8-1-2003

Urinary CCN2 (CTGF) as a possible predictor of diabetic nephropathy: Preliminary report

Bruce L. Riser
Henry Ford Health

Pedro Cortes
Henry Ford Health

Mark DeNichilo
Henry Ford Health

Poornima V. Deshmukh
Henry Ford Health

Parminder S. S. Chahal
Henry Ford Health

See next page for additional authors

Recommended Citation

Riser BL, Cortes P, DeNichilo M, Deshmukh PV, Chahal PS, Mohammed AK, Yee J, Kahkonen D. Urinary CCN2 (CTGF) as a possible predictor of diabetic nephropathy: Preliminary report. Kidney International 2003; 64(2):451-458.

Authors
Bruce L. Riser, Pedro Cortes, Mark DeNichilo, Poornima V. Deshmukh, Parminder S. S. Chahal, Ali K. Mohammed, Jerry Yee, and Dorothy Kahkonen

Kidney International, Vol. 64 (2003), pp. 451–458

Urinary CCN2 (CTGF) as a possible predictor of diabetic nephropathy: Preliminary report

Bruce L. Riser, Pedro Cortes, Mark DeNichilo,[1] Poornima V. Deshmukh, Parminder S. Chahal, Ali K. Mohammed, Jerry Yee, and Dorothy Kahkonen

Department of Medicine, Division of Nephrology and Hypertension and Division of Endocrinology and Metabolism, Henry Ford Health Center, Detroit, Michigan

Urinary CCN2 (CTGF) as a possible predictor of diabetic nephropathy: Preliminary report.

Background. It is currently impossible to reliably predict which diabetic patients will develop nephropathy and progress to kidney failure. Microalbuminuria, often regarded as a predictor of overt diabetic renal disease is, in fact, an indicator of established glomerular damage. We have shown that glomerular expression of the prosclerotic cytokine CCN2 (CTGF) is greatly up-regulated early in experimental and in human diabetes and mesangial cell exposure to CCN2 increases its production of extracellular matrix (ECM) molecules responsible for glomerulosclerosis. As an early marker, we therefore investigated the presence of CCN2 in urine and the relationship to diabetes and/or renal disease in an experimental model of diabetes and in a limited patient population.

Methods. Urine samples from (*1*) healthy rats, (*2*) rats made diabetic by streptozotocin (STZ), (*3*) healthy human volunteers, (*4*) diabetic patients with renal disease, and (*5*) diabetic patients without renal disease were examined by Western blotting and/or enzyme-linked immunosorbent assay (ELISA) for qualitative and quantitative analysis of the of CCN2.

Results. Low levels of urinary CCN2 were present in healthy, control rats, but were increased approximately sevenfold overall in STZ-diabetic animals. CCN2 levels were the highest at week 3 of diabetes, then decreased with time, but remained significantly elevated over controls even after 32 weeks. Consistently low levels of urinary CCN2 were also detected in healthy volunteers (mean value, 7.1 CCN2/mg creatinine). However, levels were elevated approximately sixfold in the majority of diabetic patients with nephropathy. A small number of the diabetic patients not yet exhibiting evidence of renal involvement demonstrated CCN2 urinary levels that were ninefold greater than controls. The remaining normoalbuminuric diabetic patients demonstrated CCN2 levels indistinguishable from those of healthy volunteers. Analysis by Western blotting confirmed the identity of the urinary CCN2. A molecular species equivalent to full-length CCN2 (37/39 kD doublet) was present in healthy controls. In contrast, the nephropathic group demonstrated multiple CCN2 bands.

Conclusion. These findings support our hypothesis that CCN2 is up-regulated early in the evolution of glomerulosclerosis, including that of diabetes. We contend that urinary CCN2 may both stage nephropathy and predict those patients who are destined for progressive glomerulosclerosis and end-stage renal disease (ESRD). Cross-sectional and prospective studies of larger, well-defined diabetic patients groups will be required to prove this hypothesis, and are ongoing.

Kidney failure resulting from progressive glomerulosclerosis is the leading cause of morbidity and mortality among patients with type 1 and type 2 diabetes [1, 2]. Current therapy with angiotensin-converting enzyme (ACE) inhibitors or angiotensin II receptor blockers, the drug classes of choice, effectively slow the progression of disease [3–6]. Nevertheless, such treatments are not justified in all newly diagnosed diabetic patients because only approximately 30% to 40% of these develop progressive renal disease, and the long-term side effects of these drugs are uncertain [6, 7]. Currently, diabetic patients are monitored for microalbuminuria. Persistent microalbuminuria is a marker of widespread vascular damage [8] and it has been regarded as an indicator of incipient nephropathy in type 1 and type 2 diabetes [9, 10]. However, the actual level of microalbuminuria may not necessarily predict the development of overt, clinical nephropathy, particularly among patients with a long duration of diabetes [9]. In addition, by the time microalbuminuria is detected, structural renal lesions are often already present [10–12]. Therefore, there is a great need for an early marker, or predictor, of nephropathy among diabetic patients. Patients with an earlier diagnosis would then likely benefit from an earlier initiation of therapy.

Human CCN2 (hCCN2), formerly designated human connective tissue growth factor (hCTGF) [13], is a 349 amino acid polypeptide that was first identified as a product of human umbilical vein endothelial cells, and which is chemotactic and mitogenic for fibroblasts [14]. It is now known to be one of the seven cysteine-rich secreted

[1] Mark DeNichilo's current address is TGR Biosciences, Adelaide, Australia.

