Increased Urinary Type IV Collagen Marks the Development of Glomerular Pathology in Diabetic *d/db* Mice

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The diabetic *db/db* mouse exhibits increased albumin excretion soon after the onset of obesity and hyperglycemia, and later manifests glomerular mesangial matrix expansion resembling that found in human diabetic nephropathy. Since the glomerular lesion in this rodent model of type 2 diabetes is associated with renal overexpression of mRNA encoding type IV collagen, we postulated that changes in the urinary excretion of collagen IV may reflect developing glomerular pathology. To explore this hypothesis, we monitored urinary collagen IV (measured by immunoassay) in *db/db* mice during the course of evolution of nephropathy. At age 8 weeks, collagen IV excretion was not different in diabetic compared to nondiabetic animals despite marked albuminuria, but was significantly increased in *db/db* compared to *db/m* mice at age 12 and 16 weeks. Serum levels of collagen IV did not significantly differ between normal versus diabetic mice at any age. Glomerular morphometry revealed mesangial matrix expansion at age 12 weeks, coincident with the rise in collagen IV excretion, which became more marked at age 16 weeks in association with reduced creatinine clearance and elevated serum creatinine. The findings suggest that increased urinary type IV collagen is a better indicator than albuminuria of developing glomerular matrix accumulation that results in compromised renal filtration function.

THE WELL-DESCRIBED structural abnormalities in diabetic nephropathy include an accumulation of glomerular mesangial matrix and a thickened basement membrane.[1-5] Activation of protein kinase C (PKC) and stimulation of the fibrogenic transforming growth factor-β (TGF-β) system by diabetogenic factors such as hyperglycemia and increased protein glycation have been pathogenetically linked to this process.[6-11] The *db/db* mouse, a rodent model of genetic diabetes, manifests glomerular changes resembling those found in human diabetes, and the evolution of renal dysfunction in this model parallels that in human diabetic nephropathy.[12,13] Increased albumin excretion, which is believed to represent leaky nephrons,[14,15] appears soon after the establishment of hyperglycemia, and is followed several weeks later by pronounced glomerular mesangial matrix expansion.[16-18] As in the human disease, a rise in serum creatinine indicates the transition from leaky to occluded glomeruli in which filtration is reduced.[19-21] This resemblance in nature and chronology of renal involvement to that occurring in the human disease has made the *db/db* mouse useful for investigations concerning the pathogenesis of diabetic nephropathy.[16,17] It also provides an animal model for the identification of surrogate markers that could serve as early indicators of renal involvement in diabetes, and for the assessment of intervention therapies that might influence the course of renal disease in diabetes.[16-19,22-25]

Glomerulopathy in the *db/db* mouse is associated with renal overproduction of the extracellular matrix (ECM) proteins fibronectin and type IV collagen and increased expression of the mRNAs encoding these proteins.[17,18,22] Some studies have suggested that these changes might be accompanied by the excretion of increased amounts of collagen IV in the urine, which could provide a useful marker for the diagnosis of diabetic nephropathy.[26-29] However, these studies disagree as to whether changes in collagen IV excretion antedate[27,29] or postdate[26,28] microalbuminuria and whether there are corresponding changes in serum concentrations of this analyte. To address this issue, we monitored urinary albumin and collagen IV excretion as nephropathy evolved in *db/db* mice and assessed changes in relation to glomerular histopathology and renal function. The findings indicate urinary collagen IV increases with age in *db/db* but not in nondiabetic *db/m* mice, and that this increase follows an earlier appearance of albuminuria and portends a reduction in filtration surface that is marked by a declining creatinine clearance, a rising serum creatinine, and progressive expansion of periodic acid Schiff (PAS)-positive mesangial matrix.

MATERIALS AND METHODS

Experimental Animals

Male diabetic *db/db* mice and age-matched male nondiabetic *db/m* mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Mice were designated *db/db* by the vendor on the basis of the appearance of obesity, which is usually detectable at about 5 weeks of age. Animals were weighed and hyperglycemia was confirmed in the diabetic mice after arrival in our laboratory. Animals were provided food and water ad libitum, weighed at regular intervals, and the persistence of hyperglycemia in *db/db* mice and normoglycemia in *db/m* mice during the course of the experiment was documented. Blood for measurement of serum glucose, creatinine and collagen IV was periodically obtained from the retro-orbital sinus. Twenty-four–hour urine specimens were collected from each animal at the indicated ages by individual placement in metabolic cages, with washing of the collection apparatus with a measured volume of distilled water in a spray bottle to ensure collection of residual droplets. Kidneys were harvested when animals were killed at the indicated ages. One kidney from each animal was fixed by immersion in 10% neutral buffered formalin and embedded in parafilm for histologic examination; cortex from the other kidney was separated by dissection and snap frozen for RNA extraction. All animal experimentation was conducted in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals.

