Abstract. Connective tissue growth factor (CTGF) is a peptide secreted by cultured endothelial cells and fibroblasts when stimulated by transforming growth factor-β (TGF-β), and is overexpressed during fibrotic processes in coronary arteries and in skin. To determine whether CTGF is implicated in the pathogenesis of diabetic glomerulosclerosis, cultured rat mesangial cells (MC) as well as kidney cortex and microdissected glomeruli were examined from obese, diabetic db/db mice and their normal counterparts. Exposure of MC to recombinant human CTGF significantly increased fibronectin and collagen type I production. Furthermore, unstimulated MC expressed low levels of CTGF message and secreted minimal amounts of CTGF protein (36 to 38 kD) into the media. However, sodium heparin treatment resulted in a greater than fourfold increase in CTGF protein (18 kD) containing the heparin-binding domain. The induction of CTGF protein by a high glucose concentration was mediated by TGF-β, since a TGF-β-neutralizing antibody blocked this stimulation. In vivo studies using quantitative reverse transcription-PCR demonstrated that although CTGF transcripts were low in the glomeruli of control mice, expression was increased 28-fold after approximately 3.5 mo of diabetes. This change occurred early in the course of diabetic nephropathy when mesangial expansion was mild, and interstitial disease and proteinuria were absent. A substantially reduced elevation of CTGF mRNA (twofold) observed in whole kidney cortices indicated that the primary alteration of CTGF expression was in the glomerulus. These results suggest that CTGF upregulation is an important factor in the pathogenesis of mesangial matrix accumulation and progressive glomerulosclerosis, acting downstream of TGF-β.

Acute imbalances in the expression, distribution, and/or biologic activities of cytokines such as transforming growth factor-β (TGF-β) and platelet-derived growth factor (PDGF) are an important part of the initial response to renal injury and are necessary for the increased collagen formation that is part of normal wound healing (1,2). However, a sustained imbalance in these factors may lead to progressive renal sclerosis with eventual organ failure. The evidence for such an effect appears strongest for TGF-β. The exposure of either cultured mesangial cells (MC) or glomeruli to this cytokine results in increased extracellular matrix (ECM) production (3,4), mirroring the overaccumulation of mesangial matrix components that characterizes the lesion in vivo (5). Furthermore, TGF-β activity is increased in a variety of both human and experimental forms of glomerulosclerosis (6–8), and ECM production is induced after the transfection and overexpression of the TGF-β gene in the rat kidney (9). Finally, treatment with anti-TGF-β antibody attenuates the enhanced glomerular ECM gene expression that occurs in experimental glomerulonephritis (10) and in experimental diabetic glomerulosclerosis (11).

Two factors that appear responsible for the sustained overexpression of TGF-β are a high extracellular glucose concentration and altered glomerular hemodynamics. Exposure of MC to increased glucose concentrations stimulates the production and binding of TGF-β1 (12–14) in concert with induction of ECM production (15,16). The stimulation of TGF-β1 production is attributed, at least in part, to transcriptional activation involving a region in the TGF-β1 promoter containing a glucose-response element (17). The mechanisms for the hemodynamically induced TGF-β activity are also becoming apparent. In diabetes and in the remnant kidney, the impairment of
glomerular pressure autoregulation (18) results in the exposure of the capillary bed to the large moment-to-moment variations in systemic BP (19). Due to the elasticity of the glomerulus, this increased capillary pressure produces expansion of the structure and MC mechanical strain (20). The consequence of this mechanical stimulation is increased production, release, activation, and binding of TGF-β1, as shown in cultured MC subjected to cyclic stretching (14,21). This same treatment also results in increased synthesis and accumulation of mesangial matrix components, fibronectin, laminin, and collagen types I and IV (20). Furthermore, the marked induction of MC collagen synthesis by high glucose levels, or the combined effects of high glucose and cyclic stretching are significantly reduced by neutralization of TGF-β (13,22).

A cytokine that may act downstream of TGF-β to regulate matrix metabolism is connective tissue growth factor (CTGF). Human CTGF (hCTGF) was first identified as a product of human umbilical vein endothelial cells that was chemotactic and mitogenic for fibroblasts (23). It is now known to be one of the seven cysteine-rich secreted proteins that comprise the CTGF family (24). These related proteins are comprised of four modules and one signal peptide (24). The four modules include an insulin-like growth factor-binding domain, a von Willebrand factor type C repeat likely involved in oligomerization, a thrombospondin type I repeat thought to be involved in binding to the ECM, and a C-terminal module that may be involved in receptor binding. The human, mouse, and rat CTGF are highly conserved proteins with greater than 90% amino acid homology and a molecular mass of approximately 37 kD (24).

The biologic activities of CTGF have been incompletely studied. However, in the aggregate, an important emerging role for CTGF is that of a prosclerotic mediator in both dermal fibrosis and coronary atherosclerosis. Recombinant human CTGF are highly conserved proteins with greater than 90% identity to produce CTGF, and in coronary disease, CTGF mRNA is expressed 50- to 100-fold higher in atherosclerotic plaques than in the normal arterial wall (30).

Although CTGF is causally implicated in dermal fibrosis and atherosclerosis via its apparent stimulation by TGF-β, very little is known about the level and control of its expression during the development of glomerulosclerosis. Recently, kidney biopsy specimens from patients with various forms of renal disease were examined for CTGF mRNA by in situ hybridization and compared to areas of normal tissue in kidneys with localized renal tumors (31). A qualitative assessment indicated that compared to the normal tissue, glomerular CTGF message appeared as upregulated in lesions of crescentic glomerulonephritis, IgA nephropathy, focal and segmental glomerulosclerosis, and diabetic nephropathy. Based on the above evidence, we hypothesized that CTGF may be an important mediator of ECM accumulation in diabetic glomerulosclerosis. To test this theory, we studied the expression and matrix-inducing effects of CTGF in cultured MC. In addition, we examined glomerular and whole renal CTGF activity in experimental diabetes and its relationship to the level of mRNA expression for ECM components and the degree of mesangial expansion and proteinuria.

