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Glomerular mRNAs in human type 1 diabetes: Biochemical evidence for microalbuminuria as a manifestation of diabetic nephropathy

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Glomerular mRNAs in human type 1 diabetes: Biochemical evidence for microalbuminuria as a manifestation of diabetic nephropathy.

Background. In patients with type 1 diabetes, some consider microalbuminuria to be a predictor of diabetic nephropathy while others believe it is an early feature of diabetic nephropathy.

Methods. Levels of mRNAs that are of pathogenetic relevance in diabetic nephropathy were compared in glomeruli isolated from microalbuminuric and overtly proteinuric subjects and in control normoalbuminuric diabetic subjects and living renal transplant donors.

Results. In subjects with microalbuminuria and overt proteinuria, glomerular mRNAs were virtually identical and approximately twofold higher for connective tissue growth factor (CTGF; $P < 0.01$) and collagen α2(IV) ($P < 0.03$) compared to living renal donors and normoalbuminuric patients. Glomerular glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels were not significantly different among the groups ($P = 0.4$). Weak but statistically significant correlations were noted between CTGF mRNA and albuminuria (assessed by rank), fractional mesangial surface area, and a composite renal biopsy index. Glomerular CTGF mRNA correlated inversely with creatinine clearance. Glomerular collagen α2(IV) mRNA levels correlated with albuminuria (by rank) and less strongly with fractional mesangial area.

Conclusion. To our knowledge, these data provide the first biochemical evidence demonstrating that the glomeruli of microalbuminuric patients and those with overt proteinuria do not differ significantly. The data support the concept that microalbuminuria is not "predictive" of diabetic nephropathy, but rather is an earlier point in the spectrum of diabetic nephropathy.

Ground breaking work in the late 1970s and early 1980s identified microalbuminuria as a marker of incipient diabetic nephropathy in patients with type 1 diabetes [1–3]. However, more recent data, particularly from renal biopsies, suggest that microalbuminuria is not truly a "predictor," but rather is the earliest clinically identifiable sign of diabetic nephropathy [4–6]. Biochemical similarities in the glomeruli of subjects with microalbuminuria and those with overt proteinuria support the postulate that microalbuminuria is an early point in the spectrum of diabetic nephropathy, rather than a "predictor" of it. We posited that (*1*) In diabetic nephropathy due to type 1 diabetes, relevant glomerular mRNAs would be increased compared to those measured in living renal donors (LRD) and normoalbuminuric diabetic subjects; and (2) If microalbuminuria represents an early phase in the spectrum of overt diabetic nephropathy, mRNA marker values would be similar in glomeruli from microalbuminuric subjects and in those with overt proteinuria.

To test these hypotheses, we performed a cross-sectional study measuring glomerular type IV collagen and connective tissue growth factor (CTGF) mRNAs in patients with type 1 diabetes who had either normoalbuminuria, microalbuminuria, or overt proteinuria. Living renal donors (LRD) served as controls.

METHODS

Patients

This protocol was approved by the Harbor-UCLA Research and Education Institute Investigational Review Board. Renal biopsies were performed for research and not clinical purposes. Thirty-three renal biopsies were utilized for mRNA measurements: living renal donors ($N = 10$); normoalbuminurics ($N = 12$); microalbuminurics ($N = 5$); and overt nephropathy ($N = 6$). In three subjects, material was not available to test all of the markers. Normoalbuminuria was defined as the presence of <20 μg/min albuminuria on at least two out

Key words: Type 1 diabetes, diabetic nephropathy, type IV collagen, connective tissue growth factor, mesangial expansion.

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of three timed overnight urine collections and microalbuminuria as 20 to 200 μg/min albuminuria. Overt proteinuria was defined as ≥500 mg proteinuria on at least one 24-hour urine collection or ≥300 mg albuminuria on an average of three timed overnight collections.

Clinical data

The following tests were measured in the hospital clinical laboratory: serum creatinine, creatinine clearance, 24-hour urine protein and creatinine, plasma glucose within three hours prior to biopsy, and hemoglobin A_{1c} levels (the latter two measures in the diabetics only). Mean arterial pressure was calculated using measurements taken during the outpatient visit prior to the renal donation or biopsy. Renal size was measured by ultrasound in the diabetic patients and by intravenous urography or angiography in transplant donors.

