

Differential Expression of Nephrin According to Glomerular Size in Early Diabetic Kidney Disease

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ABSTRACT

Diabetic nephropathy (DN) is clinically characterized by proteinuria. Many studies tried to demonstrate a relationship between proteinuria and changes in nephrin in various forms of glomerular diseases including DN, but the results are not consistent. Glomerular hypertrophy occurs in DN, yet hypertrophy does not develop in all glomeruli concurrently. For investigation of the differences in nephrin expression according to glomerular size, glomeruli were isolated from 10 control and 10 streptozotocin-induced diabetic rats at 6 wk after the induction of diabetes by a sieving technique using sieves with pore sizes of 250, 150, 125, and 75 μm . Glomeruli then were classified into large glomeruli (LG; on the 125- μm sieve) and small glomeruli (SG; on the 75- μm sieve) groups. Glomerular volumes were determined using an image analyzer, and mRNA and protein expression was determined by real-time PCR and Western blot, respectively. The mean volumes of diabetic LG ($1.51 \pm 0.06 \times 10^6 \mu\text{m}^3$) and control LG ($1.37 \pm 0.05 \times 10^6 \mu\text{m}^3$) were significantly higher than those of diabetic SG ($0.94 \pm 0.03 \times 10^6 \mu\text{m}^3$) and control SG ($0.87 \pm 0.03 \times 10^6 \mu\text{m}^3$; $P < 0.01$). Nephrin mRNA expression was significantly reduced in the diabetic LG group compared with the diabetic SG and control glomeruli groups ($P < 0.05$). In contrast, nephrin mRNA expression was significantly higher in the diabetic SG group compared with the diabetic LG and control glomeruli groups ($P < 0.05$). Even after correction for 18s rRNA and Wilms' tumor-1 mRNA expression, the differences in nephrin mRNA expression remained significant. The expression of nephrin protein showed a similar pattern to the mRNA expression. In conclusion, these data suggest that the nephrin gene is differentially expressed according to glomerular size. Furthermore, more hypertrophied glomeruli with lesser nephrin expression may be responsible for albuminuria in the early stage of DN.

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Diabetic nephropathy (DN), the leading cause of ESRD in the United States,¹ is clinically characterized by proteinuria.² The underlying pathologic change responsible for proteinuria in various glomerular diseases is the loss of size-selective and/or charge-selective properties of the glomerular filtration barrier.^{3–7} The glomerular filtration barrier is composed of three layers: A fenestrated endothelial layer, a glomerular basement membrane (GBM), and podocyte foot processes connected by a slit diaphragm. Traditionally, the GBM has been considered a coarse filter that restricts large molecules,

whereas the slit diaphragm was thought to function as a fine filter that contributes to ultimate size selec-

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tivity, permitting permeability only to molecules that are smaller than albumin.⁸

Nephrin, a product of the *NPHS1* gene that is mutated in patients with congenital nephrotic syndrome of the Finnish type,⁹ was the first protein demonstrated to comprise the slit diaphragm.^{10,11} Experiments that were designed to demonstrate a relationship between changes in nephrin expression and/or localization and proteinuria in various forms of glomerular diseases, including DN, have been inconsistent.^{12–19} A reduction in nephrin expression has been observed in experimental glomerular diseases¹² and in adult patients with primary acquired nephrotic syndrome¹⁵ but not in pediatric patients with glomerular diseases.¹⁶ In cases of experimental DN, one study demonstrated a reduction in nephrin mRNA and protein expressions in streptozotocin-induced diabetic spontaneously hypertensive rats,¹⁴ whereas another study in streptozotocin-induced diabetic rats and in nonobese diabetic mice revealed an increase in nephrin mRNA levels.¹³ The reasons for the divergence of changes in nephrin expression in DN are not clear, but differences between species, duration of diabetes, or accompanying hypertension may contribute to these disparities.

