Temporal expression profile and distribution pattern indicate a role of connective tissue growth factor (CTGF/CCN-2) in diabetic nephropathy in mice

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Departments of 1Pathology, 2Nephrology and Hypertension, and 3Metabolic and Endocrine Diseases, University Medical Center Utrecht, Utrecht; 4Fibrogen, Incorporated, South San Francisco, California; and 5Department of Pathology, Academic Medical Center, Amsterdam, The Netherlands

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Roestenberg, Peggy, Frans A. van Nieuwenhoven, Jaap A. Joles, Claudia Trischberger, Paula P. Martens, Noelynn Oliver, Jan Aten, Jo W. Höppener, and Roel Goldschmeding. Temporal expression profile and distribution pattern indicate a role of connective tissue growth factor (CTGF/CCN-2) in diabetic nephropathy in mice. Am J Physiol Renal Physiol 290: F1344–F1354, 2006. First published December 27, 2005; doi:10.1152/ajprenal.00174.2005.—Connective tissue growth factor (CTGF) is overexpressed in diabetic nephropathy (DN) and has therefore been implicated in its pathogenesis. The objective of the present study was to determine the tissue distribution of increased CTGF expression and the relationship of plasma, urinary, and renal CTGF levels to the development and severity of DN. We studied the relationship between CTGF and renal pathology in streptozotocin (STZ)-induced diabetes in C57BL/6J mice. Diabetic and age-matched control mice were killed after 1, 2, 4, and 9 wk of diabetes. In addition, key parameters of diabetes and DN were analyzed in 10-mo-old diabetic ob/ob mice and their ob/+ littermates. STZ-induced diabetic mice showed a significantly increased urinary albumin excretion after 1 wk and increased mesangial matrix score after 2 wk. Increased renal fibronectin, fibronectin ED-A, and collagen IVα1 expression, as well as elevated plasma creatinine levels, were observed after 9 wk. After 2 wk, CTGF mRNA was upregulated threefold in the renal cortex. By 9 wk, CTGF mRNA was also increased in the heart and liver. In contrast, transforming growth factor-β1 mRNA content was significantly increased only in the kidney by 9 wk. Renal CTGF expression was mainly localized in podocytes and parietal glomerular epithelial cells, and less prominent in mesangial cells. In addition, plasma CTGF levels and urinary CTGF excretion were increased in diabetic mice. Moreover, albuminuria strongly correlated with urinary CTGF excretion (R = 0.83, P < 0.0001). Increased CTGF expression was also demonstrated in type 2 diabetic ob/ob mice, which points to a causal relationship between diabetes and CTGF and thus argues against a role of STZ in this process. The observed relationship of podocyte and urinary CTGF to markers of DN suggests a pathogenic role of CTGF in the development of DN.

diabetes; podocyte; fibrosis; kidney

DIABETIC NEPHROPATHY (DN) is a major complication of diabetes mellitus (DM) and an important cause of morbidity and mortality in diabetic patients. Early characteristics of DN are albuminuria, renal and glomerular hypertrophy, extracellular matrix (ECM) accumulation, and thickening of the glomerular basement membrane (GBM) (30). Growth factors play an important role in the development of diabetic complications (6, 10, 18, 43). One of the major growth factors involved in ECM accumulation in many fibrotic disorders, including DN, is transforming growth factor-β (TGF-β) (6). Inhibition of TGF-β results in prevention of fibrosis under diabetic conditions (59). However, TGF-β has also important antiproliferative and anti-inflammatory effects, which makes it a less suitable target for therapeutic intervention of DN.

Connective tissue growth factor (CTGF), also known as CCN-2 (7), is a profibrotic growth factor critically involved in TGF-β-induced (18) but also in TGF-β-independent fibrotic processes (28, 49). In renal cells in vitro, CTGF can be induced by TGF-β, mechanical strain, and diabetic conditions, such as high levels of glucose and AGEs (5, 15, 18, 30, 32, 48). In vivo, overexpression of CTGF has been reported in renal tissue of both diabetic patients and animals with DN (25, 38, 51). Aminoguanidine, an inhibitor of advanced glycation end product formation, reduced CTGF and fibronectin overexpression in streptozotocin (STZ)-induced diabetic rats (47).