Materials and Methods

Cells and Tissue Culture

The MC, previously characterized and described by us, were a cloned line derived from outgrowths of Fischer rat glomeruli (13). Upon serial passage, they continue to express key markers of MC (13). The medium used was RPMI 1640 with penicillin and streptomycin and, unless otherwise noted, 5 mM glucose. MC growth medium contained 20% Nu-serum from Collaborative Research (Bedford, MA). Unless otherwise noted, MC were cultured for approximately 4 d in growth medium and, when reaching confluence, were washed twice with serum-free medium and incubated for 24 to 48 h under serum-deprived (0.5% fetal calf serum [FCS]) conditions. The cultures were then incubated for a designated period in fresh maintenance medium (0.5% FCS), with or without experimental treatments. At the concentration of FCS used in these studies, there was no active TGF-β1, -2, or -3 detectable in the fresh medium, as determined by a highly sensitive mink lung bioassay (our unpublished observation). The renal fibroblasts used in Northern analysis were mouse tubulointerstitial fibroblasts, as described by Alvarez et al. (32).

Experiments testing the effects of mechanical strain were carried out as described previously (13). In brief, MC were seeded into 6-well, Flex I plates (5 \times 10^4 cells/25-mm diameter well) with flexible collagen-coated bottoms from Flexcell International (McKeesport, PA) and cultured in 1 ml/well of growth medium. After 24 h, experimental cultures were subjected to cyclic stretching while the control cultures were maintained in a static environment under identical conditions. In an attempt to mimic conditions of MC stretch during possible low frequency oscillations in intraglomerular pressure, all experiments were carried out using alternating cycles of 10-s stretch and 10-s relaxation (50 mHz) (33). Vacuum intensity was set to provide an average elongation of approximately 8% over the entire culture surface (34).

Animals and Specimen Collection

Diabetic male db/db mice and their nondiabetic db/m littermates were from Jackson Laboratories (Bar Harbor, ME). The db/db mouse carries a defective receptor gene for leptin, a key weight control hormone (35). These mice become obese at 3 to 4 wk of age and...
develop hyperglycemia. Associated nephropathy includes proteinuria and mesangial expansion with increased mesangial matrix that develops by 5 to 7 mo (36). In the present experiments, mice were sacrificed at the age of 5 mo, when incipient glomerular lesions were expected to occur. Blood glucose levels were determined during the study and at sacrifice, using a colorimetric method based on the glucose oxidase-peroxidase reaction and supplied in a kit form (Glucose Procedure No. 510 kit) from Sigma Diagnostics (St. Louis, MO). After 24-h acclimation to metabolic cages, two consecutive 24-h urine samples were collected. At the end of the collection period, the lower part of the cage including the collection funnel was rinsed with distilled water and the final sample volume was recorded. Protein concentration in the urine was measured according to the method of Bradford (37).

After anesthesia by an oxygen/ether mixture, the abdominal cavity was opened, a 23-gauge needle was inserted into the aorta, and the kidneys were perfused with 4 ml of ice-cold perfusion buffer (RPMI with 4% bovine serum albumin (BSA)) containing 10 mM vanadyl ribonucleoside complex, an RNase inhibitor from Life Technologies/ BRL (Grand Island, NY). Chilled 0.9% saline was poured over the kidneys during this perfusion. The kidneys were then removed and the right kidney was frozen in liquid nitrogen for subsequent RNA extraction and Northern analysis. Fine sagittal slices of the left kidney were rapidly obtained. One section was fixed in 3.8% paraformaldehyde, embedded in paraffin, and stained with periodic-acid Schiff (PAS) for light microscopic evaluation. The remaining slices were used for glomerular microdissection and reverse transcription (RT)-PCR of the isolated glomeruli. The methods used were a modification of those described previously by Peten et al. for determination of glomerular mRNA levels (38). In brief, tissue sections were placed in a buffer of Hanks’ balanced salt solution containing 10 mM vanadyl ribonucleoside complex, then 50 glomeruli were dissected from each kidney in less than 50 min. The glomeruli were next transferred to a PCR tube with 30 µl of rinse buffer (Hanks’ balanced salt solution containing 5 mM dithiothreitol and 50 U/ml human placental ribonuclelease inhibitor from Boehringer Mannheim [Indianapolis, IN]). After centrifugation, the supernatant was removed and microscopically examined for the accidental presence of glomeruli. Seven microliters of a lysis solution (rinse buffer containing 2% Triton X-100) was added, and the samples were stored at −70°C until processed. All of these procedures were carried out at 4°C.

Experimental samples from control and diabetic mice were thawed on ice, then subjected to two additional freeze/thaw cycles to lyse the glomeruli. The RT reaction was then carried out using a cDNA synthesis kit from Boehringer Mannheim, with oligo(dT) as a primer. Reactions containing glomeruli, but without added reverse transcriptase, served as negative controls. The reaction mixture was incubated for 60 min at 42°C, then chilled to 4°C for 10 min. Samples were then diluted 1:10 in distilled water and frozen at −70°C until PCR was done.

**Evaluation of Renal Tissue by Light Microscopy**

Five to six nonconsecutive 6-µm sections per kidney were PAS-stained and examined. Mesangial sclerosis was scored on a scale of 0 to 4: (0), no lesion; (1), minimal mesangial expansion; (2), mesangial expansion and/or basement membrane thickening; (3), marked mesangial thickening, some collapsed lumina, occasional lobule with full sclerosis; (4), diffuse collapse of capillary lumina and sclerosis involving 75% or more of the tuft). A total of 100 to 150 glomeruli per kidney was scored by an observer blinded to the origin of the specimens. Only glomerular profiles showing a mesangial region that could be unequivocally evaluated were scored.