Glomerular microdissection and RT-PCR measurements

Microdissection and reverse transcription-polymerase chain reaction (RT-PCR) measurements were performed as previously described [7, 8] with minor modifications. Renal biopsy was performed in the standard manner and three to four renal cores obtained. Specimens were immediately aliquoted at the bedside for fixation in alcoholic Bouin's solution for light microscopy, snap frozen in isopentane for immunohistochemistry, or placed in glutaraldehyde for electron microscopy. One to 1.5 of the cores were placed in vanadyl ribonucleoside complex (VRC) for preservation of the RNA, and brought to a microdissecting microscope for collection within 10 to 20 minutes of the biopsy. For the living renal donors, the same protocol was followed, except that the biopsies were performed by the transplant surgeon after the kidney was excised from the donor and before it was placed in the host. Microdissection was usually completed within 30 minutes. Glomeruli were microdissected at 4°C in VRC, rinsed, aliquoted four per tube in RNAse inhibitor, solubilized in triton buffer, lysed by freezing and thawing, and reverse transcribed (RT) to cDNA utilizing a Boehringer Mannheim cDNA synthesis kit (Boehringer Mannheim, Mannheim, Germany). Standard polymerase chain reaction (PCR) was performed to check the adequacy of the RT procedure and satisfactory samples from all glomeruli from a given patient were pooled. cDNA from one fifth of a glomerulus per reaction tube was used to measure collagen and CTGF mRNA, and one tenth of a glomerulus for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Control samples were simultaneously run in which the RT enzyme was omitted. Quantitative competitive PCR was performed using the GeneAmp DNA amplification kit (Perkin Elmer Cetus Corp., Norwalk, CT, USA). In each tube containing test cDNA and amplification reagents, serial dilutions of mutated collagen α2(IV) or mimic CTGF template cDNA were added, and the reaction mixture amplified. For collagen α2(IV) mRNA, PCR proceeded as follows: (*1*) 94°C × 3 minutes; (*2*) 42 cycles of the following sequential steps: 94°C × one minute; 60°C × one minute, and 72°C × 3 minutes; and (*3*) 72°C × 7 minutes. For CTGF the protocol was modified as follows: (*1*) 95°C × 9 minutes; (*2*) 36 cycles of the following sequential steps: 95°C × 30 seconds; 67°C × 30 seconds; 95°C × 30 seconds; and (*3*) 72°C × 7 minutes. For GAPDH, PCR proceeded as per the manufacturer's protocol (Clontech, Palo Alto, CA, USA). The RT-PCR products were separated by electrophoresis, the band densities analyzed by laser densitometry (Helena Laboratories, Beaumont, TX, USA), the values log transformed, and a log-linear regression analysis was performed against the mutant or mimic concentration for each PCR tube. The quantity of cDNA in the test sample was defined as that amount at which the mutant or mimic and wild-type optical density bands were equal.

Primers

The primers for human collagen α2(IV) were TAT TCC TTC CTC ATG CAC ACG GCG (sense) and CCA ATT TTT GGG TTG GCA CC (antisense) [7, 8]. The primers and competitive mutant for CTGF were ATG TCT CCG TAC ATC TTC CTG TAG T (sense) and GAG TGG GTG TGT GAC GAG CCC AAG G (antisense) [9]. Primers and competitive mutant for human GAPDH (G3PDH) were purchased from Clontech.

Morphological and morphometric assessments

Renal biopsies were scored semiquantitatively for the degree of injury by a pathologist who was unaware of the clinical circumstances of the patient from whom the tissue was obtained. Biopsies were graded on a scale of 0 to 3 in increments of 0.5 for changes in the glomeruli, tubulointerstitium, and arterioles. The individual scores were added to give a final renal biopsy index. The degree of diabetic glomerulosclerosis (based on diffuse and/or nodular increases in mesangial matrix material, amount of segmental sclerosis, insudates, capillary microaneurysms, and capillary collapse or obliteration) was semiquantitated as none, mild, moderate, or severe (scored 0 to 3). Arterioles and arteries were assessed as having no lesions, mild, moderate, or severe involvement based on the amount of hyalinization, muscular wall thickening, and intimal fibrosis. The extent of tubular atrophy and interstitial fibrosis were similarly scored. The three biopsy scores were added yielding a renal biopsy index describing pathological changes in each biopsy.

