasma and urinary levels of CTGF were significantly elevated in nondiabetic proteinuric CKD patients. Antiproteinuric intervention was associated with a stepwise reduction in urinary CTGF in proportion to the reduction in proteinuria but did not affect the elevated plasma CTGF levels.

CTGF is strongly implicated in diabetic renal fibrosis and injury (4–12). The elevated levels of CTGF in plasma and urine in our patients suggest that CTGF may also play a role in the pathophysiology of nondiabetic CKD and its extrarenal complications, as a biomarker and/or as a pathogenic factor.

The source of the urinary CTGF is of interest. Because of their small size (<38 kD), CTGF and the fragments thereof are predicted to be cleared from plasma by glomerular filtration (21,22). Consequently, glomerular filtration of elevated plasma CTGF may be one of the causes of the elevated urinary CTGF levels in our patients who had a relatively preserved renal function. Second, the elevated urinary CTGF in our proteinuric patients may result from proteinuria-induced proximal tubular saturation or dysfunction (22,23). Accordingly, the reduction in urinary CTGF during antiproteinuric therapy could reflect amelioration of tubular dysfunction by reduction in proteinuria, as also observed for other proximal tubular markers like kidney injury molecule 1 (KIM-1) (24,25). Third, local pro-

Table 2. Clinical parameters during ARB, LS, and diuretics

<table>
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<th>Placebo</th>
<th>ARB</th>
<th>ARB+Diuretics</th>
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<tr>
<td><strong>Urinary Na+ excretion (mmol/24 h)</strong></td>
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<tr>
<td>regular sodium diet</td>
<td>200 ± 10</td>
<td>197 ± 11</td>
<td>193 ± 11</td>
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<tr>
<td>low sodium diet</td>
<td>90 ± 10a</td>
<td>92 ± 8c</td>
<td>93 ± 8e</td>
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<tr>
<td><strong>Systolic blood pressure (mmHg)</strong></td>
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<tr>
<td>regular sodium diet</td>
<td>143 ± 4b</td>
<td>135 ± 3</td>
<td>125 ± 3c,df</td>
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<tr>
<td>low sodium diet</td>
<td>137 ± 3</td>
<td>128 ± 3c,df</td>
<td>121 ± 2c,df</td>
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<tr>
<td><strong>Diastolic blood pressure (mmHg)</strong></td>
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<tr>
<td>regular sodium diet</td>
<td>86 ± 2b</td>
<td>80 ± 2</td>
<td>75 ± 1c,f</td>
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<tr>
<td>low sodium diet</td>
<td>83 ± 1b</td>
<td>78 ± 1</td>
<td>74 ± 1c,f</td>
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<td><strong>Body weight (kg)</strong></td>
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<tr>
<td>regular sodium diet</td>
<td>91 ± 3</td>
<td>90 ± 3d</td>
<td>89 ± 3a,d,f</td>
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<tr>
<td>low sodium diet</td>
<td>89 ± 3a</td>
<td>88 ± 3a,d,e</td>
<td>88 ± 3a,de</td>
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<tr>
<td><strong>Creatinine clearance (ml/min)</strong></td>
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<td></td>
<td></td>
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<tr>
<td>regular sodium diet</td>
<td>89 ± 5</td>
<td>94 ± 6d</td>
<td>86 ± 6c</td>
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<tr>
<td>low sodium diet</td>
<td>82 ± 6</td>
<td>83 ± 7c</td>
<td>75 ± 5b</td>
</tr>
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</table>

The data are shown as means ± SE. ARB, angiotensin-receptor blockade; LS, low sodium diet.

*P < 0.05 versus placebo + RS.
*P < 0.05 versus all periods.
*P < 0.05 versus ARB + RS.
*P < 0.05 versus placebo + LS.
*P < 0.05 versus ARB + diuretics + RS.
*P < 0.05 versus ARB + LS.
duction of CTGF in the kidney, e.g., downstream of angiotensin II (26,27) and high sodium intake (28–30), may be a determinant of the elevated urinary CTGF levels as well. Local CTGF production in the kidney has been observed in animal experiments and human biopsies (4–10,12,31,32). In addition to renal CTGF production, enhanced CTGF ultrafiltration and impaired tubular CTGF reabsorption may also increase the exposure to CTGF of the proximal and distal nephron, respectively, and thus contribute to a profibrotic microenvironment (33–35).