### Competitive PCR and Northern Blotting

All PCR was performed using the GeneAmp DNA amplification kit from Perkin-Elmer-Cetus (Norwalk, CT) and a Perkin-Elmer 9600 thermal cycler. For quantification, a competitive PCR reaction was run using a cDNA mimic. Thirty-eight cycles of replication were used. Five PCR tubes were set up for each sample. Each tube in a series contained a fixed amount of the wild-type cDNA along with decreasing concentrations of the mimic cDNA. The products were separated by agarose gel electrophoresis and visualized by ethidium bromide staining. Bands were digitized by scanning densitometry using a Howtek, Scansammyter 3+ Densitometer (Hudson, NH) and quantified with image analysis using NIH Image, version 1.59, from Twilight Clone BBS (Silver Spring, MD). A plot of the ratio of wild-type/mimic versus the reciprocal of the input mutant concentration was constructed, and the amount of glomerular cDNA was determined from the resulting linear regression. Northern analysis was carried out as described previously (21), following pulverization of samples in a liquid nitrogen cooled stainless-steel mortar and homogenization in 1.0 ml of RNA Stat-60 reagent from Tel-Test, Inc. (Friendswood, TX). Probes for individual mRNA, the corresponding cDNA, were labeled with 32P by random hexamer priming using the Sigma Prime-1 kit from Sigma Chemical. Autoradiograms were digitized by scanning densitometry and quantified as above.

### Primers, Probes, and cDNA Mimics

Primers for CTGF were designed and synthesized based on conserved sequences between the human and mouse CTGF (fisp 12) gene. They were F: 5′-GAG TGG GTG TGT GAC GAG CCC AA G G 3′- and R: 5′-ATG TCT CCG TAC ATC TTC CTG TAG T-3′. The amplification product was 558 bp in size. The sequence was confirmed by cloning into pcR script from Invitrogen (Carlsbad, CA). Two clones were sequenced and were identical. A competitive cDNA mimic was produced using a PCR mimic construction kit (K1700-1) from Clontech Laboratories (Palo Alto, CA). For each mimic, two composite primers (3′ and 5′) were first made containing the target gene sequence (CTGF) plus a 20-nucleotide stretch designed to hybridize to opposite strands of a heterologous DNA fragment provided in the kit. The desired primer sequences were then incorporated into this fragment during a PCR amplification. A dilution of the first PCR reaction was then amplified using only the gene-specific primers, ensuring that all mimic molecules had complete gene-specific sequences. The mimic was then purified by passage through CHROMA SPIN TE-100 columns from Clontech. By this method, the size (200 to 650 bp) could be adjusted by choosing the appropriate sequences of the generic DNA fragment for the composite primers. The resulting cDNA competes on an equal basis for the same primers in the same reaction. Amplifier of the CTGF mimic was 496 bp in size.

Primers for rat fibronectin cDNA were F: 5′-TGC CAC TGT TCT CCT ACG TG-3′ and R: 5′-ATG CTT TGA CCC TTA CAC GG-3′. A competitive mimic for fibronectin was constructed as described above. Products of amplification were approximately 312 (sample) and 474 bp (mimic). Primers for GAPDH were obtained from Clontech and produced an amplification fragment of 985 bp. A GAPDH competitive mimic was constructed as described above, and produced a fragment of 604 bp. The cDNA probe for Northern analysis was from a sequence of human CTGF shared by rat and mouse. The cDNA probe for TGF-β1 (pRTGF/β1) was a gift from Drs. Su Wen Qian and Anita Roberts at the National Cancer Institute (Bethesda, MD). The fibronectin cDNA probe (39) was a gift from Dr. Subhas Chakrabarty at The University of Texas.
Production of Recombinant CTGF and anti-CTGF Antibodies

Recombinant human CTGF protein (rhCTGF) was generated using a baculoviral expression system. In brief, the human CTGF open-reading frame (1047 bp) was amplified using primers engineered with BamHI sites immediately flanking the ATG start codon and TGA stop codon (forward primer 5′-GCT CCG CCC GCA GTG GGA TCC ATG ACC GCC GGC-3′; reverse primer 5′-GGA TCC GGA TCC TCA TGC CAT GTC TCC GTA-3′). Clone DB60R32 was used as template (23), which contains the entire 2075-bp CTGF cDNA. The amplified product was subcloned into the BamHI site of pFastBac1 from Life Technologies/BRL, analyzed for insert orientation, and verified by sequencing of both DNA strands. Generation of recombinant baculovirus containing the CTGF cDNA was performed as outlined by Life Technologies/BRL (pFastBac expression system). Recombinant baculovirus stocks were isolated, expanded to high virus titer, and used to infect High Five insect cells (Invitrogen, Carlsbad, CA) for expression of CTGF. The recombinant CTGF was purified by heparin-Sepharose affinity chromatography as described previously (24). Peak fractions containing rhCTGF were determined by immunoblotting and Coomassie staining of sodium dodecyl sulfate (SDS)-polyacrylamide gels (25).

Two anti-CTGF antibodies were used. The first, anti-CTGF polyclonal pAb839, was prepared through a contract service with Quality Controlled Biochemicals (Hopkinton, MA) by immunizing rabbits with a keyhole limpet hemocyanin-coupled synthetic peptide corresponding to amino acids 329 to 343 (CPG DND IFE SLY YRK) that is unique to the carboxy terminus of CTGF. The production of antibody was monitored by enzyme-linked immunosorbent assay (ELISA) with the peptide conjugated to BSA and absorbed to plastic. The anti-CTGF antibodies were affinity-purified by passage through a CPG DND IFE SLY YRK-Sepharose peptide column using standard protocols (40). Peptide-blocking studies confirmed the monospecificity of pAb839 for CTGF in Western immunoblotting assays. Western immunoblot analysis also revealed that pAb839 recognized CTGF only in a reduced conformation. The second antibody, plgY3 polyclonal, was raised in chickens by immunizing with purified baculovirus-derived full-length rhCTGF protein as described previously (41), and was subsequently affinity-purified through a rhCTGF-Sepharose column.

ELISA

The amount of specific ECM components secreted into the culture medium was quantified by ELISA, using a described procedure (20). It was previously determined in MC cultures that media containing 0.5 to 1% FCS was optimal for the recovery of fibronectin and collagen (20). Experimental samples of culture medium were tested in triplicate. Purified matrix components, diluted in the same medium, were run (0.5 to 500 ng/well) as standards. All antisera were tested for specificity before their use by immunoblotting, with and without blocking, using the ECM standards. Color intensity was measured with a TiterTek Multiscan MCC/340 from Flow Laboratories (McLean, VA), and the results were analyzed using a curve-fitter computer program from Interactive Microware (State College, PA).

