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

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Progression of diabetic nephropathy can be monitored by quantifying urinary connective tissue growth factor

Yasuhiko Ito, Hirotake Kasuga, Yoshiro Fujita, Akio Tanaka, Yukio Yuzawa, Noelynn Oliver, Roel Goldschmeding, Jan Aten, Jan J Weening, Seiichi Matsuo

manuscript in preparation
Expression of the prosclerotic Connective Tissue Growth Factor (CTGF/CCN2) is upregulated in progressive renal diseases. We examined excretion of CTGF in the urine of patients (n=333) with type 2 diabetes (T2DM) or non-diabetic renal diseases. Urinary CTGF (U-CTGF) excretion was significantly higher in renal insufficiency and renal failure of T2DM nephropathy (T2DN) and nephrosclerosis than in healthy controls or less progressive glomerular diseases with heavy proteinuria, such as minimal change nephrotic syndrome and membranous nephropathy. In T2DN, U-CTGF correlated well with proteinuria and was significantly higher than in non-diabetic progressive renal diseases. Sequential monitoring of 11 T2DM patients showed good association of a reduction of U-CTGF with decline or stabilization of renal function and response to therapy. Compared with non-DM renal disease patients, the tubulo-interstitium of T2DNrenal biopsies contained more CTGF mRNA-positive cells. In vitro, high glucose and advanced glycosylation end products (AGE) enhanced expression of CTGF mRNA in proximal tubular epithelial cells and fibroblasts. Hyperglycemia- and AGE-elevated tubulo-interstitial expression of CTGF might promote increased U-CTGF levels, and contribute to accelerating functional deterioration in DN. In T2DN, increased U-CTGF levels appear to be related temporally and functionally to diabetic derangements in the kidney thereby reflecting severity of renal dysfunction and progression of damage.
INTRODUCTION

Chronic kidney disease is a world wide public health problem. In the United States, Europe and Japan, the incidence and prevalence of kidney failure have doubled in the past 10 years, and the condition is associated with poor outcomes and high cost (1, 2). In both the USA and Japan more than 40 percent of patients starting dialysis therapy in 2004 had diabetic nephropathy (2). Type 2 diabetic patients are about nine times more prevalent than type 1 diabetes, accounting in part for the greater contribution of T2DN to end stage renal failure incidence. High dose of Angiotensin Converting Enzyme inhibitor (ACEI) and Angiotensin II Receptor Blocker (ARB) are reported to be useful to prevent deterioration of renal function (3, 4). Accurate markers that indicate the effectiveness and optimal doses of therapy are important and necessary when patients are treated with these medicines.

Fibrosis is the final common pathway of almost all forms of renal diseases that progress to end stage renal failure. Many factors including platelet-derived growth factor (PDGF), transforming growth factor-β (TGF-β) and Connective tissue growth factor (CTGF/CCN2) are considered to promote tissue scarring. In this respect, CTGF is thought to be of key importance (5-7). CTGF is over-expressed in human and experimental fibrotic renal diseases, including diabetic nephropathy and proliferative glomerulonephritides (8-11). CTGF may accelerate and maintain development of fibrosis and act as a mediator in epithelial-to-mesenchymal transition (EMT) (12), in some aspects downstream of TGF-β (9, 13) and in others independent of TGF-β (5)(10)(14, 15). CTGF promotes insulin-like growth factor-1 (IGF-1) and TGF-β signaling whereas signaling by bone morphogenetic protein-4 (BMP4) and vascular endothelial growth factor (VEGF) is inhibited. Overexpression of CTGF might thereby contribute to development of diabetic nephropathy (16-18). Therefore, CTGF is considered to be an attractive target for the modulation of matrix overproduction in diabetic nephropathy (DN) and other fibrotic diseases (5, 6, 8, 19). Recently, U-CTGF was reported to be a possible marker of the progression of type 1 DN and of diabetic nephropathy in an animal model (20-22). However, at present it is unclear whether U-CTGF can be used to monitor or predict development of nephropathy in T2DM patients and patients with non-diabetic glomerular diseases.

The aim of the present study was to investigate production and excretion of CTGF in the urine as a measure of severity and predictor of progression of renal dysfunction in type 2 diabetic nephropathy (T2DN) and non-diabetic renal diseases. Our data are consistent with the concept of CTGF overexpression as a key pathogenic factor in renal disease and further indicate its potential use to evaluate renal disease progression and response to therapy.
MATERIALS AND METHODS