Most studies on the glomerular expression of certain molecules have been performed with glomeruli that were isolated by either microdissection or a sieving technique. At the time of microdissection, especially in studies with diabetic glomeruli, it is apparent that all of the glomeruli are not the same in size. A difference in gene expression according to the size of glomeruli may exist but has never been studied. We hypothesized that differential gene expression might occur depending on glomerular size and that the differences in glomerular size as a result of diverse isolation techniques could lead to apparent discordance of gene expression. In this study, we investigated the differences in nephrin expression between relatively small and large glomeruli that were isolated from diabetic rats. We focused on the expression of nephrin, the most important filtration barrier-associated molecule, because of the conflicting reports on its expression patterns in DN.

RESULTS

Animal Data

All rats gained weight during the 6-wk experimental period, but weight gain was higher in control compared with diabetic rats ($P < 0.01$). The ratio of kidney weight to body weight in diabetic rats ($1.07 \pm 0.04\%$) was significantly higher than in control rats ($0.64 \pm 0.02\%$; $P < 0.05$).

The mean blood glucose levels of control and diabetic rats were 101.7 ± 1.9 and 483.3 ± 7.3 mg/dl, respectively ($P < 0.01$). Compared with the control group (0.29 ± 0.04 mg/d), 24-h urinary albumin excretion was significantly higher in the diabetic group (1.09 ± 0.17 mg/d; $P < 0.05$; Table 1).

Table 1. Body weight, kidney weight, kidney weight/body weight, blood glucose, and 24-h urinary albumin excretion of the two groups

Parameter	Control (n = 10)	Diabetic (n = 10)
Body weight after 6 wk (g)	418.00 ± 5.00	295.00 ± 4.00 ^a
Kidney weight (g)	2.69 ± 0.05	3.15 ± 0.07 ^b
Kidney weight/body weight (%)	0.64 ± 0.02	1.07 ± 0.04 ^b
Blood glucose (mg/dl)	101.70 ± 1.90	483.30 ± 7.30 ^a
24-h urinary albumin excretion (mg/d)	0.29 ± 0.04	1.09 ± 0.17 ^b

^a $P < 0.01$ versus control.

^b $P < 0.05$ versus control.

Glomerular Volume

We first examined the volume of glomeruli that were found on the 125- and 75- μm sieves. The mean volumes of diabetic large glomeruli (LG; $1.51 \pm 0.06 \times 10^6 \mu\text{m}^3$) and control LG ($1.37 \pm 0.05 \times 10^6 \mu\text{m}^3$) were significantly higher than those of diabetic small glomeruli (SG; $0.94 \pm 0.03 \times 10^6 \mu\text{m}^3$) and control SG ($0.87 \pm 0.03 \times 10^6 \mu\text{m}^3$; $P < 0.01$; Figures 1 and 2). The proportions of decapsulated glomeruli on the 125- μm sieve in control and diabetic rats were 89.6 ± 3.4 and $91.3 \pm 5.7\%$ and on the 75- μm sieve were 92.9 ± 2.7 and $92.0 \pm 3.1\%$, respectively.

Glomerular Nephrin mRNA Expression

We next examined the changes of glomerular nephrin mRNA expression according to the size of glomeruli. Nephrin mRNA expression was significantly reduced in diabetic LG (0.49 ± 0.10) compared with diabetic SG (1.71 ± 0.21) and control glomeruli (SG reference value 1; LG 1.05 ± 0.16 ; $P < 0.05$), whereas its expression was significantly higher in diabetic SG compared with diabetic LG and control glomeruli ($P < 0.05$; Figure 3A). In contrast, there was no statistically significant difference in glomerular 18s rRNA and Wilms' tumor-1 (WT-1) mRNA expression among the four groups (data not shown). Even after correction for 18s rRNA expression, the differences in nephrin mRNA expression between groups remained significant ($P < 0.05$; Figure 3B).

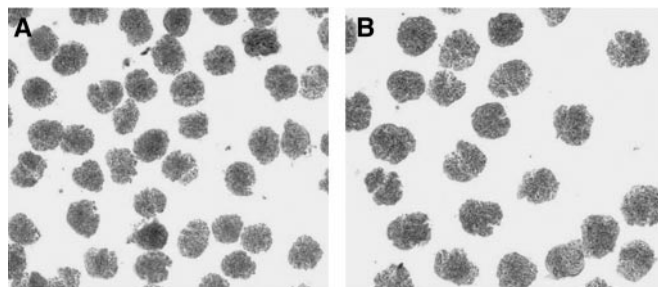


Figure 1. A representative photograph of sieved glomeruli from a diabetic rat. Diabetic large glomeruli (DM-LG; on the 125- μm sieve; B) were significantly larger than diabetic small glomeruli (DM-SG; on the 75- μm sieve; A). Magnification, $\times 100$.

