Kidney International, Vol. 57 (2000), pp. 2084–2092

Glomerular type IV collagen in patients with diabetic nephropathy with and without additional glomerular disease

Sharon G. Adler, Stella Feld, Liliane Striker, Gary Striker, Janine LaPage, Ciro Esposito, Jamil Aboulhosn, Lilly Barba, Dae Ryong Cha, and Cynthia C. Nast

Harbor-UCLA Research and Education Institute, Torrance, California; National Institutes of Health, Bethesda, Maryland; and Cedars-Sinai Medical Center, Los Angeles, USA

Glomerular type IV collagen in patients with diabetic nephropathy with and without additional glomerular disease.

Background. Type IV collagen is a constituent of mesangial matrix and is increased in amount in many forms of glomerular injury.

Methods. We performed renal biopsies in patients who (*1*) were donating a kidney to a relative (LRD, $N = 6$), (*2*) had diabetic glomerulopathy with or without nephrosclerosis (DM, $N = 6$), or (*3*) had diabetic glomerulopathy with a superimposed glomerular lesion (DM+, $N = 5$). Glomerular collagen α2(IV) and control glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNAs were measured, and the former correlated with clinical and morphological data to assess its usefulness in reflecting glomerular injury.

Results. Collagen α2(IV) mRNA levels were lowest in LRD (2.9 ± 0.6 attomol/glomerulus), higher in DM (5.9 ± 1.6, $P = 0.05$), and highest in DM+ (12.7 ± 2.8 attm/glomerulus, $P < 0.05$ vs. LRD and vs. DM). Control GAPDH mRNA levels were not significantly different ($P > 0.05$). Levels of proteinuria, serum creatinine, and glomerular size did not correlate with collagen α2(IV) mRNA levels. The fractional mesangial area and the fractional mesangial area occupied by type IV collagen were higher in both diabetic groups than in LRD ($P < 10^{-6}$), but the intensity of type IV collagen staining in the diabetic patients was significantly less than that seen in the LRD ($P < 0.01$). In DM+ patients, extramesangial type IV collagen was present. Fractional mesangial area and glomerular collagen α2(IV) mRNA levels correlated ($r = 0.45$, $P < 0.05$).

Conclusion. These data are consistent with a view of diabetic nephropathy as a lesion of increased α2 type IV collagen transcription, increased total amount of collagen present, but decreased mesangial density relative to other matrix molecules. These data further demonstrate that glomerular injury superimposed on diabetic nephropathy contributes to additional structural damage by inducing increased synthesis of type IV collagen at extramesangial sites.

Diabetic nephropathy with or without arteriolar nephrosclerosis is the likely diagnosis in diabetic patients with: (*1*) a disease duration of more than seven years who have greater than 500 mg of proteinuria in a 24-hour urine specimen; (*2*) whose urinary sediment is acellular; and (*3*) who have diabetic retinopathy. Renal biopsies are rarely performed under such clinical conditions.

Atypical clinical or laboratory features suggest the need for a renal biopsy, and as many as one third demonstrate findings in addition to or instead of diabetic nephropathy [1–4]. However, there is controversy as to whether nondiabetic glomerular lesions occur at greater than chance frequency in diabetic patients [5, 6]. Nevertheless, anecdotal reports of diabetic patients with superimposed or alternative lesions are frequently reported, albeit more commonly in type 2 than type 1 diabetes [2, 7–22]. Often, no clinical action is taken based on these biopsies.

Collagen α2(IV) mRNA and protein were chosen as markers of glomerular injury for this study because increased type IV collagen has been linked to the development of glomerulosclerosis in experimental [18–27] and human [17, 28–46] diabetic and nondiabetic renal disease. The study thus had three goals. The first was to determine whether glomerular collagen α2(IV) mRNA levels and immunohistochemically identified glomerular type IV collagen are increased in patients with diabetic nephropathy compared with healthy individuals. Previous studies performed to examine this question in humans used either no control tissue [39] or the tissue of patients undergoing nephrectomy for renal carcinomas [28]. Second, we wished to determine whether type IV collagen and its mRNA are differentially expressed in patients with diabetic nephropathy alone compared with patients who have both diabetic nephropathy and superimposed renal lesions. A third goal was to demonstrate that the reverse transcription-polymerase chain reaction (RT-PCR) data obtained from glomeruli microdissected from percutaneous renal biopsies would be sufficient in small numbers of patients per experimental group to yield statistically significant results, thereby validating the usefulness of the technique for clinical purposes, and also potentially, as a surrogate outcome in clinical trials.

Key words: diabetic nephropathy, type IV collagen, glomerulonephritis, collagen α2(IV) mRNA.

Received for publication October 20, 1998
and in revised form October 8, 1999
Accepted for publication December 5, 1999

METHODS

Patients

Three groups of patients were studied. The first group was comprised of living-related renal transplant donors (LRD, $N = 6$), all of whom had normal renal biopsies. The second group consisted of patients with diabetic glomerulopathy (DM, $N = 6$). Four of the DM patients had type 1 and two had type 2 diabetes. In these, the glomerular lesions were characterized by thickened glomerular basement membranes (GBMs) and diffuse mesangial expansion. Some had Kimmelstiel-Wilson nodules and/or nephrosclerosis. The third group of patients consisted of diabetic individuals (type 1, $N = 3$; type 2, $N = 2$) with additional glomerular disease (DM+, $N = 5$). All DM+ patients had at least thickened glomerular basement membranes (GBMs) and some mesangial widening, as evidence of diabetic renal disease. Some also had Kimmelstiel-Wilson nodules and nephrosclerosis. Superimposed renal disorders were IgA nephropathy with or without crescents, anti-GBM disease with crescents, immune complex glomerulonephritis, and Tamm-Horsfall protein in Bowman's space in a patient with renal cell carcinoma but without anatomic evidence of overt obstruction. For the purposes of this study, the presence of arteriosclerosis and/or nephrosclerosis in patients with diabetes mellitus was not considered as an additional or alternative diagnosis.

Clinical data

Serum creatinine, 24-hour urine protein and creatinine, and hemoglobin A1c measurements (in the diabetics only) were measured in the hospital clinical laboratory. The 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 urographic measurement in transplant donors.

