Increased Collagen IV Excretion in Diabetes

A marker of compromised filtration function

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OBJECTIVE — Increased albumin excretion in diabetes is believed to be derived from hemodynamic and/or permeability abnormalities, whereas mesangial matrix expansion gives rise to the reduction in glomerular filtration surface and decline in renal function in diabetic nephropathy. We postulated that the overproduction of extracellular matrix proteins underlying glomerulosclerosis in diabetes might be associated with the excretion of increased amounts of type IV collagen in the urine.

RESEARCH DESIGN AND METHODS — To explore this hypothesis, we measured the urinary excretion of (human) collagen IV by immunoassay in 65 patients with type 1 or type 2 diabetes and various degrees of albuminuria and examined its relationship to filtration function assessed by the reciprocal of the serum creatinine (RSC).

RESULTS — Collagen IV excretion showed a significant ($P < 0.001$) inverse correlation ($r = -0.62$) with the RSC, and this correlation pertained regardless of whether albumin excretion was in the low ($\leq 100 \mu g/mg$ creatinine; $r = -0.73$) or high ($>100 \mu g/mg$; $r = -0.53$) range. In contrast, albumin excretion showed insignificant correlation with either collagen IV excretion ($r = 0.12$) or with the RSC ($r = -0.20$). Urinary collagen IV was significantly higher ($P < 0.05$) in patients with an RSC value $\leq 100 (28.3 \pm 2.4$ ng/mg creatinine) than in patients with an RSC value $>100 (16.0 \pm 0.8$ ng/mg creatinine).

CONCLUSIONS — Because not all patients with microalbuminuria progress to declining renal function and some patients who develop nephropathy do not manifest albuminuria, the findings in this cross-sectional analysis suggest that measurement of urine collagen IV may be a useful noninvasive indicator to detect diabetic renal disease entering a phase of compromised renal function.

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Abbreviations: A:C, albumin:creatinine; EIA, enzyme immunosorbent assay; RSC, reciprocal of the serum creatinine; TGF, transforming growth factor.
RESEARCH DESIGN AND METHODS — The study group consisted of 65 subjects (aged 55.3 ± 1.4 years) with type 1 or type 2 diabetes (American Diabetes Association criteria) who had a wide range of duration of diabetes (5–35 years) and who gave informed consent to participate in the study, which was approved by the Institutional Review Board. The mean age was lower (41.4 ± 1.9 years) and the mean duration of diabetes was longer in type 1 diabetic patients than in type 2 diabetic patients. All patients were currently being treated and were receiving insulin, oral hypoglycemic agents, or both. A total of 30% of the patients with type 2 diabetes and two patients with type 1 diabetes had been taking an ACE inhibitor or angiotensin receptor antagonist and had been treated with such for at least 6 months. Measurement of HbA1c levels (high-performance liquid chromatography analysis) and fasting glucose and serum creatinine concentrations was performed in a commercial laboratory on two blood samples, obtained at a 1-month interval, and the values were averaged in each patient. Urine samples for measurement of albumin, creatinine, and type IV collagen also were obtained twice at a 1-month interval in each patient, and the values were averaged. Reciprocal of the serum creatinine was calculated from the formula

\[ \frac{1}{\text{SCr}} \times 100, \]

where the serum creatinine value is taken as milligrams per deciliter. Because the purpose of the study was to assess the relationship between urinary collagen IV and early decline in renal function, serum creatinine between 0.5 and 1.7 mg/dl (RSC 200–259) was required for inclusion.

Determination and definition of microalbuminuria
Urinary albumin was measured in random urine samples by a competitive enzyme-linked immunosorbent assay in which albumin in standard or sample competes in a soluble phase with human albumin immobilized onto plastic microtiter wells for binding to horseradish peroxidase–conjugated antibody to human albumin (24). The assay is sensitive to 0.1 μg and shows linearity with the log of concentration between 10 and 1,000 μg/ml. Intra-assay and interassay coefficients of variation are 3 and 4–6%, respectively. Creatinine was measured in the same sample by a colorimetric method (Sigma, St. Louis, MO) to calculate the urine albumin:creatinine (A:C) ratio. This ratio is regarded as one of the best accepted means for assessing urinary albumin excretion and is recommended by the American Diabetes Association (3) and by Warram and colleagues (25–28), who have used it to establish sex-specific ranges delineating normoalbuminuria, microalbuminuria, and overt proteinuria and levels of microalbuminuria associated with declining glomerular filtration function (25). Warram’s definition of microalbuminuria provides a lower limit of 17 μg/mg for men and a lower limit of 25 μg/mg for women (25,28).