Key words: chronic kidney disease, diagnostic, renal fibrosis, prognostic indicator.

Received for publication August 9, 2002
and in revised form December 16, 2002
Accepted for publication April 7, 2003

proteins that comprise the CCN family (named for its original members, cry61, CTGF and NOV) [15]. The CCN proteins are composed of four modules and one signal peptide [15]. The four modules include an insulin-like growth factor (IGF) binding domain, a von Willebrand factor type C module, a thrombospondin type 1 repeat, and a C-terminal module. However, the function of each of the modules is not yet clear. Human, mouse, and rat CCN2 are highly conserved proteins with greater than 90% homology at the amino acid level [15].

An important emerging role for CCN2 is that of a prosclerotic mediator. In renal biopsy specimens from patients with various forms of renal disease, including crescentic glomerulonephritis, immunoglobulin A (IgA) nephropathy, focal and segmental glomerulosclerosis, and diabetic nephropathy, it has been shown that *CCN2* gene expression is up-regulated [16]. Our laboratory has previously demonstrated that cultured mesangial cells express low levels of CCN2 mRNA and CCN2 protein [17]. However, exposure of these cells to high glucose concentrations, mechanical strain, or transforming growth factor-β (TGF-β), all causal factors in diabetic nephropathy [18, 19], resulted in increased levels of *CCN2* gene expression and protein secretion [17]. In vivo studies using the *db/db* mouse model of type 2 diabetes demonstrated by quantitative reverse transcription-polymerase chain reaction (RT-PCR) that CCN2 transcripts were low in the glomeruli of control mice, whereas this expression was increased 28-fold early in the course of diabetic nephropathy at a time when mesangial expansion was mild and proteinuria was within the control range. Measurements of concomitant CCN2 mRNA in the whole renal cortex indicated that the primary alteration of CCN2 renal expression was in the glomeruli [17]. Taken collectively, these results suggested that CCN2 up-regulation is an important early factor in the pathogenesis of extracellular matrix (ECM) accumulation that characterizes diabetic and nondiabetic glomerulosclerosis. In the present study, we have investigated the possibility that CCN2 is an early urinary prosclerotic factor, the excretion level of which can be correlated with the development of glomerulosclerotic process in diabetic nephropathy. This preliminary report supports the idea that measurement of urinary CCN2 in diabetic patients may serve to predict the future development of nephrologic complications. Confirmation of this hypothesis will be determined in a study current ongoing expanded patient study.

METHODS

Recombinant CCN2 and anti-CCN2 antibodies

Recombinant human CCN2 protein (rhCCN2) was generated as previously described [17] in a baculoviral expression system and purified by heparin sepharose affinity chromatography. Two anti-CCN2 antibodies, which we have previously described, were used [17]. For Western blotting, the antibody used was anti-CCN2 polyclonal pAb839, originally generated against amino acids 329-343 (CPG DND IFE SLY YRK) and unique to the CCN2 carboxy terminus. Peptide blocking studies confirmed the specificity of pAb839 for CCN2 in immunoblotting assays. For enzyme-linked immmunosorbent assay (ELISA), the antibody used was polyclonal pIgY3, produced by immunizing chickens with purified baculovirus-derived full-length rhCCN2 protein.

ELISA for urinary CCN2

A modified ELISA was used to quantify CCN2 levels in urine [17]. In brief, the wells of a microtiter plate were coated with undiluted urine or rhCCN2 standard (50 μL/well) for 2 hours at room temperature, washed with phosphate-buffered saline (PBS), and then incubated with pIgY3 antibody in blocking buffer (1%BSA, 0.05% Tween 20 in PBS) for 60 minutes. After washing with PBS, a peroxidase-conjugated rabbit anti-chicken IgG (Zymed Laboratories, Inc., South San Francisco, CA, USA) was added. The substrate, TMB-ELISA from Gibco (Carlsbad, CA, USA) was added and the color intensity read at 620 nm with a Titertek Multiscan MCC/340 (Flow Laboratories, McLean, VA, USA).

Western blotting for CCN2

Western analysis for CCN2 protein was carried out as previously described for cell culture supernatants [17]. Briefly, CCN2 in urine samples was heparin sepharose–extracted and specimens then boiled in sodium dodecyl sulfate (SDS) sample buffer under nonreducing or reducing conditions [17]. The proteins were resolved on 4% to 20% SDS-polyacrylamide gels, transferred to nitrocellulose, and then probed for CCN2 by incubation with anti-CCN2 antibody. The secondary antibody was an horseradish peroxidase (HRP)-conjugated rabbit anti-chicken IgG. Immunoreactivity was detected with a chemiluminescent system (SuperSignal; Pierce, Rockford, IL, USA).

Animals and induction of experimental diabetes

Insulin-deficient diabetes was induced in male Fischer rats (150 to 180 g) by a single intravenous injection of 55 mg/kg streptozotocin (STZ) (Sigma Chemical Co., St. Louis, MO, USA) immediately following dissolution in acidified saline. An equal volume of buffer was injected into control animals. To maintain body weight and prevent ketoacidosis, hyperglycemia was partially corrected (300 to 350 mg/dL) with daily administration of Ilentin II insulin (Eli Lilly, Indianapolis, IN, USA). All procedures were carried out with appropriate institutional approval following the guidelines for the use and care of animals. Animals were housed in accordance with good practice guidelines established by AALAC-approved animal care

facilities of Henry Ford Hospital. Animals were fed standard Purina rat chow (Ralston Purina Co., St. Louis, MO, USA) and permitted free access to water.