Glomerular microdissection and RT-PCR measurements

Microdissection and RT-PCR measurements were performed as previously described [28, 30], with minor modifications. Glomeruli from the renal core were microdissected at 4°C in vanadyl ribonucleoside complex, rinsed, aliquotted four per tube in RNAse inhibitor, solubilized in triton buffer, lyzed by freezing and thawing, and reverse transcribed to cDNA using a Boehringer Mannheim cDNA synthesis kit (Mannheim, Germany). Standard 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 for collagen α2(IV) 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 template cDNA were added, and the reaction mixture was amplified. For collagen α2(IV) mRNA, PCR proceeded as follows: (*1*) 94°C × 3 minutes; (*2*) 42 cycles of 94°C × one minute, 60°C × one minute, and 72°C × three minutes; and (*3*) 72°C × seven minutes. For GAPDH, PCR was done according to the manufacturer's protocol (Clontech, Palo Alto, CA, USA). The RT-PCR products were separated by electrophoresis. The band densities were analyzed by laser densitometry (Helena Laboratories, Beaumont, TX, USA). The values were log transformed, and a log-linear regression analysis was performed against the mutant concentration for each PCR tube. The quantity of cDNA in the test sample was defined as that amount at which the mutant 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). Results with these primers and the competitive mutant have been previously reported [28, 30]. The primers and competitive mutant for human GAPDH (G3PDH) were purchased from Clontech.

Morphological assessments

Renal biopsy tissue was manually divided, and tissue was fixed in alcoholic Bouin's solution, Zeus solution, or glutaraldehyde for evaluation by light, immunofluorescence, and electron microscopy in the standard fashion (47, 48). Glomerular surface area was measured using Micrometer V1.0 software (Cell Analysis Systems, Inc., Oak Brook, IL, USA). Glomerular tuft volume (GTV) was calculated based on the following formula: $GTV = 1.25 (\text{surface area})^{3/2}$ [21]. Depending on the adequacy of the biopsy, these measures were based on 3 to 25 glomeruli per subject.

Morphometric measures of fractional mesangial area and the fractional collagen IV area

Glomeruli were stained either with periodic acid methenamine silver ("Jones" stain) or with monoclonal mouse antihuman type IV collagen antibody (Dako, Carpinteria, CA, USA) by an avidin-biotin immunoperoxidase method. Each glomerulus was photographed and subjected to point counting by superimposing a grid over the photo montages and identifying the glomerular region under the area where grid lines intersected. The mesangial region (comprised of mesangial cells, nuclei,

and matrix as a single region) and the mesangial staining for type IV collagen were quantitated by this method. Fractional mesangial area (A_{FM}) and the fractional type IV collagen area ($A_{FTypeIV}$) were calculated by dividing the total number of points per glomerulus for these regions by the total number (N) of glomerular points counted and multiplying by 100. Equations 1 and 2 describe these calculations:

$$A_{FM} = \frac{(\text{mesangial points per glomerulus})}{(\text{Total } N \text{ glomerular tuft points})} \times 100 \quad \text{(Eq. 1)}$$

$$A_{FTypeIV} = \frac{(\text{mesangial type IV points per glomerulus})}{(\text{Total } N \text{ glomerular tuft points})} \times 100 \quad \text{(Eq. 2)}$$

Immunoperoxidase staining intensity was also evaluated semiquantitatively on a scale of 0 to 4+.

In situ hybridization for collagen IV

In situ hybridization was performed by the method of Suzuki et al on frozen renal sections from each patient [46]. Negative controls included pretreating the tissue with RNAse after proteinase digestion, followed by prehybridization and hybridization. In addition, the in situ procedure was carried out substituting buffer for the antisense probe. Staining was evaluated on a scale of 0 to 4+.

Statistics

Results were analyzed using Access software and 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's test and Student's t-test. Pearson's correlation coefficient was used to test relationships between sets of data. Results are expressed as the mean ± SEM.

RESULTS

Clinical data

Clinical parameters are summarized in Table 1. Renal size was measured ultrasonographically in the DM groups and angiographically in the living related donor (LRD), and thus, the values are not directly comparable. However, renal length in LRD was 11.7 ± 0.73 cm. In DM patients, the renal length was 11.5 ± 0.5 cm, and in DM+, it was 12.5 ± 0.7 cm. Angiotensin-converting enzyme inhibitor use was limited to two patients, both in the DM group.

Glomerular RT-PCR measurements

Glomerular collagen α2(IV) mRNA levels were lowest in LRD (2.9 ± 0.6 attomol/glomerulus), higher in DM (5.9 ± 1.6 attomol/glomerulus, $P = 0.05$), and highest in DM+ (12.7 ± 2.8 attomol/glomerulus, $P < 0.05$ LRD vs. DM+ and DM vs. DM+; Fig. 1). Glomerular GAPDH mRNA levels were not significantly different among the groups ($P = 0.6$; Fig. 2).

Morphological assessments

Representative biopsies are shown in Figure 3. Biopsy material in LRD showed normal kidney in all cases. The DM biopsies showed a spectrum of diabetic changes ranging from mild to moderately diffuse increases in mesangial matrix to marked nodules of mesangial matrix. Capillary basement membranes were thickened to varying degrees. All biopsies had some degree of tubular atrophy with interstitial fibrosis and arteriolar hyalinization of the walls. DM+ biopsies had a broader spectrum of morphologic diabetic alterations, ranging from a very mild, diffuse increase in mesangial matrix and mild capillary basement membrane thickening to marked mesangial nodules and severe thickening of the basement membranes. Biopsies with IgA nephropathy and immune complex glomerulonephritis showed mild to moderate mesangial hypercellularity with IgA or IgM, respectively, on immunofluorescence, and electron dense deposits. One biopsy with IgA deposits had 30% fresh to fibrocellular crescents. The biopsy demonstrating anti-GBM antibody nephritis had 80% fresh to fibrocellular crescents. The single nephrectomy specimen revealed Tamm-Horsfall protein in glomerular urinary spaces, indicating obstruction or reflux. Tubular atrophy and interstitial fibrosis varied from rarely present to involving one third of the renal parenchyma.