Upregulation of growth factors in several glomerular cells has been implicated in DN. For example, increased TGF-β and PDGF-BB expression in diabetic glomeruli seems mainly localized to the mesangial cells (21, 33, 46), but increased TGF-β expression has also been reported for glomerular endothelial cells (8). VEGF overexpression, on the other hand, is found mainly in podocytes (23, 46). In DN, the main site of renal CTGF overexpression is the glomerulus (25, 38, 51), but it is not known which cells within the glomerulus contribute most importantly to this. Cell-specific variation in glomerular expression and activity of growth factors has important implications for the pathogenic role of these factors. Understanding the kinetics of renal CTGF expression and the magnitude of increased CTGF levels in plasma and urine is important for interpreting the source of CTGF in these different compartments and for targeting therapeutic intervention strategies.

Urinary CTGF excretion is increased in STZ-induced diabetic rats as well as in patients with DN (14, 37). In addition, plasma CTGF levels are increased in patients with DN and correlate with both HbA1c and other parameters of DN (39). Thus far, however, no integrative analysis has been performed on CTGF expression levels in plasma, urine, and renal tissue in relation to the development of DN. Therefore, we set out to...
explore the cellular origin of CTGF and its relationship to plasma, urinary, and renal CTGF levels in time, in association with development and severity of DN.

METHODS

Animals. Two models of diabetes were studied. In the first model, diabetes was induced in female C57BL/6J mice (12 wk old, Harlan, Horst, The Netherlands) by a single intraperitoneal injection of STZ (200 mg/kg, Sigma, St. Louis, MO) dissolved in 10 mM sodium citrate buffer (pH 4.6). Control animals were injected with sodium citrate buffer alone. All animals were housed in a room with constant temperature, on a 12:12-h light-dark cycle and fed a standard pellet laboratory chow and water ad libitum. Presence of diabetes was determined 3 days after STZ injection by measurement of blood glucose levels (Medisense Precision Xtra apparatus, Abbott, Bedford, IL). When necessary, as indicated by a decrease in body weight of >10% within 2 days together with blood glucose levels >20 mmol/l, slow-release insulin pellets (Linshin Canada, Ontario, Quebec) were implanted subcutaneously. Every other week and before death, STZ-diabetic (STZ/DM) mice were kept in metabolic cages for 4 h and urine was collected and stored at −70°C. Mice were killed after 1, 2, 4, and 9 wk of DM. Control animals were killed 2 and 9 wk after citrate injection.

In the second model, genetically determined leptin deficiency in homozygous ob/ob mice causes insulin resistance and hyperglycemia already at a young age (58). Transgenic mice expressing human amyloid polypeptide in their pancreatic islets develop islet amyloid, particularly male animals when crossed back with ob/ob mice (22). Female hiAPP-transgenic ob/ob mice (n = 5, C57BL/6J background) and their nondiabetic hiAPP-transgenic ob/+ littermates (n = 6) were studied when 10 mo old. Urine was collected in metabolic cages before death.

In both models, blood was collected in EDTA, and plasma was stored at −70°C. Body and organ weights were determined. Tissues were frozen in liquid nitrogen or fixed in buffered formalin (10%) and then embedded in paraffin for light microscopy. The experiments were performed with the approval of the Animal Ethical Commission of the University of Utrecht.

Analysis of plasma and urine. Plasma fructosamine levels were measured using a commercially available test (F. Hoffmann-La Roche, Basel, Switzerland). Albumin levels in urine were determined by means of sandwich ELISA using a goat anti-mouse albumin antibody (Bethyl Laboratories, Montgomery, TX). Creatinine concentrations in plasma and urine were determined using an enzymatic assay (J2L Elitech, Labarthe Inard, France).

Mesangial matrix score. The amount of mesangial matrix was estimated in periodic acid-Schiff-stained sections from all animals. In each slide, 25 glomeruli were scored with a range from 1 to 4 by 2 persons, both blinded to the origin of the slide. The mean of both scores was used as the mesangial matrix score (MMS).

Quantitative RT-PCR. The mRNA levels of CTGF, TGF-β1, and several ECM components in total renal cortex were quantified by real-time quantitative PCR. For this purpose, RNA was isolated from frozen sections of renal cortex using a RNeasy minikit (Qiagen, Hilden, Germany). cDNA synthesis was performed on 3 μg of RNA using oligo-dT12-18 and Superscript RT (Invitrogen, Carlsbad, CA). Quantitative PCR was performed using a SYBR Green kit (Applied Biosystems, Foster City, CA) and analyzed on an ABI PRISM 7700 Sequence Detector System (Applied Biosystems). Genes and primer sequences are listed in Table 1. The thermal cycling comprised a denaturation step at 95°C for 10 min followed by 45 cycles of 95°C for 15 s and 60°C for 1 min. To examine whether only one single PCR product was generated, the PCR products were analyzed using a heat dissociation protocol. Quantitative values were calculated from the threshold PCR cycle numbers, which were derived from the exponential phase of each PCR reaction. The relative mRNA level in each sample was normalized for TATA box-binding protein mRNA content (4).