Patient profile
We measured U-CTGF in 333 study subjects with type 2 diabetes, MCNS, MN, IgAN, FSGS, nephrosclerosis, hypertension, other chronic glomerulonephritis and healthy control subjects. This study has been approved by the Ethics Committee for Human Research of the Faculty of Medicine, Nagoya University, Nagoya Kyoritsu Hospital and Chubu Rosai hospital, and all patients provided their informed consent prior to participation in the study. The patients with type 2 diabetes mellitus were classified into five groups, defined as normoalbuminuria (<30 mg/day), microalbuminuria (30-300 mg/day), macroalbuminuria (>300 mg/day), renal insufficiency (DMN.RI; serum creatinine level 1.3-1.9 mg/dl), and renal failure (DMN.CRF; serum creatinine level >2.0 mg/dl). We also analyzed U-CTGF in patient groups based on the CKD classification (Stage 1, kidney damage with normal or increased GFR (≥90 ml/min per 1.73 m²); Stage 2, kidney damage with mildly decreased GFR (60 to 89 ml/min per 1.73 m²); Stage 3, moderately decreased GFR (30 to 59 ml/min per 1.73 m²); Stage 4, severely decreased GFR (15 to 29 ml/min per 1.73 m²); and stage 5, kidney failure (<15 ml/min per 1.73 m²)) (41). We collected samples without cessation of medication. For the diabetic patients, 30 percent of those with normoalbuminuria and 75 percent with diabetic nephropathy received antihypertensive drugs, similar rates as previously reported (42). Patients’ profiles are given in Table 1. MCNS and MN patients were evaluated in both nephrotic syndrome and remission state (proteinuria less than 1 g/day). Diagnosis of hypertensive nephrosclerosis was performed clinically by the presence of long standing hypertension with renal insufficiency in the absence of evidence of pyelonephritis, glomerulonephritis, and metabolic diseases.

Collection of Urine
For each determination in the urine, early morning voided spot urine samples were used. For urinary CTGF 9.5 ml of fresh urine was mixed with 0.5 ml of 0.5M EDTA. This mixture was centrifuged at 2000 rpm for 10 min and stored at -70°C until analysis.

Measurement of markers for glycaemic control and nephropathy.
Proteinuria was measured by pyrogallol red-molybdate complex method (Roche Diagnostics, Tokyo). Creatinine and Hemoglobin A1c were measured with enzymatic assay and immunoassay respectively. Creatinine clearance (CCr, ml/min) was estimated with the Cockroft-Gault formula and was used as GFR (43).

Enzyme-linked immunosorbent assay (ELISA) for U-CTGF
Urine content of CTGF was determined by a sandwich ELISA using 2 distinct monoclonal antibodies against the CTGF protein as described (FibroGen, Inc., South San Francisco, CA) (21, 32, 44). The catching and detecting monoclonal antibodies bind distinct
epitopes on the N-terminal half of the protein. This assay detects both CTGF N-terminal half fragments and the full length CTGF protein with detection limit of approximately 0.30 ng/ml (8 pmol/L). The antibodies used for this assay show no cross reactivity with other CCN proteins (cyr61 and nov).

**Combined detection of CTGF mRNA and type IV collagen protein in the diabetic nephropathy**

In situ hybridization (ISH) in combination with immunohistology was performed on the same sections to detect simultaneously CTGF mRNA and type IV collagen protein using previously described methods (8-10). Renal biopsy specimens from 11 DN patients who were undergoing diagnostic evaluation were used. CCr for 9 patients was <50 ml/min, which is compatible with that of the renal insufficiency or renal failure groups studied for U-CTGF. CCr for the remaining 5 patients was > 50 ml/min. General patient characteristics are summarized in Table 3. CTGF mRNA expression in the tubular epithelial cells was analyzed and reported as “CTGF tubular expression score” by observers who were unaware of the CCr of each patient, using a semi-quantitative classification as follows: 0 = no expression, 1 = some positive cells in focal area, 2 = some positive cells in diffuse area or many positive cells in focal area, 3 = many positive cells in diffuse area. We also examined the “CTGF tubular expression score” in 58 kidney samples from 3 diabetic patients and 55 non-diabetic patients, used in a previous study (8). Sensitivity of detection for CTGF mRNA was similar between the two ISH experiments.

**AGE synthesis**

AGE were synthesized in vitro, as previously described (45). BSA (fraction V; Sigma, St. Louis, MO) at 50 mg/ml was coincubated in sterile PBS containing antibiotics and protease inhibitors with 0.5 mol/l D-glucose, in the presence or absence of 0.1 mol/l aminoguanidine (Sigma) for 10 weeks at 37 °C according to the protocol by Pugliese (45). At the end of the incubation period, all preparations were extensively dialyzed. Measurements of AGE levels by ELISA using anti-AGE antibody (Kumamoto Immunochemical Laboratory, Japan) showed twenty times increased AGE values in BSA exposed to glucose compared with native BSA, whereas aminoguanidine reduced AGE levels by 10 %.