An indirect ELISA was used to quantify CTGF levels in the conditioned media. In brief, microtiter wells were coated with media samples or the rhCTGF standard (50 μl/well) for 2 h at room temperature in a 96-well plate. The wells were washed 4 times with Dulbecco’s phosphate-buffered saline (D-PBS) and then incubated with pIgY3 antibody at 1.25 μg/ml (50 μl/well) in blocking buffer (1% BSA, 0.05% Tween 20 in D-PBS) for 60 min. After thorough washing with D-PBS, a horseradish peroxidase (HRP)-conjugated rabbit anti-chicken IgG from Zymed Laboratories (South San Francisco, CA) was added to all wells at a 1:6000 dilution in blocking buffer for 30 min. The substrate, TMB-ELISA from Life Technologies/BRL, was added at room temperature for 15 min, the reaction was stopped with 1 M sulfuric acid, and the color developed was read at 450 nm in an ELISA multispec spectrophotometer ( Molecular Dynamics, Sunnyvale, CA). The amount of CTGF protein present in samples was determined using a logarithmic standard curve with serial dilutions (3 pg to 3 ng/well) of rhCTGF standard antigen.

Heparin-Sepharose Precipitation and Immunoblotting

To analyze for CTGF protein expression, conditioned media were collected and the heparin-binding proteins were precipitated by end-over-end mixing for 4 h at 4°C with heparin-Sepharose CL-6B beads from Pharmacia (Piscataway, NJ). The beads were washed three times with ice-cold radioimmunoprecipitation assay lysis buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, and 2 mM ethylenediaminetetra-acetic acid). The bound proteins were then eluted by boiling in SDS sample buffer (62 mM Tris-HCl, pH 6.8, 2.3% SDS, 10% glycerol and bromphenol blue) for 5 min under either nonreducing or reducing conditions (containing 5% mercaptoethanol). The eluted heparin-binding proteins were resolved in 4 to 20% SDS-polyacrylamide gels and electrophoretically transferred to nitrocellulose filters from Schleicher & Schuell (Keene, NH) for 2 h at 140 mA. The filters were blocked with blocking buffer (TTBS; 150 mM NaCl, 50 mM Tris, 0.2% Tween 20, 5% BSA, pH 7.4) for 2 h at room temperature and then probed for CTGF by incubation for 40 min with anti-CTGF antibody at 0.5 μg/ml in blocking buffer. After extensive washing at 37°C, the filters were incubated with either HRP-conjugated donkey anti-rabbit IgG from Amersham (Arlington Heights, IL) or HRP-conjugated rabbit anti-chicken IgG from Zymed Laboratories at a 1:12,000 dilution in blocking buffer. Immunoreactivity was detected by using the Supersignal chemiluminescent substrate from Pierce (Rockford, IL), according to the manufacturer’s instructions.

Additional Reagents

Purified ECM components used as standards were rat collagen I obtained from Upstate Biotechnology (Lake Placid, NY) and rat fibronectin from Chemicon International (Temecula, CA). The corresponding antibodies, polyclonal anti-rat collagen I and anti-rat fibronectin from Chemicon International, were used in ELISA. In preliminary experiments, the former antibody did not cross-react with fibronectin or laminin, whereas the latter does not cross-react with collagen I or laminin. The TGF-β used for stimulation experiments was human TGF-β2 and was a gift from Celsrix Corp. (Santa Clara, CA). This recombinant cytokine was produced in Chinese hamster ovary cells, then purified by previously reported techniques (42). A monoclonal antibody (1.D 11.1) that neutralizes TGF-β1, -2, and -3 was a generous gift of Genzyme Corp. (Cambridge, MA).

Statistical Analyses

Data were expressed as means ± SEM. For tissue culture data, unless otherwise noted, differences between two groups were evaluated using a paired t test. A paired test was used because of the cloned nature of the MC studied. In the case in which results were normalized to corresponding control values, the data were analyzed by a one-sample t test with a hypothesized mean of 100% to compare the test group with the control. A paired two-sample t test was used to examine differences between three test groups. In both cases, a Holm’s test was then applied post hoc to adjust for multiple compar-
isons (43). For histologic data, the mean sclerosis score was calculated in the glomeruli of each kidney, and the statistical difference between the diabetic and control groups was determined using a nonpaired t test.

Results

CTGF-Induced Changes in ECM Production by MC

We first carried out experiments designed to determine the effects of exogenous CTGF on the MC production of ECM. To do this, serum-depleted cells were exposed for 48 h to media containing 20 ng/ml rhCTGF. For comparison purposes, additional cultures were incubated in media without exogenous CTGF, but containing either 2 ng/ml TGF-β, or 20 mM glucose. As anticipated, exogenous TGF-β and the high glucose concentration increased the amount of secreted fibronectin by 23 and 30%, respectively, over that of controls (Figure 1A). The presence of exogenous CTGF in the media also effectively stimulated fibronectin secretion (45%). Like fibronectin, the quantity of secreted collagen type I was also increased by CTGF (64%), as well as by TGF-β (50%) or high glucose (22%) (Figure 1B).

Renal and MC CTGF Expression: Regulation by TGF-β

We next sought to determine whether CTGF mRNA was expressed by cultured rat MC and then compared the results with those from whole kidney. Northern analysis demonstrated a single 2.4-kb CTGF transcript in MC and whole kidney, but in contrast no detectable message was evident in cultured kidney fibroblasts (Figure 2). When compared with other tissues, the most abundant expression was in the kidney and was approximately 20-fold higher than in the brain.

To determine whether TGF-β was a regulatory factor in MC expression of CTGF message, cells were serum-depleted and exposed to 2 ng/ml TGF-β for 24 h, and the mRNA was probed. Changes in TGF-β transcript levels were also monitored. Exogenous TGF-β exposure increased the expression of CTGF mRNA more than fourfold (Figure 3, A and B), whereas TGF-β1 mRNA was increased 80% (Figure 3, A and C). To determine whether CTGF was capable of regulating its own expression, or that of TGF-β, MC were also exposed to 20 ng/ml rhCTGF. This treatment failed to alter the level of TGF-β mRNA (Figure 3, A and C), but in contrast, it strongly autoinduced CTGF message (Figure 3, A and B).