CTGF ELISA of plasma and urine samples. CTGF levels in plasma and urine were determined by means of a sandwich ELISA using an affinity-purified polyclonal antibody and a monoclonal antibody. These two antibodies react with distinct epitopes in the NH2-terminal half of the CTGF protein (CTGF-N; FibroGen, South San Francisco, CA).

Microtitre plates were coated overnight at 4°C with 50 μl of capture goat-anti-CTGF-N polyclonal antibody (10 μg/ml) in coating buffer (0.05 M sodium bicarbonate, pH 9.6). Subsequently, the plates were blocked with 1% BSA in PBS for 2 h at room temperature and washed with wash buffer (PBS/0.05% Tween 20). Plasma samples were diluted 1:10 and urine samples 1:2 in assay buffer (50 mM Tris, pH 8.0, 0.1% BSA; 50 ng/ml sodium heparin, 0.1% Triton X-100) and 100 μl of diluted sample were added to each well. Purified recombinant human CTGF (FibroGen) was used as a standard. Plates were incubated for 2 h at room temperature, washed, and incubated with 100 μl (4 μg/ml in assay buffer) monoclonal human anti-CTGF-N antibody produced by transgenic mice generating human antibodies (FibroGen). Plates were washed and incubated with 100 μl/well goat anti-human IgG alkaline phosphatase (diluted 1:2,000 in assay buffer, Sigma). Plates were washed again, and 100 μl of substrate solution containing p-nitrophenylphosphate (Sigma) were added to each well. After a 20- to 30-min incubation in the dark, absorbance was read at 405 nm on a Bio-Rad microplate reader. As this ELISA cannot discriminate between full-length and different NH2-terminal fragments of CTGF, all levels are expressed as picomoles per liter.

CTGF immunohistochemistry. Four-micrometer sections were cut from formalin-fixed and paraffin-embedded tissue samples and mounted on silan-coated slides. Slides were fixed overnight at 56°C in an incubator. Sections were deparaffinized and rehydrated. A 3-min incubation of the slides at 37°C with protease XXIV (4.4 U/ml phosphate, Sigma) was performed for epitope retrieval. After 2 min of washing with distilled water, slides were treated for 20 min with a 1.5% hydrogen peroxide solution in PBS to block endogenous peroxidase activity. Tissue sections were washed three times for 3 min in PBS-Tween and incubated for 1 h with 8 μg/ml CTGF-specific human monoclonal antibody (FibroGen) in PBS/1% BSA. Sections were rinsed three times for 3 min in PBS-Tween and incubated for 30 min with rabbit-α-human IgG (DakoCytomation, Copenhagen, Denmark) diluted 1:1,500 in PBS/1% BSA containing 5% normal mouse serum (DakoCytomation). After three times 3 min of washing with PBS-Tween, sections were incubated with goat α-rabbit Powervision-PO

Table 1. Primer sequences used in quantitative RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTGF</td>
<td>5′-CACAGAGTGGAGCCGCTGTTC-3′</td>
<td>5′-GATGCACATCTTCTCCTTTAATG-3′</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>5′-GCAACATGTTAGCACTTACAGA-3′</td>
<td>5′-GACGTCAAAGACAGGCTACTCA-3′</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>5′-CAGGAGGCCCAGAGTGAAGAC-3′</td>
<td>5′-GAGAAATAATTCTCTGAGCTATGCCTACT-3′</td>
</tr>
<tr>
<td>Collagen IV</td>
<td>5′-ATTCTCCTCTGTAAGCACC-3′</td>
<td>5′-TGGGCGCTTCTGACATCTC-3′</td>
</tr>
<tr>
<td>TBP</td>
<td>5′-CGAGGAGCCAAAGTGAAGAC-3′</td>
<td>5′-GAGAAATAATTCTCTGAGCTATGCCTACT-3′</td>
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</tbody>
</table>

CTGF, connective tissue growth factor; TGF, transforming growth factor; TBP, TATA binding protein.
RESULTS

General characteristics of mouse models of diabetes. After injection with STZ, ~75% of the mice developed hyperglycemia with blood glucose levels exceeding 15 mmol/l within 3 days. Mice that did not develop hyperglycemia within 1 wk were excluded from the study. General characteristics of STZ-DM and ob/ob, as well as of appropriate nondiabetic control mice, are presented in Tables 2 and 3. Mean blood glucose levels of STZ-DM mice exceeded the upper limit of quantification (27.8 mmol/l) 1 wk after development of diabetes and remained elevated throughout the study. Blood glucose levels in control mice ranged from 4 to 8 mmol/l. Insulin implants, providing a constant release of insulin, stabilized the condition of the STZ-DM mice, although blood glucose levels in these animals showed a high variation during the day. Also, in ob/ob mice blood glucose levels at 10 mo of age were increased above the upper limit of quantification (27.8 mmol/l).

