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Urinary excretion of glycosaminoglycans and albumin in experimental diabetes mellitus

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Diabetes mellitus was induced in one group of rats by a single injection of streptozotocin. The glycemia, the body weight, and the blood systolic pressure were measured every week, and the 24 h urine volume and urinary excretions of creatinine, albumin and glycosaminoglycans were measured every 2 weeks. At the end of the experiment (12 weeks) the weight and the glycosaminoglycan composition of the kidneys were determined. All the diabetic animals were hyperglycemic, hypertense, and did not gain weight during all the experimental period. Albuminuria appeared from the second week on. Rat urine was shown to contain heparan sulfate, chondroitin sulfate, and dermatan sulfate, and the glycosaminoglycan excretion decreased in all diabetic animals. The onset of the change in glycosaminoglycan excretion rate was a very early event, appearing in the second week after diabetes induction. The main glycosaminoglycan found in normal rat kidney was heparan sulfate and, in contrast to the urine, the total kidney glycosaminoglycans increased in diabetic kidney, due to chondroitin sulfate and dermatan sulfate accumulation. The heparan sulfate concentration (per tissue dry weight) did not change. Our results suggest that quantification of urinary glycosaminoglycans may be a useful tool for the early diagnosis of diabetic nephropathy.

Key words: albuminuria/diabetes mellitus/glycosaminoglycan/kidney/urine

Introduction

A proteoglycan is a protein substituted with at least one glycosaminoglycan chain. Six classes of glycosaminoglycans are now recognized: chondroitin sulfate, dermatan sulfate, heparan sulfate, keratan sulfate, heparin, and hyaluronic acid. They share some common features: the long unbranched heteropolysaccharide chains are made up largely of disaccharide repeating units, in which one sugar is a hexosamine (D-glucosamine or D-galactosamine) and the other is either a neutral sugar (D-galactose, in keratan sulfate) or a uronic acid (D-glucuronic or L-iduronic acid). Other constituents of glycosaminoglycans are sulfate groups, linked either by ester bonds to the sugar residues or by amide bonds to the amino group of glucosamine. The carboxyl groups from the uronic acids and the sulfate groups contribute to the highly polyanionic nature of glycosaminoglycans. With the exception of hyaluronic acid, which is not sulfated and has not been shown to exist covalently attached to protein, every glycosaminoglycan is linked covalently to a protein, forming proteoglycans (reviews in Vogel, 1994; Iozzo, 1998).

Proteoglycans are widely distributed in animal tissues (Dietrich *et al.*, 1976; Cássaro and Dietrich, 1977) and their biological roles are very diversified, ranging from mechanical support functions to more intricate effects on various cellular processes such as cell adhesion and recognition, motility, and proliferation (Dietrich *et al.*, 1977b, 1982; Dietrich, 1984). Proteoglycans may occur in the intracellular compartment (usually in secretory granules), at the cell surface, or in the extracellular matrix (Vogel, 1994; Iozzo, 1998). Three major classes of proteoglycans may be distinguished in the extracellular matrix: the large aggregating proteoglycans, which interact with hyaluronic acid, the small leucine-rich proteoglycans, also called "fibrilar proteoglycans" because some of them interact with fibrilar collagens, and the basement membrane proteoglycans. Perlecan is the main basement membrane proteoglycan, present in virtually all vascularized tissues. The name designates a family of multidomain proteins, expressed by different cell types, that most often carry covalently linked heparan sulfate side chains (Iozzo *et al.*, 1994).

In long term diabetes mellitus, among the most clinically significant complications are the microangiopathies, which are disruption of the normal function of vascular capillary beds. A hallmark of these pathological processes is a significant thickening of microvascular basement membrane, with chronic progression with time. Paradoxically, this thickening of basement membranes is accompanied by a loss of function, allowing charged serum molecules which are normally retained within the circulation to pass across the matrix. This produces systemic effects in skin and muscle, but problems facing investigators are those familiar to matrix researchers: the affected structure in most vascular beds are too diffusely distributed to yield adequate material for biochemical analyses. Most studies therefore focused on the kidney, since the glomerulus provides concentrate and abundant amounts of capillary basement membrane. Furthermore, one of the most marked clinical consequences of diabetes is observed in the renal glomerulus, resulting in diabetic nephropathy (Templeton, 1989). A number of approaches have been

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Table I. Characteristics of the rats 12 weeks after induction of diabetes mellitus

	Group	
Parameter	Control (n = 12)	Diabetes mellitus (n = 13)
Body weight (g)	358 ± 21	240 ± 42
Glycemia (mg/dl)	72 ± 15	426 ± 65
Urinary creatinine (mg/ml)	1.88 ± 0.14	0.28 ± 0.19
24 h urine volume (ml)	12 ± 1	88 ± 7
Systolic blood pressure (mm Hg)	127 ± 2	171 ± 8
Wet kidney weight (g)	1.00 ± 0.12	1.20 ± 0.11
Urinary albumin (mg/24 h)	0.5 ± 0.1	2.1 ± 0.5

The numbers represent mean values ± SD.

developed in the investigation of these complications, many involving experimental models of diabetes which are either induced by chemical destruction of pancreatic islet cells or by the use of strains of animals bred to be genetically susceptible to the disease. Nevertheless, a close examination of the kidney reveals the potential complexity that proteoglycans may have in a very small region. The glomerular capillary wall consists of the epithelial and endothelial coats and glomerular extracellular matrices, which are composed of basement membrane and mesangial matrix. Cell surface proteoglycans as well as extracellular matrix proteoglycans, both in glomerular basement membrane and in mesangial matrix, are present (Kanwar *et al.*, 1983a,b; Stow and Farquhar, 1985, 1987; Klein *et al.*, 1986). The mesangial matrix has been less investigated than the basement membrane, although the two matrices appear to differ with respect to their glycosaminoglycan composition (Kanwar *et al.*, 1983a,b; Farquhar, 1991).

A role for heparan sulfate in creating the normal permeability barriers of glomerular basement membrane was suggested by *in situ* digestion with glycosaminoglycan-degrading enzymes, followed by perfusion with native ferritin (Kanwar *et al.*, 1980).