Patients

Consecutive patients making routine visits to either the nephrology or the endocrinology clinics of Henry Ford Hospital were studied. Excluded were patients with diagnoses other than diabetes. Patients were divided into two groups. Those with renal insufficiency (serum creatinine >1.8 mg/dL) and/or clinical proteinuria (>30 mg/dL protein, by dipstick) were placed the renal disease group, whereas patients with neither proteinuria nor renal insufficiency were placed in the group without renal disease. In some patients, only a single protein value was available, but in those cases the value was from the same collection as was used for the CCN determination. During the same period, samples were obtained from healthy volunteers with no history of diabetes, hypertension, or renal disease. Consent was obtained after the nature of the procedure and study was explained. No patients, who were asked, refused to take part in the study. For the quantitative study of urinary CCN2, the mean age was 58.9 ± 16.5 years and 51.8 ± 12.42 years (±SD), respectively, for groups with and without renal disease. In the group of seven patients with renal disease, six patients were hypertensive, four required insulin, and two were being treated with an ACE inhibitor or an angiotensin receptor blocker. In the group of six patients without renal disease, three were hypertensive, four required insulin, and two were being treated with an ACE inhibitor. For the qualitative study of urinary CCN2, four patients were studied, all required insulin, two had renal insufficiency and/or clinical proteinuria, three were receiving ACE inhibitor therapy, and three were hypertensive. Healthy volunteers had no history of diabetes, hypertension, or renal disease.

Collection and processing of urine samples

For animal studies, rats were placed in metabolic cages for a 24-hour adjustment period before duplicate 24-hour urine samples were obtained. The data from two consecutive 24-hour collections in each individual animal were pooled. For human studies, freshly voided urine was collected from patients during their routine visits to either the nephrology or the endocrinology clinics of Henry Ford Hospital. During the same period, samples were obtained from healthy volunteers with no history of diabetes, hypertension, or renal disease. Consent was obtained after the nature of the procedure and study was explained. All samples were maintained at 4°C during handling (maximum 6 hours for human samples and 2 hours for animal samples) before storage at −70°C. Samples were later thawed, centrifuged, and assayed directly, by ELISA, or following heparin sepharose–extraction by immunoblotting. Urine creatinine values were determined with a VITROS 250 analyzer (Johnson and Johnson, Rochester, NY, USA). Albumin levels in rat urine samples were determined by competitive ELISA using Nephrat (Exocell, Philadelphia, PA, USA).

Statistical analysis

Ratio estimator methods were used to compare CTGF/creatinine levels and albumin/creatinine levels in the two animal groups at each time point. Time points within a group were compared using ratio estimator methods taking into account the paired nature of the data. The data were transformed using a natural log transformation to account for nonnormality and Hochberg's method was used to adjust for multiple comparisons [20]. Statistical analysis of the CTGF/creatinine levels in the human data was performed by analysis of variance followed by *t* tests for pair-wise comparisons. A natural log transform of the data was used to correct nonnormality within groups. The analysis of variance (ANOVA) of Welch [21] and the variance approximation of Satterthwaite [22] were used to account for the difference in variance between groups. The method of Hochberg [20] was used to adjust for multiple comparisons.

RESULTS

Urinary CCN2 in normal and diabetic rats

To determine if CCN2 was present in urine and the effect of diabetes, 12 rats were made diabetic by STZ injection and urine samples collected after 3, 8, 11, 16, and 32 weeks of diabetes. Urine samples were also collected at these same periods from six age-matched healthy controls. CCN2 levels were determined by ELISA and expressed as the ratio of urinary creatinine. We found that overall concentrations of the cytokine among controls were 6.75 ± 1.1 ng CCN2/mg creatinine as compared to 49.0 ± 11.8 ng CCN2/mg creatinine (±SD) in diabetic animals (mean of all collections). This represented an average sevenfold increase in CCN2 following the onset of diabetes. The time course study showed that urinary CCN2 was present at consistently low levels at all time points in control animals (Fig. 1). In contrast, CCN2 amounts were substantially elevated shortly after the induction of diabetes. The levels in diabetic animals remained significantly elevated compared to age-matched controls during the entire study period (Fig. 1). However, with diabetes, there was a gradual decrease in the amount of CCN2 excreted with time. That is, the excretion at 32 weeks following diabetes was significantly lower than that at 3 weeks. The amount of urinary CCN2 in healthy animals also decreased significantly with time, although to a lesser degree (Fig. 1).

To determine the relationship between CCN2 and urinary albumin excretion, albumin was measured and also normalized for creatinine in the same sample. Unlike that observed for CCN2, albumin levels were consistently low

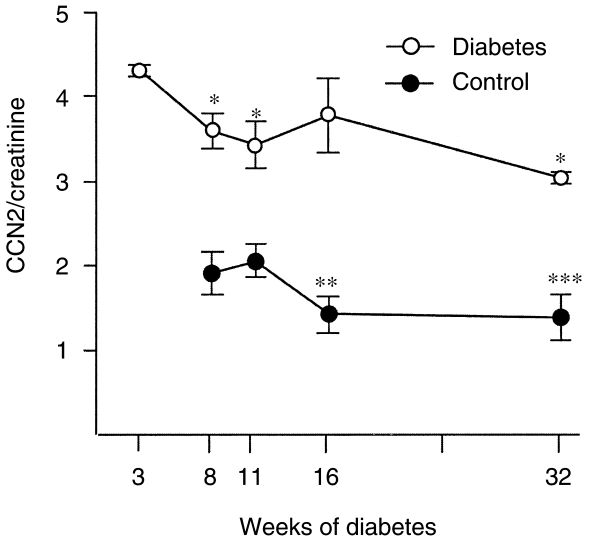

Fig. 1. Rat urinary CCN2 and the influence of diabetes. Concentrations in two consecutive 24-hour urine samples were measured by enzyme-linked immunosorbent assay (ELISA) and normalized to the amount of creatinine in the same sample. All CCN2 values (mean log ± SE) in the experimental group ($N = 12$) were significantly different than those in the control group ($N = 6$) at the corresponding periods ($P < 0.0001$). Within each group, CCN2 levels decreased significantly over time. *$P <$ 0.0006 versus 3 weeks; **$P < 0.0001$ versus 11 weeks; ***$P < 0.032$ versus 8 weeks and 11 weeks.