To next demonstrate whether the low CTGF mRNA expression in unstimulated MC was associated with a detectable production of the corresponding protein, and to determine the effects of TGF-β, cells were serum-depleted and then cultured for an additional 24 h in fresh maintenance medium in the presence or absence of 2 ng/ml exogenous TGF-β. The conditioned medium was subsequently heparin-Sepharose-precipitated and analyzed by immunoblotting using two different anti-CTGF antibodies. Immunoblotting with pIgY3 antibody, raised against the full-length rhCTGF, demonstrated that under basal, unstimulated conditions, MC secreted very small amounts of CTGF (Figure 4A). However, upon exposure to TGF-β, the secretion of CTGF protein was markedly stimulated. The predominant product detected in these cultures migrated to the same position as the recombinant standard. Immunoblotting of the same samples with pAb839 antibody, raised against a 15 amino acid sequence unique to CTGF, confirmed the identity of protein detected (Figure 4B). This latter antibody, while highly specific for CTGF, is of low comparative affinity, binding CTGF only when samples are analyzed under reduced conditions. For that reason, pAb839 antibody was not used in subsequent immunoblotting analyses.

The CTGF protein detected in MC cultures above represents a free molecule present in the media. The existence of a heparin-binding domain within CTGF suggests that a substantial portion of the synthesized and released protein exists bound to proteoglycans, or to fibronectin, present on the cell surface or

Figure 1. Effects of exogenous connective tissue growth factor (CTGF) on mesangial cell (MC) secretion of extracellular matrix (ECM). Cells grown in a 5 mM glucose medium were serum-depleted for 48 h, then incubated for an additional 48 h in media containing: (1) 5 mM glucose; (2) 5 mM glucose plus 2 ng/ml transforming growth factor-β (TGF-β); (3) 5 mM glucose plus 20 ng/ml recombinant human CTGF (rhCTGF); or (4) 20 mM glucose. The quantities of fibronectin (A) or collagen type I (B) contained in the media at the end of incubation were determined by enzyme-linked immunosorbent assay (ELISA). Bars represent means of six separate cultures in a representative experiment. Each value shown is the percentage increase over the corresponding control. *P < 0.05 versus control.
in the ECM. To ascertain whether this was the case, and to
determine the time course for appearance of CTGF in the
extracellular environment under unstimulated conditions, MC
cultures were serum-deprived and then fresh maintenance me-
dia containing 50 μg/ml sodium heparin were added. Conditioned media were collected after defined incubation periods,
the majority of the sample was pooled, and heparin-sulfate was
precipitated. Immunoblotting of the 4-h samples produced faint
CTGF bands at approximately 36 and 39 kD (Figure 5A). The
intensities of these bands increased sharply by 24 h and re-
maind elevated throughout the 72-h incubation period. At 48
and 72 h, when the full-length CTGF bands were intense, a
faint additional band with an electrophoretic mobility of ap-
proximately 20 kD could also be seen. As demonstrated pre-
viously, in the absence of sodium heparin, the CTGF present in
the media was barely detectable, suggesting that the majority
of CTGF protein produced was bound to the cell and/or sub-
strate. Because immunoblotting is largely a qualitative assay,
individual supernatants were also evaluated by ELISA before
their pooling and precipitation, and the results were expressed
on a per cell basis. This highly quantitative assay demonstrated
a time-dependent increase in CTGF, with approximately 7
ng/10⁶ cells being secreted in a 24-h period (Figure 5B). The
amount of CTGF secreted in the medium during the total 72-h
period was reduced to 20% in the absence of heparin.

We next reexamined the regulation of secreted CTGF by
TGF-β, this time in the presence of heparin. Accordingly, MC
were serum-deprived as before, then incubated for 48 h in
maintenance media containing 50 μg/ml sodium heparin and 2
ng/ml TGF-β. Immunoblotting of pooled, precipitated media
samples indicated that TGF-β markedly increased the secretion
of full-length (36 to 39 kD) CTGF (Figure 6A). However, even
more pronounced was the induction of the molecule(s) appear-
ing at 18 to 20 kD. This smaller moiety corresponds in size to
half of the full-length CTGF molecule. Quantitative analysis
by ELISA of the individual samples, before being pooled and
precipitated for immunoblotting, demonstrated a 2.5-fold en-
hancement of total secreted CTGF in response to TGF-β treat-
ment (Figure 6B).
MC CTGF Expression: Regulation by Glucose Concentrations

To determine whether CTGF expression might also be altered by the ambient concentration of glucose, MC cultures continuously grown in 5 mM glucose were incubated for 14 d in growth media containing 35 mM glucose. This time was chosen because our previous studies showed that this period was required for the full induction of ECM protein production. As observed previously, MC grown in medium containing 5 mM glucose concentration demonstrated minimal levels of CTGF message (Figure 7). However, after the long-term exposure to an increased glucose concentration, MC transcripts for CTGF were markedly upregulated (Figure 7), reaching a sevenfold level above control, as determined by quantitative image analysis (data not shown).

To examine the effects of high glucose exposure on the secretion of CTGF protein, we used serum-deprived cultures, shortened the exposure time to 48 h, and included sodium heparin in the medium. This protocol allowed a comparison to the effects of TGF-β. Immunoblotting of pooled and precipitated media samples indicated that exposure to 20 mM glucose increased the amount of CTGF secreted (Figure 6A). Interestingly, however, this stimulation appeared to be limited to the full-length molecule only. Quantification of secreted CTGF protein by ELISA, before pooling and precipitation, demonstrated a twofold induction by high extracellular glucose levels (Figure 6B), an increase similar to that induced by TGF-β, under the experimental conditions selected. To determine if the observed increase in CTGF could be due to an osmolar effect, we repeated these experiments using mannitol. Under these conditions, there was no induction of CTGF release as mea-
sured by ELISA (5 mM glucose, 2.39 ± 0.28 ng/10^6 cells; 5 mM glucose plus 15 mM mannitol, 1.94 ± 0.32), and no change in the distribution of CTGF forms secreted as determined by immunoblotting (data not shown).