Both the glomerular basement membrane and the mesangial matrix are increased in area in diabetic nephropathy (Mauer *et al.*, 1992) and this expansion is, possibly, a major contributor to diabetic renal failure. Mesangial cells exposed to high glucose concentrations have shown increased synthesis of extracellular matrix components, such as laminin, fibronectin and type IV collagen (Ayo *et al.*, 1991). Silbiger *et al.* (1993) reported that the total amount of proteoglycans did not change, but a reduction in their charge density was observed. These findings suggest that changes in extracellular matrix organization and metabolism could be involved in the pathogenesis of diabetic nephropathy, with alteration in filtration features. Previous data from our laboratory have shown, in the 12th week after strepzotocin injection, mesangial matrix expansion, a certain degree of hypercellularity and Armani-Ebstein changes at both proximal and distal renal tubules in diabetic Wistar rats. At the same time (12th week post diabetes induction) small increases in plasma creatinine concentration (Ramos, 1988) and urinary albumin excretion also occurred. Definitive and progressive albuminuria was detected in all animals only 18–20 weeks after the streptozotocin injection

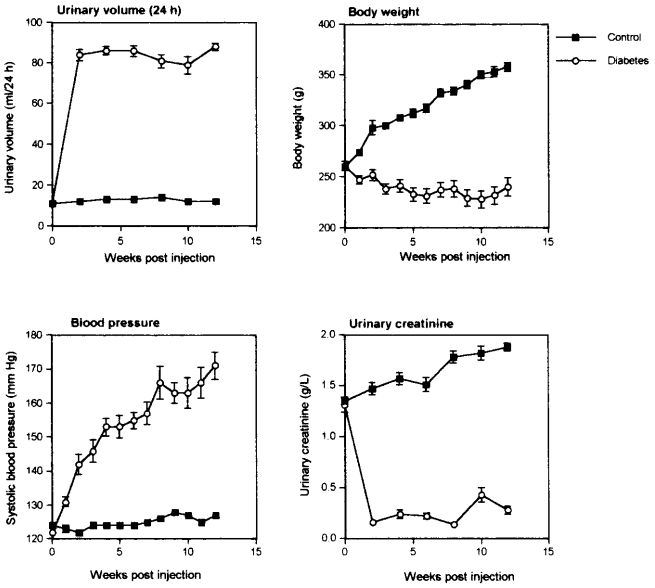

Fig. 1. Twenty-four hour urine volume, body weight, systolic blood pressure, and urinary creatinine concentration in normal (Control) and diabetic (Diabetes) rats. Each point represents mean ± SE.

(Bossolan *et al.*, 1991). These data indicate the stage of alterations that exist in the kidney at the 12th weeks in the experimental model here used. Since basement membrane thickening and mesangial expansion seem to precede the symptoms of renal dysfunction, such as albuminuria and changes in glomerular filtration rate, the aim of the present study was to investigate whether the urinary excretion of glycosaminoglycans might reflect these biochemical alterations, permitting early diagnosis of diabetic nephropathy.

Results

Characterization of streptozotocin-diabetic rats

Diabetes was induced in a group of animals by exposure to streptozotocin. Table I shows that 12 weeks after streptozotocin injection, all the experimental rats presented increased blood glucose concentration, decreased urinary creatinine concentration and increased 24 h urine volume, increased systolic blood pressure and albuminuria, as compared to age-matched controls. These animals also presented enlargement of the kidneys and, in contrast to the control animals, which gained weight, all the diabetic rats demonstrated a progressive decrease in body weight. Figure 1 shows that these changes began on the first week after streptozotocin exposure. The decrease in body weight and urinary creatinine concentration, as well as the increase in 24 h urine volume and systolic blood pressure remained significant, at $p < 0.001$ or better, from the second week on for the streptozotocin-exposed animals (Figure 1). The blood glucose levels were also increased to >200 mg/dl beginning on the first week after injection and maintained until the end of the experiment, as controlled by glycemic levels monitoring every week. All these data indicate that injection of streptozotocin resulted in the rapid development of diabetes.

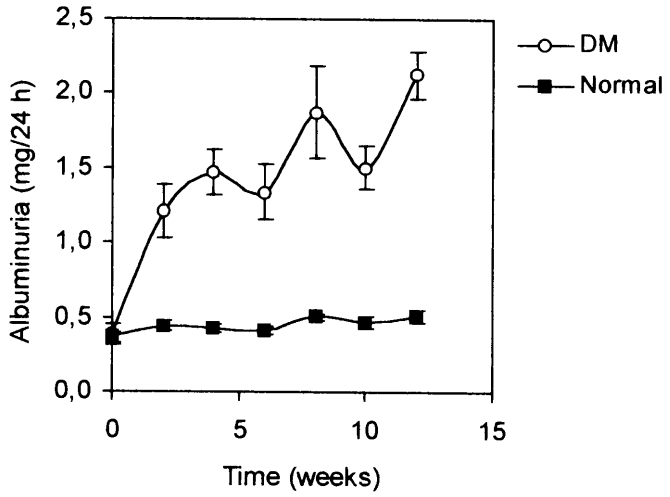

Fig. 2. Albumin excretion in normal (Control) and streptozotocin-exposed diabetic (Diabetes) rats. Each point represents mean ± SE.

Albuminuria

Figure 2 shows the mean values and the standard error of albuminuria (expressed as mg/24 h) for control and diabetic rats and Figure 3 shows some of the individual data obtained. The albumin excretion in diabetic rats increased, beginning on the second week ($p < 0.001$, Figure 2) but, for a few animals, albuminuria appeared only on the 12th week (Figure 3).

Urinary glycosaminoglycans

The urinary glycosaminoglycan excretion (expressed both as mg/l and as µg/24 h) was greatly decreased in the diabetic animals (Figures 4), from the second week on. Figure 5 shows some of the individual data obtained. All the diabetic animals presented lower glycosaminoglycan excretion on a daily basis from the 4th week on.

Agarose gel electrophoresis of rat urinary glycosaminoglycans is shown in Figure 6. Three bands migrating as chondroitin sulfate (CS), dermatan sulfate (DS), and heparan sulfate (HS) were identified. The band migrating as chondroitin sulfate was totally degraded by chondroitinase AC and the band migrating as heparan sulfate was susceptible to heparitinases I and II from *Flavobacterium heparinum*. The band migrating as dermatan sulfate was degraded by chondroitinase B and was partially degraded by chondroitinase AC, indicating that this dermatan sulfate is composed by both glucuronic acid and iduronic acid-containing disaccharide units (Población and Michelacci, 1986). Table II shows the concentrations (expressed as µg/24 h) and the relative proportions of these urinary glycosaminoglycans in the control and experimental groups. In some instances it was difficult to separate dermatan sulfate and chondroitin sulfate; so, they were quantified together. The glycosaminoglycan excretion was decreased to about 1/10 of the control in the "Diabetes" group, expressed as mg/l of urine (Figure 4), and to 1/5 as expressed as µg/24 h (Figure 4 and Table II). Both heparan sulfate and chondroitin sulfate/dermatan sulfate decreased in diabetic animals from the 2nd week on after streptozotocin exposure.