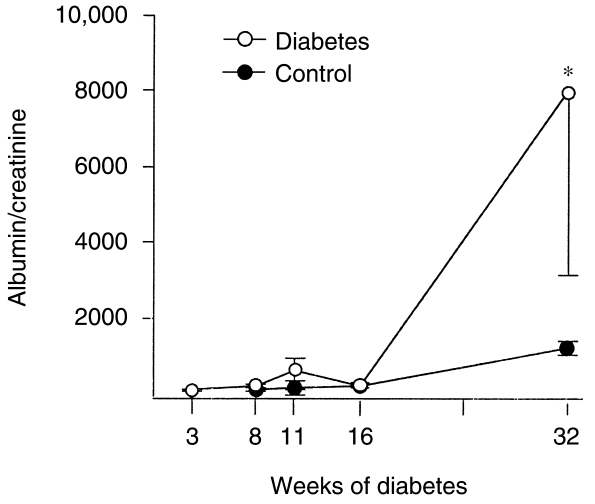

Fig. 2. Urinary CCN2 albumin levels in streptozotocin (STZ)-diabetic and control rats. Concentrations of albumin were measured following urine collection as described in Figure 1. The value (mean ± SE.) of albumin in diabetic animals ($N = 12$) was significantly greater at 32 weeks than the corresponding control ($N = 6$) group ($P = 0.042$).

in both groups until 32 weeks (Fig. 2). At this latter period, urinary albumin levels rose substantially in the diabetic group, resulting in significant ($P = 0.042$) differences as compared to albumin excretion in controls (Fig. 2).

Urinary excretion of CCN2 in control and diabetic patients

To determine if the increase in CCN2 excretion observed in STZ-diabetic animals reflected changes in humans with diabetes, we quantified urinary CCN2 excretion in healthy controls, diabetic patients, and diabetic patients with renal disease. CCN2 levels were determined to be consistently low among the healthy volunteers (mean, 7.1 ± 3.0 ng/mg creatinine) (±SD). Interestingly, however, in the diabetic patient group without renal disease, four of six patients demonstrated CCN2 levels equal to those of healthy volunteers, whereas the other two excreted substantially elevated (average ninefold) amounts of the protein (Fig. 3). Of the four patients in this group whose albumin/creatinine ratios were available, all were normoalbuminuric, including the two patients with high levels of urinary CCN2. Neither of the two patients with elevated CCN2 levels was receiving ACE inhibitor therapy. In the group of diabetic patients with renal disease, five of seven demonstrated significantly elevated CCN2 levels (mean of five, 42.1 ± 13.6 ng/mg creatinine or a sixfold increase over controls) (Fig. 3). Two of the five patients with renal disease and high CCN2 levels were receiving ACE inhibitor therapy, whereas neither of the two patients with renal disease but low CCN2 values was receiving such therapy. To compare the three groups, including all samples, an analysis of variance with the log transformation of the data was used. The values from the volunteer group did not differ from the diabetic patient group without renal disease as a whole (control, 1.90 ± 0.45 SD; patient, 2.56 ± 1.25 SD, $P = 0.35$). However, there was a significant difference between the control group and the diabetic patients with renal disease (control, 1.90 ± 0.45 SD; patient, 3.18 ± 0.99, $P = 0.01$).

To identify the molecular form of CCN2 present and the stability of CCN2 in urine, a sample was collected from a healthy volunteer then divided into five aliquots. Increasing quantities of rhCCN2 were added to four aliquots, whereas a fifth received diluent only. The aliquots were incubated at 37°C for 4 hours then frozen at –70°C. Samples were subsequently processed and analyzed by Western blotting in parallel with rhCCN2 in buffer alone. The sample with urine alone demonstrated the presence of CCN2, appearing as a 37/39 kD doublet (Fig. 4). This molecular form is characteristic of the full-length CCN2 molcule produced by cells in culture [17]. Preserved integrity of CCN2 was indicated by the expected progressive increase in amounts of full-length CCN2 recovered. Notably, only exceedingly high concentrations of added CCN2 demonstrated the presence of a lower-molecular-weight CCN2 fragment, in this case, equal to the half the full-length molecule (Fig. 4).