Collagen IV immunoassay
The immunoassay for quantitation of type IV collagen used type IV collagen purified from human placenta (Collaborative Biomedical Products, Bedford, MA) and rabbit anti-human type IV collagen antibody (Biodesign, Kennebunk, ME), according to previously described methodology (29). The antigen was coated onto plastic microtiter wells (125 ng/well) in carbonate-bicarbonate coupling buffer (pH 9.6), and the wells were blocked with 0.1% bovine serum albumin in glycine coupling buffer, pH 8.5, containing 0.05% Proclin (Supelco, Bellefonte, PA). To initiate the assay, plates were washed with enzyme immunosorbent assay (EIA) buffer (0.15 mol/l NaCl, 10 mmol/l triethanolamine, pH 6.8, containing 0.01% Tween-20) and blotted by inversion. A total of 200 μl of a solution containing standard or sample and anti-human collagen IV antibody in 10% fetal calf serum, 100 mmol/l Tris HCl, pH 7.5, was added to the wells and allowed to react for 1 h at room temperature. This solution was prepared by adding equal volumes of standard or sample, diluted in EIA buffer, and the primary antibody (1:2,000 dilution in the same buffer) to microfuge tubes and preincubating overnight at room temperature. The preincubation step enhances binding of antibody to antigen in the soluble phase, reducing binding to coated antigen when competing antigen is present in low concentrations and allowing measurement in unconcentrated specimens. Horseradish peroxidase–conjugated goat anti-rabbit IgG (1:2,000 dilution in 10% bovine serum albumin, 100 mmol/l Tris HCl) was then added to the wells, and the incubation was continued for another hour. The plates were washed and developed with TMBBlue substrate, stopped, and read as described above. The assay was sensitive to 5 ng/ml and showed a linear inverse relationship with the log of concentration between 5 and −2,000 ng/ml. Intra-assay and interassay coefficients of variation were ≤9%, and the average intridual variation between the two specimens was 13%. The anti-collagen IV antibody showed no reactivity with other human urinary proteins (the principal one being albumin). Glucose up to 50 mmol/l did not interfere in the assay. None of the subjects had ketonuria or evidence of urinary tract infection.

Statistical analysis
Statistical analysis was performed using unpaired t tests to compare means. Correlations between analyses were determined by linear regression analysis.

RESULTS — The clinical characteristics of the study population are summarized in Table 1. As a group, the patients had suboptimal control of diabetes with a mean fasting glucose concentration of 11.8 ± 0.8 mmol/l (range 5.4–21.9) and a mean HbA1c of 8.9% (range 5.4–12.7). The mean HbA1c was significantly higher in patients with type 1 diabetes than in those with type 2 diabetes (9.45 ± 0.50 and 8.60 ± 0.25%, respectively). Serum
creatinine concentrations were \( \leq 1.7 \) mg/dl \((\leq 150 \mu\text{mol}/\text{L})\) in all patients, and the mean \( \pm \) SEM value for the RSC was 113 \( \pm 3 \). The mean urine albumin excretion was 216 \( \mu\text{g}/\text{mg creatinine} \), a value falling clearly within the microalbuminuric range. Four patients had values \(<20 \mu\text{g}/\text{mg}\), and 10 patients had values in the overt proteinuric range \((>350 \mu\text{g}/\text{mg})\). The mean A:C ratio was 181.0 \( \pm 46.5 \) in patients with type 1 diabetes and 228.9 \( \pm 67.0 \) in patients with type 2 diabetes, and the median A:C ratios were 79.2 and 93.6, respectively.

Urine excretion of collagen IV ranged from 6.8 to 52.8 \( \mu\text{g}/\text{mg creatinine} \) (mean 20.9 \( \mu\text{g}/\text{mg} \)). The mean value for urine collagen IV in 12 volunteers with albumin excretion \(<10 \mu\text{g}/\text{mg creatinine} \) was 6.6 \( \mu\text{g}/\text{mg creatinine} \) (range 4.3–12.8; intra-individual variability \(<9\%\) ). Collagen IV excretion showed no significant correlation with albumin excretion \((r = 0.12)\) with analysis of 64 of 65 patients, excluding an outlier with A:C ratio of 3,080 but had a significant inverse correlation \((r = -0.62)\) with RSC values (Fig. 1). In the 65 patients studied, urine collagen IV did not correlate with HbA\(_{1c}\) levels \((r = -0.03)\).

Considering the data of Warram et al. (25), which suggest that a decline in RSC begins to be detectable when A:C ratios exceed 100 \( \mu\text{g}/\text{mg} \), the patients in this study were divided into two groups according to albumin excretion. The mean \( \pm \) SEM RSC in subjects with an A:C ratio \( \leq 100 \mu\text{g}/\text{mg} \) was 115 \( \pm 4 \) and 109 \( \pm 5 \) in patients with an A:C ratio \( > 100 \) \((P < 0.05)\). Urine collagen IV showed a significant inverse correlation with RSC values in patients with either low \((r = -0.73)\) or high \((r = -0.53)\) albumin excretion (Fig. 2). In contrast, there was no significant correlation between A:C ratios and RSC values, whether analyzed as all patients (Fig. 3) or separated into patients with low versus high albumin excretion.