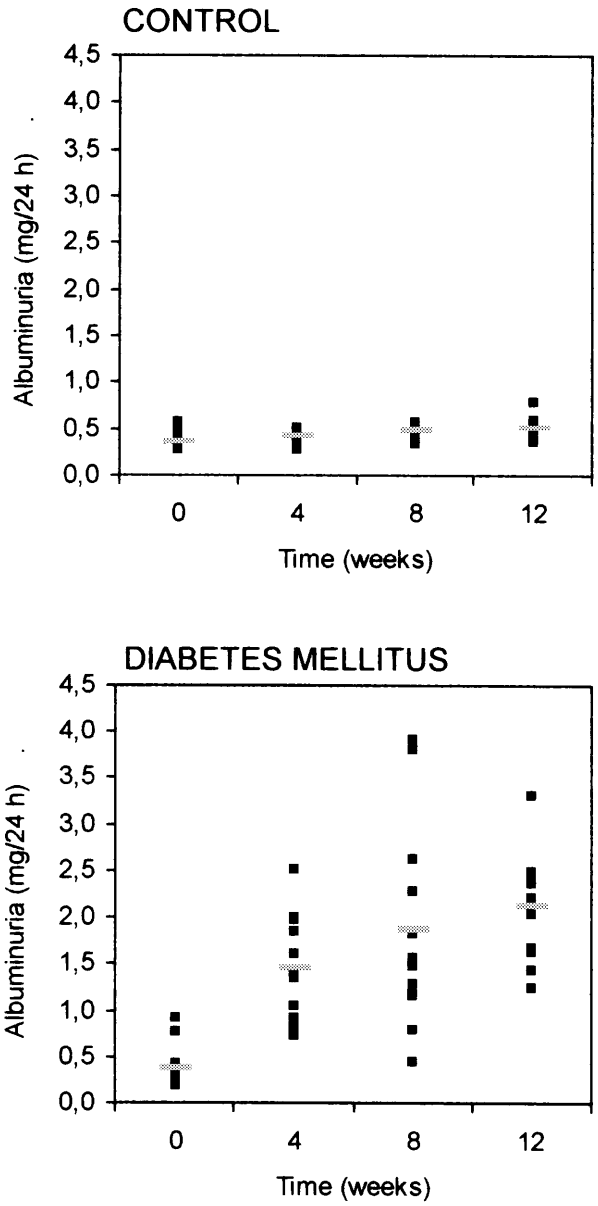

Fig. 3. Albuminuria in normal and diabetic rats. Individual data for 24 h urinary albumin excretion immediately before (0), and 4, 8, and 12 weeks after streptozotocin administration to normal rats. The control animals received placebo. The horizontal gray lines indicate the mean values.

Kidney glycosaminoglycans

At the end of experiment (12 weeks), the kidneys were removed from the control and experimental animals, weighted (Table I), and the glycosaminoglycans were extracted and submitted to agarose gel electrophoresis (Figure 7). In normal rats, only one band appeared, which was identified as heparan sulfate by a combination of agarose gel electrophoresis and enzymatic degradation with specific mucopolysaccharidase. Only trace amounts of dermatan sulfate (<1% of total) were identified. In the kidneys from diabetic rats, the heparan sulfate was also the main glycosaminoglycan, but small amounts of dermatan sulfate and chondroitin sulfate were also present. Table III shows the numerical data obtained. Although the total

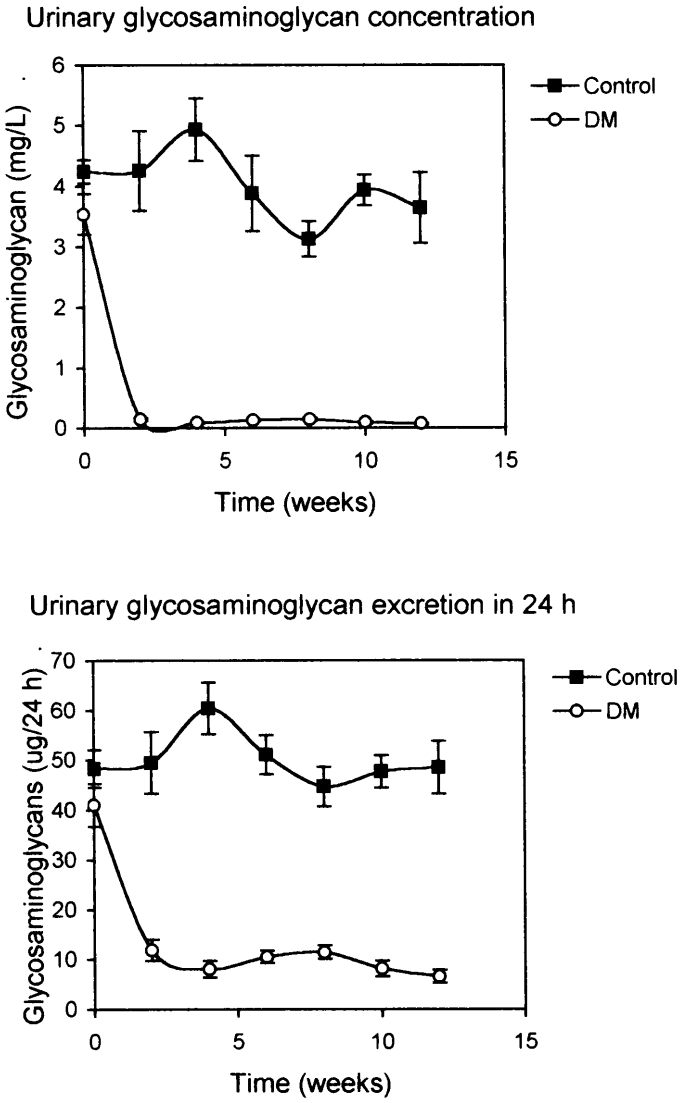

Fig. 4. Urinary glycosaminoglycan concentration and 24 h glycosaminoglycan excretion of normal (Control) and streptozotocin-exposed diabetic (DM) rats. Each point represents mean ± standard error.