Finally, we investigated the possibility that the molecular form of urinary CCN2 might be altered among diabetic patients with established nephropathy. Accordingly, urine samples from four type 1 diabetic patients with

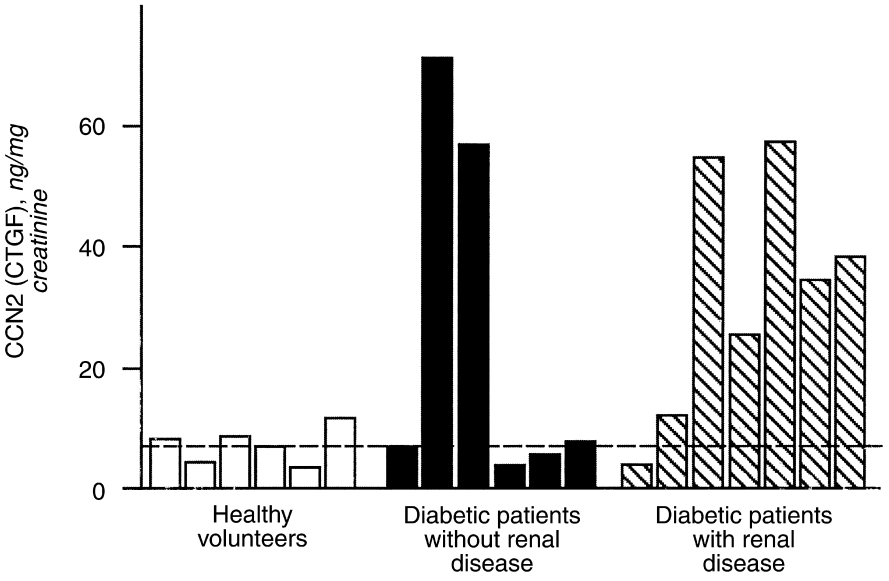

Fig. 3. Measurement of urinary CCN2 from patients and healthy volunteers. Urinary CCN2 levels were measured by enzyme-linked immunosorbent assay (ELISA). Each bar represents the determination from an individual patient sample run in triplicate. The dashed line indicates the mean value for all healthy volunteers.

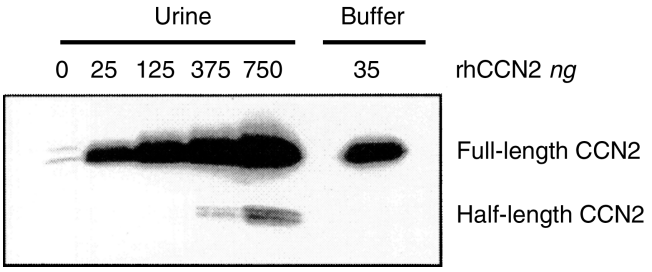

Fig. 4. Identification of urinary CCN2 protein in a healthy donor and the stability of added recombinant molecule. Urine was collected from a healthy donor who demonstrated detectable CCN2 level by enzyme-linked immunosorbent assay (ELISA). The sample was divided into five, 25 mL aliquots. Increasing amounts of recombinant human CCN2 (rhCCN2) (25 to 750 ng) were added to four of the aliquots. As a control, buffer only was added to the fifth aliquot. Immunoblotting was performed using CCN2-specific antibody.

renal disease and three healthy volunteers were collected. The CCN2 in urine samples was concentrated by heparin-sepharose extraction, then analyzed by Western blotting. Immunoreactive CCN2 appeared in three different molecular forms. A 37/39 kD doublet representing full-length CCN2 was present in two of four patient samples (Fig. 5, lanes A and D) and one control sample (Fig. 5, lane G). Interestingly, a large-molecular-weight band of approximately 200 kD was present in each patient sample, but appeared only as a very faint band in a single control sample (Fig. 5). Also present was a small CCN2 fragment of approximately 9 to12 kD. This CCN2 fragment appeared in the urine of three of the four diabetic patients (Fig. 5, lanes A, C, and D), but was absent in all healthy individuals (Fig. 5, lanes E, F, and G). This smaller moiety is equivalent to the heparin-binding C-terminal quarter fragment of CCN2.

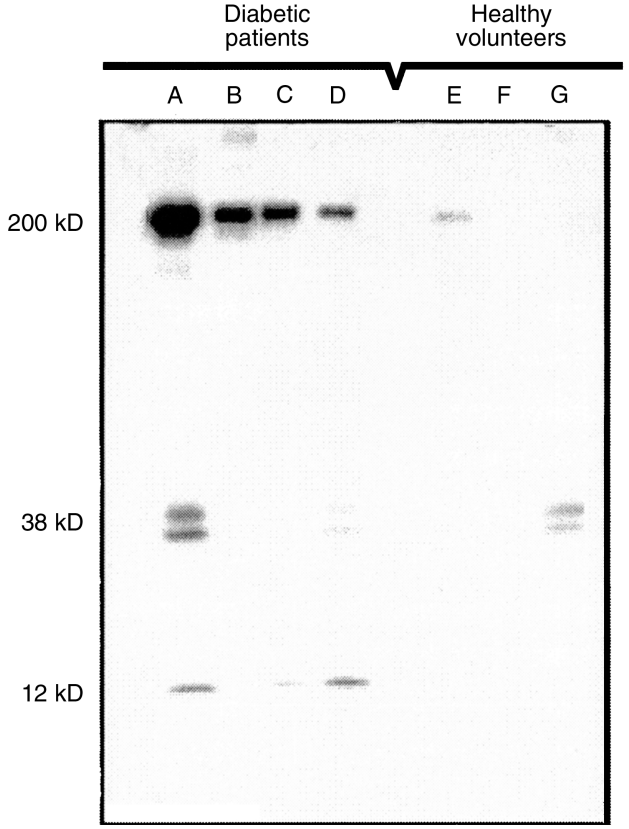

Fig. 5. Immunoblot analysis of urinary CCN2 from healthy volunteers and diabetic patients. Representative urine samples from four unselected patients (lanes A to D) collected during routine visits and three healthy volunteers (lanes E to G) were processed and examined for CCN2 by immunoblotting using CCN2-specific antibody.