The plasma fructosamine level, an indicator of metabolic control (2), was significantly higher in STZ-DM and ob/ob mice compared with their respective controls. In 9-wk STZ-DM mice, all receiving insulin, plasma fructosamine levels were lower compared with earlier time points but still significantly elevated compared with those in control mice. STZ-DM mice lost weight during the experiment, whereas control mice gained weight due to normal growth. After an initial drop of ~10%, body weight of the STZ-DM mice receiving insulin pellets remained almost constant throughout the study. As no differences in key parameters were observed between 2- and 9-wk control mice, these mice served as one control group; ob/ob mice became progressively obese but remained clinically stable without insulin supplementation.

Table 2. General characteristics of STZ-DM and control mice

<table>
<thead>
<tr>
<th>STZ-DM</th>
<th>Duration of DM, wk</th>
<th>n</th>
<th>Control</th>
<th>1</th>
<th>2</th>
<th>4</th>
<th>9</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td></td>
<td></td>
<td>14</td>
<td>6</td>
<td>5</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>Blood glucose, mmol/l</td>
<td>6.8±0.4</td>
<td></td>
<td>&gt;27.8</td>
<td>329±7*</td>
<td>314±25*</td>
<td>366±14*</td>
<td>275±13*</td>
</tr>
<tr>
<td>Plasma fructosamine, μmol/l</td>
<td>183±3</td>
<td></td>
<td>&gt;27.8</td>
<td>19.9±0.1</td>
<td>20.4±0.3</td>
<td>22±0.2</td>
<td>22.1±0.1</td>
</tr>
<tr>
<td>Body wt at day 0, g</td>
<td>21.5±0.2</td>
<td></td>
<td>&gt;27.8</td>
<td>16.4±0.4*</td>
<td>17.8±0.4*</td>
<td>19.3±0.6*</td>
<td>21.5±0.2*</td>
</tr>
<tr>
<td>Body wt/body wt day 0, %</td>
<td>113±2</td>
<td></td>
<td>&gt;27.8</td>
<td>83±2*</td>
<td>87±2*</td>
<td>87±3*</td>
<td>97±1*</td>
</tr>
<tr>
<td>Kidney wt, mg</td>
<td>136±2</td>
<td></td>
<td>&gt;27.8</td>
<td>150±7</td>
<td>167±4*</td>
<td>163±9*</td>
<td>148±6</td>
</tr>
<tr>
<td>Kidney wt/body wt, %</td>
<td>1.12±0.02</td>
<td></td>
<td>&gt;27.8</td>
<td>1.82±0.08*</td>
<td>1.88±0.07*</td>
<td>1.69±0.11*</td>
<td>1.38±0.06</td>
</tr>
<tr>
<td>Plasma creatinine, mg/dl</td>
<td>0.18±0.02</td>
<td></td>
<td>&gt;27.8</td>
<td>0.36±0.05*</td>
<td>0.35±0.03*</td>
<td>0.30±0.05</td>
<td>0.26±0.2*</td>
</tr>
<tr>
<td>Albuminuria, mg/g creatinine</td>
<td>27±6</td>
<td></td>
<td>&gt;27.8</td>
<td>731±229*</td>
<td>223±97</td>
<td>968±248*</td>
<td>113±35*</td>
</tr>
</tbody>
</table>

Values are means ± SE. n, No. of mice; STZ-DM, streptozotocin-induced diabetes mellitus. *P < 0.5 compared with nondiabetic control.