Glomerular surface area was measured using a point counting method. All non-sclerotic intact glomeruli were photographed and a grid was superimposed on each picture. All points falling within the glomerular tufts were

Table 1. Physiological data in patients with type 1 diabetes

	LRD	NORMO-DM	MICRO-DM	OP-DM	*P*
Age *years*	38 ± 2	36 ± 2	35 ± 3	33 ± 4	0.6
Range	(28–47)	(27–48)	(25–42)	(23–53)	
Duration DM *years*	0	20 ± 1	16 ± 2	22 ± 3	0.16
Range		(15–27)	(9–23)	(14–29)	
HbA1c %	ND	8.0% ± 0.4	10.3% ± 0.9	9.1% ± 0.8	0.054
Serum creatinine *mg/dL*	0.8 ± 0.05	0.9 ± 0.06	0.9 ± 0.07	1.7 ± 0.3	<0.001
Creatinine clearance *mL/min*	121 ± 8	100 ± 5	102 ± 8	71 ± 17	<0.05
Mean arterial pressure *mm Hg*	91 ± 2	87 ± 2	89 ± 4	97 ± 5	0.7
Renal size *cm*	11.9 ± 0.7	11.3 ± 0.3	11.2 ± 0.3	11.6 ± 0.5	0.16

Data are expressed as mean ± SEM. Abbreviations are: LRD, living renal donors; DM, diabetes mellitus; NORMO, normoalbuminuria; MICRO, microalabuminuria; OP, overt proteinuria; HbA1c, hemoglobin A1c.

counted, including capillary walls and lumina, mesangial regions including overlying basement membranes, and visceral epithelial cells. Bowman's space, Bowman's capsule and parietal epithelial cells were not included. The mean number of points in the living donor biopsy glomeruli was taken as the mean control surface area equal to 1. All glomerular surface areas were then calculated and expressed as a percent relative to the average living donor biopsy glomeruli as:

$$\frac{\text{Total number of points in the glomerulus} \times 100}{\text{Mean number of glomerular points in living donor glomeruli}} \quad \text{(Eq. 1)}$$

The fractional mesangial area was measured with a similar point counting method as previously described [8].

Statistics

Results were analyzed using Access software, an Excel Spreadsheet (Microsoft, Seattle, WA, USA), and the StatMost software package (DataMost Corp, Salt Lake City, UT, USA). The significance of analysis of variance (ANOVA) testing was ascertained using the Student-Neuman-Keul test and Student *t* test. Fisher's exact test was used to assess non-parametric comparisons among groups. The strength of relationships between sets of data was tested utilizing either the Pearson correlation coefficient or Spearman rank-order correlation test. Results are expressed as the mean ± standard error of the mean (SEM). Significance is assigned at the level of $P \leq 0.05$.

RESULTS

Clinical data

Clinical parameters are summarized in Table 1. There was no significant difference in the age of the patients at biopsy, the duration of diabetes among the groups, the glycosylated hemoglobin level, or the plasma glucose measured within three hours before the biopsy in the diabetic subjects. Mean arterial pressure also was not significantly higher in the subjects with overt proteinuria compared to the other three groups, but the difference nevertheless may be clinically significant. Not surprisingly, serum creatinine was higher and creatinine clearance lower in subjects with overt proteinuria compared to the other three groups. Antihypertensive agents were not taken, except by one subject with overt nephropathy who was on a low dose of an angiotensin-converting enzyme (ACE) inhibitor.

Albuminuria was measured on timed overnight collections for the initial group assignment prior to biopsy in normoalbuminuric and microalbuminuric patients with diabetes, but was extrapolated to an expected 24-hour excretion rate to enhance comparability among groups. In living renal donors, the measurement was variably for albuminuria or proteinuria and the values ranged from a minimum of 4.4 mg albumin/day to a maximum of 158 mg protein/day. Mean urinary albumin excretion in the normoalbuminuric patients was 9.6 ± 2.2 mg albumin/day (range 3.8 to 26.6 mg albumin/day). Mean urinary albumin excretion in the microalbuminuric patients was 56.0 ± 17.5 mg albumin/day (range 24.8 to 109.5 mg albumin/day). Mean proteinuria in the patients with overt nephropathy was 4.27 ± 1.49 g/day (range 0.52 to 9.04 g/day).