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Analytical Procedures

Glucose was determined by the glucose oxidase method (Sigma Chemical Co, St Louis, MO). Creatinine was measured by the picric acid colorimetric procedure (Sigma). Urine albumin was measured with a competitive enzyme-linked immunoassay that has been described previously.[16] In brief, murine albumin was immobilized onto plastic microtiter wells and incubated with standard (mouse albumin) or sample in the presence of antimurine albumin antibody. After washing, development and detection were accomplished with horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG, and tetramethylbenzidine(TM) Blue substrate (microwell peroxidase system). The reaction was stopped with 2 mol/L H_2SO_4 and the absorbances in the wells were read at 450 nm. Absorbance was inversely proportional to albumin concentration. The assay was sensitive to 15 ng/well, and showed linearity with the log of concentration between 0.3 to 10 μg/mL.

The immunoassay for quantitation of type IV collagen employed murine type IV collagen purified from Engelbreth-Holm-Swarm (EHS) tumor (Collaborative Biomedical Products, Bedford, MA) and rabbit antimurine type IV collagen antibody (BioDesign, Kennebunk ME), as previously described.[30] The murine 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 (BSA) in glycine coupling buffer, pH 8.5, containing 0.05% Proclin (Supelco, Bellefonte, PA). To initiate the assay, plates were washed with a solution of 0.15 mol/L NaCl, 10 mmol/L triethanolamine, pH 6.8, containing 0.01% Tween-20 (EIA buffer), and blotted by inversion. Two hundred microliters of a solution containing the standard or sample and rabbit antimurine collagen IV antibody in 10% fetal calf serum, 100 mmol/L Tris HCl, pH 7.5, were added to the wells and allowed to react for one hour 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 same buffer) to microfuge tubes, and preincubating the mixture overnight at room temperature. The preincubation step enhances binding of antibody to antigen in soluble phase, reducing binding to coated antigen when competing antigen is present at low concentrations. HRP-conjugated goat anti-rabbit IgG (1:2,000 dilution in 10% BSA, 100 mmol/LTris HCl) was then added to the wells and the incubation continued for another hour. The plates were washed and developed with TMBlue substrate, stopped, and read as described above. Absorbance was inversely proportional to collagen IV concentration. The assay was sensitive to 5 ng/mL and showed linearity with the log of concentration between 5 and 2,000 ng/mL. Intra- and inter-assay coefficients of variation were $\leq$9%. The anti-collagen IV antibody showed no reactivity with other mouse urinary proteins (the principal one being albumin). Glucose up to 50 mmol/L did not interfere in the assay.

Glomerular Pathology

Random sections (3 μm thick) of the renal cortex were stained with PAS and subjected to quantitative glomerular morphometry, which is considered the best index of accumulation of collagen IV and other collagen types in the ECM. Sections were coded and read by an observer unaware of the experimental group from which they derived. At least 10 glomeruli in the outer cortex were selected at random in PAS-stained sections from each *db/db* and *db/m* animal. Mesangial matrix was identified as PAS-positive material in the mesangial region, exercising care to exclude cellular elements. Values obtained with this method are consistent with those reported by other investigators using light and/or electron microscopic methods. The mesangial matrix area was calculated as the fraction of total glomerular tuft cross-sectional area as previously described.[17,18] Increased mesangial fractional volume is considered a better indicator of declining filtration function than is width of the glomerular basement membrane.[1,5,31]

RNA Hybridization Analysis

Northern blots of RNA extracted from renal cortex were hybridized with ^{32}P-labeled probe encoding murine α1 (IV) collagen and the membranes were autoradiographed according to the methods previously described in detail.[11,17,18] The blots were then stripped with 5 mmol/L Tris, 0.2 mmol/L EDTA, and 5% sodium pyrophosphate at 62°C for 2 hours, and subsequently rehybridized with cDNA fragment encoding for mouse ribosomal protein (mrpL32) as a loading standard to account for small variations in RNA loading and transfer.[18] Exposed films were scanned with a laser densitometer, and mRNA levels were calculated relative to those of mrpL32. This ratio was assigned an arbitrary value of 1.0 in Northern blots from 8 week *db/m* mice.