Because the stimulation of ECM production in MC by high glucose has been shown to be mediated in large part by TGF-β (14,15), we wanted to determine whether this cytokine is also responsible for the observed effect on CTGF production. Accordingly, MC cultured for 14 d in the presence of either 5 or 20 mM glucose were seeded and grown under the same glucose conditions for an additional 8-d period. On day 4, the cultures were serum-deprived and half of the cultures received 20 μg/ml of an antibody that neutralizes TGF-β1, -2, and -3 activity. Fresh antibody was added daily, and the media was replaced 24 h before collection. Media from individual culture wells were tested by ELISA. Fresh antibody was added daily, and the media was replaced 24 h before collection. Media from individual culture wells were tested by ELISA.

Figure 8. TGF-β blockade of high glucose-induced CTGF production. MC cultured for 14 d in the presence of either 5 or 20 mM glucose were seeded and grown under the same glucose conditions for an additional 8-d period. On day 4, the cultures were serum-deprived and half of the cultures received 20 μg/ml anti-TGF-β antibody. Fresh antibody was added daily, and the media was replaced 24 h before collection. Media from individual culture wells were tested by ELISA. n = 4. *P < 0.05 versus 5 mM glucose; **P < 0.05 versus 20 mM glucose without TGF-β antibody.

Although the constitutive secretion of CTGF in the presence of normal concentrations of glucose also appeared somewhat reduced by the presence of TGF-β antibody, this change was not statistically significant (P = 0.09). Also nonsignificant (P = 0.075) was the difference in CTGF levels in normal glucose- and high glucose-treated cells when TGF-β was neutralized (Figure 8).

**MC CTGF Expression: Regulation by Cyclic Mechanical Strain**

To determine whether cyclic mechanical strain was also a factor capable of altering MC expression, cells were seeded
into collagen-coated flexible-bottom plates, then after overnight incubation, they were subjected to either stretch or maintained under static conditions. At the designated periods, the cells were lysed and total RNA was extracted and probed for CTGF transcripts. Cyclic stretching induced a rapid and marked increase in the CTGF message (Figure 9). Quantitative image analysis of the Northern blot showed that levels of CTGF mRNA increased more than twofold by 4 h and remained elevated at this level after 8 h of stretching. In separate experiments, CTGF transcripts were significantly increased even after 48 h of stretch (not shown).

Experiments were also conducted to investigate whether the mechanically induced CTGF mRNA levels were accompanied by an upregulation in the secretion of CTGF protein. To this end, MC were seeded as described above and the next day subjected to 24 or 48 h of cyclic stretching. The media was replaced 24 h before harvest and heparin was added. Quantification of CTGF by ELISA demonstrated that cyclic strain failed to alter the amount of secreted CTGF (Figure 10A). Separate experiments showed a similar lack of effect after 4 and 8 h of stretch (data not shown). Additional studies were then performed in which MC cultures were grown to confluence (4 d), serum-depleted, then subjected to 24 or 48 h of cyclic stretching in the presence of sodium heparin. Again, cyclic strain failed to alter the amount of CTGF secreted during a 24-h period into the media (Figure 10B, bottom). Immunoblotting of heparin-precipitated pooled samples demonstrated that the molecular species of the secreted CTGF were also unchanged by stretch (Figure 10B, top).

**CTGF Expression in Experimental Diabetic Nephropathy**

To determine whether CTGF is upregulated in early diabetic nephropathy, studies were carried out on diabetic db/db mice, and the results were compared to those from age-matched nondiabetic db/m littermates. At 5 mo of age, approximately 3.5 mo after the onset of diabetes, animals were evaluated for

![Figure 9. Effect of cyclic stretching on the MC expression of CTGF transcripts. Cells cultured overnight on collagen-coated flexible-bottomed dishes were subjected to cyclic stretching or control, static conditions. At the indicated periods, RNA was extracted and probed for CTGF message. Each lane represents the results of samples pooled from 24 different culture wells.](image)

![Figure 10. CTGF protein secretion in response to mechanical strain. (A) MC were cultured under cyclic stretching conditions as described in Figure 9, except that heparin was added to the medium during the final 24 h of culture. Samples represent the CTGF analyses by ELISA in media during the last 24 h of culture. (B) MC were cultured to confluence, serum-depleted, and then subjected to cyclic stretching in the presence of heparin. The quantities of CTGF protein contained in the final 24-h incubation media were analyzed by ELISA (bottom panel). Immunoblotting results of duplicate, pooled (two cultures/sample) heparin-Sepharose-treated samples in a representative experiment are shown (top panel). In both bar graphs, n = 4. Experimental groups were not significantly different from controls (P > 0.05).](image)

blood glucose levels, total weight, proteinuria, and mesangial expansion. At the time of sacrifice, mean blood glucose levels, as well as body weights, were significantly greater in the db/db animals (Table 1). Inspection of the renal tissue by light microscopy demonstrated that the diabetic animals exhibited noticeable, but minimal, glomerular changes consistent with early diabetic glomerulosclerosis, i.e., mild mesangial matrix expansion without apparent tubulointerstitial disease (Figure
In addition, the level of proteinuria was not significantly greater than in controls (Table 1). Semiquantitative analysis (Table 1) of the glomerular changes demonstrated that the observed mesangial expansion in the diabetic animals was indeed consistent but of mild intensity, a value of 0 representing no lesion and a value of 1 representing minimal mesangial expansion in the majority of glomeruli, without basement membrane thickening.

Northern analysis of whole kidney RNA indicated that the CTGF message levels were markedly increased in four out of five diabetic mice (Figure 12A). These changes were mirrored by parallel changes in fibronectin transcript levels. Quantification of results yielded a mean 103% increase in CTGF expression, while fibronectin levels were 80% greater than in the controls (Figure 12B). Analysis of microdissected glomeruli by quantitative RT-PCR identified a low, but measurable, transcript level of CTGF in the glomeruli of control animals (Figure 13). However, this level was dramatically increased (27-fold) in the mice with diabetes. The upregulation of glomerular CTGF mRNA was accompanied by a nearly fivefold increase in the amount of fibronectin mRNA. These large differences were not due to dissimilar glomerular size resulting from diabetic hypertrophy, since the level of GAPDH message was not significantly increased in diabetic animals compared to controls (control, $1.39 \pm 0.524 \times 10^{-3}$ attomoles/glomerulus; diabetic, $2.59 \pm 0.307$ attomoles/glomerulus; $P > 0.05$).