Obsolescent glomeruli were rare in the LRD (1.4 ± 1.4% of the total number of glomeruli present, range 0 to 8.3% for the group) and were more common in the DM (15.1 ± 11.0% of total glomeruli, range 0 to 68.4%) and DM+ groups (8.4 ± 3.5% of total glomeruli, range 1.0 to 20%). Segmental sclerosis was absent in LRD (0 ± 0) and was more common in the DM group (17.3 ± 7.0% of glomeruli present on biopsy, range 0 to 40%) and the DM+ group (3.6 ± 2.5% of glomeruli present on biopsy, range 0 to 12.5%). The GTV was larger in DM (3.27 ± 0.38 × 10^6 μ^3) and DM+ (3.88 ± 0.34 × 10^6 μ^3) than in LRD (1.40 ± 0.18 × 10^6 μ^3, $P < 0.05$ DM and DM+ vs. LRD). Although there was more than a twofold difference in collagen α2(IV) mRNA levels between the DM and DM+ groups, the difference between the GTV measurements between the two groups did not reach statistical significance ($P = 0.075$). A significant relationship between glomerular collagen α2(IV) mRNA levels and GTV measurements in the DM and DM+ groups was not found ($r = 0.5$, $P = 0.1$).

Table 1. Clinical characteristics

Group	Age *years*	HbA1c %	Serum creatinine *mg/dL*	$U_{protein/creatinine}$	Urine protein *g/24 h*	MAP *mm Hg*
LRD	33.7 ± 3.4 (28–46)	ND	0.8 ± 0.04 (0.6–0.9)	0.09 ± 0.03	0.12 ± 0.0 (0.03–0.23)	87.2 ± 2.0 (82–95)
DM	44.0 ± 5.6 (29–62)	8.8 ± 0.6 (7.9–10.7)	1.4 ± 0.3 (0.5–2.6)	5.03 ± 1.79[a]	4.43 ± 1.48[a] (0.52–9.04)	102.8 ± 6.1 (83–121)
DM+	40.2 ± 6.4 (25–58)	7.5 ± 1.9 (4.0–12.7)	2.4 ± 0.8 (0.7–4.7)	3.6 ± 1.58[a]	5.84 ± 2.30[a] (2.40–12.4)	101.8 ± 14.2 (67–136)

Values expressed as mean ± SEM. Ranges of values for each group are in parentheses. Abbreviations are: LRD, living-related transplant donor; DM, diabetic nephropathy alone; DM+, diabetic nephropathy with superimposed lesions; Hb, hemaglobin; $U_{protein/creatinine}$, the ratio of urinary protein divided by creatinine; MAP, mean arterial pressure.

[a] $P < 0.05$ compared with LRD

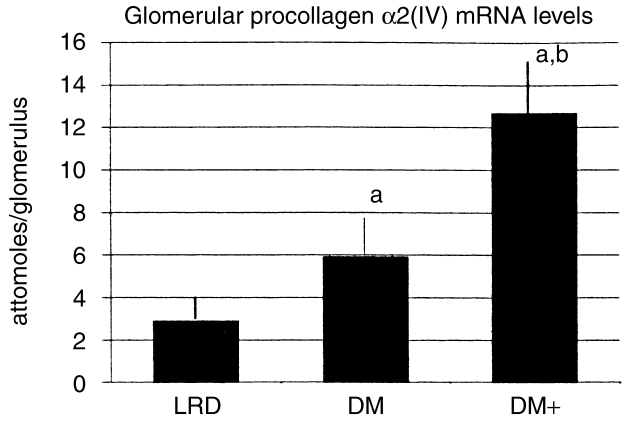

Fig. 1. Glomerular collagen α2(IV) mRNA levels were lowest in living related transplant donor kidneys (LRD), higher in diabetic nephropathy alone (DM), and highest in diabetic nephropathy with superimposed lesions (DM+). [a] $P \leq 0.05$ vs. LRD; [b] $P < 0.05$ vs. DM.

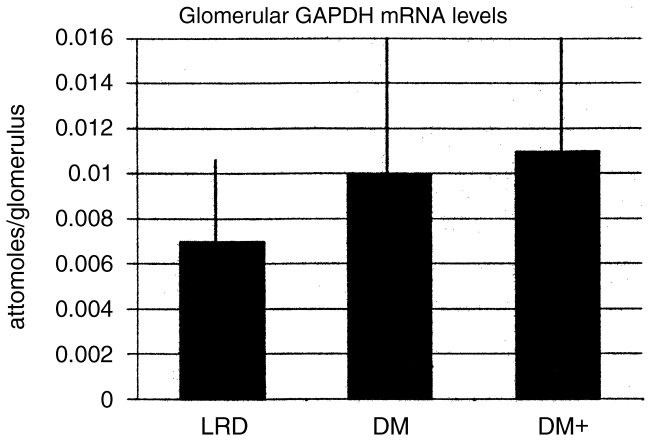

Fig. 2. Glomerular GAPDH mRNA levels were not significantly different in kidneys from patients in the LRD, DM, or DM+ groups.

Morphometric measures of fractional mesangial area and the fractional collagen IV area

The fractional mesangial area in LRD was 18.8 ± 0.9% compared with 45.7 ± 1.6% in DM patients and 41.2 ± 0.8% in DM+ patients ($P < 10^{-6}$, DM and DM+ vs. LRD; Fig. 4). Similarly, the fractional mesangial area staining for type IV collagen was less in LRD (18.2 ± 1.3%) compared with DM (49.0 ± 1.3%) and DM+ (41.5 ± 6.5%, $P < 10^{-6}$, DM and DM+ vs. LRD). There was a statistically significant correlation between the glomerular collagen α2(IV) mRNA levels and the fractional mesangial area ($r = 0.49$, $P = 0.044$), thus demonstrating a relationship between the mRNA measure employed and histologic glomerular damage (Fig. 5). A similar relationship between collagen α2(IV) mRNA and the fractional mesangial area staining for type IV collagen was also appreciated ($r = 0.45$, $P = 0.07$). In contrast, the intensity of type IV collagen staining was significantly stronger in LRD (4.0 ± 0.0) compared with DM (2.5 ± 0.3) and DM+ (2.6 ± 0.6, $P < 10^{-6}$, DM and DM+ vs. LRD; Fig. 6).

In situ hybridization for collagen IV

Glomeruli with mild diffuse mesangial expansion contained mesangial cells with 1 to 2+ cytoplasmic staining for type IV collagen mRNA (Fig. 7A). As mesangial expansion increased, the expression of type IV collagen was reduced, with nodules containing only rare mesangial cells showing trace staining (Fig. 7B), similar to that described previously [42]. Glomeruli from DM+ patients contained mesangial cells staining appropriately for the degree of diabetic involvement. In DM+ patients without crescents, there was variable focal epithelial cell cytoplasmic 1+ staining for type IV collagen mRNA. In patients with crescentic lesions, there was extensive 3+ staining of cells within the urinary space (Fig. 7B), while adjacent glomeruli without crescents had focal 3+ staining of epithelial cells (Fig. 7C).