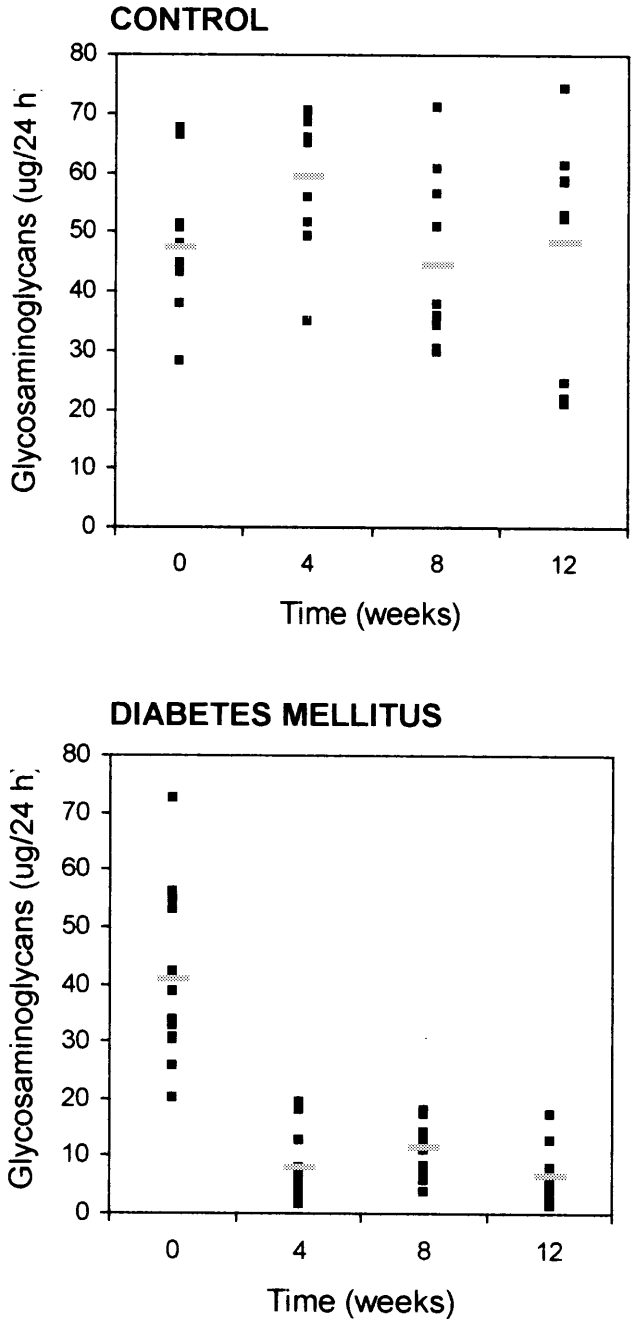

Fig. 5. Urinary glycosaminoglycan excretion in normal (Control) and diabetic rats. Individual data for 24 h urinary glycosaminoglycan excretion immediately before (0) and 4, 8, and 12 weeks after streptozotocin administration to normal rats are shown. The control animals received placebo. The horizontal gray lines indicate the mean values.

glycosaminoglycan concentration increased in the experimental group, the heparan sulfate concentration (expressed as mg/g of dry tissue) was unaltered. The increase was due to accumulation of dermatan sulfate and chondroitin sulfate in the kidney.

The kidney heparan sulfate and the urinary heparan sulfate presented similar molecular weights, in the order of 25 kDa. In contrast, the chondroitin/dermatan sulfates presented lower molecular weights (5 kDa), suggesting that they were partially degraded before excretion.

Discussion

Basement membrane thickening and mesangial expansion characterize the renal involvement in diabetic nephropathy (Osterby *et al.*, 1997; Daimon and Koni, 1998; Toth and Takebayashi, 1998; Matsumae *et al.*, 1999), which is a serious and increasingly burdensome disease for both the diabetic individual and the society. Some authors suggested that thickening is due to accumulation of basement membrane material deposited by successive cell layers (Vracko, 1974), while others proposed either a decreased susceptibility of diabetic basement membrane to degradation (Knecht *et al.*, 1987; Brownlee *et al.*, 1988), or overexpression of basement membrane components, such as laminin and type IV collagen (Asselot-Chapel *et al.*, 1996).

Table II. Urinary glycosaminoglycan excretion at the beginning of the experiment (Time 0) and 2, 4, 6, 8, 10, and 12 weeks after induction of diabetes mellitus in rats

	Time (weeks)						
	0	2	4	6	8	10	12
Control							
Total GAG[a]	47.4 ± 3.8	49.5 ± 6.2	59.5 ± 5.2	51.1 ± 3.9	44.7 ± 3.9	47.6 ± 3.3	48.5 ± 5.3
HS (%)	57	59	60	59	58	57	56
DS+CS (%)	43	41	40	41	42	43	44
Diabetes mellitus							
Total GAG[a]	41.1 ± 4.2	11.9[b] ± 2.1	8.1[b] ± 1.7	10.5[b] ± 1.3	11.5[b] ± 1.3	8.2[b] ± 1.6	6.6 **± 1.3
HS (%)	56	58	60	58	59	59	60
DS+CS (%)	44	42	40	42	41	41	40

[a]Expressed as μg/24 h (average ± SE).

We have previously shown that cultured mesangial cells synthesize a mixture of dermatan sulfate and heparan sulfate and most of the dermatan sulfate is released to the culture medium. Mesangial cells from rats which had been diabetic for 4 months incorporate twice the amount of ^{35}S-sulfate in glycosaminoglycans, especially dermatan sulfate, as compared to mesangial cells from normal rats. This increase is proportional to the duration of diabetes, since the mesangial cells from animals that had been diabetic for 5 or 6 months presented higher ^{35}S-incorporations in glycosaminoglycans, particularly dermatan sulfate, than cells from animals which had been diabetic for 4 months (Hadad *et al.*, 1996). The dermatan sulfate was secreted to the medium as two proteoglycans, identified as members of the small matrix proteoglycan decorin/biglycan family (Krusius and Ruoslahti, 1986; Fisher *et al.*, 1989; Border *et al.*, 1990; Harper and Mason, 1994). The extracellular matrix proteoglycans and glycosaminoglycans are supposed to play a role in the control of cell proliferation (Dietrich, 1984) and decorin is known to bind collagen (Oldberg and Rouslahti, 1982). So, decorin and other extracellular matrix proteoglycans, such as biglycan, may functionally facilitate the assembly of extracellular matrix (Ruoslahti and Pierschacher, 1987). The stimulation of proteoglycan production in mesangial cells obtained from diabetic rats may be related to the accumulation of dermatan sulfate and chondroitin sulfate here reported and possibly to the mesangial matrix expansion observed in diabetes mellitus. Taken together, these data suggest morphological and biochemical alterations in renal extracellular matrix.

The present study was designed to investigate whether the urinary excretion of glycosaminoglycans might reflect these biochemical alterations in diabetic nephropathy. All the diabetic animals were hyperglycemic, hypertense, and did not gain weight during all the experimental period. Albuminuria appeared from the second week on.