Table 3. General characteristics of nondiabetic ob/+ and diabetic ob/ob mice

<table>
<thead>
<tr>
<th>ob/+</th>
<th>ob/ob</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blood glucose, mmol/l</td>
<td>9.7±0.6</td>
<td>&gt;27.8</td>
</tr>
<tr>
<td>Plasma fructosamine, μmol/l</td>
<td>180±2</td>
<td>319±13</td>
</tr>
<tr>
<td>Body wt, g</td>
<td>41±1.0</td>
<td>49.1±2.9</td>
</tr>
<tr>
<td>Kidney wt, mg</td>
<td>175±5</td>
<td>208±6</td>
</tr>
<tr>
<td>Kidney wt/body wt, %</td>
<td>0.42±0.02</td>
<td>0.42±0.02</td>
</tr>
<tr>
<td>Plasma creatinine, mg/dl</td>
<td>0.29±0.08</td>
<td>0.43±0.04</td>
</tr>
<tr>
<td>Albuminuria, mg/g creatinine</td>
<td>69.10±0.09</td>
<td>653±200</td>
</tr>
<tr>
<td>Plasma CTGF, pmol/l</td>
<td>483±44</td>
<td>4,320±1,607</td>
</tr>
<tr>
<td>Urinary CTGF, pmol/g creatinine</td>
<td>36±6</td>
<td>9,099±2,283</td>
</tr>
</tbody>
</table>

Values are means ± SE. n, No. of mice.
Development of nephropathy. Already after 1 wk of STZ-DM, a significant increase in plasma creatinine was observed, which persisted at 2, 4, and 9 wk. Especially at the earliest time points, the rise in plasma creatinine might have been caused, at least in part, by dehydration. Because we have no data on actual creatinine clearance, it remains unclear whether the elevated plasma creatinines at later time points reflect loss of renal function. STZ-DM mice showed an increase in albuminuria as early as 1 wk after development of DM, and albuminuria persisted during the experiment. However, large variations in albuminuria levels were observed among individual STZ-DM mice (Fig. 1, A and B). Kidney mass was significantly increased after 2 and 4 wk of STZ-DM and had subsequently decreased at 9 wk (Table 2), indicating a fast but transient development of renal hypertrophy after induction of DM. In contrast, the mesangial matrix expanded progressively over time, also from 2 wk onward in STZ-DM mice (Fig. 1, C and D). Besides glomerular alterations, tubulointerstitial damage was observed, as exemplified by epithelial simplification and degeneration and the presence of protein casts and cellular infiltrate (Fig. 1C).

Because hyperglycemia may lead to upregulation of specific ECM proteins (31), quantitative RT-PCR was performed for total fibronectin, its fibrosis-associated splice variant fibronectin ED-A, and collagen IVα1 on total renal cortex RNA of control mice and of diabetic mice. After 2 and 4 wk of STZ-DM, renal mRNA expression of all these ECM genes tended to increase, which became significant at 9 wk of STZ-DM (Fig. 2).

Together, these functional and structural data confirm development of nephropathy in our mouse model of STZ-induced diabetes mellitus.

At 10 mo of age ob/ob mice also showed albuminuria, but no significant increase in plasma creatinine and relative kidney weight was observed compared with the nondiabetic ob/+ littermates (Table 3).

Increased CTGF expression in diabetic animals. Plasma CTGF levels were significantly increased in 9-wk STZ-DM animals compared with controls (P < 0.0001, Fig. 3A). Urinary CTGF levels of most control mice were below the detection limit of the sandwich ELISA (<50 pmol/l). However, in STZ-DM mice, urinary CTGF levels were readily detectable. After correction for urinary creatinine excretion, urinary CTGF levels appeared significantly increased at all time points (Fig. 3B). In consecutive urine samples from the group of 9-wk STZ-DM mice, CTGF levels were significantly increased at all time points (Fig. 3C). Plasma CTGF levels increased >10-fold in three of five ob/ob mice (Fig. 3D). Urinary CTGF excretion in ob/ob mice increased 15–400 times compared with their nondiabetic ob/+ littermates (Fig. 3E).

CTGF in situ hybridization as well as CTGF immunohistochemistry was performed in paraffin sections of renal tissue to determine the localization of CTGF expression. In healthy control animals, some glomerular cells as well as some cells in vessel walls expressed CTGF mRNA, but hardly any renal CTGF protein expression was observed. However, in STZ-DM mice, both renal CTGF mRNA and protein expression were strongly increased (Fig. 4, A and B). In 1-, 2-, and 4-wk STZ-DM mice, CTGF protein appeared mainly localized in podocytes (Fig. 4B). In 9-wk STZ-DM mice, however, in addition to CTGF expression in podocytes, Bowman’s capsule and mesangial cells also appeared to be CTGF positive. In these mice, thickening of Bowman’s capsule and occasional glomerulosclerosis were observed (Fig. 4B). Similarly, in ob/+ mice, CTGF protein expression was increased and mainly localized in podocytes.