**Discussion**

This study provides the first evidence demonstrating the production of CTGF protein by glomerular cells and its role as a potentially important factor in the pathogenesis of diabetic glomerulosclerosis. In diabetic glomerulosclerosis, two major, well-known casual factors are hyperglycemia and glomerular hypertension. Recent studies have demonstrated that elevated glucose concentrations and hypertension-induced glomerular mechanical strain stimulate the overproduction of ECM, a change that is at least in part mediated through the metabolic action of TGF-β (13,15,16,22). CTGF, a newly described factor that promotes ECM deposition and fibrosis in the skin, appears to act downstream of TGF-β to induce ECM produc-

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**Table 1. Measurement of changes in db/db mice at 5 mo**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Control db/m</th>
<th>Diabetic db/db</th>
<th>$P$ Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood glucose</td>
<td>$142 \pm 19.0$ mg/dl (n = 8)</td>
<td>$485 \pm 58.0$ (n = 6)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Weight</td>
<td>$29.2 \pm 1.00$ g (n = 8)</td>
<td>$43.1 \pm 7.30$ (n = 6)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Proteinuria</td>
<td>$2.32 \pm 1.07$ mg/24 h (n = 10)</td>
<td>$2.78 \pm 0.93$ (n = 9)</td>
<td>0.330</td>
</tr>
<tr>
<td>Glomerular sclerosis</td>
<td>$0.101 \pm 0.048$ (n = 17)</td>
<td>$0.649 \pm 0.369$ (n = 6)</td>
<td>0.003</td>
</tr>
</tbody>
</table>

* Protein excretion was determined in two consecutive 24-h urine collections. The mean value of these two collections was taken as the degree of proteinuria. A total of 100 to 150 glomeruli per kidney were scored on a scale of 0 to 4 as described in Materials and Methods. All values are means ± SEM.

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*Figure 11. Glomerular disease associated with diabetes in db/db mice. Renal cortical sections from control (left) db/m or diabetic (right) db/db mice at 5 mo of age were stained with periodic acid-Schiff for light microscopy examination. Shown is an example of glomeruli demonstrating the most severe mesangial expansion observed in the diabetic group.*
tion, but whether TGF-β action is obligatory for its overexpression is uncertain. In addition, the role of CTGF in the development of glomerulosclerosis has not been studied. Here, we demonstrate that CTGF stimulates cultured MC to produce the ECM components fibronectin and collagen type I, and that this induction of ECM formation by CTGF is triggered by increased glucose concentrations and exogenous TGF-β. Although findings similar to these have not been previously reported in glomerular cells, Frazier et al. (29), using qualitative pulse labeling techniques, demonstrated that rhCTGF treatment increases, as does TGF-β, the synthesis of ECM components by a fibroblast cell line. In addition, the relevance of these findings was demonstrated with the observation that injection of CTGF into the skin induces the formation of fibrous tissue (25).

In this study, we demonstrate that CTGF mRNA is expressed in the whole kidney of normal animals, and that its level is high compared with the heart and brain. This suggests that endogenously produced cytokine may be involved in normal turnover of renal ECM. However, the low levels of constitutive CTGF mRNA expression demonstrated in cultured MC suggest that this cell type may have a controlling mechanism for CTGF formation different from that in the cells forming the bulk of the renal tissue, i.e., tubular epithelial cells. The low expression of CTGF mRNA observed in MC under unstimulated conditions is associated with an apparent release of small quantities of CTGF protein into the culture medium. The CTGF protein was present as 36 and 38 kD molecular species. The larger protein is equivalent in size to the full-length CTGF molecule predicted from gene analysis, whereas the smaller peptide may represent a differential N-glycosylation in the CTGF N-terminal half. We have observed that in both insect and mammalian cells pretreated with tunicamycin, which inhibits the N-glycosylation of glycoproteins, the larger CTGF band is reduced in its migration to localize with the smaller moiety (unpublished observation). These molecular species observed in MC are similar in size to that secreted by vascular endothelial and fibroblast cells (23,41,44). The small amounts of CTGF detected in the conditioned medium of MC cultures are not the result of low levels of its synthesis, but rather due to the restricted release of the protein into medium. This was indicated by the ability of sodium-heparin to dramatically increase the levels of CTGF protein measured in the media. Our results suggest that as much as 80% of the CTGF synthesized by MC remains cell- or matrix-bound. This finding is also supported by preliminary receptor studies showing that exogenous heparin is able to displace iodinated CTGF bound to the MC surface (J. Nesbitt, personal communication). In a quantitative assay developed for the current study, it is shown

Figure 12. Induction of CTGF and fibronectin transcripts in whole kidneys of diabetic db/db mice. At 5 mo of age, total RNA was extracted from whole kidneys of control nondiabetic (C) or diabetic (D) mice and probed for CTGF mRNA by Northern analysis (A). Results of Northern analyses were quantified by densitometric analysis (B). $n = 5$. *$P < 0.05$ versus control.

Figure 13. Effects of diabetes on the glomerular expression of CTGF and fibronectin transcript levels in db/db mice. Analyses were carried out in samples of 50 glomeruli microdissected from each kidney of five control (db/m) and five diabetic (db/db) mice. After competitive reverse transcription-PCR, the amount of CTGF mRNA was determined and expressed per glomerulus. *$P < 0.05$ versus control.
that in the presence of heparin, MC secrete approximately 7 ng of CTGF per 10^6 cells in each 24-h period.