**Culture of fibroblast and tubular epithelial cells**

Established cell-lines of rat kidney fibroblast (NRK/49F) and of human renal proximal tubular epithelial cells (RPTEC) were obtained from American Type Culture Collection (Manassas, VA) and were cultured as described previously (24, 46). The cultures were washed twice with serum free medium at 70 to 80% confluent condition, cultured in serum free medium for 24 hours, and then changed to incubate with 200 μg/ml AGE-BSA or 25 mM D-glucose. Cells were harvested after exposure periods of 0, 6, 24 and 48 hours. At harvesting, the cells were washed with PBS and lysed with TRIzol (Life
Table 1-A – Patient profile

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Age (yr)</th>
<th>Gender (male/female)</th>
<th>SCR (mg/dl)</th>
<th>CCr (mL/min)</th>
<th>U-prot/UCr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>28</td>
<td>43.9 ± 10.4</td>
<td>20/8</td>
<td>0.80 ± 0.18</td>
<td>98.1 ± 4.4</td>
<td>0.10 ± 0.06</td>
</tr>
<tr>
<td>DM normoalbuminuria</td>
<td>20</td>
<td>56.4 ± 9.1</td>
<td>17/3</td>
<td>1.11 ± 0.14</td>
<td>76.8 ± 4.0</td>
<td>0.15 ± 0.05</td>
</tr>
<tr>
<td>DM microalbuminuria</td>
<td>23</td>
<td>63.7 ± 9.6</td>
<td>15/8</td>
<td>1.11 ± 0.18</td>
<td>67.9 ± 3.9</td>
<td>0.28 ± 0.22</td>
</tr>
<tr>
<td>DM macroalbuminuria</td>
<td>17</td>
<td>58.8 ± 12.1</td>
<td>7/10</td>
<td>0.98 ± 0.13</td>
<td>71.7 ± 7.0</td>
<td>1.83 ± 1.36</td>
</tr>
<tr>
<td>DM renal insufficiency</td>
<td>41</td>
<td>63.6 ± 10.7</td>
<td>31/10</td>
<td>1.62 ± 0.21</td>
<td>38.4 ± 2.4</td>
<td>3.85 ± 2.52</td>
</tr>
<tr>
<td>DM CRF</td>
<td>74</td>
<td>65.1 ± 8.9</td>
<td>56/18</td>
<td>3.59 ± 1.70</td>
<td>19.9 ± 1.2</td>
<td>4.82 ± 3.69</td>
</tr>
<tr>
<td>Hypertension</td>
<td>19</td>
<td>64.4 ± 12.6</td>
<td>11/8</td>
<td>0.76 ± 0.21</td>
<td>86.8 ± 2.1</td>
<td>0.18 ± 0.11</td>
</tr>
<tr>
<td>Nephrosclerosis</td>
<td>16</td>
<td>76.3 ± 5.5</td>
<td>10/6</td>
<td>1.88 ± 0.71</td>
<td>26.4 ± 4.4</td>
<td>0.58 ± 0.57</td>
</tr>
<tr>
<td>MCNS/MN nephrotic S.</td>
<td>7</td>
<td>49.3 ± 18.9</td>
<td>4/3</td>
<td>0.86 ± 0.19</td>
<td>75.7 ± 9.1</td>
<td>3.49 ± 1.81</td>
</tr>
<tr>
<td>MCNS/MN remission</td>
<td>14</td>
<td>61.7 ± 11.9</td>
<td>7/7</td>
<td>1.07 ± 0.83</td>
<td>68.8 ± 8.9</td>
<td>0.58 ± 0.65</td>
</tr>
<tr>
<td>IgA nephropathy</td>
<td>25</td>
<td>58.0 ± 14.7</td>
<td>21/4</td>
<td>1.98 ± 1.34</td>
<td>42.9 ± 7.3</td>
<td>1.61 ± 1.57</td>
</tr>
<tr>
<td>FSGS</td>
<td>21</td>
<td>56.1 ± 15.7</td>
<td>17/4</td>
<td>1.52 ± 1.21</td>
<td>52.2 ± 5.1</td>
<td>2.13 ± 1.78</td>
</tr>
<tr>
<td>CGN</td>
<td>17</td>
<td>54.9 ± 12.4</td>
<td>9/8</td>
<td>1.12 ± 0.71</td>
<td>80.0 ± 10.1</td>
<td>0.55 ± 0.35</td>
</tr>
<tr>
<td>Others</td>
<td>11</td>
<td>62.8 ± 3.0</td>
<td>10/1</td>
<td>1.50 ± 0.08</td>
<td>40.8 ± 5.3</td>
<td>3.95 ± 0.14</td>
</tr>
</tbody>
</table>

Table 1

A: Patient profile – Clinical characteristics of patients evaluated for CTGF excretion in the urine
B: Diabetic patients – Clinical characteristics and data of patients with diabetes mellitus