Retinopathy was ascertained historically and confirmed by physical examination. Of the patients in the normoalbuminuric group, 2 of 10 had no retinopathy, 6 of 10 had background diabetic retinopathy (BDR), and 2 of 10 had proliferative retinopathy (PDR). In the microalbuminuric group, 1 of 5 had no retinopathy, 1 of 5 had BDR, and 3 had PDR. In the group with overt proteinuria, 2 of 6 had mild to moderate BDR and 4 of 6 had PDR.

Glomerular RT-PCR measurements

Glomerular collagen α2(IV) mRNA levels were low in living renal donors (1.97 ± 0.52 attm/glomerulus), similar in normoalbuminurics (1.5 ± 0.28 attm/glomerulus), and higher in microalbuminurics (3.98 ± 1.49 attm/glomerulus) and overtly proteinuric subjects (4.56 ± 1.56 attm/glomerulus; $P < 0.03$). The patterns for glomer-

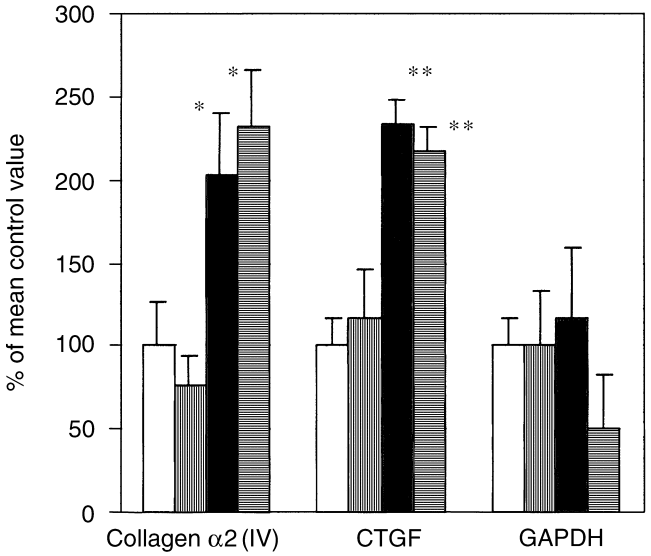

Fig. 1. Glomerular collagen α2(IV) and connective tissue growth factor (CTGF) mRNA levels are nearly identical in microalbuminuric (MA; □) and overtly proteinuric subjects (▨) and higher than in living renal donors (LRD; ■) and normoalbuminuric subjects (NA; ▤). Glomerular GAPDH mRNA levels were not significantly different. *$P < 0.03$ and **$P < 0.01$ vs. LRD and NA.