DISCUSSION

This study had three major goals. The first was to clarify the roles of classical type IV collagen mRNA and protein as valid markers for mesangial expansion and glomerular injury in patients with a spectrum of diabetic

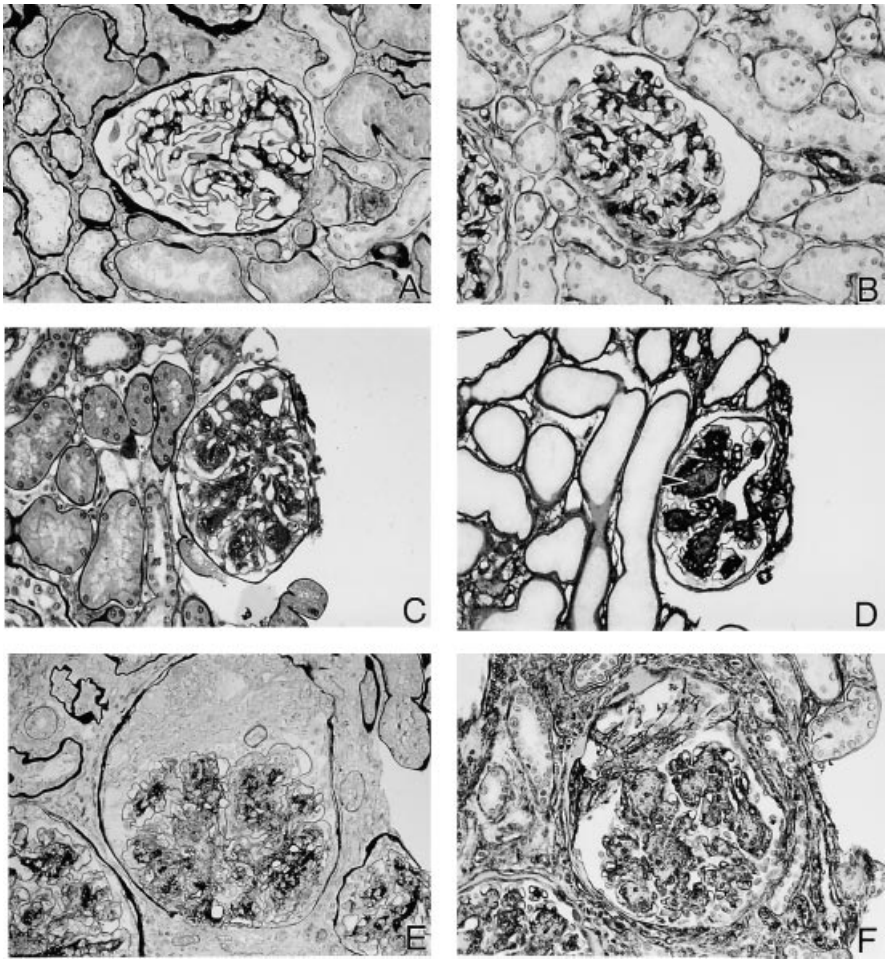

Fig. 3. Representative glomeruli from patients from the LRD, DM, and DM+ groups. (*A*) Glomeruli from LRD donor biopsy. There are normal mesangial regions and capillary walls (periodic acid-methenamine silver stain). (*B*) Type IV collagen stain shows no increase in collagen, and the staining intensity is normal (similar to capillary basement membranes). (*C*) Glomeruli from DM. There is diabetic glomerulosclerosis characterized by diffuse increase in mesangial matrix material with reduced cellularity and thickened capillary basement membranes (periodic acid-methenamine silver). (D) There is increased mesangial type IV staining with reduction of staining intensity, more pronounced in the central mesangial regions (arrowhead). (*E*) Glomeruli from DM with superimposed crescentic glomerulonephritis. There is a background of diabetic glomerulosclerosis with a nodular increase in mesangial matrix material and focal thickening of capillary basement membranes. The urinary space contains a fresh crescent composed of cells and fibrin, and there are focal leukocytes in capillary lumina (periodic acid-methenamine silver). (*F*) There is an increase in mesangial area staining for type IV collagen with central areas of reduced staining intensity. Collagen is also evident within the fibrocellular crescent (A–F ×170).

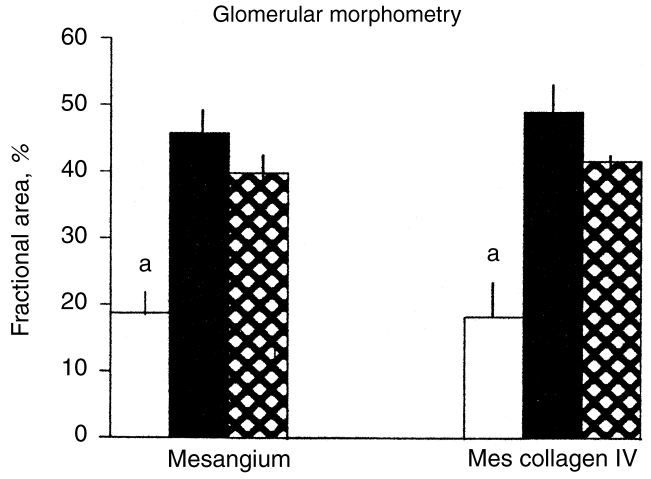

Fig. 4. The fractional mesangial area (mesangium) and the fractional mesangial area staining for type IV collagen (Mes collagen IV) were higher in the DM (■) and DM+ (⊠) groups than in LRD (□; $^{a}P < 10^{-6}$).