To investigate the level of renal CTGF mRNA expression, quantitative PCR was performed on cDNA of (total) renal cortex of STZ-DM and control mice. As early as 2 wk of STZ-DM, CTGF mRNA expression was upregulated threefold and remained at that level until 9 wk (Fig. 5A). Upregulation of TGF-β1 expression, an important inducer of CTGF expression, has been described under diabetic conditions. Therefore, we also determined TGF-β1 mRNA expression in these animals. Renal cortical TGF-β1 expression was increased twofold at 1, 2, and 4 wk after development of diabetes (Fig. 5B).

Quantitative PCR was also performed on other organs of 9-wk diabetic animals. This revealed that CTGF mRNA expression was also significantly increased in the heart and liver but not in the spleen of 9-wk diabetic mice (Fig. 5C). To obtain an impression of the contribution of the kidney to total (systemic) CTGF expression, we multiplied relative CTGF mRNA abundance by organ mass (Fig. 5E). These calculations indicate that, at least in terms of mRNA, the kidneys might contribute up to twice as much as the liver and three times as much as the heart to total CTGF expression. At 9 wk, also the relative and absolute TGF-β1 mRNA expression in the renal cortex, but not in the heart, liver, and spleen, was increased compared with controls (Fig. 5, D and F).

Urinary CTGF level is correlated with nephropathy. One of the main questions of this study was whether CTGF is associated with the development of DN. Because increased albuminuria is the main early characteristic of DN, we assessed the possible correlation between urinary CTGF excretion and albuminuria in the collected urine samples of the STZ-DM mice (1, 2, 4, and 9 wk). Urinary CTGF excretion was significantly correlated with urinary albumin excretion in these animals (Fig. 6, R = 0.86, P < 0.0001). The number of samples from ob/ob mice was too small for such an assessment.

DISCUSSION

The objective of the present study was to determine the temporal profile of the renal CTGF expression pattern in murine experimental DM and the relationship of plasma, urinary, and renal CTGF levels to the development and severity of DN.

Pancreatic β-cell depletion by STZ injection in C57BL/6J mice has been used extensively as a model for type 1 diabetes and might also be applicable in future studies in genetically modified mice, many of which are on a C57BL/6J background (44). A disadvantage of this model has been that within a few weeks after induction of DM, most of the animals die from severe wasting and dehydration due to extreme hyperglycemia and insulin deficiency.

We used insulin implants to prevent severe dehydration and maintain sufficient metabolic control for at least 9 wk, despite
Fig. 1. Development of diabetic nephropathy in streptozotocin-induced diabetic (STZ-DM) mice. 

**A:** albuminuria in control mice and diabetic mice 1, 2, 4, and 9 wk after development of diabetes mellitus (DM). 

**B:** course of albuminuria in diabetic mice. The dashed line represents mean ± 2 SD of control. 

**C:** periodic acid-Schiff-stained kidney sections of control and diabetic mice 1, 2, 4, and 9 wk after development of DM showing increasing kidney damage in diabetic animals. All pictures are taken at similar magnification. 

**D:** mesangial matrix score as measure for glomerular ECM accumulation of control and diabetic mice 1, 2, 4, and 9 wk after development of DM. Values are means ± SE.
the persistence of high blood glucose levels and elevated plasma fructosamine levels. With this approach, the mice developed progressive albuminuria, matrix expansion, and elevated plasma creatinine. These disturbances were already present after 1 or 2 wk, demonstrating that, in this model of insulin-treated STZ-DM, the onset of nephropathy is rapid and progressive. STZ has intrinsic toxic effects on the kidney, especially the tubular epithelium. This might be an important confounder when DN in STZ-treated mice is studied. However, it has been established that in rats in which treatment with STZ was followed by pancreatic islet or sham transplantation, increased kidney mass and albuminuria as well as decreased GFR were mainly caused by the diabetic status and not by STZ (35). Nevertheless, it remains important to control for a possible confounding effect in a study of nephropathy in STZ-induced diabetes. Therefore, we reproduced analyses of key parameters in a genetic model of type 2 DM, hIAPP-transgenic ob/ob mice (22). This revealed that, although severe hyalinosis and nodular lesions were not observed, the upregulation of CTGF is a phenomenon that occurs in close association with renal deterioration in two independent models of murine diabetes.