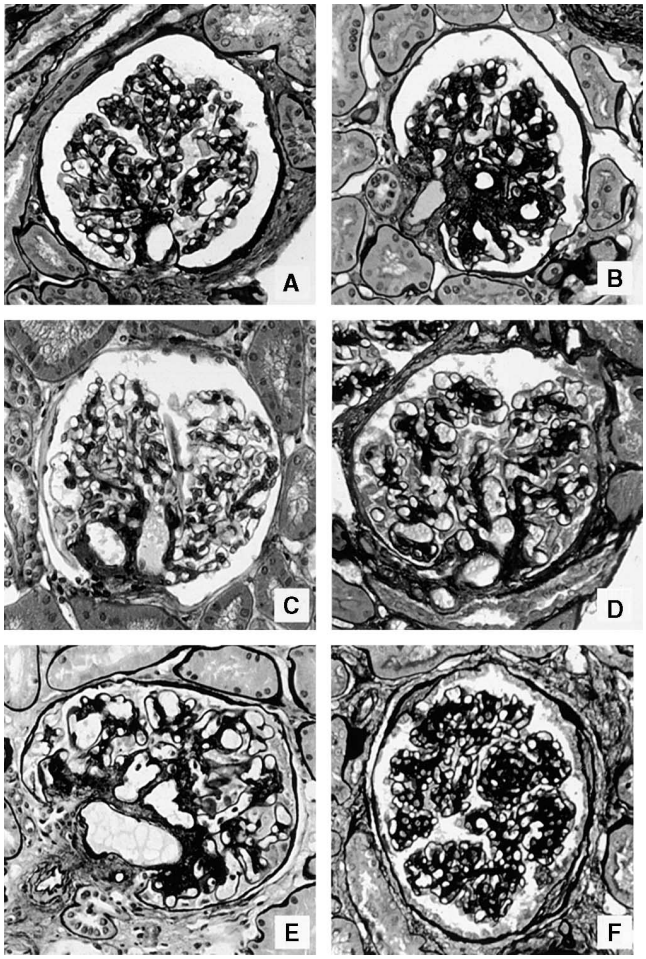

Fig. 2. Diabetic patients in each group showing the range from least (*A, C, E*) to most (*B, D, F*) diabetic involvement, including mesangial matrix expansion and capillary wall thickening, in glomeruli without segmental sclerosis. (A, B) Normoalbuminuric. (C, D) Microalbuminuric. (E, F) Overt proteinuria. (A, B, D–F, periodic acid-methenamine silver; C, Masson's trichrome. All ×150)

ular CTGF mRNA levels were similar: lowest in living renal donors (0.06 ± 0.01 attm/glomerulus), similar in normoalbuminurics (0.07 ± 0.02 attm/glomerulus), and higher in microalbuminurics (0.14 ± 0.02 attm/glomerulus) and overtly proteinuric subjects (0.13 ± 0.02 attm/glomerulus; $P < 0.01$). Glomerular GAPDH mRNA levels were not significantly different among the groups. Figure 1 shows these results expressed as a percent of the mean control (living renal donor) value. The correlation coefficient between the collagen α2(IV) and the CTGF mRNA levels was 0.38 ($P = 0.03$)

Morphological and morphometric assessments

The degree of mesangial expansion varied considerably within the given diagnostic categories of normoalbuminuria, microalbuminuria, and overt proteinuria and spanned from little or none to marked proteinuria, resulting in a substantial overlap among the groups (Fig. 2 A–E). Despite the overlap, the morphometric measurement of fractional mesangial area was lowest in the living renal donors (19.0 ± 1.8%), and progressively higher in normoalbuminurics (27.3 ± 8.5%), microalbuminurics (31.9 ± 6.5%), and patients with overt proteinuria (44.2 ± 1.8%; $P < 0.001$; Fig. 3). Glomerular surface area was expressed as a % of the mean control value observed in living renal donors. The values were similar in living renal donors and normoalbuminuric diabetics (100 ± 5.8% and 98.3 ± 6.6%, respectively), and were higher in microalbuminuric subjects and those with overt proteinuria (121.0 ± 10.3% and 125.5 ± 7.2%, respectively; $P < 0.05$; Fig. 4).

Correlational relationships between functional or biopsy data and the mRNA values

Functional correlations. Proteinuria was measured as either urinary total protein or as albuminuria. Therefore, for analytical purposes, subjects were ranked according to amount of proteinuria or albuminuria. Four values were omitted because they could not be ranked due to incompatibility of the urinary measurements. Glomerular collagen α2(IV) mRNA was weakly but statistically significantly correlated with the ranking of diabetic patients according to amount of albuminuria/proteinuria ($r = 0.38$, $P < 0.05$) and CTGF mRNA correlated more strongly ($r = 0.68$, $P < 0.0001$; Fig. 5). Glomerular CTGF mRNA also correlated negatively and inversely with cre-