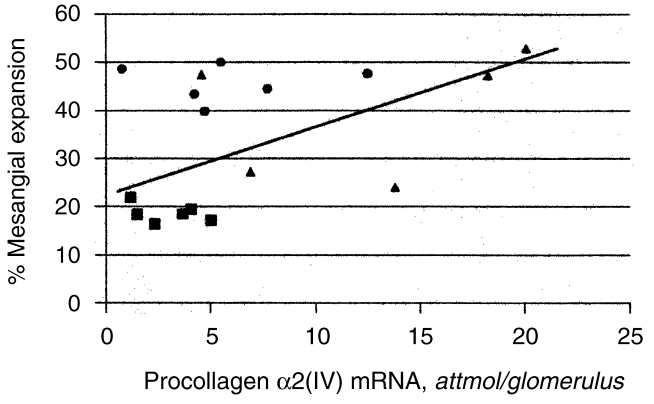

Fig. 5. There was a weak ($r = 0.45$) but statistically significant ($P < 0.05$) correlation between glomerular collagen α2(IV) mRNA levels and morphometrically measured mesangial expansion. Symbols are: (■) LRD; (●) DM; (▲) DM+ ($P < 0.05$; r = 0.45).

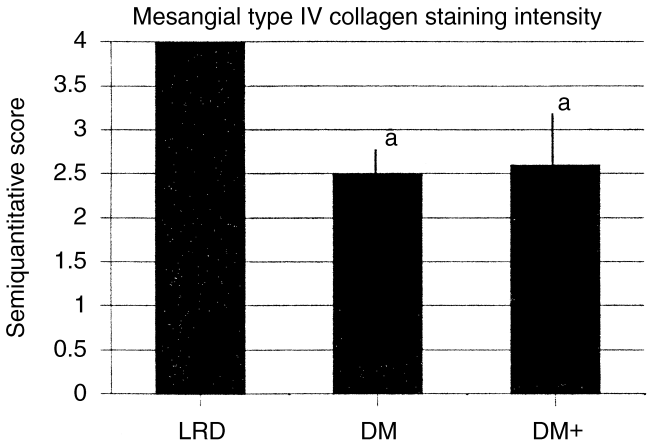

Fig. 6. The intensity of type IV collagen staining was higher in LRD than in the DM or DM+ groups ($P < 0.01$).

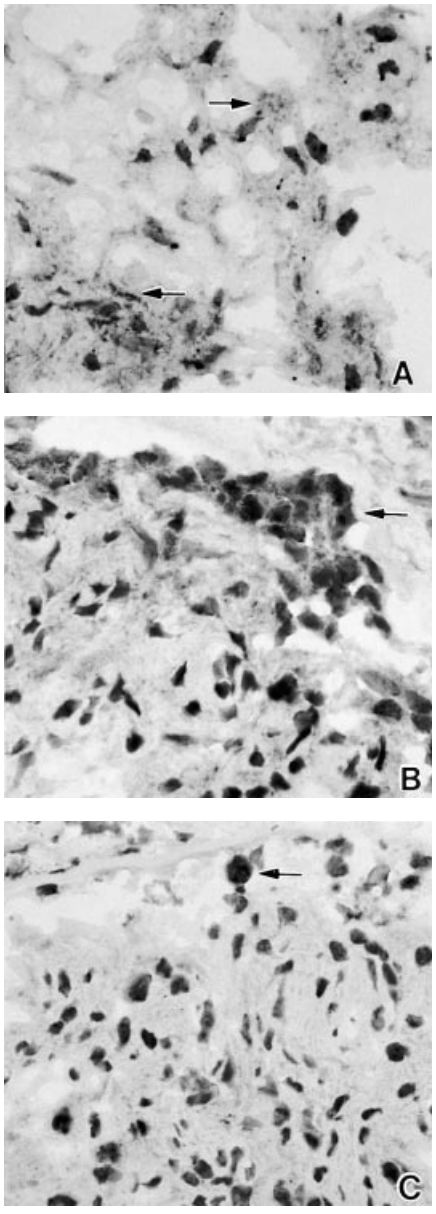

Fig. 7. In situ hybridization for type IV collagen mRNA in glomeruli of DM and DM+ patients. (*A*) DM patient with mild diffuse increase in mesangial matrix. There is 1 to 2+ scattered staining of mesangial cells (arrows). (*B*) DM+ patient with moderate mesangial matrix increase and a segmental crescent. There is 3+ staining of crescentic cells within the urinary space (arrow). (*C*) Glomerulus without a crescent in DM+ crescentic patient. An epithelial cell has 3+ cytoplasmic staining (arrow). The underlying diabetic lesion is moderate to severe with nodule formation; note that there is no staining for type IV collagen mRNA in mesangial cells within the nodules, as has been described previously.

nephropathy from mild to relatively histologically advanced. The second was to determine whether additional glomerular lesions superimposed on diabetic glomerulopathy were associated with an increased expression of glomerular α2 type IV collagen mRNA and protein compared with patients with DM alone. In both cases, this study found in the affirmative, demonstrating increased glomerular collagen α2(IV) mRNA levels in patients with histologically apparent diabetic nephropathy and further increments in patients with diabetic nephropathy plus superimposed glomerular disorders, while no significant differences were observed among groups for the control mRNA GAPDH. Statistically significant differences in collagen α2(IV) mRNA levels were obtained with as few as five or six patients in each clinically defined group, confirming the third major goal, thus demonstrating the usefulness of this technique in showing differences among small numbers of patients. The latter confirms four other published studies using human glomeruli from patients and examining collagen α2(IV), transforming growth factor-β1, and matrix metalloproteinase-2 mRNAs [28, 39, 49, 50]. Our studies further demonstrated a correlation between fractional mesangial expansion and collagen α2(IV) mRNA levels, underscoring the relationship between histologic glomerular damage and collagen α2(IV) mRNA levels. The immunohistochemical studies showed that the distribution of type IV collagen was increased within a widened mesangium, but was decreased in density in diabetic nephropathy.

Comparative data to other human glomerular studies using RT-PCR

Our data confirm and extend two previously published reports evaluating the expression of type IV collagen mRNA and protein in diabetic nephropathy in patients. In one, increments in glomerular α2 type IV collagen relative to α3 type IV collagen mRNA were demonstrated [28]. In the other, collagen α2(IV) mRNA was increased in specimens with glomerulosclerosis, independent of the underlying histopathological diagnosis [39]. In the former report, the increment in α2 type IV collagen mRNA was expressed as a ratio relative to a α3 type

IV collagen mRNA [28], and in the latter as a ratio relative to β-actin [39]. In one, the control specimens were uninvolved areas of tissue from patients undergoing nephrectomy for renal cancers [28]; in the other, control tissue was not studied [39]. The current report is the first study in humans in which glomerular mRNA for α2 type IV collagen was statistically significantly elevated, expressed on a per glomerulus basis, concomitant with no demonstrable difference in a housekeeping gene, and compared with normal healthy human glomeruli.