General and clinical parameters of healthy controls and renal diseases (mean ± SE; DM = diabetic mellitus, CRF = chronic renal failure, MCNS = minimal change nephritic syndrome, MN = membranous nephropathy, FSGS = focal segmental glomerulosclerosis, CGN = chronic glomerulonephritis).
<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>DM history (years)</th>
<th>HbA1C (%)</th>
<th>ACEI</th>
<th>ARB</th>
<th>Ca-Antagonist</th>
<th>Diuretics</th>
</tr>
</thead>
<tbody>
<tr>
<td>DM normoalbuminuria</td>
<td>20</td>
<td>5.2 ± 0.89</td>
<td>8.5 ± 0.36</td>
<td>3</td>
<td>0</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>DM microalbuminuria</td>
<td>23</td>
<td>10.1 ± 1.05</td>
<td>8.2 ± 0.32</td>
<td>4</td>
<td>2</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>DM macroalbuminuria</td>
<td>17</td>
<td>15.9 ± 1.70</td>
<td>7.4 ± 0.30</td>
<td>11</td>
<td>16</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>DM renal insufficiency</td>
<td>41</td>
<td>17.7 ± 1.02</td>
<td>8.5 ± 0.48</td>
<td>18</td>
<td>35</td>
<td>29</td>
<td>32</td>
</tr>
<tr>
<td>DM CRF</td>
<td>74</td>
<td>18.2 ± 0.84</td>
<td>7.3 ± 0.31</td>
<td>13</td>
<td>35</td>
<td>66</td>
<td>53</td>
</tr>
</tbody>
</table>

### Table 2-A U-CTGF levels of T2DM patients classified according to the CKD stages

<table>
<thead>
<tr>
<th>CKD stage</th>
<th>Age (yr)</th>
<th>Gender (male/female)</th>
<th>Body weight (kg)</th>
<th>CCr (mL/min)</th>
<th>U-P/U-Cre</th>
<th>U-CTGF/U-Cr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage1</td>
<td>55.2±4.1</td>
<td>9 / 1</td>
<td>70.5±3.9</td>
<td>102.5±2.7</td>
<td>0.8±0.2</td>
<td>2.1±0.4</td>
</tr>
<tr>
<td>Stage2</td>
<td>51.2±1.6</td>
<td>21 / 5</td>
<td>71.2±8.0</td>
<td>75.9±1.6</td>
<td>1.7±0.5</td>
<td>4.0±0.7</td>
</tr>
<tr>
<td>Stage3</td>
<td>64.1±1.3</td>
<td>43 / 20</td>
<td>58.2±1.2</td>
<td>41.5±1.1</td>
<td>2.3±0.3</td>
<td>15.9±3.3</td>
</tr>
<tr>
<td>Stage4</td>
<td>67.3±0.9</td>
<td>41 / 13</td>
<td>58.11.2</td>
<td>22.5±0.6</td>
<td>4.0±0.5</td>
<td>79.9±15.3</td>
</tr>
<tr>
<td>Stage5</td>
<td>66.1±1.8</td>
<td>12 / 10</td>
<td>56.9±2.3</td>
<td>10.9±9.6</td>
<td>6.42±0.8</td>
<td>213.5±23.1</td>
</tr>
</tbody>
</table>

Values are represented as mean ± SE.
* P<0.05 as compared with the stage 1
** P<0.001 as compared with the stages 2 and 3
*** P<0.0001 as compared with the stage 1, 2, 3 and 4

### Table 2-B U-CTGF levels of non-diabetic patients classified according to the CKD stages

<table>
<thead>
<tr>
<th>CKD</th>
<th>Age (yr)</th>
<th>Gender (male/female)</th>
<th>Body weight (kg)</th>
<th>CCr (mL/min)</th>
<th>U-P/U-cre</th>
<th>U-CTGF/U-Cr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage1</td>
<td>44.1±3.1</td>
<td>7 / 9</td>
<td>63.0±3.6</td>
<td>121.9±8.1</td>
<td>1.0±0.3</td>
<td>3.4±0.5</td>
</tr>
<tr>
<td>Stage2</td>
<td>55.1±2.0</td>
<td>22 / 9</td>
<td>58.6±1.4</td>
<td>71.6±1.6</td>
<td>1.4±0.3</td>
<td>3.6±0.4</td>
</tr>
<tr>
<td>Stage3</td>
<td>66.5±1.8</td>
<td>27 / 6</td>
<td>57.9±1.0</td>
<td>44.2±1.4</td>
<td>1.0±0.2</td>
<td>12.7±4.7</td>
</tr>
<tr>
<td>Stage4</td>
<td>71.6±2.0</td>
<td>8 / 6</td>
<td>53.0±3.0</td>
<td>24.3±1.3</td>
<td>1.4±0.5</td>
<td>18.7±7.4</td>
</tr>
<tr>
<td>Stage5</td>
<td>76.9±2.0</td>
<td>6 / 7</td>
<td>45.3±2.4</td>
<td>8.2±0.9</td>
<td>3.4±1.1</td>
<td>188.4±96.0</td>
</tr>
</tbody>
</table>