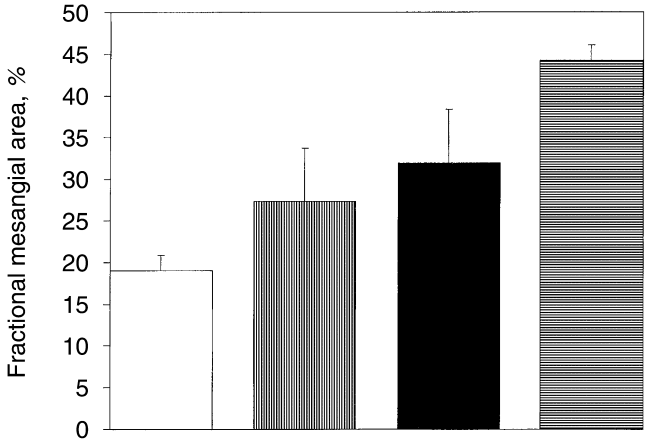

Fig. 3. Fractional mesangial area demonstrates considerable overlap among the groups as classified by albuminuria or proteinuria. Abbreviations are: LRD, living renal donors (□); NA, normoalbuminuric subjects with type 1 diabetes (▥); MA, microalbuminuric subjects with type 1 diabetes (■); Overt, subjects with type 1 diabetes and overt proteinuria (▤). The overall difference in mean values among groups was $P < 0.001$. The difference between LRD and NA, MA, and subjects with overt proteinuria was $P = 0.013$, $P < 0.00001$, and $P < 10^{-7}$, respectively. The difference between NA and MA was not significant ($P = 0.18$), but the difference between NA and subjects with overt proteinuria was significantly different ($P < 10^{-6}$). The difference between MA and subjects with overt proteinuria was $P < 0.001$.

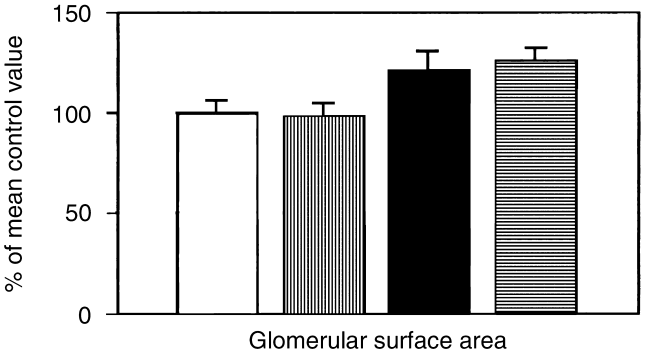

Fig. 4. Glomerular surface area expressed as a % of the mean value measured in living renal donors. Abbreviations are: LRD, living renal donors (□); NA, normoalbuminuric subjects with type 1 diabetes (▥); MA, microalbuminuric subjects with type 1 diabetes (■); Overt, subjects with type 1 diabetes and overt proteinuria (▤). The overall difference in mean values among groups was $P < 0.05$. There were no significant differences between LRD and NA or between MA and subjects with overt proteinuria. Significant differences were observed between LRD and MA ($P < 0.02$), LRD and subjects with overt proteinuria ($P < 0.002$), NA and MA ($P < 0.02$), and NA and subjects with overt proteinuria ($P < 0.002$).

atinine clearance (r = -0.34, $P = 0.057$), but collagen α2(IV) mRNA did not (data not shown).

Pathology correlates. Glomerular CTGF mRNA, but not collagen α2(IV) mRNA, correlated with the renal biopsy index ($r = 0.45$, $P < 0.05$), comprised of a composite score representing changes in mesangial matrix, arteriolar hyalinosis, and tubulointerstitial fibrosis and atrophy (data not shown). Neither CTGF nor collagen α2(IV) mRNA correlated with the individual scores for mesangial matrix expansion, tubulointerstitial fibrosis, arteriolar hyalinosis, or the % of completely sclerotic glomeruli. Glomerular CTGF mRNA also correlated weakly with fractional mesangial area measured by point counting ($r = 0.4$, $P = 0.04$; data not shown). Glomerular collagen α2(IV) mRNA did not correlate with measured fractional mesangial area ($r = 0.23$, $P = 0.23$) when all samples were considered, but reached significant values when only the diabetic subjects were considered for analysis ($r = 0.44$, $P = 0.05$; data not shown).