It is apparent from our results that the chronic diabetic state induced by streptozotocin resulted in marked decrease of urinary glycosaminoglycan excretion, on a daily basis. The onset of this feature was a very early event in the development of diabetes. Both heparan sulfate and chondroitin/dermatan sulfate concentrations were decreased. Decreased excretion of glycosaminoglycans have also been observed by others in patients with diabetic nephropathy and other glomerular diseases (Tencer *et al.*, 1997).

The urinary heparan sulfate presents modal molecular weight similar to the kidney heparan sulfate, suggesting that

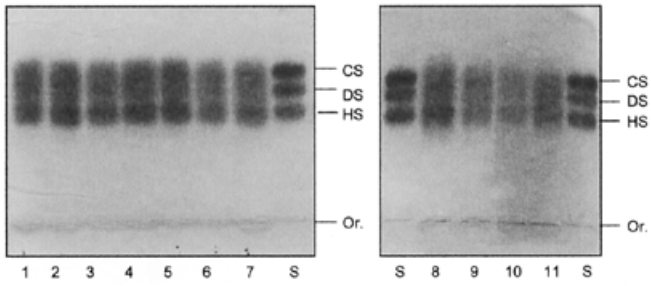

Fig. 6. Agarose gel electrophoresis of rat urinary glycosaminoglycans from normal and diabetic rats. The glycosaminoglycans were extracted from urine samples (10 ml for nondiabetic rats and 50 ml for diabetic rats) as described in *Materials and methods*. The dried material was resuspended in 50 μl, and 5 μl aliquots were applied to agarose gel slabs and submitted to electrophoresis as described previously (Dietrich *et al.*, 1977a). The glycosaminoglycans were stained by toluidine blue. Or, origin; S, mixture of standard glycosaminoglycans; CS, chondroitin sulfate; DS, dermatan sulfate; and HS, heparan sulfate. 1–7, Urine samples from normal rats; 8–11, urine samples from diabetic rats.

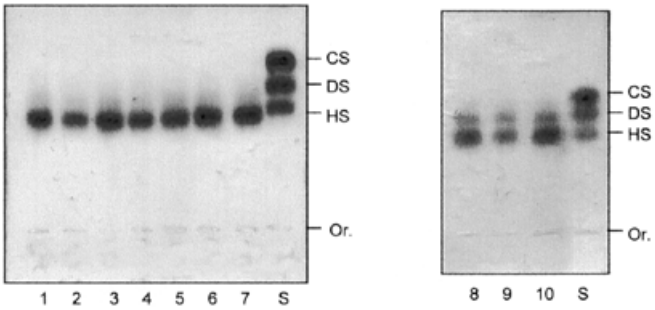

Fig. 7. Agarose gel electrophoresis of kidney glycosaminoglycans from normal and diabetic rats. Or, Origin; S, mixture of standard glycosaminoglycans; CS, chondroitin sulfate; DS, dermatan sulfate; and HS, heparan sulfate. 1–7, Kidneys from normal rats; 8–10, kidneys from diabetic rats.

Table III. Kidney glycosaminoglycans from normal and diabetic rats

	Glycosaminoglycans						
	Total amounts	Heparan sulfate		Dermatan sulfate		Chondroitin sulfate	
Group	(mg/g dry tissue)	(mg/g dry tissue)	(%)	(mg/g dry tissue)	(%)	(mg/g dry tissue)	(%)
Control	1.88	1.86	99	0.02	1	n.d.[a]	0
Diabetes mellitus	2.14	1.86	87	0.17	8	0.11	5

[a]n.d., Not detected.

the urinary heparan sulfate could be, at least in part, of renal origin. In contrast, only trace amounts of dermatan sulfate are present in normal rat kidney, while chondroitin sulfate was not detected, suggesting that the urinary chondroitin/dermatan sulfate does not come from the kidney. Although a decrease in glomerular heparan sulfate concentration in diabetic nephropathy has been reported by other authors (Templeton, 1989; Tamsma *et al.*, 1994; van den Born *et al.*, 1995), we did not find any changes in the heparan sulfate amounts (per tissue dry weight). Nevertheless, it is possible that the structural organization of the expanded basement membrane and mesangial matrix lead to a looser arrangement of proteoglycans, affecting the tissue filtration properties. Many authors reported the upregulation and overexpression of glomerular extracellular matrix components in diabetic nephropathy and other models of glomerulonephritis (Del Prete *et al.*, 1998; Kamata *et al.*, 1990; Mizuno *et al.*, 1999). Nevertheless, the synthesis of these components may be unbalanced. For instance, the expression of type IV collagen α3(IV), α4(IV), and α5(IV) chains is dissociated from the α1(IV) and α2(IV) expression in the kidney of diabetic mice (Funabiki *et al.*, 1998). This unbalanced synthesis may affect the type IV collagen assembly and contribute to the basement membrane thickening. This may also be related to the increased urinary excretion of type IV collagen observed in non-insulin-dependent diabetes mellitus (Kado *et al.*, 1996).

The decreased urinary glycosaminoglycan concentration could also be related to the decreased glycosaminoglycan contents of other tissues in diabetic animals reported by Cechowska-Pasko *et al.* (1996). Altered urinary excretion of other extracellular matrix macromolecular components, such as laminin, collagen type IV and fibronectin was also reported in diabetic nephropathy (Jaackle-Meyer *et al.*, 1995).

We believe our results show a potentially important value for the urinary glycosaminoglycan measurement in the early diagnosis of diabetic nephropathy, appearing together with (or even before) the onset of albuminuria.

Materials and methods

Materials

Chondroitin 4-sulfate (from whale cartilage), chondroitin 6-sulfate (from shark cartilage), dermatan sulfate (from hog skin), streptozotocin, papain, Sigma Diagnostic Creatinine Kit (cat. no. 555-A), and Amberlite IRA-900 were purchased from Sigma Chemical Co. (St. Louis, MO). Heparan sulfate (from bovine pancreas), heparitinases I and II, and chondroitinases AC and B (from *Flavobacterium heparinum*) were prepared by methods previously described (Dietrich and Nader, 1974; Michelacci *et al.*, 1987; Nader *et al.*, 1990). Agarose (standard, low M_r) and the protein molecular size markers were purchased from Bio-Rad Laboratories (Richmond, CA). Acrylamide, N,N-methylenebisacrylamide, N,N,N′,N′-tetramethylethylenediamine, 1,3-diaminopropane and ethylenediamine were from Aldrich Chemical Co. (Milwaukee, WI).