Values are represented as mean ± SE.
* P<0.005 as compared with the stage 5
** P<0.001 as compared with the stages 1, 2 and 3
We confirmed that CTGF mRNA expression peaked at 48 hours in both cell lines. Based on these experiments, incubation time chosen for subsequent studies was 48 hours for each experiment. Glucose-modified BSA, unmodified BSA or aminoguanidine-treated glucose-modified BSA was added to culture media at 200 μg/ml. Separately, cells were exposed to fresh serum free medium with 100 mg/dl (5.6 mM) D-glucose, 450 mg/dl (25 mM) D-glucose or 100 mg/dl D-glucose plus 350 mg/dl D-mannitol to achieve equal final medium osmolarity as that 450 mg/dl D-glucose. Cells were harvested after 48 hours and processed for RNA assessment.

**RNA extraction and Northern blot analysis**

Total RNA was prepared from cultured cells using TRIzol reagent (Life Technologies) and quantified by spectrophotometry. Fifteen μg of total RNA was size separated by electrophoresis in a 1% agarose-0.34 M formaldehyde gel, transferred to Hybond-N membrane (Amersham, NJ) and UV-crosslinked. After assessing the integrity of RNA, RNA hybridization with 0.6-kb CTGF cDNA labelled with (32P)-dNTP (Amersham) was performed at 65 °C for 1 h. The membranes were washed at room temperature and at 65°C. Bound radioactivity was documented by Imaging Plate (Fuji Film, Tokyo, Japan). To control for equivalent loading of RNA rehybridization was performed with oligonucleotide 5'-ACGGTATCTGATCGTCTTCGAACC-3' for 18s-ribosomal RNA (47). Data were analysed using BA Station software (Fuji Film, Tokyo, Japan) and are expressed as CTGF/18S ratios.

**Statistical analysis**

All values were presented as mean±SE. Statistical analysis for comparison among groups of patients was performed by one way analysis of variance (ANOVA). When significant difference was present, statistical analysis was further performed using Scheffe F test between two groups. Relationship between U-CTGF and GFR were analyzed by Least Squares method. A comparison between diabetes and non-diabetes groups for CTGF tubular expression score was evaluated by Mann-Whitney test. Differences were considered to be statistically significant if \( P < 0.05 \). All analysis was done using SPSS (Chicago, IL).

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**Table 3 – Diabetic nephropathy renal biopsy cases**

<table>
<thead>
<tr>
<th>Group</th>
<th>CCr (ml/min)</th>
<th>n</th>
<th>Gender</th>
<th>Age</th>
<th>DM type1/type2</th>
<th>DM history (years)</th>
<th>proteinuria (g/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DM ≥50ml/min</td>
<td>5</td>
<td>5</td>
<td>/0</td>
<td>64.5±4.6</td>
<td>1 / 4</td>
<td>17.7±4.3</td>
<td>6.66±2.6</td>
</tr>
<tr>
<td>DM &lt;50ml/min</td>
<td>9</td>
<td>5</td>
<td>/4</td>
<td>57.9±4.2</td>
<td>1 / 8</td>
<td>18.0±6.5</td>
<td>10.8±1.4</td>
</tr>
</tbody>
</table>

Characteristics of patients with diabetes mellitus, classified according to renal function, of whom renal biopsies were studied for CTGF mRNA expression by in situ hybridization. Values represent mean±SE.
RESULTS

Urinary CTGF levels are increased in chronic renal dysfunction: cross-sectional studies

U-CTGF in renal diseases and CKD stages

In a cross-sectional comparison of patients with various renal disorders and control individuals (n=333, Table 1) CTGF levels, normalized for creatinine content, in single morning urine samples (U-CTGF) were significantly higher in patients with DM renal insufficiency (DMN.RI) and renal failure of DM nephropathy (DMN.CRF) and nephrosclerosis than in healthy controls. In contrast, U-CTGF was not elevated in glomerular diseases characterized by non-proliferative lesions with heavy proteinuria, such as minimal change nephrotic syndrome (MCNS) and membranous nephropathy (MN). Also in patients with hypertension without renal dysfunction, U-CTGF was similar to control levels. However, U-CTGF was increased in patients with nephrosclerosis associated with renal dysfunction (Figure 1). Urinary excretion levels of CTGF were significantly higher in diabetic nephropathy patients with chronic kidney disease (CKD) stages 4 and 5 compared to those with CKD stages 1, 2 and 3; also in non-diabetic patients, those with CKD stage 5 showed significantly higher U-CTGF (Tables 2A and 2B, respectively).