Lack of effect of glomerular hypertrophy on collagen mRNA level

It is theoretically possible that nonspecific increments in mRNA caused by glomerular hypertrophy could otherwise confound data interpretation in diabetic glomeruli. Whereas collagen α2(IV) mRNA increased twofold per glomerulus in DM patients and fourfold per glomerulus in the DM+ patients, GAPDH levels were similar despite the increased size of the diabetic glomeruli. Furthermore, there was no significant relationship between glomerular size and collagen α2(IV) mRNA levels among the samples in this study, substantiating the independence of glomerular mRNA from glomerular size. Taken together, these data dissociate glomerular mRNA levels from glomerular size in diabetes and underscore the specificity of the increment in α2 type IV collagen mRNA.

Glomerular collagen IV in DM increased in amount but decreased in density, and increased early but decreased in the most advanced lesions

Immunohistochemical studies in diabetic humans have been inconsistent. Early immunohistochemical studies, as well as a few more recent studies, reported increased type IV collagen in the expanded mesangium of biopsies from patients with diabetic nephropathy, and also sometimes in Kimmelstiel-Wilson nodules [31, 32, 37, 40, 41, 45]. Others, using both immunohistochemical and immunoelectron microscopic techniques, demonstrated that type IV collagen disappears or is present at a lesser density in patients with moderate or advanced diabetic nephropathy [40, 42–44]. This is reported to be particularly true for those with a more rapid course [44]. In the studies reported herein, immunoperoxidase staining for type IV collagen confirms the finding of diminished density of type IV collagen in widened mesangial regions in diabetic nephropathy. However, the overall increase in mesangial area and the diffuse representation of type IV collagen throughout the widened mesangial region suggest an overall increase in type IV collagen in the enlarged diabetic glomerulus. This is consistent with studies previously reported by Zhu et al in which the total type IV collagen per glomerulus, as assessed by immunoelectron microscopy, was increased but the density was decreased [44]. There was also a tendency for type IV collagen and its mRNA to be more abundantly expressed, as assessed immunohistochemically in mild compared with more advanced lesions, as previously described [40]. Taken together, these data are consistent with the interpretation that in the mesangium, the moderately advanced diabetic nephropathy lesion is characterized by an increase in the transcription and translation of α2 type IV collagen, but a decrease in quantity relative to other matrix molecules. Furthermore, in the most advanced lesions, there is a tendency toward lesser collagen expression.

Increased glomerular collagen IV mRNA in patients with DM and superimposed glomerular lesions

These results also demonstrate that glomerular collagen α2 (IV) mRNA levels are higher when there are glomerular lesions superimposed on diabetic nephropathy compared with values measured in diabetic nephropathy alone. This was shown in patients with diabetic glomerulopathy and superimposed IgA nephropathy with and without crescents, Goodpasture's syndrome, mesangial proliferative immune complex glomerulonephritis, or obstruction from renal cell carcinoma causing Tamm-Horsfall protein to be present in Bowman's space. These findings parallel the larger increments in type IV collagen mRNA levels seen in animal models with crescentic glomerulonephritis [51], compared with the more modest increments seen in animal models of diabetic nephropathy [52, 53]. The higher collagen α2(IV) mRNA levels in the DM+ group appear to be due to the recruitment of additional cells, particularly glomerular epithelial cells and cells comprising crescents, which contribute to the excess in collagen α2(IV) mRNA, as demonstrated by in situ hybridization.

Superimposition of another glomerular lesion on diabetic nephropathy is a well-recognized phenomenon, particularly but not exclusively in patients with type 2 diabetes [7, 8, 11–15, 17, 54]. However, there is a reticence to identify any but the most aggressive of additional glomerular lesions by renal biopsy. This is due both to a reluctance to use corticosteroids in these patients and to a belief by many that the ultimate prognosis of these individuals will usually be determined by their underlying diabetic renal disease. The current findings demonstrate a twofold further increase in glomerular collagen α2(IV) mRNA levels in these patients compared with those with isolated diabetic nephropathy and suggest that superimposed renal lesions may accelerate glomerular injury at least in part by further increasing type IV collagen synthesis at extramesangial sites.

In summary, this study demonstrated that collagen α2(IV) mRNA levels, the fractional mesangial area, and the fractional area staining for type IV collagen are increased in patients with diabetic nephropathy and are further increased in patients with superimposed glomer-

ular lesions, although the density of type IV collagen in diabetic nephropathy is diminished. Increments in mRNA and protein reflected the presence of mesangial expansion in diabetic patients, but also the addition of extramesangial sites of collagen IV synthesis in patients with superimposed glomerular injury. The ability of RT-PCR to discern differences between groups with small numbers of patients in each group suggests that the technique may be valuable both in measuring surrogate outcomes in clinical trials and in importing additional prognostic information in the evaluation of routine renal biopsy material.

ACKNOWLEDGMENTS

This work was supported by grants from the American Diabetes Association (S.A.), the Juvenile Diabetes Foundation (S.A.), and the National Institutes of Health (Clinical Research Center Grant MO1 RR00425). It was reported in abstract form at the meetings of the American Diabetes Association, Chicago, June 1998, and the American Society of Nephrology, Philadelphia, October 1998.

Reprint requests to Sharon G. Adler, M.D., Division of Nephrology and Hypertension, Harbor-UCLA Medical Center, 1000 West Carson Street, Box 406, Torrance, California 90509, USA.
E-mail: sadler@rei.edu