DISCUSSION

This study demonstrates, for the first time, the presence of the prosclerotic cytokine CCN2 (formerly named CTGF) in urine. When normalized for urine creatinine, low levels of CCN2 were present in normal rats. However, with the establishment of experimental diabetes, there was a sevenfold elevation in the level of total CCN2. These levels exceeded controls for the entire 32-week period of observation. However, they declined slowly and significantly with time after their initial rapid elevation by week 3. The finding that CCN2 levels in nondiabetic animals also declined with time suggested an effect of normal aging. However, the drop was not entirely independent of diabetes, since the decline observed in diabetic rats was substantially greater than that among nondiabetic controls. This increase in urinary CCN2 preceded the time that we previously reported for the up-regulation of CCN2 message in tissue of *db/db* diabetic mice at $3^1/_2$ months of disease [17]. However, earlier examinations of mRNA levels were not performed. Based on the findings presented here, one might expect renal CCN2 transcript levels in *db/db* diabetic mice to be elevated considerably sooner than $3^1/_2$ months. Murphy et al [23] found measurable CCN2 mRNA in the renal cortex of STZ-diabetic rats 4 months after surgery when, in this model, mesangial expansion and proteinuria were present, but did not report on earlier time periods.

Our initial investigations in human beings, using a limited patient population, showed that urinary CCN2 levels were consistently low among healthy volunteers, whereas the majority of those with diabetes and nephropathy demonstrated on average a sixfold increase in excretion. The finding that CCN2 levels in two of the patients within this group were not different from those in the control group was not unexpected and suggests that in diabetic nephropathy other factors may affect excretion. One factor may be the stage of progression. This is supported by our animal study where there was an early elevation in urinary CCN2 levels following the onset of diabetes, with values subsequently decreasing over time and approaching those of normal control animals after 32 weeks. Another factor that may influence urinary CCN2 excretion is patient medication. There appeared to be no indication that those receiving ACE inhibitor or angiotensin receptor blocker therapy demonstrated reduced CCN2 excretion. However, the patient population was too small to accurately determine an effect. An effect of such therapeutics on urinary CTGF excretion will be important in future expanded determinations.

In the patient group with diabetes but with no renal disease, two of six patients demonstrated marked elevation in CCN2 excretion (eight- and tenfold over controls). The remaining four patients showed CCN2 levels within the range of the normal volunteers. Both of the patients with high CCN2 excretion were normoalbuminuric at the time of assay. Although not yet proven, these findings support our hypothesis that increased urinary CCN2 may precede progressive nephropathy. The mean levels in those diabetic patents with elevated urinary CCN2, but without nephropathy, were considerably higher than the mean levels in diabetic patients with renal disease. This is in agreement with the concept that in susceptible patients, CCN2 levels are elevated early, and then fall as the renal disease becomes established. The possibility that elevated CCN2 may occur in response to diabetes, but independent of renal alterations, cannot be ruled out at this time. However, this seems unlikely when interpreted in the context of our findings that in experimental diabetes, alterations in available renal CCN2 are associated with the early development of glomerulosclerosis, and CCN2 in vitro stimulates ECM synthesis by renal cells [24]. Further, we recently determined by RT-PCR amplification of mRNA from renal biopsies that patients with type 1 diabetes have glomerular CCN2 transcripts that are elevated in the presence of microalbuminuria and overt proteinuria [25]. We found a positive correlation between glomerular CCN2 mRNA levels and albuminuria and fractional mesangial area [25]. Collectively, this supports the idea that the changes in urinary CCN2 levels observed in the present study reflect changes in synthesis occurring in the kidney.

In immunoblotting studies, we found that among healthy volunteers the low urinary CCN2 levels as detected by ELISA produced either a weak doublet band at 37/39 kD or no detectable band. The larger protein of this doublet is equivalent in size to the full-length CCN2 molecule predicted from gene analysis. The smaller peptide may represent differential N-glycosylation of CCN2 [17]. The full-length CCN2 forms detected in normal urine are like those previously reported in fibroblasts, endothelial, and mesangial cells [14, 17, 26] and suggests that at least the majority of urinary CCN2 remains intact. In support of this notion, we found that rhCCN2, when added to normal urine, was recoverable as full-length molecules. The finding of a large, 200 kD, immunoreactive species in all urine samples from type 1 diabetic patients was unanticipated. This band was present as a weak signal in only one of the healthy volunteers. The identity of this large molecule is currently unknown, but most likely represents CTGF complexed to a second urinary protein. A small molecule equal in size to the quarter-length fragment of CCN2 was also present in three samples. Interestingly, this represented three of the four patients in the study. Ball et al [27] had reported a similar small fragment in the uterine cavity of pig and Steffen et al [26] reported a similar fragment in human and mouse fibroblast cultures. We have previously demonstrated the presence of smaller-molecular-weight forms of CCN2, equivalent to both the half- and quarter-length fragments in mesangial cell cultures stimulated

by TGF-β [17]. To date, the biologic function(s) of these fragments has not been established. It is also unknown whether these forms arise as the result of proteolysis of full-length CCN2, or represent components of one larger molecule to be assembled. Determining whether the urinary quarter-length fragment of CCN2 is characteristic of diabetic nephropathy is compelling and will require more detailed investigation.