DISCUSSION

Historically, microalbuminuria was proposed as a predictor of the subsequent development of nephropathy in patients with type 1 diabetes [1–3]. It is now variably viewed as a predictor of diabetic nephropathy or as the earliest clinically measurable feature of it [4–6]. Our data show that glomerular CTGF and collagen α2(IV) mRNA levels in microalbuminuric and overtly proteinuric subjects are similar in magnitude and are higher than the measured values in normoalbuminuric type 1 diabetic subjects and living renal donors. Thus, these data provide the first biochemical evidence that in type 1 diabetes, microalbuminuria, rather than being a predictor of the subsequent development of overt diabetic nephropathy, is pathogenetically similar to overt nephropathy, and therefore is more appropriately viewed as an earlier point in the spectrum of actual diabetic nephropathy. These studies also showed positive correlations between glomerular CTGF mRNA levels and albuminuria, fractional mesangial area, and a renal biopsy index including composite scores for changes in arterioles, glomeruli, and the tubulointerstitium. CTGF mRNA values correlated negatively with creatinine clearance. Finally, the data show similar correlations between collagen α2(IV) mRNA and albuminuria, and a weaker association with fractional mesangial area.

Type IV collagen and CTGF mRNA: Markers for defining pathogenetic identity and comparisons to other studies

We chose to study type IV collagen and CTGF mRNA as markers because there is good evidence that these molecules play a significant role in the pathogenesis of diabetic nephropathy. Type IV collagen accumulation and mRNA increments have been documented not only in the glomeruli of rats and mice with experimental diabetes [10–12], but also in the glomeruli of diabetic patients [8, 13–19], although in the latter there may be a total increment in mesangial type IV collagen protein at the same time that the density of type IV collagen declines

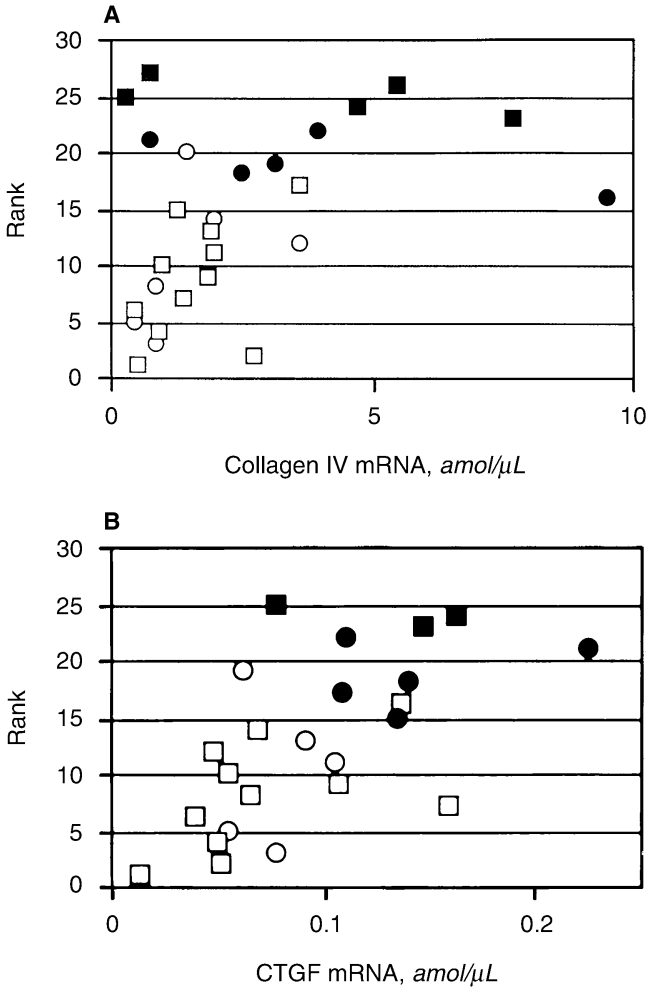

Fig. 5. (*A*) There was a significant correlation (r = 0.4) between a ranking of urinary albumin or protein in individual subjects and their glomerular collagen α2(IV) mRNA levels (P = 0.036). (*B*) There was a significant correlation (r = 0.55) between a ranking of urinary albumin or protein in individual subjects and their glomerular CTGF mRNA levels (P = 0.0036).