REFERENCES

1. Mak SK, Gwi E, Chan KW, Wong PN, Lo KY, Lee KF, Wong AK: Clinical predictors of non-diabetic renal disease in patients with non-insulin dependent diabetes mellitus. *Nephrol Dial Transplant* 12:2588–2591, 1997
2. Matsumura N, Hanatani M, Nishino T, Ishihara K, Kishimoto T, Tonomura Y, Shiiki H, Kanauchi M, Dohi K: The clinicopathological significance of hematuria in diabetics. *Nippon Jinzo Gakkai Shi* 36:1036–1045, 1994
3. Hironaka K, Makino H, Ikeda S, Haramoto T, Ota Z: Nondiabetic renal disease complicating diabetic nephropathy. *J Diabetes Complications* 5:148–149, 1991
4. Gambara V, Mecca G, Remuzzi G, Bertani T: Heterogeneous nature of renal lesions in type II diabetes. *J Am Soc Nephrol* 3:1458–1466, 1993
5. Olsen S, Mogensen CE: How often is NIDDM complicated with non-diabetic renal disease? An analysis of renal biopsies and the literature. *Diabetologia* 39:1638–1645, 1996
6. Waldherr R, Ilkenhans C, Ritz E: How frequent is glomerulonephritis in diabetes mellitus type II? *Clin Nephrol* 37:271–273, 1992
7. Kitazawa M, Tomosugi N, Ishii T, Hotta F, Nishizawa M, Itou T, Nakano S, Kigoshi T, Ishikawa I, Uchida K: Rapidly progressive glomerulonephritis concomitant with diabetic nephropathy. *Intern Med* 36:906–911, 1997
8. Ahuja TS, Velasco A, Deiss WJ, Indrikovs AJ, Rajaraman S: Diabetic nephropathy with anti-GBM nephritis. *Am J Kidney Dis* 31:127–130, 1998
9. Robinson LA, Howell DN, Wigfall DR, Foreman JW: Appearance of immune complex glomerulonephritis following the onset of type I diabetes mellitus in a child. *Am J Kidney Dis* 30:713–716, 1997
10. Hong CY, Chia KS: Markers of diabetic nephropathy. *J Diabetes Complications* 12:43–60, 1998
11. Yuksel B, Noyan A, Anarat A, Gonlusen G, Ozer G: Membranoproliferative glomerulonephritis associated with insulin-dependent diabetes mellitus: A case report. (letter) *Nephron* 73:716–717, 1996
12. Ferluga D, Hvala A, Vizjak A, Koselj-Kajtna M, Mihelic-Brcic M: Immunotactoid glomerulopathy with unusually thick extracellular microtubules and nodular glomerulosclerosis in a diabetic patient. *Pathol Res Pract* 191:585–596, 1995
13. Orfila C, Lepert JC, Modesto A, Pipy B, Suc JM: Henoch-Schonlein purpura in a patient with diabetic nephropathy. *Am J Kidney Dis* 24:509–514, 1994
14. Venkataraman TV, Knickerbocker F, Sheldon CV: Unusual causes of renal failure in diabetics: Two case studies. *J Okla State Med Assoc* 83:164–168, 1990
15. Gans R, Ueda Y, Ito S, Kohli R, Min I, Shafi M, Brentjens J: The occurrence of IgA-nephropathy in patients with diabetes mellitus may not be coincidental: A report of five cases. *Am J Kidney Dis* 20:255–260, 1992
16. Furuta T, Seino J, Saito T, Sato H, Agatsuma J, Ootaka T, Satoh T, Yoshinaga K: Insulin deposits in membranous nephropathy associated with diabetes mellitus. *Clin Nephrol* 37:65–69, 1992
17. Gallego N, Olivares F, Mampaso F, Gonzalo A, Barrio R, Estepa R, Ortuno J: Membranous nephropathy, antitubular basement membrane antibodies and alveolar hemorrhage in a diabetic child. *Child Nephrol Urol* 10:154–157, 1990
18. Yang CW, Striker GE, Chen WY, Kopchick JJ, Striker LJ: Differential expression of glomerular extracellular matrix and growth factor mRNA in rapid and slowly progressive glomerulosclerosis: Studies in mice transgenic for native or mutated growth hormone. *Lab Invest* 76:467–476, 1997
19. Jacot TA, Striker GE, Stetler-Stevenson M, Striker LJ: Mesangial cells from transgenic mice with progressive glomerulosclerosis exhibit stable, phenotypic changes including undetectable MMP-9 and increased type IV collagen. *Lab Invest* 75:791–799, 1996
20. Lee GS, Nast CC, Peng SC, Artishevsky A, Ihm CG, Guillermo R, Levin PS, Glassock RJ, Lapage J, Adler SG: Differential response of glomerular epithelial and mesangial cells after subtotal nephrectomy. *Kidney Int* 53:1389–1398, 1998
21. Lombet JR, Adler SG, Anderson PS, Nast CC, Olsen DR, Glassock RJ: Sex vulnerability in the subtotal nephrectomy model of glomerulosclerosis in the rat. *J Lab Clin Med* 114:66–74, 1989
22. Ebihara I, Suzuki S, Nakamura T, Fukui M, Yaguchi Y, Tomino Y, Koide H: Extracellular matrix component mRNA expression in glomeruli in experimental focal glomerulosclerosis. *J Am Soc Nephrol* 3:1387–1397, 1993
23. Floege J, Alpers CE, Burns MW, Pritzl P, Gordon K, Couser WG, Johnson RJ: Glomerular cells, extracellular matrix accumulation, and the development of glomerulosclerosis in the remnant kidney model. *Lab Invest* 66:485–497, 1992
24. Kopp JB, Klotman ME, Adler SH, Bruggeman LA, Dickie P, Marinos NJ, Eckhaus M, Bryant JL, Notkins AL, Klotman PE: Progressive glomerulosclerosis and enhanced renal accumulation of basement membrane components in mice transgenic for human immunodeficiency virus type 1 genes. *Proc Natl Acad Sci USA* 89:1577–1581, 1992
25. Floege J, Johnson RJ, Gordon K, Iida H, Pritzl P, Yoshimura A, Campbell C, Alpers CE, Couser WG: Increased synthesis of extracellular matrix in mesangial proliferative nephritis. *Kidney Int* 40:477–488, 1991
26. Bergijk EC, Van Alderwegen IE, Baelde HJ, De Heer E, Funabiki K, Miyai H, Killen PD, Kalluri RK, Bruijn JA: Differential expression of collagen IV isoforms in experimental glomerulosclerosis. *J Pathol* 184:307–315, 1998
27. Bergijk EC, Baelde HJ, De Heer E, Killen PD, Bruijn JA: Role of the extracellular matrix in the development of glomerulosclerosis in experimental chronic serum sickness. *Exp Nephrol* 3:338–347, 1995
28. Esposito C, Striker LJ, Patel A, Peten E, Liu ZH, Sakai H, Striker GE: Molecular analysis of glomerular diseases in renal biopsies: Initial results of a collaborative international study: The International Study Group for Molecular Study of Kidney Biopsies. *Proc Assoc Am Phys* 108:209–217, 1996
29. Wu K, Setty S, Mauer SM, Killen P, Nagase H, Michael AF, Tsilibary EC: Altered kidney matrix gene expression in early stages of experimental diabetes. *Acta Anat (Basel)* 158:155–165, 1997
30. Esposito C, Patel A, Liu ZH, Striker GE, Striker LJ: Involvement of synthesis and degradation pathways of collagen type IV in human glomerulosclerosis: Molecular analysis by in situ reverse