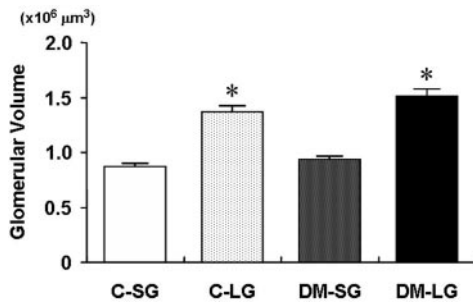


Figure 2. Mean glomerular volume in control small glomeruli (C-SG), control large glomeruli (C-LG), DM-SG, and DM-LG groups. The mean volumes of DM-LG ($1.51 \pm 0.06 \times 10^6 \mu\text{m}^3$) and C-LG ($1.37 \pm 0.05 \times 10^6 \mu\text{m}^3$) were significantly higher than those of DM-SG ($0.94 \pm 0.03 \times 10^6 \mu\text{m}^3$) and C-SG ($0.87 \pm 0.03 \times 10^6 \mu\text{m}^3$). * $P < 0.01$ versus C-SG and DM-SG.

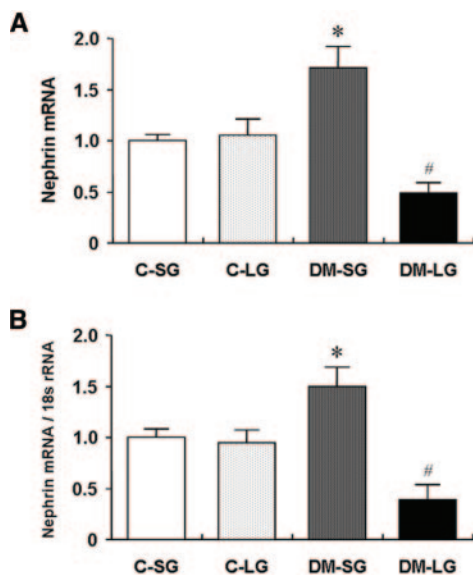


Figure 3. (A) Nephtrin mRNA expression in C-SG, C-LG, DM-SG, and DM-LG groups. Nephtrin mRNA expression was significantly reduced in DM-LG (0.49 ± 0.10) compared with DM-SG (1.71 ± 0.21) and control glomeruli (C-SG reference value 1; C-LG 1.05 ± 0.16), whereas its expression was significantly higher in DM-SG compared with DM-LG and control glomeruli. Data are expressed as fold changes of nephtrin mRNA expression relative to C-SG. (B) Even after correction for 18s rRNA expression, the differences in nephtrin mRNA expression between groups remained significant. * $P < 0.05$ versus control glomeruli and DM-LG; # $P < 0.05$ versus control glomeruli and DM-SG.

Glomerular Nephtrin Protein Expression

Figure 4 shows a representative Western blot with the lysates of control SG, control LG, diabetic SG, and diabetic LG at 6 wk after streptozotocin injection. Glomerular nephtrin, both extracellular and intracellular, protein expression was increased in diabetic SG, whereas there was a significant reduction in nephtrin protein expression in diabetic LG compared with control SG and control LG, as shown by nephtrin mRNA expression. Densitometric quantification revealed 56 and 92% in-

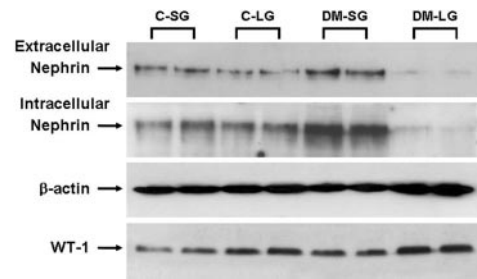


Figure 4. A representative Western blot with the lysates of C-SG, C-LG, DM-SG, and DM-LG. Both extracellular nephtrin (corresponding to amino acids 974 to 987 mapping within an extracellular fibronectin domain) and intracellular nephtrin (corresponding to amino acids 1243 to 1256 mapping within intracellular domain) protein expression were increased in DM-SG, whereas there was a significant reduction in nephtrin protein expression in DM-LG compared with C-SG and C-LG. β -Actin and Wilms' tumor-1 (WT-1) protein expression tended to be higher in both C-LG and DM-LG but did not reach statistical significance.