Experimental animals

Male Wistar rats, 12–14 weeks of age (220–260 g body weight), were separated in two groups: "Control" and "Diabetes." Diabetes was induced in 21 rats by a single injection of streptozotocin (50 mg/kg body weight). The drug was dissolved in 300 µl of 10 mM sodium citrate buffer (pH 4.5) and injected into the tail vein. These animals were fed standard laboratory chow and a 5% glucose solution *ad libitum*, for 48 h. Afterwards, the glucose solution was replaced by water. The glycemia was measured (Glycofilm, Bayer Diagnostics MFG Ltd., Bridgent, Mild Glamorgan, England) and all the diabetic animals presented blood glucose concentrations higher than 200 mg/dl. Twelve age-matched animals that served as controls ("Control" group) were fed standard laboratory chow and water *ad libitum*. The systolic blood pressure was measured in conscious rats by tail-cuff method. The body weight, and the blood glucose of the animals were measured weekly and every 2 weeks each rat was placed in a metabolic cage (model 650–0350, Nalgene Company, Rochester, NJ) for 24 h urine collection. Total volume was measured, the urine was centrifuged to remove debris, and used for the determination of albumin, creatinine, and glycosaminoglycans. Two animals of the "Diabetes" group died during the experimental period and six diabetic animals presented urinary infections and were excluded. All the diabetic rats maintained glycemia higher than 200 mg/dl during all the experimental period.

Quantification of urinary albumin, protein and creatinine

Albumin urinary excretion was determined by the radial immunodiffusion method based on precipitation with rabbit antibodies against rat albumin (Mancini *et al.*, 1965). The diameter of the precipitation halo was measured after 48 h incubation and compared to a standard curve of rat albumin.

Protein was measured by the Coomassie blue method, described by Spector (1978) and urinary creatinine was measured by the picric acid reaction in alkaline conditions (Sigma creatinine kit).

Isolation, identification, and quantification of kidney glycosaminoglycans

At the end of the experiment (12 weeks), the rat kidneys were removed, ground in 10 volumes of acetone and, after standing overnight at room temperature, the fragments were collected by centrifugation and dried under vacuum. The dried material was resuspended in 0.08 M phosphate-cysteine buffer pH 6.5, containing 0.02 M EDTA and 100 mg/l papain (10 ml/g of dry material) and incubated at 50°C overnight. The incubation mixtures were cooled in an ice bath and trichloroacetic acid and NaCl were added to final concentrations of 5% and 1 M, respectively. After standing 15 min in the ice bath, the precipitate formed was removed, the pH of the supernatant was adjusted to 7 and two volumes of ethanol were added. The precipitate formed after 24 h at –20°C was collected by centrifugation and dried. The dried material was resuspended in water and the glycosaminoglycans were identified by a combination of agarose gel electrophoresis in 0.05 M 1,3-diaminopropane-acetate buffer (pH 9), and enzymatic degradation with specific mucopolysaccharidases, as already described (Dietrich *et al.*, 1977a).

The enzymatic degradations with chondroitinases B and AC from *Flavobacterium heparinum* were carried out as described previously (Petricevich and Michelacci, 1990). Briefly, aliquots of the glycosaminoglycans (50–100 μg) were incubated with 2×10^{-4} units of either chondroitinase B or chondroitinase AC or a mixture of chondroitinases B and AC, in 0.05 M ethylenediamine-acetate buffer, pH 8.0, in a final volume of 20 μl. One unit of enzyme was defined as the amount that degrades 1 μmol of substrate (expressed as disaccharide) per min., at the optimum temperature for each enzyme. After 6 h incubation at either 20°C (for chondroitinase B or the mixture of chondroitinases B + AC) or 37°C (for chondroitinase AC), the incubation mixtures were spotted on Whatman no. 1 paper and chromatographed in isobutyric acid:1.25 M NH_4OH (5:3, v/v) for 24 h. The products formed were visualized by silver nitrate staining of the paper chromatograms and quantified by densitometry.

For degradations with heparitinases I and II from *Flavobacterium heparinum*, aliquots of the glycosaminoglycans (50–100 μg) were incubated with 2×10^{-4} units of either heparitinases I or II or a mixture of both enzymes, in 0.05 M ethylenediamine-acetate buffer, pH 7.0, in a final volume of 20 μl. One unit of enzyme was defined as the amount that degrades 1 μmol of substrate (expressed as disaccharide) per min. After 24 h incubation at 30°C, the incubation mixtures were spotted on Whatman no. 1 paper and chromatographed as above described for chondroitinases. The products formed were visualized by silver nitrate staining of the paper chromatograms and quantified by densitometry.

Isolation, identification, and quantification of urinary glycosaminoglycans

To assess the urinary glycosaminoglycan concentration, samples of urine (10 ml for normal rats and 50 ml for diabetic rats) were incubated with 400 μl of Amberlite IRA 900 at 60°C, under agitation (urine of normal rats was diluted with 10 ml of distilled water). After 18–24 h, the resin was collected, exhaustively washed with hot water (60°C) and the glycosaminoglycans were eluted in a step wise fashion with 0.3 M NaCl (1 ml), 0.3 M NaCl (0.5 ml), 1 M NaCl (1 ml), and 1 M NaCl (0.5 ml). To the supernatants, four volumes of ethanol were added and the precipitates formed at –20°C overnight were collected by centrifugation and dried. The dried material was resuspended in water and analyzed as described in the previous section.

Polyacrylamide gel electrophoresis of the glycosaminoglycans was performed in a BRL vertical mini-system. Samples for SDS–PAGE were mixed with sample buffer and submitted to electrophoresis as already described (Laemmli, 1970). Aliquots (5 μl) were applied to 6% gels and run for 45–60 min at constant power. Gels were stained with toluidine blue.

Statistical analysis

Parametric and nonparametric statistical tests were used. Student's two-sided t test for paired samples and the Wilcoxon nonparametric sample rank test for independent groups were used to compare the mean difference in body weight, blood glucose concentration, urinary creatinine concentration, 24 h urine volume, systolic blood pressure, albuminuria, and the urinary glycosaminoglycans of the experimental and control groups. The significance level is indicated in each experiment.

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References

Asselot-Chapel,C., Borchiellini,C., Labat-Robert,J. and Kern,P. (1996) Expression of laminin and type IV collagen by basement membrane-producing EHS tumors in streptozotocin-induced diabetic mice: *in vivo* modulation by low-molecular-weight heparin fragments. *Biochem. Pharmacol.*, **52**, 1695–1701.

Ayo,S.H., Radnik,R.A., Glass,W.F., Garoni,J.A., Rampt,E.R., Appling,D.R. and Kreisberg,J.I. (1991) Increased extracellular matrix synthesis and mRNA in mesangial cells grown in high glucose medium. *Am. J. Physiol.*, **269**, F185–F191.