transcription and competitive polymerase chain reaction. *Contrib Nephrol* 118:12–16, 1996
31. Tamsma JT, Van Den Born J, Bruijn JA, Assmann KJ, Weening JJ, Berden JH, Wieslander J, Schrama E, Hermans J, Veerkamp JH: Expression of glomerular extracellular matrix components in human diabetic nephropathy: Decrease of heparan sulphate in the glomerular basement membrane. *Diabetologia* 37:313–320, 1994
32. Nerlich A, Schleicher E: Immunohistochemical localization of extracellular matrix components in human diabetic glomerular lesions. *Am J Pathol* 139:889–899, 1991
33. Morel-Maroger Striker L, Killen PD, Chi E, Striker GE: The composition of glomerulosclerosis. I. Studies in focal sclerosis, crescentic glomerulonephritis, and membranoproliferative glomerulonephritis. *Lab Invest* 51:181–192, 1984
34. Suzuki Y: Constituents of the extracellular matrices in diabetic glomerulosclerosis. *Nippon Jinzo Gakkai Shi* 31:1047–1054, 1989
35. Funabiki K, Horikoshi S, Tomino Y, Nagai Y, Koide H: Immunohistochemical analysis of extracellular components in the glomerular sclerosis of patients with glomerulonephritis. *Clin Nephrol* 34:239–246, 1990
36. Buyukbabani N, Droz D: Distribution of the extracellular matrix components in human glomerular lesions. *J Pathol* 172:199–207, 1994
37. Ikeda S, Makino H, Haramoto T, Shikata K, Kumagai I, Ota Z: Changes in glomerular extracellular matrices components in diabetic nephropathy. *J Diabetes Complications* 5:186–188, 1991
38. Cai YI, Sich M, Beziau A, Kleppel MM, Gubler MC: Collagen distribution in focal and segmental glomerulosclerosis: An immunofluorescence and ultrastructural immunogold study. *J Pathol* 179:188–196, 1996
39. Yang CW, Hsueh S, Wu MS, Lai PC, Huang JY, Wu CH, Hu SA, Chen JF, Huang CC: Glomerular transforming growth factor-beta1 mRNA as a marker of glomerulosclerosis-application in renal biopsies. *Nephron* 77:290–297, 1997
40. Falk RJ, Scheinman JI, Mauer SM, Michael AF: Polyantigenic expansion of basement membrane constituents in diabetic nephropathy. *Diabetes* 32(Suppl 2):34–39, 1983
41. Woodrow D, Moss J, Shore I, Spiro RG: Diabetic glomerulosclerosis: Immunogold ultrastructural studies on the glomerular distribution of type IV collagen and heparan sulphate proteoglycan. *J Pathol* 167:49–58, 1992
42. Kim Y, Kleppel MM, Butkowski R, Mauer SM, Wieslander J, Michael AF: Differential expression of basement membrane collagen chains in diabetic nephropathy. *Am J Pathol* 138:413–420, 1991
43. Yagame M, Kim Y, Zhu D, Suzuki D, Eguchi K, Nomoto Y, Sakai H, Groppoli T, Steffes MW, Mauer SM: Differential distribution of type IV collagen chains in patients with diabetic nephropathy in non-insulin-dependent diabetes mellitus. *Nephron* 70:42–48, 1995
44. Zhu D, Kim Y, Steffes MW, Groppoli TJ, Butkowski RJ, Mauer SM: Glomerular distribution of type IV collagen in diabetes by high resolution quantitative immunochemistry. *Kidney Int* 45:425–433, 1994
45. Razzaque MS, Koji T, Taguchi T, Harada T, Nakane PK: In situ localization of type III and type IV collagen-expressing cells in human diabetic nephropathy. *J Pathol* 174:131–138, 1994
46. Suzuki D, Miyazaki M, Jinde K, Koji T, Yagame M, Endoh M, Nomoto Y, Sakai H: In situ hybridization studies of matrix metalloproteinase-3, tissue inhibitor of metalloproteinase-1 and type IV collagen in diabetic nephropathy. *Kidney Int* 52:111–119, 1997
47. Cohen AH: Morphology of renal tubular hyaline casts. *Lab Invest* 44:280–287, 1998
48. Cohen AH, Border WA, Rajfer J, Dumke A, Glassock RJ: Interstitial Tamm-Horsfall protein in rejecting renal allografts: Identification and morphological pattern of injury. *Lab Invest* 50:519–525, 1984
49. del Prete D, Anglani F, Forino M, Ceol M, Fioretto P, Nosadini R, Baggio B, Gambaro G: Down-regulation of glomerular matrix metalloproteinase-2 gene in human NIDDM. *Diabetologia* 40:1449–1454, 1997
50. Iwano M, Kubo A, Nishino T, Sato H, Nishioka H, Akai Y, Kurioka H, Fujii Y, Kanauchi M, Shiiki H, Dohi K: Quantification of glomerular TGF-beta 1 mRNA in patients with diabetes mellitus. *Kidney Int* 49:1120–1126, 1996
51. Merritt SE, Killen PD, Phan SH, Wiggins RC: Analysis of α 1 (I) procollagen, α 1 (IV) collagen, and β-actin mRNA in glomerulus and cortex of rabbits with experimental anti-glomerular basement membrane disease: Evidence from early extraglomerular collagen biosynthesis. *Lab Invest* 63:762–769, 1990
52. Fukui M, Nakamura T, Ebihara I, Shirato I, Tomino Y, Koide H: ECM gene expression and its modulation by insulin in diabetic rats. *Diabetes* 41:1520–1527, 1992
53. Park IS, Kiyomoto H, Abboud SL, Abboud HE: Expression of transforming growth factor-beta and type IV collagen in early streptozotocin-induced diabetes. *Diabetes* 46:473–480, 1997
54. Maeda Y, Tomura S, Kato K, Owada A, Imai K, Koyano T, Shimokama T, Watanabe T, Shiigai T: Churg-Strauss syndrome associated with necrotizing crescentic glomerulonephritis in a diabetic patient. *Intern Med* 36:68–72, 1997