Statistical Analysis

Statistical analysis was performed using unpaired *t* tests for comparison of the means.

RESULTS

General characteristics of the experimental animals used in this study conformed with those known to be associated with this model, and are summarized in Table 1. All of the *db/db* mice were obese upon arrival in our laboratory, with a mean body weight that was significantly greater than that in age-matched nondiabetic *db/m* controls and that remained significantly higher compared to *db/m* animals throughout the experimental period, which concluded at age 16 weeks. Diabetic mice stopped gaining weight between 12-16 weeks of age, consistent with entry into an insulin-deficient catabolic state that develops in this rodent model.[32-34] Nondiabetic *db/m* controls continued to gain weight during the experimental period. Blood glucose concentrations in *db/db* mice were abnormally elevated throughout the study period, and remained in the normal range in *db/m* mice.

Urinary albumin excretion was significantly greater in *db/db* compared to *db/m* animals by age 8 weeks, and remained significantly elevated relative to nondiabetic control mice for the remainder of the experimental period (Table 1). The values for albumin excretion were consistent with those reported by other investigators using similar methodology.[25]

Figure 1 depicts representative results of the collagen IV immunoassay, and illustrates the relationship between absorbance and log concentration of murine collagen IV without or

Table 1. Experimental Animal Data

	Age		
	8 Weeks	12 Weeks	16 Weeks
Body weight (g)			
db/m	20.0 ± 0.5	27.1 ± 0.6*	27.5 ± 0.4
db/db	38.7 ± 1.2†	42.2 ± 2.2*†	38.7 ± 3.2†
Blood glucose (mmol/L)			
db/m	5.8 ± 0.6	5.8 ± 0.5	6.1 ± 0.4
db/db	14.2 ± 2.1†	23.8 ± 1.8*†	28.3 ± 2.1*†
Urine albumin (μg/24 hr)			
db/m	6 ± 3	4 ± 2	21 ± 8
db/db	247 ± 41†	249 ± 34†	303 ± 30*†

NOTE. n = 6 in each experimental group.

*$P \leq .05$ compared to value at preceding age in same experimental group.

†$P < .05$ compared to *db/m* control.

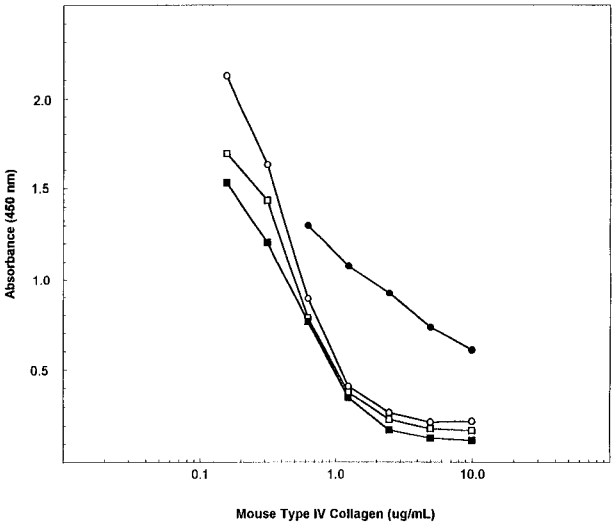

Fig 1. Immunoassay for type IV collagen performed by incubating antigen and primary antibody for 2 hours in antigen-coated microtiter wells (●), and by preincubating antigen and primary antibody overnight before placement into antigen-coated wells (○). The standard curve retains the same relationship when performed in urine from nondiabetic (△) or diabetic (■) animals.

with the preincubation step. Incubation for 2 hours conducted with the murine antigen and primary antibody added in soluble phase directly to the wells yielded a linear inverse relationship with 0.156 to 10 μg/mL of antigen. Extending the period of incubation with antigen and primary antibody added directly to the wells did not enhance sensitivity. However, overnight preincubation of murine collagen IV with primary antibody in microfuge tubes, followed by placement of an aliquot of this mixture into the coated wells, extended the sensitivity to 5 ng/mL. This range was sufficient for measurement of collagen IV concentrations in unconcentrated urine specimens that were collected as described above. The relationship between absorbance and log concentration remained linear in the indicated range when the standard curve was performed in urine from nondiabetic or diabetic mice, being shifted slightly to the left due to endogenous collagen IV in the sample (Fig 1). Addition of graded amounts of authentic exogenous collagen IV to serially diluted specimens proportionately shifted the curve, with recovery according to the change in absorbance values of greater than 80% of the amount added.