Border,W.A., Okuda,S., Languino,L.R. and Ruoslahti,E. (1990) Transforming growth factor-β regulates production of proteoglycans by mesangial cells. *Kidney Int.*, **37**, 689–695.

Bossolan,D., Ginoza,M., Santos,A.C., Tavares,A., Marson,O., Ribeiro,A.B. and Kohlmann Jr.,O. (1991) Insulin therapy in streptozotocin (STZ)-induced diabetes melitus (DM): effects upon blood pressure (BP) and microalbuminuria. *Hypertension*, **17**, 461.

Brownlee,M., Cerami,A. and Vlassara,H. (1988) Advanced glycosylation end products in tissue and the biochemical basis of diabetic complications. *N. Engl. J. Med.*, **318**, 1315–1321.

Cássaro,C.M.F. and Dietrich,C.P. (1977) Distribution of sulfated mucopolysaccharides in invertebrates. *J. Biol. Chem.*, **252**, 2254–2261.

Cechowska-Pasko,M., Palka,J. and Bankowski,E. (1996) Decrease in the glycosaminoglycan content in the skin of diabetic rats. The role of IGF-I, IGF-binding proteins and proteolytic activity. *Mol. Cell. Biochem.*, **154**, 1–8.

Daimon,S. and Koni,I. (1998) Glomerular enlargement in the progression of mesangial proliferative glomerulonephritis. *Clin. Nephrol.*, **49**, 145–152.

Del Prete,D., Anglani,F., Ceol,M., D'Angelo,A., Forino,M., Vianello,D., Baggio,B. and Gambaro,G. (1998) Molecular biology of diabetic glomerulosclerosis. *Nephrol. Dial. Transplant.*, **13** (Suppl 8), 20–25.

Dietrich,C.P. (1984) A model for cell–cell recognition and control of cell growth mediated by sulfated glycosaminoglycans. *Brazilian J. Med. Biol. Res.*, **17**, 5–15.

Dietrich,C.P. and Nader,H.B. (1974) Fractionation and properties of four heparitin sulfates from beef lung tissue. Isolation and partial characterization of a homogeneous species of heparitin sulfate. *Biochim. Biophys. Acta*, **343**, 34–44.

Dietrich,C.P., Sampaio,L.O. and Toledo,O.M.S. (1976) Characteristic distribution of sulfated mucopolysaccharides in different tissues and in their respective mitochondria. *Biochem. Biophys. Res. Commun.*, **71**, 1–10.

Dietrich,C.P., McDuffie,N. and Sampaio,L.O. (1977a) Identification of acidic mucopolysaccharides by agarose gel electrophoresis. *J. Chromatogr.*, **130**, 299–304.

Dietrich,C.P., Sampaio,L.O., Toledo,O.M.S. and Cássaro,C.M.F. (1977b) Cell recognition and adhesiveness. A possible role for the sulfated mucopolysaccharides. *Biochem. Biophys. Res. Commun.*, **75**, 329–336.

Dietrich,C.P., Armelin,H.A., Nogueira,Y.L., Nader,H.B. and Michelacci,Y.M. (1982) Turnover, change of composition with rate of cell growth and effect of phenylxyloside on synthesis and structure of cell surface sulfated glycosaminoglycans of normal and transformed cells. *Biochim. Biophys. Acta*, **717**, 387–397.

Farquhar,M.G. (1991) The glomerular basement membrane: a selective macromolecular filter. In Hay,E.D. (ed.), *Cell Biology of Extracellular Matrix*, second edition. Plenum, New York, pp. 365–418.

Fisher,L.W., Termine,J.D. and Young,M.F. (1989) Deduced protein sequence of bone small proteoglycan I (biglycan) shows homology with proteoglycan II (decorin) from several nonconnective tissues in a variety of species. *J. Biol. Chem.*, **264**, 4571–4576.

Funabiki,K., Makita,Y., Yamamoto,M., Shike,T., Fukui,M., Sumiyoshi,Y. and Tomino,Y. (1998) Dissociated expression of collagen type IV subchains in diabetic kidneys of KKAy mice. *Nephron*, **80**, 208–213.

Hadad,S.J., Michelacci,Y.M. and Schor,N. (1996) Proteoglycans and glycosaminoglycans synthesized *in vitro* by mesangial cells from normal and diabetic rats. *Biochim. Biophys. Acta*, **1290**, 18–28.

Harper,K. and Mason,R. (1994) Increased expression of decorin but not of biglycan in hyperglucaemic cultures of human mesangial cells. *Biochem. Soc. Trans.*, **22**, 184S.

Iozzo,R.V. (1998) Matrix proteoglycans: from molecular design to cellular function. *Annu. Rev. Biochem.*, **67**, 609–652.

Iozzo,R.V., Cohen,I.R., Grassel,S. and Murdoch,A.D. (1994) The biology of perlecan: the multifaceted heparan sulphate proteoglycan of basement membranes and pericellular matrices. *Biochem. J.*, **302**, 625–639.

Jaackle-Meyer,I., Szukics,B., Neubauer,K., Metze,V., Petzoldt,R. and Stolte,H. (1995) Extracellular matrix proteins as early markers in diabetic nephropathy. *Eur. J. Clin. Chem. Clin. Biochem.*, **33**, 211–219.

Kado,S., Aoki,A., Wada,S., Katayama,Y., Kugai,N., Yoshizawa,N. and Nagata,N. (1996) Urinary type IV collagen as a marker for early diabetic nephropathy. *Diabetes Res. Clin. Pract.*, **31**, 103–108.

Kamata,T., Muso,E., Yashiro,M., Kawamura,T., Oyama,A., Matsushima,H., Takeuchi,E., Yoshida,H. and Sasayama,S. (1990) Up-regulation of glomerular extracellular matrix and transforming growth factor-beta expression in RF/J mice. *Kidney Int.*, **55**, 864–876.

Kanwar,Y.S., Linker,A. and Farquhar,M.G. (1980) Increased permeability of the glomerular basement membrane to ferritin after removal of glycosaminoglycans (heparan sulfate) by enzyme digestion. *J. Cell Biol.*, **86**, 688–693.

Kanwar,Y.S., Jakubowski,M.L. and Rosenzweig,L.J. (1983a) Distribution of sulfated glycosaminoglycans in the glomerular basement membrane and mesangial matrix. *Eur. J. Cell Biol.*, **31**, 290–295.

Kanwar,Y.S., Rosenzweig,L.J. and Jakubowski,M.L. (1983b) Distribution of *de novo* synthesized sulfated glycosaminoglycans in the glomerular basement membrane and mesangial matrix. *Lab. Invest.*, **49**, 216–225.