U-CTGF and proteinuria

Urinary CTGF was analyzed in patients with type 2 DM that were classified to the level of proteinuria as described by Remuzzi and Bertani (23). U-CTGF levels and urinary-protein to creatinine ratios were positively correlated (Figures 2A, 2B). Patients with a urinary protein to creatinine ratio of less than 1.0 (Group I) are considered to be at lower risk for decline in GFR. Compared to those with a ratio of 1.0 or more, Group I showed lower excretion rates of CTGF (Figure 2A). In contrast, U-CTGF in patients with MCNS and MN was not increased, even with heavy proteinuria (Figure 2B). U-CTGF was much higher in patients with nephrosclerosis than in DM patients with similar levels of proteinuria (Figure 2B).

U-CTGF and Creatinine Clearance (CCr)

In patients with diabetic and non-diabetic renal diseases, a fall in CCr was associated with a proportionate rise in U-CTGF (Figure 3A). With moderate reduction of CCr (CCr ≥ 50 ml/min), U-CTGF was at most slightly increased. However, as CCr decreased below half normal values (CCr < 50 ml/min), the increase in U-CTGF became hyperbolic coinciding with increased serum creatinine levels. This incremental change of U-CTGF was enhanced at the DM N.RI and DM N.CRF. An inverse relationship between CTGF excretion and CCr was demonstrated in both diabetic and non-diabetic groups. In this analysis, we found that as CCr fell to 50 ml/min, there was a trend towards higher U-CTGF levels in patients with DM when compared to non-diabetic patients with similar CCr (Figure 3A). As summarized in Figure 3B, U-CTGF was significantly higher
Urinary CTGF as a predictor of diabetic nephropathy

Urinary CTGF levels reflect development of chronic renal dysfunction: serial studies

In order to test the utility of U-CTGF as an indicator of disease progression and/or response to therapy, we performed successive monitoring of U-CTGF in 10 patients with DN and in 1 patient with IgA N whose CCr were less than 50 ml/min. Increase of U-CTGF correlated well with the extent of renal deterioration in 7 diabetic patients whose renal function became worse (data for 3 patients are shown in Figures 4A, 4B, and 4C). In contrast, U-CTGF did not change or decreased in 2 diabetic patients whose renal function gradually improved or became stable during ACEI or ARB treatment (Figures 4D, 4E). In 2 patients (1 DM, 1 IgA N) whose renal function was stable for 18 months, U-CTGF levels did not change significantly (data not shown). These findings indicate that U-CTGF could be used to monitor the response to therapy in patients with chronic renal diseases, in particular in DN.

Tubular CTGF mRNA expression is increased in diabetic nephropathy

To explain the higher U-CTGF levels in DN patients compared to non-diabetic patients with similar creatinine clearance, we hypothesize that the diabetic condition enhances the local renal production of CTGF. We previously reported that CTGF was up-regulated in glomeruli of diabetic glomerulosclerosis as well as non-diabetic diseases that are prone to deteriorating renal function (8). In these studies, CTGF mRNA-positive cells were identified in the tubulo-interstitial fibrotic area and most of these were fibroblasts (8, 24). For the present investigation, CTGF mRNA-positive cells were detected in renal biopsies by in situ hybridization and assigned a semi-quantitative “tubular CTGF expression score” by visual inspection. Renal biopsies of 14 DN cases (Table 3) as well as samples from our previous study [53 cases of non-DM kidney diseases and 5 controls

Figure 1. CTGF excretion levels in the urine of healthy controls, diabetic patients and various renal diseases. *P< 0.01, ###P< 0.0001, as compared with the normal control individuals, applying Scheffe F test for multiple comparison after one way ANOVA.

in DM nephropathy compared to non-DM nephropathy if CCr is less than 30 ml/min (p<0.05). If the kidney is a major source of U-CTGF, these findings suggest that renal CTGF production is higher in DN than in other non-DM diseases.
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Figure 2-A:
Relationship between U-CTGF and the extent of proteinuria in T2DM patients.
### P < 0.0001, as compared with group I (U-protein/U-Cr < 1), applying Scheffe F test for multiple comparison after one way ANOVA.

Figure 2-B:
Relationship between U-CTGF and proteinuria in T2DM patients (a, b), nephrosclerosis (c, d), and MCNS and MN (e, f). Shaded area in a, c and e is enlarged in b, d, and f, respectively.
Linear regression analyses:
DM: \( Y = 4.98 + 16.86 \times X; \ R^2 = 0.312; \)
Nephrosclerosis: \( Y = -12.74 + 98.72 \times X; \ R^2 = 0.792; \)
Minimal Change Nephrotic Syndrome (MCNS) and Membranous Nephropathy (MN):
\( Y = 4.40 - 0.79 \times X; \ R^2 = 0.235 \)
Normal range of U-CTGF excretion is indicated by the dashed lines in b, d, and f.
(8)] were analyzed. For the non-DM patients, CTGF mRNA expression in tubular epithelial cells was only detected in three cases: FSGS, IgA N and chronic transplant rejection. In the DN samples, increased tubular CTGF mRNA expression was observed. CTGF transcripts were detected in some tubular epithelial cells in cortical areas with just slight tubulo-interstitial damage (Figures 5A, 5B) and, in severely injured areas (Figures 5C, 5D), tubular cells positive for CTGF mRNA were even more prevalent. In both GFR ≥50 ml/min and <50 ml/min groups, the “tubular CTGF expression score” was significantly higher in DN than in non-diabetic diseases (Figure 5F).