Using the approach of Warram et al. (28), who segmented microalbuminuric patients into four groups according to HbA\(_{1c}\) levels in assessing risk for progressive disease, we evaluated albumin excretion and RSC values in our study population similarly divided into groups according to HbA\(_{1c}\). The mean A:C ratio was lower in patients with HbA\(_{1c}\) between 5.1 and 5.9\% \((128 \pm 27; n = 21)\) and between 9.0 and 9.9\% \((98 \pm 21; n = 10)\) than in patients in the other two categories of HbA\(_{1c}\) values \((330 \pm 175, n = 17;\) HbA\(_{1c}\) 8.0–8.9\%; and 278 \( \pm 65, n = 17;\) HbA\(_{1c}\) 10–12.7\%). However, the mean RSC value in patients with HbA\(_{1c}\) >10\% was significantly higher at 129 \( \pm 10 \) \((P < 0.05)\) than in patients with HbA\(_{1c}\) levels in the other three categories \((108 \pm 4, 104 \pm 3, \) and 109 \( \pm 8\)\), suggesting that hyperfiltration in conjunction with poor...
glucose control contributed to increased albumin excretion in this group. Nine of the patients in this HbA_1c_ category had type 1 diabetes.

To further explore the relationship between RSC as a measure of declining filtration function and urinary collagen IV as a marker of glomerular matrix expansion compromising the filtration surface area, subjects were divided into two groups: RSC ≤100 (corresponding to a serum creatinine = 1.0 mg/dl or ≥88 μmol/l) and RSC >100 (corresponding to a serum creatinine <1.0 mg/dl or <88 μmol/l). This value was arbitrarily selected from results in published studies, indicating that decline in filtration function with time can begin to be detected at this level (25,30,31). HbA_1c_ values were 8.25 ± 0.36 and 8.36 ± 0.42, respectively, in these two groups. Three patients with type 1 diabetes and 23 patients with type 2 diabetes had RSC values ≤100. Urinary collagen IV excretion was significantly higher (28.3 ± 2.4 ng/mg creatinine) in patients with RSC ≤100 than in patients with RSC >100 (16.0 ± 0.8 ng/mg creatinine). The mean ± SEM urine albumin excretion in patients with RSC >100 was 145.2 ± 26.9 (n = 39) and was 321.3 ± 116.7 (n = 26) in patients with RSC ≤100.

**CONCLUSIONS** — The principal findings in this study are that the excretion of type IV collagen is increased in diabetic patients with albuminuria and that urinary collagen IV levels in these patients correlate inversely with the RSC. These results suggest that an increase in collagen IV excretion accompanies the overproduction of this extracellular matrix protein in the course of the development of diabetic nephropathy. As expansion of the glomerular extracellular matrix encroaches on the glomerular filtration surface area, filtration function begins to decline, as evidenced by decreasing RSC. The increased collagen IV excretion may reflect the clinical onset of this process. Long-term studies are needed to confirm whether overt nephropathy is more likely to develop in albuminuric patients with elevated versus normal urinary collagen IV excretion.

Other investigators have reported that the excretion of the fibrogenic cytokine transforming growth factor (TGF)-β1, which has been pathogenetically implicated in diabetic nephropathy, and of collagen IV as markers of renal fibrogenesis is elevated in microalbuminuric diabetic patients (32). Increased renal production of TGF-β1 in human diabetics, evidenced by overexpression of the cytokine and by TGF-β1 concentrations in renal vein effluent, also has been described (33,34). The relatively modest increase in TGF-β1 excretion that was observed in patients with microalbuminuria or with overt proteinuria did not correlate with the much larger (sixfold) increase in collagen IV excretion in these patients (32), suggesting that factors other than TGF-β1 are important in the enhanced matrix production and/or that altered renal handling of matrix proteins may be responsible for the elevated collagen excretion. However, collagen IV excretion has been shown to be elevated in the diabetic db/db mouse, a rodent model of genetic diabetes that develops glomerular pathology similar to that which occurs in human diabetes (35). Notably, the increase in collagen IV excretion in db/db mice postdated the development of albuminuria, which becomes manifest soon after the onset of hyperglycemia, and coincided with histopathological evidence of glomerular matrix accumulation and the onset of a decreasing creatinine clearance. These findings are consistent with the interpretation that collagen IV excretion is a marker of glomerular basement membrane synthesis and matrix remodeling.

Although the data of Warram et al. (25) suggest that decline in RSC correlates with increasing albuminuria at an A:C ratio >100, we did not find a significant negative correlation between A:C ratios and RSC values in patients with either higher (A:C >100) or lower (A:C ≤100) levels of albumin excretion. However, urinary collagen IV showed a significant inverse relationship with the RSC in patients in whom the A:C ratio was either ≤100 or >100. The significant correlation of collagen IV excretion with RSC values in patients with A:C ratios ≤100 μg/mg is of special interest because it suggests that, in patients with lower levels of microalbuminuria and especially in type 2 diabetic patients (who represented ~70% of our study population), urinary collagen IV excretion may be a better indicator than the A:C ratio of impending decline in filtration function. The findings in this cross-sectional study support the hypothesis that elevated collagen IV excretion is an indicator of diabetic renal disease entering a phase of compromised filtration function. Longitudinal studies in which filtration function is carefully monitored in patients with type 1 and type 2 diabetes are needed for confirmation.
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References