Klein,D.J., Brown,D.M. and Oegema,T.R., Jr. (1986) Partial characterization of heparan and dermatan sulfate proteoglycans synthesized by normal rat glomeruli. *J. Biol. Chem.*, **261**, 16636–16652.

Knecht,R., Leber,R. and Hasslacher,C. (1987) Degradation of glomerular basement membrane in diabetes. I. Susceptibility of diabetic and nondiabetic basement membrane to proteolytic degradation of isolated glomeruli. *Res. Exp. Med.*, **187**, 323–328.

Krusius,T. and Ruoslahti,E. (1986) Primary structure of an extracellular matrix proteoglycan core protein deduced from cloned cDNA. *Proc. Natl Acad. Sci. USA*, **83**, 7683–7687.

Laemmli,U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680–685.

Mancini,G., Carbonara,A.O. and Heremans,J.F. (1965) Immunochemical quantitation of antigens by single radial immunodifusion. *Immunochemistry*, **2**, 235–254.

Matsumae,T., Jimi,S., Uesugi,N., Takebayashi,S. and Naito,S. (1999) Clinical and morphometrical interrelationships in patients with overt nephropathy induced by non-insulin-dependent diabetes mellitus. A light- and electron-microscopy study. *Nephron*, **81**, 41–48.

Mauer,S.N., Lane,P., Hattori,M., Fioretto,P. and Steffes,M.W. (1992) Renal structure and function in insulin-dependent diabetes mellitus and type I membranoproliferative glomerulonephritis in humans. *J. Am. Soc. Nephrol.*, **2**, S181–S184.

Michelacci,Y.M., Horton,D.S.P.Q. and Población,C.A. (1987) Isolation and characterization of an induced chondroitinase ABC from *Flavobacterium heparinum*. *Biochim. Biophys. Acta*, **923**, 291–301.

Mizuno,S., Mizuno-Horikawa,Y., Yue,B.F., Okamoto,M. and Kurosawa,T. (1999) Nephrotic mice (ICGN strain): a model of diffuse mesangial sclerosis in infantile nephrotic syndrome. *Am. J. Nephrol.*, **19**, 73–82.

Nader,H.B., Porcionatto,M.A., Tersariol,I.L.S., Pinhal,M.A.S., Oliveira,F.W., Moraes,C.T. and Dietrich,C.P. (1990) Purification and substrate specificity of heparitinase I and heparitinase II from *Flavobacterium heparinum*. *J. Biol. Chem.*, **265**, 16807–16813.

Oldberg,A. and Ruoslahti,E. (1982) Interaction between chondroitin sulfate proteoglycan, fibronectin and collagen. *J. Biol. Chem.*, **257**, 4859–4863.

Osterby,R., Asplund,J., Bangstad,H.J., Nyberg,G., Rudberg,S., Viberti,G. and Walker,J.D. (1997) Glomerular volume and the glomerular vascular pole area in patients with insulin-dependent diabetes mellitus. *Virchows Arch.*, **431**, 351–357.

Petricevich,V.L. and Michelacci,Y.M. (1990) Proteoglycans synthesized *in vitro* by nude and normal mouse peritoneal macrophages. *Biochim. Biophys. Acta*, **1053**, 135–143.

Población,C.A. and Michelacci,Y.M. (1986) Structural differences of dermatan sulfates from different origins. *Carbohydr. Res.*, **147**, 87–100.

Ramos,O.L. (1988) *Diabetes* mellitus and hypertension. State of Art Lecture. *Hypertension*, **11** (Suppl I), I14–I18.

Ruoslahti,E. and Pierschacher,M.D. (1987) New perspectives in cell adhesion: EGD and integrins. *Science*, **238**, 491–497.

Silbiger,S., Crowley,S., Shan,Z., Brownlee,M., Satriano,J. and Schlondorff,D. (1993) Non-enzymatic glycation of mesangial matrix and prolonged exposure of mesangial matrix to elevated glucose reduces collagen synthesis and proteoglycan charge. *Kidney Int.*, **43**, 853–864.

Spector,T. (1978) Refinement of the Coomassie blue method of protein quantitation. A simple and linear spectrophotometric assay of 0.5 to 5 mg of protein. *Anal. Biochem.*, **86**, 142–145.

Stow,J.L. and Farquhar,M.G. (1985) Basement membrane heparan sulfate proteoglycans are concentrated in the lamina rara and in the podocytes of the rat renal glomerulus. *Proc. Natl. Acad. Sci. USA*, **82**, 3296–3300.

Stow,J.L. and Farquhar,M.G. (1987) Distinctive populations of basement membrane and cell membrane heparan sulfate proteoglycans are produced by culture cell lines. *J. Cell Biol.*, **105**, 529–539.

Tamsma,J.T., van den Born,J., Bruijn,J.A., Assmann,K.J.M., Weening,J.J., Berden,J.H.M., Wieslander,J., Schrama,E., Hermans,J., Veerkamp,J.H., Lemkes,H.H.P.J. and van der Woude,F.J. (1994) Expression of glomerular extracellular matrix components in human diabetic nephropathy: decrease of heparan sulphate in the glomerular basement membrane. *Diabetologogia*, **37**, 313–320.

Templeton,D.M. (1989) Retention of glomerular basement membrane-proteoglycans accompanying loss of anionic site staining in experimental diabetes. *Lab. Invest.*, **61**, 202–211.

Tencer,J., Torffvit,O., Bjornsson,S., Thysell,H., Grubb,A. and Rippe,B. (1997) Decreased excretion of glycosaminoglycans in patients with primary glomerular diseases. *Clin. Nephrol.*, **48**, 212–219.

Thomas,G.J., Mason,R.M. and Davies,M. (1991) Characterization of proteoglycans synthesized by human adult glomerular mesangial cells in culture. *Biochem. J.*, **277**, 81–88.

Toth,T. and Takebayashi,S. (1998) Glomerular hypertrophy as a prognostic marker in childhood IgA nephropathy. *Nephron*, **80**, 285–291.

van den Born,J., van Kraats,A.A., Bakker,M.A.K., Assmann,K.J.M., Dijkman,H.B.P.M., van der Laak,J.A.W.M. and Berden,J.H.M. (1995) Reduction of heparan sulphate-associated anionic sites in the glomerular basement membrane of rats with streptozotocin-induced diabetic nephropathy. *Diabetologia*, **38**, 1169–1175.

Vogel,K.G. (1994) Glycosaminoglycans and Proteoglycans. In *Extracellular Matrix Assembly and Structure*. Academic Press, New York.

Vracko,R. (1974) Basal lamina layering in diabetes mellitus. *Diabetes*, **23**, 94–104.