Urine excretion of collagen IV was not significantly different in *db/db* compared to *db/m* mice at age 8 weeks (Fig 2), despite the presence of significant albuminuria. Collagen IV excretion rose with age in diabetic animals, reaching significantly higher levels in *db/db* mice compared to controls at age 12 weeks and remaining significantly increased thereafter (Fig 2). The correspondence of elevated urinary collagen IV excretion with increasing albumin excretion in the last 4 weeks of the study is consistent with the view espoused by Warram et al,[35] who found that reduction in filtration function, which reflects encroachment of the glomerular filtration surface by expanding mesangium, is observed when albuminuria exceeds 100 μg/mg creatinine. The discordant relationship between albumin versus collagen excretion at the younger age is consistent with the interpretation that the mechanisms underlying elevated excretion of these two proteins is different, with the early appearance of albuminuria reflecting hyperfiltration (see below) and/or nonspecific damage to the filtration barrier and the later appearance of increased urinary collagen reflecting overproduction of matrix. Serum concentrations of type IV collagen did not differ between nondiabetic and diabetic animals (Fig 2), consistent with the interpretation that the observed increase in urinary collagen IV is of renal origin.

Early in the course of diabetes and coincident with the appearance of albuminuria, creatinine clearance values in *db/db* mice were significantly greater than those in the nondiabetic controls, consistent with hyperfiltration (Fig 3). At age 12 weeks, creatinine clearance in *db/db* mice did not differ significantly from that in *db/m* controls (4.43 ± 0.51 *v* 4.02 ± 0.79, not significant). With longer duration of diabetes, creatinine clearance fell, reaching levels below those of *db/m* mice, as serum creatinine concentrations rose in *db/db* mice (Figs 3 and 4) .

Renal glomeruli in specimens obtained from *db/db* mice

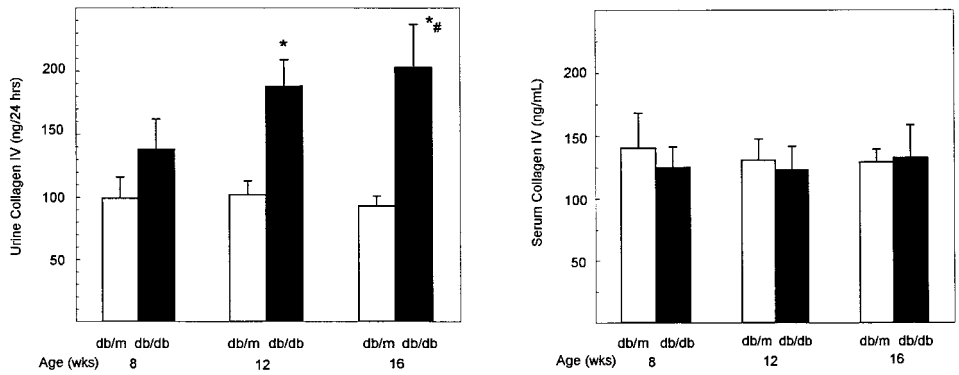

Fig 2. Urinary collagen IV excretion (left) and serum collagen IV concentrations (right) measured in nondiabetic *db/m* and diabetic *db/db* mice at the indicated ages. Results represent mean ± SEM of 6 animals per group. Range of urine collagen IV values in *db/m v db/db* mice was 84 to 123 *v* 63 to 228 at 8 weeks, 75 to 112 *v* 100 to 300 at 12 weeks, and 42 to 140 *v* 110 to 398 at 16 weeks. * $P \leq .05$ compared to *db/m* controls at same age. # $P \leq .05$ compared to value at 8 weeks in same experimental group.

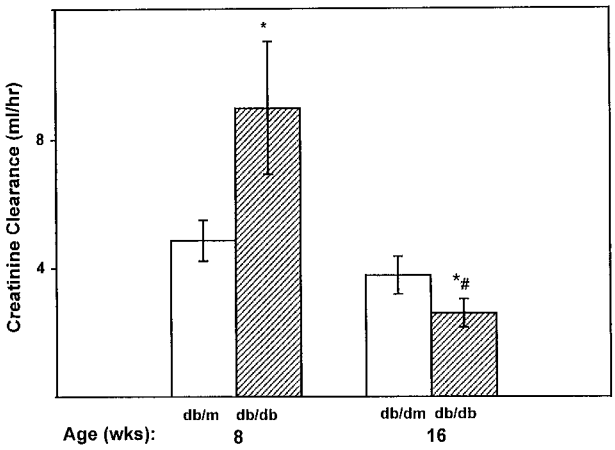

Fig 3. Creatinine clearance in *db/m* and *db/db* mice at the indicated ages. *P ≤ .05 compared to *db/m* controls. #P ≤ .05 compared to value at preceding time in same experimental group.