[8, 13]. In three previously published studies, glomerular type IV collagen mRNA was increased in diabetic subjects compared to controls [7, 8, 20]. In one, diabetes was not restricted to type 1 diabetes, nephropathy was not restricted to diabetes alone, and all of the subjects studied had more than 500 mg/day of proteinuria [8]. In the second, the glomerular mRNA measurements did not include comparison to a housekeeping gene, but instead reported a ratio of two different collagen IV chains [7]. The latter also lacked normal kidney controls [7]. In a third, type IV collagen mRNA was increased in all specimens studied with glomerulosclerosis (including diabetic nephropathy), irrespective of the underlying histopathology, and control tissue was not studied [20]. Thus, this is the only report examining glomerular type IV collagen mRNA levels in diabetic subjects to focus this type of evaluation on type 1 diabetes exclusively. It also is the only one to examine subjects with normoalbuminuria and microalbuminuria as well as subjects with overt proteinuria and to compare these results to the findings in normal glomeruli. Thus, we were able to analyze the findings in relation to the clinical stage of diabetic nephropathy.

Connective tissue growth factor is a cysteine-rich peptide that is secreted by fibroblasts, endothelial cells, and mesangial cells after stimulation with transforming growth factor-β (TGF-β). It mediates at least some of the downstream effects of TGF-β, including cell proliferation and collagen synthesis [9, 21]. CTGF is implicated in the pathogenesis of diabetic nephropathy. Riser et al showed increased CTGF mRNA and protein in mesangial cells exposed to high medium glucose concentration or to the exogenous provision of TGF-β, both features characteristic of the diabetic state [9]. Elevated CTGF mRNA levels also were demonstrated in the glomeruli of diabetic mice with early nephropathy characterized by mild mesangial expansion [9]. In a recent preliminary report, Umezono et al also demonstrated increased CTGF assessed immunohistochemically in diabetic subjects with mild or moderate nephropathy, albeit with diminished glomerular CTGF with more advanced nephropathy (abstract; Umezono et al, *J Am Soc Nephrol* 11:123A, 2000). Thus, the relevance of the findings demonstrating identity in glomerular CTGF mRNA values in microalbuminuric and overtly proteinuric type 1 diabetic subjects is underscored by the importance of CTGF as a pathogenetic marker for diabetic nephropathy.

Lack of effect of glomerular hypertrophy on type IV collagen and CTGF mRNA levels

It is theoretically possible that the increments in type IV collagen and CTGF mRNA might have reflected only glomerular hypertrophy, thus confounding data interpretation. We believe that this is not the case for the following reasons. Firstly, there was a similar twofold increment in both marker mRNAs studied in subjects with microalbuminuria and overt nephropathy, despite no significant increment in the control marker GAPDH mRNA, and actually even a numerical decline (albeit not statistically significant) in GAPDH mRNA in the subjects with overt nephropathy and the largest glomeruli. Thus, increments in mRNA in large glomeruli are not a generalized phenomenon, but rather reflect specific changes. Secondly, in this study, as we already had reported in subjects with both type 1 and type 2 diabetes assessing type IV collagen mRNA alone [8], there were poor correlations between the mRNA measurements and glomerular size. Taken together, the data dissociate these glomerular mRNA changes from glomerular size, and emphasize the specificity of the increments observed in type IV collagen and CTGF mRNA levels.

Correlations between mRNA levels and functional and histopathological changes

Somewhat weak but statistically significant correlations were noted between glomerular CTGF mRNA levels and albuminuria (expressed by rank), fractional mesangial area, and an index of renal injury comprised of composite scores for changes in arterioles, glomeruli, and the tubulointerstitium. There was also a negative correlation between CTGF mRNA values and creatinine clearance. Type IV collagen mRNA levels correlated with albuminuria (expressed by rank) and weakly with fractional mesangial area. A weak correlation between collagen IV mRNA levels and fractional mesangial volume in separate diabetic subjects was reported by us previously [8]. Despite the relatively small number of patients studied in each of the groups, these correlations nevertheless confirm the physiological relevance of the markers chosen. However, they by no means prove that either CTGF and/or collagen $\alpha 2$(IV) mRNA are necessarily the only or even the best markers of disease progression in microalbuminuric and overtly proteinuric glomeruli. Instead, the data suggest the not too surprising conclusion that the development of diabetic nephropathy is a complex process, and the relative weakness of these statistically significant correlations suggest that many other markers are likely to play important roles as well.

Our data do not rule out the possibility that other factors not assessed in these analyses contribute to the differences in the clinical phenotypes of our subjects as defined by degrees of albuminuria.

However, these data provide the first biochemical evidence supporting the view that microalbuminuria in type 1 diabetes represents an early point in the spectrum of actual diabetic nephropathy.

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