We did not determine that urinary CCN2 was produced in the kidney. Its size and charge predict that any CCN2 present in the circulation would be freely filtered in the glomerulus. However, we have clearly shown that the *db/db* mouse greatly augments renal *CCN2* gene expression by $3^1/_2$ months following the onset of diabetes, thus implying local synthesis [17, 24]. This renal CCN2 expression appears to be concentrated in the glomerulus since isolated glomeruli demonstrate a 27-fold increase in transcript levels compared to only a doubling of cortical CCN2 transcripts. Conceivably, the mesangial cell could be responsible for a large proportion of the CCN2 observed in urine. Also, in our previous study, we reported that mesangial cells constitutively express little CCN2 mRNA or protein. However, following exposure to promoters of diabetic nephropathy, including TGF-β, high levels of glucose, or mechanical strain, there is a marked increase in CCN2 expression. In another model, at 4 weeks following partial nephrectomy in rats, CCN2 expression was reported in regions of proliferating interstitial stromal fibroblasts and myofibroblasts, whereas expression was limited to a few interstitial and glomerular epithelial cells in control organs [28]. Along these lines, cultured renal fibroblasts have been shown to up-regulate expression of CCN2 following stimulation by TGF-β or lysophosphatidic acid (LPA) [29]. Further, patients with various forms of renal disease, including crescentic glomerulonephritis, IgA nephropathy, focal and segmental glomerulosclerosis, and diabetic nephropathy, have been demonstrated to express increased CCN2 mRNA as compared to that found in control renal tissue [16].

The accumulation of ECM characterizes diabetic nephropathy and involves not only increased synthesis but also altered turnover. Therefore, the measurement of matrix molecules as well as proteinase/proteinase inhibitors in the serum [30] or urine [31] have recently been considered as possible markers of nephropathy. However, as with microalbuminuria, changes in these markers may not be significant until relatively late in the progression of disease [10–12]. Monitoring TGF-β in the urine or serum seems attractive since its up-regulation in the glomerulus is early and is causal for changes in ECM accumulation [32–36]. Work along these lines is being pursued and may prove fruitful [31, 37]. However, the highly ubiquitous nature of this growth factor and the existence of multiple circulating forms that contrast with active local forms present difficulties in the interpretation of results.

CONCLUSION

Our findings demonstrate unequivocally that CCN2 excretion is abnormal in the urine of diabetic humans and rats. Furthermore, the molecular forms of CCN2 excreted are altered in diabetes. These findings support our hypothesis that CCN2 is up-regulated early in diabetic patients that are destined for nephropathy, and then diminishes during the evolution of progressive renal disease. We contend that the measurement of urinary CCN2 may allow one to predict and stage patients destined for progressive diabetic glomerulosclerosis and ESRD. Ultimately, proof of concept will require expanded patient studies, including a longitudinal analysis beginning prior to the development of microalbuminuria. Such a study is underway in our laboratory.

ACKNOWLEDGMENTS

This work was supported by grants to B.L.R. from the Juvenile Diabetes Research Foundation (1-2001-380) and the American Diabetes Association (D11090). We thank Ms. Gisela Deuter for her administrative support and Ms. Melisa Riser for the preparation of figures.

Reprint requests to Bruce L. Riser, A.B., M.S., Ph.D., Baxter Healthcare, Renal Division, 1620 Waukegan Rd., MPGR-A2N, McGaw Park, IL 60085.
E-mail: bruce_riser@baxter.com

REFERENCES

1. Dorman JS, Laporte RE, Kuller LH, *et al*: The Pittsburgh insulin-dependent diabetes mellitus (IDDM) morbidity and mortality study. Mortality results. *Diabetes* 33:271–276, 1984
2. Andersen AR, Christiansen JS, Andersen JK, *et al*: Diabetic nephropathy in type 1 (insulin-dependent) diabetes: An epidemiological study. *Diabetologia* 25:496–501, 1983
3. Lewis EJ, Hunsicker LG, Bain RP, Rohde RD: The effect of angiotensin-converting-enzyme inhibition on diabetic nephropathy. The Collaborative Study Group. *N Engl J Med* 329:1456–1462, 1993
4. Lewis EJ, Hunsicker LG, Clarke WR, *et al*: Renoprotective effect of the angiotensin-receptor antagonist irbesartan in patients with nephropathy due to type 2 diabetes. *N Engl J Med* 345:851–860, 2001
5. Brenner BM, Cooper ME, de Zeeuw D, *et al*: Effects of losartan on renal and cardiovascular outcomes in patients with type 2 diabetes and nephropathy. *N Engl J Med* 345:861–869, 2001
6. Parving HH, Lehnert H, Brochner-Mortensen J, *et al*: The effect of irbesartan on the development of diabetic nephropathy in patients with type 2 diabetes. *N Engl J Med* 345:870–878, 2001
7. Parving HH, Hommel E: Prognosis in diabetic nephropathy. *Br Med J* 299:230–233, 1989
8. Stehouwer CD, Nauta JJ, Zeldenrust GC, *et al*: Urinary albumin excretion, cardiovascular disease, and endothelial dysfunction in non-insulin-dependent diabetes mellitus. *Lancet* 340:319–323, 1992
9. Bojestig M, Arnqvist HJ, Karlberg BE, Ludvigsson J: Glycemic control and prognosis in type I diabetic patients with microalbuminuria. *Diabetes Care* 19:313–317, 1996
10. Mogensen CE, Keane WF, Bennett PH, *et al*: Prevention of diabetic renal disease with special reference to microalbuminuria. *Lancet* 346:1080–1084, 1995
11. Ruggenenti P, Remuzzi G: Nephropathy of type-2 diabetes mellitus. *J Am Soc Nephrol* 9:2157–2169, 1998
12. Fioretto P, Steffes MW, Sutherland DE, Mauer M: Sequential renal biopsies in insulin-dependent diabetic patients: Structural factors associated with clinical progression. *Kidney Int* 48:1929–1935, 1995
13. Ayer-Lelievre C, Brigstock D, Lau L, *et al*: Report and abstracts