**Advanced glycosylation end products (AGE)-BSA and high glucose increase CTGF mRNA expression in fibroblast and tubular epithelial cells**

In the rat fibroblast cell line NRK/49F, a significant increase of CTGF mRNA was observed after 48 hours of exposure to AGE-BSA (Figure 6A). Furthermore, a significant increase of CTGF mRNA was not observed in NRK/49F during this timeframe when cells were
Figure 4
Serial measurement of U-CTGF in individual DN patients with either progressive loss of renal function (A, B, C) or improving or stable renal function (D, E). U-CTGF levels correlate well with changes of renal function. Urinary CTGF (triangles, dashed line), serum creatinine (dots, straight line).
Figure 5
Localization of CTGF mRNA in kidney tissues from DN and MCNS patients by in situ hybridization.
(A, C, E) - Double-labelling is shown for CTGF mRNA (purple color) and type IV collagen (brown color).
(B, D) - PAS stain on serial sections. (F) - CTGF tubular expression score in diabetic and non-diabetic renal diseases.

Some CTGF mRNA positive tubular epithelial cells were detected in slightly injured areas in the DN samples (A, B). In severely damaged DN kidney, CTGF mRNA was strongly increased in the proximal tubular epithelial cells as well as in the interstitial cells. In both GFR <50ml/min and ≥50ml/min conditions, CTGF mRNA expression in the tubular epithelial cells was higher in diabetic patients than in non-diabetic renal diseases (F). ### P< 0.0001, as compared with the non-diabetic patients applying Mann-Whitney test. Scale bar represents 100 μm.
Figure 6
Time course induction of CTGF mRNA by AGE-BSA (A) or high glucose (B) in NRK/49F cells. CTGF mRNA expression in NRK/49F fibroblasts after 48 hr incubation with AGE-BSA (C) or high glucose (D). CTGF mRNA expression in proximal tubular epithelial cells after 48 hr incubation with AGE-BSA (E) or high glucose (F).
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Exposure to aminoguanidine-BSA (AM-BSA) or control BSA (Figure 6C). High glucose (450 mg/dl, 25 mM) also increased CTGF mRNA expression at 48 hours of incubation (Figure 6B). High glucose induced a 2.1-fold increase in CTGF mRNA when compared with low glucose (100 mg/dl, 5.6 mM) conditions. In contrast, CTGF mRNA was not increased in NRK/49F that were cultured in low glucose conditions supplemented with 350 mg/dl mannitol (Figure 6D). Exposure to AGE-BSA and high glucose also increased content of CTGF mRNA in human proximal tubular epithelial cells. Over a 2-day time course, maximal CTGF mRNA /18S-rRNA ratios were detected at 48 h of incubation. AGE-BSA-induced stimulation was 1.8 ± 0.1 fold and consistent with results for NRK/49F fibroblasts, AGE-BSA increased CTGF mRNA expression significantly when compared with control BSA and AM-BSA (Figure 6E). Also high glucose stimulated CTGF mRNA expression in human tubular epithelial cells (3.2 ± 0.5 fold) whereas low glucose with or without mannitol did not increase CTGF mRNA expression (Figure 6F).

DISCUSSION

Proteinuria and albuminuria are prognostic indicators for various types of glomerular disease including DN. The inherent toxicity of these pathologies may be related to activation of cytokines and chemoattractant peptides, ultimately causing tubulo-interstitial injury (23, 25). In patients with chronic proteinuric nephropathies, the ratio of urinary protein to creatinine is useful to predict the rate of decline in glomerular filtration rate (GFR) (23, 25, 26). However, in diseases not characterized by heavy proteinuria, such as hypertensive nephrosclerosis, polycystic kidney and interstitial nephritis, the degree of proteinuria may not be useful to monitor response to treatment or predict disease progression. In agreement with this, patients with nephrosclerosis who are prone to deterioration of their renal function showed only mild proteinuria (proteinuria to creatinine ratio 0.58±0.57) in this study. Measurements of other urinary markers, such as TGF-β and IL-6, have been reported to provide information regarding the extent of renal dysfunction or the specific site of injury (27-31); however, the utility of these potential markers has yet to be established.