CTGF is a key growth factor linked with enhanced ECM expression in numerous physiological and pathological processes. Quantitative RT-PCR and in situ hybridization of renal tissue showed that CTGF mRNA expression was upregulated in the kidneys of STZ-DM mice. This is in agreement with previous studies in other models of DN (38, 47). However, the spatiotemporal distribution of diabetes-induced CTGF mRNA and protein expression in the kidney has not been resolved in detail (25, 45, 57). In situ hybridization and immunohistochemistry showed that CTGF expression was increased in kidneys of diabetic mice and mainly localized in the epithelial cells of the glomerulus. After 9 wk of STZ-DM, CTGF was also present in Bowman’s capsule and in the mesangial area, in addition to persistent expression in podocytes. Podocytes contribute to synthesis and maintenance of the GBM. Therefore, increased CTGF expression in podocytes might play an important role in diabetes-induced GBM thickening. In vitro, CTGF importantly contributes to ECM accumulation by rat and human mesangial cells (16, 38). Cross talk between podocytes and endothelial cells has been described for angiopoietin 1 and VEGF (41). Similarly, cross talk between podocytes and mesangial cells might also be possible, but it is not clear whether an increase in mesangial matrix in this model of DM relates to CTGF expression by podocytes or to CTGF derived from mesangial and other cells or from the circulation.

Plasma CTGF levels were variably increased in most of the diabetic mice. A comparable increase in plasma CTGF level has been observed in human DN, where this was shown to correlate with albuminuria (39). We also detected increased urinary CTGF levels in the STZ-DM mice as well as in ob/ob mice, confirming previous observations in experimental DM, as well as in human DN (14, 37). In the present study, we confirmed that urinary CTGF is correlated with albuminuria, the hallmark of DN, which is consistent with the notion that CTGF plays a pathogenic role in DN.

The markedly increased urinary CTGF excretion in 9-wk STZ-DM mice, and even more in ob/ob mice, together with the strongly increased expression of CTGF in glomerular podocytes suggest that at least part of the urinary CTGF excretion is derived from local renal production. Nevertheless, it remains difficult to identify the main source of the increased urinary CTGF levels.

Our finding that, in STZ-DM, CTGF mRNA expression is upregulated not only in the kidney but also in several other organs (Fig. 4C) and that the plasma CTGF level is upregulated twofold suggests that at least part of the increased urinary CTGF excretion is derived from renal filtration of the relatively small CTGF molecule and possibly fragments thereof. In addition, proteinuria might hamper tubular reabsorption of filtered CTGF from the primary urine, which would independently contribute to higher levels in urine, and provide a trivial explanation for the observed correlation of urinary CTGF with
Arguing against the latter, the fractional excretion of CTGF at 9 wk STZ-DM was about six times higher than the fractional excretion of albumin (data not shown). Still, although less likely in this markedly proteinuric condition, we cannot exclude the possibility that this difference relates, at least in part, to difference in molecular size and maybe charge [molecular mass of albumin is ~66 kDa compared with full-length CTGF (38 kDa) and CTGF NH2-terminal fragment (18 kDa)]. Hence, the DM-associated increase in urinary excreted CTGF could well be of mixed renal and extrarenal origin. Ultimately, experiments involving injection of labeled CTGF and CTGF fragments will be necessary to better define the contribution of plasma levels and local renal production to urinary CTGF excretion.

Comparing CTGF mRNA levels in normal human tissues by Northern blot analysis, Oemar et al. (34) found that CTGF expression is more abundant in kidney than in the heart, liver, lung, and pancreas. In control mice, we confirmed by quantitative RT-PCR that CTGF expression was highest in the kidney, followed by the left ventricular tissue of the heart, than the liver and was lowest in the spleen. In situ hybridization and immunohistochemistry of these tissues showed that in addition to the renal cortex, also the atria, but not the ventricles of the heart abundantly express CTGF mRNA and protein as was reported by Chuva de Sousa Lopes et al. (9). Overexpression of CTGF in the diabetic heart, vitreous humor, and retina has been reported previously (20, 26, 52). To see whether in our DM models upregulation of CTGF expression is kidney specific or a generalized, systemic phenomenon, we next compared CTGF mRNA levels in the kidney, heart, liver, and spleen of 9-wk STZ-DM and control mice. Besides in the kidney (2.8-fold), an increase in the relative amount of CTGF mRNA was evident in the ventricular tissue of the heart (3.5-fold) and in the liver (2.1-fold) of 9-wk STZ-DM mice. Moreover, correction for organ mass indicates that the kidneys might contribute approximately twice as much as the liver and three times as much as the heart to total (systemic) CTGF mRNA expression under diabetic conditions. Together, these data indicate that CTGF overexpression plays a central role in DM, with involvement not only in the development of DN but also in the systemic complications of DM.