Given that CTGF stimulates ECM accumulation, we have examined whether known factors implicated in the development of diabetic glomerulopathy alter CTGF mRNA expression. High extracellular glucose concentrations markedly increase the levels of CTGF mRNA as well as the production of CTGF protein in MC. In a similar manner, TGF-β also upregulates the expression of CTGF mRNA and protein. With strong upregulation, as occurred in response to TGF-β, there was a marked induction of a small molecular weight CTGF species, which according to its size (approximately 18 kD), is approximately half of the full-length CTGF molecule. The structure and biologic role of this CTGF fragment is unknown. However, its size and properties (recovery from a heparin-Sepharose column) indicate that it contains both the thrombospondin 1 and the C-terminal modules of CTGF (24). Interestingly, Brigstock and colleagues isolated novel 10-, 16-, and 20-kD heparin-binding molecules from porcine uterine secretions that reacted with CTGF antibody (45). The small molecular species demonstrated in MC following stimulation may have distinct biologic activities compared with the whole molecule, and is currently under investigation. Because TGF-β secretion in MC is stimulated by increased ambient glucose concentrations (13,15,16,22), the observed induction of CTGF by high glucose may occur indirectly, mediated by the action of TGF-β. Indeed, our neutralization studies demonstrated a direct role for the cytokine in the process, since incubation with TGF-β antibodies resulted in a complete blockade of CTGF stimulation.

Cyclic mechanical strain was also examined as a possible regulatory element in CTGF expression. Our results demonstrate that stretching is a potent stimulus for the upregulation CTGF mRNA levels. However, in contrast to the effects of high glucose or TGF-β, cell stretching does not induce significant changes in the amount or size of the secreted CTGF protein. The reason for this difference is unknown. It remains possible, although perhaps unlikely, that our inability to observe increased CTGF protein in response to stretch was due to inappropriate experimental conditions. The CTGF message level was strongly increased after 2 h of stretching, and remained elevated at 8 h. However, we found no increase in CTGF protein after 4, 8, 24, 48, or 72 h of cyclic strain. Similar results were obtained in cells cultured under both serum and serum-depleted conditions. A second possible explanation for these differences is that the response to these two stimuli follow unique pathways. In the case of cyclic strain, translational control of CTGF may be independent of transcription, as others have reported for TGF-β with certain stimuli (46). Alternatively, mechanical strain may induce CTGF synthesis while simultaneously increasing protease activity. This would result in a greater turnover of the protein without increasing CTGF accumulation in the medium. We have shown this to be the case for collagen following cyclic stretch when the glucose level is not elevated (13).

The rapid induction of CTGF mRNA following stretch suggests that TGF-β production and or activity may not be required to mediate the initial effects of mechanical strain. Cyclic strain induces TGF-β synthesis and activation, but this effect is only evident after 48 to 72 h of mechanical stimulation (21). Interestingly, we have previously shown that the high collagen turnover, without medium accumulation, occurring in MC stretched under physiologic glucose concentrations, is independent of TGF-β action (13). However, under conditions of high glucose, there is an additional enhancement of collagen synthesis, resulting in a marked net collagen accumulation that is dependent on augmented TGF-β activity. Thus, the inability of stretch to induce collagen accumulation under conditions of low glucose concentrations, even though TGF-β is upregulated, may be related to the insufficient increase in CTGF protein production. Determination of the role of CTGF in long-term high glucose-induced and stretch plus high glucose-induced ECM synthesis and accumulation will require neutralizing antibodies specific for CTGF. Similar blockade studies will also be necessary to conclude that CTGF is acting downstream of TGF-β to induce matrix production. Currently, there are no such antibodies available.

In addition to the above findings, this study also demonstrates that TGF-β and CTGF are able to autoinduce their own expression in MC. Although this autoinduction has been described previously for TGF-β in other cells (47), this is the first time it has been noted for CTGF. Furthermore, this action appears to be selective, since exogenous CTGF has no effect on TGF-β transcript levels. These findings suggest that once stimulated by TGF-β, CTGF mRNA levels in MC may remain elevated even in the absence of additional TGF-β activity, resulting in a continued enhancement of ECM synthesis and deposition. This may help to explain the prevalent inability to totally block ECM production in MC and in the mesangium by TGF-β neutralization (10–12).

The studies reported here on the quantitative glomerular expression of CTGF mRNA in db/db mice strongly suggest that CTGF action is a factor in the initiation of glomerular ECM deposition in non-insulin-deficient diabetes. Although CTGF mRNA is expressed in normal glomeruli, the levels are dramatically upregulated (28-fold) after a short period of diabetes and before the onset of overt diabetic disease. In this study, CTGF mRNA upregulation occurred at a time when glomerular fibronectin mRNA levels were increased but glomerular mesangial expansion was minimal and proteinuria insignificant. Compared with glomeruli, the much lower upregulation of CTGF observed in the whole kidney suggests that the CTGF is, at least in the early phases of nephropathy, primarily involved in the induction of the glomerular alterations. However, in the more advanced stages of diabetic nephropathy, CTGF may be also an important inducer of tubulointerstitial disease. For example, it has been shown using an in vitro model of calcium oxalate nephrolithiasis, that renal tubular epithelial cells respond to calcium oxalate by upregulating the CTGF gene along with other genes involved in matrix turnover (48). A similar response also occurs in the same cells after mechanical wounding (49). Finally, Ito et al. reported a positive relationship between the number of positive CTGF mRNA-
expressing interstitial cells and the extent of tubulointerstitial lesions in human biopsies (31).

Findings concurrent with those reported here have been described in a very recent report which, although limited to mRNA analyses, shows that CTGF is upregulated in MC following exposure to high glucose concentrations or TGF-β (50). However, in this study, high glucose-induced CTGF mRNA expression was not fully inhibited by TGF-β (50). However, in this study, high glucose-induced CTGF following exposure to high glucose concentrations or TGF-β mRNA analyses, shows that CTGF is upregulated in MC expressing interstitial cells and the extent of tubulointerstitial lesions in human biopsies (31).

In summary, these studies suggest that in addition to enhanced glomerular TGF-β expression, CTGF upregulation is an important factor in exaggerated deposition of mesangial matrix. This CTGF upregulation is likely to be maximally driven by a combination of high glucose concentrations and cellular mechanical strain via pathways that are both dependent and independent of a preceding TGF-β stimulation. This, together with the study cited above, suggests that glomerular CTGF is strongly upregulated early in the course of both insulin-dependent and insulin-independent diabetes inducing progressive glomerulosclerosis.

Acknowledgments

This work was supported by Juvenile Diabetes Foundation International Grant 1-1998-243 (to Dr. Riser). We thank Clare Hasset and K. S. Sury Sastry for their expert technical assistance.

References