killed at age 16 weeks exhibited the described picture of diffuse mesangial expansion that has been illustrated in photomicrographs published by our group.[16-18,22] On glomerular morphometry the glomerular tuft surface area and the mesangial matrix fraction (MMF) in these animals were significantly increased, with a mean MMF value approximately 3.5 times that of nondiabetic controls of the same age (Fig 5). Glomerular size was similarly increased in diabetic animals killed at 12 weeks but the mean MMF, although greater than in nondiabetic controls, was significantly less than at the more advanced age (Fig 5). The MMF was not significantly different in *db/db* versus *db/m* mice at age 8 weeks, although glomerular hypertrophy was present (Fig 5), consistent with the hyperfiltration

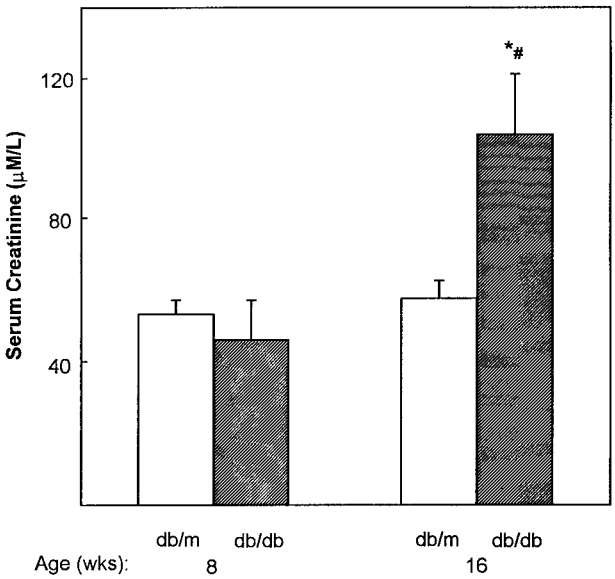

Fig 4. Serum creatinine in *db/m* and *db/db* mice at the indicated ages. *P ≤ .05 compared to *db/m* controls. #P ≤ .05 compared to value at preceding time in same experimental group.

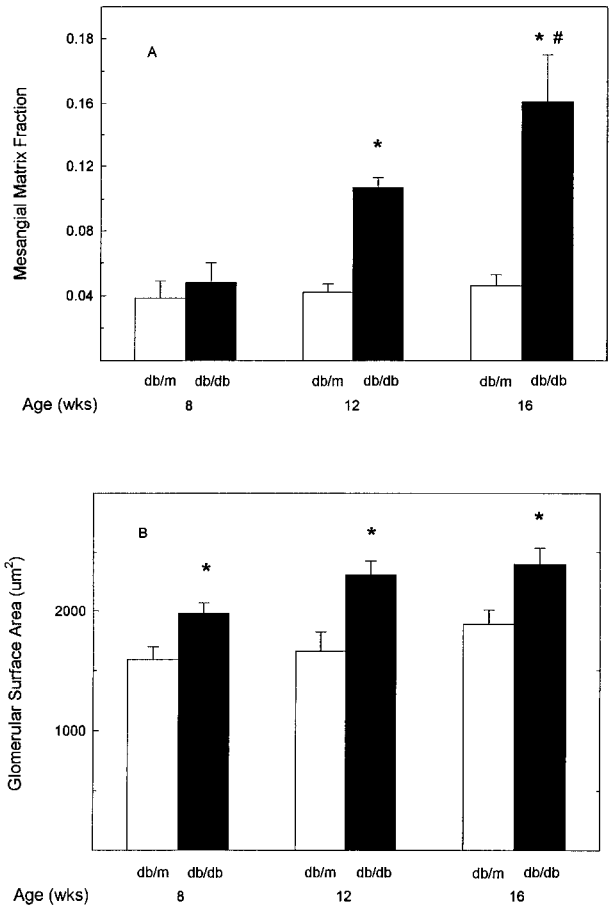

Fig 5. Mesangial matrix fraction (A) and glomerular tuft surface area (B) in glomeruli from *db/m* and *db/db* mice at the indicated ages. *P ≤ .05 compared to *db/m* controls at same age. #P ≤ .05 compared to value at preceding time in same experimental group.

that was observed in *db/db* mice at this age. Thus, a rising collagen IV excretion accompanied the appearance of measurable mesangial expansion.