In recent reports, measurement of U-CTGF has been correlated with development of nephritic complications in experimental diabetes and in type 1 diabetic patients (20-22). The magnitude of U-CTGF excretion was suggested to reflect the severity of type 1 DN (21, 22). Losartan reduced U-CTGF excretion in type 1 patients with DN whose GFR was more than 80 ml/min (32). In the present study, we analyzed U-CTGF excretion in chronic renal diseases and in T2DM patients, which is likely the major cause of DN (33, 34). In T2DM, U-CTGF levels were significantly increased at the stage of renal insufficiency. Once serum creatinine levels were increased, excretion of CTGF in the urine was dramatically elevated and correlated well with the degree of proteinuria. As control for heavy proteinuria, we measured U-CTGF in patients with
MCNS and MN. In contrast to DM, both nephrotic and non-nephrotic conditions of MCNS and MN demonstrated U-CTGF levels that were equivalent to control subjects. In contrast, U-CTGF was increased in inflammatory glomerular and tubulo-interstitial lesions, such as nephrosclerosis, IgAN, and FSGS. It is important to note that patients with nephrosclerosis, who can experience deterioration of renal function, revealed increased levels of U-CTGF in association with only slight proteinuria. In this respect, U-CTGF might be a more universal biomarker to predict prognosis than the extent of proteinuria. In addition, progression of type 2 diabetic patients, those often diagnosed at older age than type 1 diabetic patients, is influenced by several factors, such as aging, atherosclerotic changes, and hypertension, in which the associated renal injury does not induce heavy proteinuria. U-CTGF, which correlated well with proteinuria and CCr, may reflect both functional and structural damage in T2DN. This suggests U-CTGF may be a more useful predictor in T2DN.

U-CTGF levels were significantly increased when renal function, as assessed by CCr, decreased to half normal in both DM and non-DM diseases. At this stage of renal impairment, ACEI and ARB are often prescribed in order to retard the continuing renal deterioration. ACEI and ARB provide more protection from progression of renal disease in patients with more advanced renal insufficiency (35, 36). In RENAAL and COOPERATE studies, ARB or combined therapy with ACEI and ARB showed protection against the progression of renal insufficiency in patients whose mean GFR was about 40% (3, 37). Accurate markers are important to confirm the suitability of prescribing additional treatment with ACEI or ARB. Successive monitoring on the T2DM patients, whose CCr was less than 50 ml/min, showed that changes in U-CTGF levels correlated well with change in renal functioning. Therefore, sequential evaluation of U-CTGF may serve to monitor the effects of treatment on the disease process and also provide an alert to progressive renal injury.

Urinary content of CTGF could be affected by circulating CTGF, leakage from the glomerulus, and local production in the kidney. We reported that increased U-CTGF correlated best with tubulo-interstitial fibrosis in the uninephrectomized SHR models (38). Another report showed that glomerular CTGF mRNA was reported to be increased and correlated with albuminuria in the stage of microalbuminuria of type 1 DN (39) while U-CTGF had not yet increased in this stage for both type 1 (21) and type 2 diabetes. Also, we detected strong expression of CTGF mRNA in glomeruli before increase of U-CTGF. These findings suggest that U-CTGF might be much more affected by CTGF expression and production in the tubulo-interstitial lesions than in glomerular lesions. Thus, higher CTGF production by tubular epithelial cells might contribute to appreciably higher excretion levels of U-CTGF. In agreement with this hypothesis, we rarely detected CTGF mRNA in tubular epithelial cell in non-DM renal diseases, whereas in DN patients, tubular epithelial cells were frequently CTGF mRNA-positive, especially as the GFR declined below 50 ml/min. Correspondingly, CTGF expression was induced by AGE-BSA and by high glucose in cultured proximal tubular epithelial cells and fibroblasts. In addition, in tubular epithelial cells and fibroblasts CTGF expression might
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be up-regulated by TGF-β, which is induced by high glucose or AGE (40). Interestingly, an experimental rat model of DN revealed intense CTGF expression in proximal tubules (19). Proximal tubular fluid, collected by micropuncture from diabetic rats, raises the expression of CTGF in cultured proximal tubular cells (19). Taken collectively, AGE and high glucose might induce CTGF in both fibroblasts and tubular epithelial cells, leading to ECM production and accumulation in the tubulointerstitial area. Enhanced production of CTGF by these cells might contribute to increased excretion of CTGF in the urine, structural injury and rapid deterioration of renal function in type2 DN.

Recent progress in the treatment of progressive renal diseases with ACEI and ARB is striking (3, 37). However, these medicines only moderately reduce the progression of chronic renal diseases. Therapeutic approaches against specific molecular targets, such as CTGF, may have additive beneficial effects. Furthermore, as a new pharmacodynamic marker, U-CTGF may serve to indicate and predict renal injury as well as provide greater insight into the pathogenesis of progressive glomerulosclerosis and tubulo-interstitial sclerosis, especially in DN.

**DISCLOSURE**

Co-author, Noelynn Oliver, is an employee of Fibrogen Inc. which is a biotechnology company that is committed to develop drugs for fibrotic diseases. CTGF is one of their targets.

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