on the first international workshop on the CCN family of genes. *Mol Pathol* 54:105–120, 2001
14. Bradham DM, Igarashi A, Potter RL, Grotendorst GR: Connective tissue growth factor: A cysteine-rich mitogen secreted by human vascular endothelial cells is related to the SRC-induced immediate early gene product CEF-10. *J Cell Biol* 114:1285–1294, 1991
15. Bork P: The modular architecture of a new family of growth regulators related to connective tissue growth factor. *FEBS Lett* 327:125–130, 1993
16. Ito Y, Aten J, Bende RJ, *et al*: Expression of connective tissue growth factor in human renal fibrosis. *Kidney Int* 53:853–861, 1998
17. Riser BL, Denichilo M, Cortes P, *et al*: Regulation of connective tissue growth factor activity in cultured rat mesangial cells and its expression in experimental diabetic glomerulosclerosis. *J Am Soc Nephrol* 11:25–38, 2000
18. Riser BL, Cortes P, Zhao X, *et al*: Intraglomerular pressure and mesangial stretching stimulate extracellular matrix formation in the rat. *J Clin Invest* 90:1932–1943, 1992
19. Riser BL, Cortes P, Yee J: Modelling the effects of vascular stress in mesangial cells. *Curr Opin Nephrol Hypertens* 9:43–47, 2000
20. Hochberg Y, Benjamini Y: More powerful procedures for multiple significance testing. *Stat Med* 8:811–818, 1990
21. Welch B: On the comparison of several mean values: An alternative approach. *Biometrika* 38:330–336, 1951
22. Satterthwaite FE: An approximate distribution of estimates of variance components. *Biometrics Bull* 2:110–114, 1946
23. Murphy M, Godson C, Cannon S, *et al*: Suppression subtractive hybridization identifies high glucose levels as a stimulus for expression of connective tissue growth factor and other genes in human mesangial cells. *J Biol Chem* 274:5830–5834, 1999
24. Riser BL, Cortes P: Connective tissue growth factor and its regulation: A new element in diabetic glomerulosclerosis. *Ren Fail* 23: 459–470, 2001
25. Adler SG, Kang SW, Feld S, *et al*: Glomerular mRNAs in human type 1 diabetes: Biochemical evidence for microalbuminuria as a manifestation of diabetic nephropathy. *Kidney Int* 60:2330–2336, 2001
26. Steffen CL, Ball-Mirth DK, Harding PA, *et al*: Characterization of cell-associated and soluble forms of connective tissue growth factor (CTGF) produced by fibroblast cells in vitro. *Growth Factors* 15:199–213, 1998
27. Ball DK, Surveyor GA, Diehl JR, *et al*: Characterization of 16- to 20-kilodalton (kDa) connective tissue growth factors (CTGFs) and demonstration of proteolytic activity for 38-kDa CTGF in pig uterine luminal flushings. *Biol Reprod* 59:828–835, 1998
28. Frazier KS, Paredes A, Dube P, Styer E: Connective tissue growth factor expression in the rat remnant kidney model and association with tubular epithelial cells undergoing transdifferentiation. *Vet Pathol* 37:328–335, 2000
29. Heusinger-Ribeiro J, Eberlein M, Wahab NA, Goppelt-Struebe M: Expression of connective tissue growth factor in human renal fibroblasts: Regulatory roles of RhoA and cAMP. *J Am Soc Nephrol* 12:1853–1861, 2001
30. Ebihara I, Nakamura T, Shimada N, Koide H: Increased plasma metalloproteinase-9 concentrations precede development of microalbuminuria in non-insulin-dependent diabetes mellitus. *Am J Kidney Dis* 32:544–550, 1998
31. Ellis D, Forrest KY, Erbey J, Orchard TJ: Urinary measurement of transforming growth factor-beta and type IV collagen as new markers of renal injury: Application in diabetic nephropathy. *Clin Chem* 44:950–956, 1998
32. Bollineni JS, Reddi AS: Transforming growth factor-beta 1 enhances glomerular collagen synthesis in diabetic rats. *Diabetes* 42: 1673–1677, 1993
33. Border WA, Noble NA: TGF-beta in kidney fibrosis: A target for gene therapy. *Kidney Int* 51:1388–1396, 1997
34. Ziyadeh FN, Hoffman BB, Han DC, *et al*: Long-term prevention of renal insufficiency, excess matrix gene expression, and glomerular mesangial matrix expansion by treatment with monoclonal anti-transforming growth factor-beta antibody in db/db diabetic mice. *Proc Natl Acad Sci USA* 97:8015–8020, 2000
35. Chen S, Cohen MP, Ziyadeh FN: Amadori-glycated albumin in diabetic nephropathy: Pathophysiologic connections. *Kidney Int* 77:S40–44, 2000
36. Riser BL, Cortes P, Yee J, *et al*: Mechanical strain- and high glucose-induced alterations in mesangial cell collagen metabolism: Role of TGF-beta. *J Am Soc Nephrol* 9:827–836, 1998
37. Sharma K, Ziyadeh FN, Alzahabi B, *et al*: Increased renal production of transforming growth factor-beta1 in patients with type II diabetes. *Diabetes* 46:854–859, 1997