This interpretation was further supported by hybridization analysis of renal cortical α1 (IV) collagen mRNA, which demonstrated progressive increase in expression with increasing duration of diabetes. Compared to nondiabetic mice, the ratio of type IV collagen to mrpL RNA in *db/db* mice was unchanged at age 8 weeks, but was increased to 125% $\pm$ 10.2%, $P < .05$ and 150% $\pm$ 7.8%, $P < .05$ of control at age 12 weeks and 16 weeks, respectively. Expression of α1 (IV) collagen mRNA in extracts of renal cortex is a composite of tubular and glomerular elements, which undergo analogous changes in diabetes,[2,3,20] and the increased α1 (IV) mRNA may in part reflect the thickening of tubular basement membranes that we have observed in these animals. Nevertheless, the chronological concordance of this increase with expanding PAS-positive glomerular matrix is consistent with the interpretation that glomerular overexpression contributed to this increase.

DISCUSSION

Several studies have documented increased renal type IV collagen production in rodents with experimental or genetic diabetes.[3,17,36-39] In animal models with chemically induced diabetes, this increase is not regularly accompanied by the mesangial matrix expansion that characterizes the glomerular lesion in human diabetic nephropathy.[40-42] In the *db/db* mouse, however, overexpression of ECM proteins leads to glomerular pathology resembling that found in the human disease.[12,16-18,22] This feature, and the development of hyperglycemia at an early age and survival for several months without the need for treatment with exogenous insulin,[32] render this model advantageous for various experimental studies, such as investigation of in vivo influences that may be relevant to the pathogenesis of human diabetic nephropathy, of treatments that may arrest its course, and of markers that indicate early diabetic renal dysfunction and assess the efficacy of intervention therapies.

In human subjects, the excretion of collagen IV has been reported to be increased in type 1[26,43] and type 2[26-29] diabetes, without[27,29,43] or with[28,43] microalbuminuria or overt proteinuria.[26,43] Serum levels of collagen IV have been reported to be decreased,[26] increased,[44] or unchanged[28] in diabetes. The excretion of the fibrogenic cytokine TGF-β, which has been pathogenetically implicated in diabetic nephropathy, as a marker of renal fibrogenesis may be concomitantly elevated in microalbuminuric diabetic patients[43]; increased renal production of TGF-β1 in human diabetes, evidenced by overexpression of the cytokine and by TGF-β1 concentrations in renal vein effluent, also has been described.[6,45] 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 (6-fold) increase in collagen excretion in these patients,[43] suggesting that factors other than TGF-β1 may be important contributors to the enhanced matrix production in diabetes and/or that altered renal handling of matrix proteins may be responsible for the elevated collagen excretion. These considerations may pertain in the *db/db* mouse, in which excretion of TGF-β1 may be reduced despite increased glomerular expression of TGF-β1.[22,25,46] The excretion of collagen IV, a marker of glomerular basement membrane synthesis and matrix remodeling, has not been previously studied in this model.

The present results demonstrate that the excretion of collagen IV becomes elevated as renal involvement progresses in the *db/db* mouse. This increase is not accompanied by any significant change in serum concentrations of collagen IV, suggesting that it is of renal origin. The chronology of this increase post-dates the onset of increased albumin excretion, which appears soon after the onset of hyperglycemia. It antedates a rise in serum creatinine and a fall in creatinine clearance to values below those of age-matched *db/m* mice, and is associated with light microscopic evidence of glomerular matrix accumulation, manifest as an expansion of PAS-positive material in the glomerular mesangium. Whereas increased albumin excretion is believed to represent leaky nephrons without overt reduction in the single nephron glomerular filtration rate,[14,15] the expansion of mesangial matrix that encroaches upon the normal capillary network marks a reduction in the glomerular surface area available for filtration and the transition to occluded glomeruli.[20,21] The appearance of increased amounts of collagen IV in the urine may therefore reflect this transition, heralding the subsequent rise in serum creatinine and steady decline in renal function.[14,47,48] The findings suggest that urinary collagen IV excretion is an indicator of diabetic renal disease entering a phase of compromised filtration function, and that it may be a useful marker to evaluate the ability of potential therapies to arrest this process while it is at a reversible stage.

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