Urinary CTGF levels were reduced by antiproteinuric intervention, paralleling the reduction in proteinuria, with the lowest values of urinary CTGF during triple therapy. Because proteinuria may reduce proximal tubular CTGF reabsorption (22,23), the reduction in urinary CTGF may

Figure 1. | Response of proteinuria and CTGF to ARB, LS, and diuretics. Proteinuria (panel A) and CTGF levels (panel B and C) are shown as geometric means with 95% confidence intervals. Figure 1A was adapted and modified from the original study (20). ARB, angiotensin receptor blockade; LS, low sodium diet. @P < 0.05 versus all, *P < 0.05 versus healthy controls, †P < 0.05 versus placebo+RS in CKD patients, ‡P < 0.05 versus placebo+LS in CKD patients, *P < 0.05 versus ARB+RS in CKD patients, #P < 0.05 versus ARB+LS in CKD patients, □P < 0.05 versus ARB+diuretics+RS in CKD patients.

Figure 2. | The reduction of urinary CTGF parallels the reduction in proteinuria. Proteinuria and CTGF levels are shown as geometric means with 95% confidence intervals. (Panel A) Stepwise concomitant reduction of proteinuria and urinary CTGF during the six different treatment periods. 4Placebo plus regular sodium diet (RS). 5Placebo plus low sodium diet (LS). 6Angiotensin receptor blockade (ARB) plus RS. 7ARB+LS. 8ARB+RS+diuretics. 9ARB+LS+diuretics. (Panel B) Percentage change in proteinuria and urinary CTGF by LS combined with placebo (change from placebo+RS), ARB combined with RS (change from placebo+RS), diuretics combined with ARB+RS (change from ARB+RS), and ARB+diuretics+LS (change from placebo+RS), respectively.
reflect amelioration of proximal tubular dysfunction, which might be a consequence of proteinuria reduction. Such amelioration is plausible, from previously published data on this population, showing reduction of the urinary proximal tubular damage markers KIM-1 and N-acetyl-β-D-glucosaminidase (23).

More specifically, besides its antiproteinuric action, the reduction of urinary CTGF by ARB might also be independent of proteinuria, because angiotensin II can induce CTGF expression directly or through aldosterone (26,27,36). Also, dietary sodium restriction might inhibit urinary CTGF independent of proteinuria reduction, because high sodium intake promotes CTGF and transforming growth factor-β1 expression (28–30).

Plasma CTGF levels were elevated in our patients, consistent with a previously observed increase of plasma CTGF levels in patients with diabetic nephropathy (11,12,37). Because CTGF can be expressed by vascular smooth muscle cells and endothelial cells of atherosclerotic lesions (38), and also by injured myocardium (39), circulating CTGF might also reflect fibrotic activity outside the kidney as a biomarker. In addition elevated circulating CTGF might generate a systemic profibrotic environment and contribute to the pathogenesis of e.g., cardiovascular complications (13,14). Consistently, plasma CTGF was found to independently predict intima-media thickness, end-stage renal disease, and overall mortality in diabetic CKD patients (11,12).

In contrast to urinary CTGF, plasma CTGF levels in our patients were not reduced by antiproteinuric intervention, although we cannot exclude the possibility that a longer duration of treatment would have been required to reduce plasma CTGF. Because of its small size, glomerular proteinuria is not expected to affect clearance of plasma CTGF (22). Our observations suggest that proteinuria is also not directly associated with major determinants of plasma CTGF. Of note, despite the proven benefits of antiproteinuric intervention in CKD, the residual risk for cardiovascular events remains high (20,40–44). The increased level and therapy resistance of plasma CTGF in our patients might be a reflection of the ongoing cardiovascular injury in CKD patients even under appropriate anti-proteinuric therapy. Therefore, it would be interesting to see the possible effects on cardiovascular outcome of emerging therapies that reduce plasma CTGF levels (45,46).

Our study has several limitations. First, it provides short-term data only, so the effect of our data for long-term outcomes will require separate study. Another limitation is the lack of information on monotherapy with diuretics.

Conclusions

Plasma and urinary levels of CTGF are substantially elevated in nondiabetic proteinuric CKD patients, and antiproteinuric intervention lowers urinary CTGF in proportion to the reduction in proteinuria but does not affect plasma CTGF levels. Hence, CTGF may play a role in the pathophysiology of nondiabetic CKD and its extrarenal complications. Long-term studies will be needed to determine the effect of urinary and plasma CTGF levels, and their response to therapy, on outcome in nondiabetic CKD.

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Disclosures

Roel Goldschmeding has received research support from and recently spent a sabbatical as a senior research fellow at FibroGen Inc. (San Francisco, CA). FibroGen is a company that develops anti-CTGF drugs. The other authors declare no conflicts of interest.

References


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