TGF-β, one of the main inducers of CTGF, is considered an important profibrotic growth factor in the development and progression of DN (30). It is known to be overexpressed in mesangial and endothelial cells of diabetic glomeruli and has been implicated in GBM thickening and albuminuria, both
characteristics of DN (30). However, more recently, factors relevant to the DM-associated pathogenic processes of hyperglycemia and hypertension have been reported to stimulate expression of CTGF and ECM (40, 47, 53). This can occur in a TGF-β-independent manner. For example, in mouse podocytes, it has been shown that the high glucose-induced expression of the α1- and α2-subunits of collagen type IV is independent of TGF-β1 (23). In renal fibroblasts, TGF-β1-independent induction of collagen IV and fibronectin expression under high-glucose conditions has been described (27, 29). Furthermore, in rats with unilateral ureteral obstruction, CTGF antisense oligonucleotide treatment reduced the gene expression of CTGF, fibronectin, and fibronectin ED-A, whereas TGF-β1 gene upregulation was not affected. In this study, the deposition of these proteins was also reduced by the CTGF antisense treatment (56).

In our study, TGF-β1 mRNA expression was increased fourfold in renal cortex of 9-wk STZ-DM mice, but in diabetic heart, liver, and spleen the TGF-β1 level was not different from controls. This suggests that, at least in the latter organs, CTGF overexpression occurs independently of increased TGF-β1 mRNA expression levels. However, it does not preclude a critical role of TGF-β1, because the abundance and signaling activity thereof are largely regulated at the level of translation and conversion from a latent to the active form (36). Alternatively, other factors and processes that are responsible for TGF-β-independent stimulation of CTGF gene expression could mediate the observed increase in heart and liver CTGF.

Glomerular overexpression of other growth factors in DN might act in concert with increased CTGF to promote pathogenesis. For example, it is known that VEGF is highly expressed in diabetic podocytes and that inhibition of VEGF in
these cells attenuates glomerular hypertrophy, GBM thickening, and mesangial matrix expansion (11, 54). PDGF-B and its receptor are highly expressed in mesangial cells and podocytes of diabetic rats (33), and IGF-1 is yet another growth factor implicated in matrix accumulation in DN. Both CTGF and IGF-1 are produced by human renal fibroblasts and cooperate in the induction of collagen production by high glucose (28). It is known that CTGF, as a matricellular protein, can physically interact with several of these and other growth factors (49). In this way, CTGF can promote TGF-$\beta_1$ signaling and inhibit the action of BMP4 (1). CTGF can also inhibit VEGF-induced angiogenesis (24), and cooperation of CTGF and IGF-I in the induction of collagen I and -III secretion has been demonstrated in human kidney fibroblasts (29). Thus CTGF can function as a cofactor for other growth factors and modulate their biological activity in a way that contributes to the development of DN. Moreover, recent evidence indicates that, in addition to its role as a modulator of ECM, CTGF can signal through direct binding to the neurotrophin receptor TrkA (50) and through interaction with low-density lipoprotein receptor-related protein (13, 42, 55).

Different fragments of the CTGF protein have been detected in vitro and in vivo, and at least some of these have biological activity. The CTGF molecule, which consists of four modules, is mostly cleaved between modules II and III, yielding fragments of 16–20 kDa, but smaller fragments have also been

Fig. 5. CTGF and transforming growth factor (TGF)-$\beta_1$ mRNA expression in STZ-DM and control mice. A: total renal cortical CTGF mRNA expression. B: total renal cortical TGF-$\beta_1$ expression in control and 1-, 2-, 4-, and 9-wk DM mice. C: CTGF mRNA expression in kidney, heart, liver, and spleen of 9-wk diabetic and control mice. D: TGF-$\beta$ mRNA expression in kidney, heart, liver, and spleen of 9-wk diabetic and control mice. Values are means ± SE. E: CTGF mRNA expression corrected for organ mass. F: TGF-$\beta$ mRNA expression corrected for organ mass.

Fig. 6. Correlation between urinary CTGF excretion and albuminuria in STZ-DM mice. $R^2 = 0.74$, $P < 0.0001$: data from 1-, 2-, 4-, and 9-wk diabetic mice are combined.
identified (3, 19), Grotendorst et al. (17) reported distinct biological effects of CTGF fragments; the NH2-terminal part of CTGF mediates myoblast formation and collagen synthesis, whereas the COOH-terminal part is involved in fibroblast differentiation (17). However, the forms in which CTGF is present in plasma, urine, and tissue are still mainly undefined, and in addition our assays did not allow such specification.

In summary, we have shown that CTGF levels are increased in plasma, urine, and renal tissue of diabetic mice in already a very early phase of nephropathy. Moreover, the observed association of levels and distribution pattern of CTGF with markers of nephropathy lends further support to its proposed role as an early marker and key pathogenic factor in DN.

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DISCLOSURES

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