creases in extracellular and intracellular nephtrin protein expression, respectively, in diabetic SG compared with control SG ($P < 0.05$). In contrast, extracellular and intracellular nephtrin protein expression was decreased by 77 and 86%, respectively, in diabetic LG versus control SG ($P < 0.05$). There was no significant difference in nephtrin protein expression between control LG and control SG. β -Actin and WT-1 protein expression tended to be higher in both control LG and diabetic LG but did not reach statistical significance.

Total Glomerular Cells and Podocyte Numbers

Total glomerular cells and podocyte numbers were determined with the toluidine blue-stained semithin sections (Figure 5). There was a significant increase in the number of total glomerular cells in diabetic (SG 692.1 ± 21.8 ; LG 715.8 ± 18.7 /glomerulus) compared with corresponding control glomeruli (SG 654.8 ± 16.0 ; LG 689.3 ± 18.4 /glomerulus; $P < 0.05$). In addition, total glomerular cell numbers tended to be higher in diabetic LG relative to diabetic SG but did not reach statistical significance. However, the number of podocytes was comparable among the four groups (control SG 170.6 ± 6.9 ; control LG 183.6 ± 10.5 ; diabetic SG 168.8 ± 7.0 ; and diabetic LG 171.7 ± 8.8 /glomerulus).

Electron Microscopic Findings

The foot processes of diabetic LG were broader and flatter than those of the other groups. The mean foot process width (FPW) was significantly greater in diabetic LG (424.8 ± 20.7 nm) compared with control SG (306.2 ± 6.5 nm; $P < 0.001$), whereas the mean slit pore length was significantly shorter in diabetic LG (27.0 ± 1.2 nm) relative to control SG (33.4 ± 1.7 nm; $P < 0.01$). In contrast, the mean FPW and slit pore length in control LG (323.7 ± 9.4 and 34.1 ± 1.2 nm, respectively) and diabetic SG (316.3 ± 10.8 and 32.0 ± 1.5 nm, respectively) were similar to those in control SG. In addition, the number of

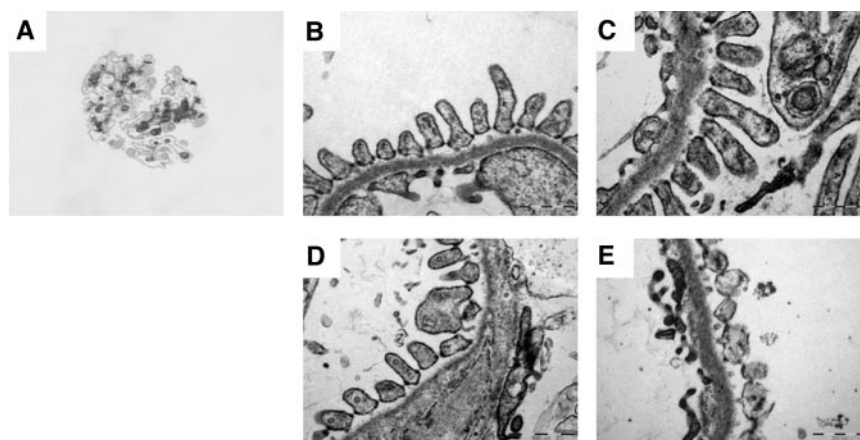


Figure 5. Representative toluidine blue–stained semithin section of DM-LG (A) and electron microscopic photographs of foot processes in C-SG (B), C-LG (C), DM-SG (D), and DM-LG (E) groups. The foot processes of DM-LG were broader and flatter than those of the other groups. The mean foot process width (FPW) was significantly greater in DM-LG, whereas the mean slit pore length was significantly shorter in DM-LG compared with the other groups. Magnifications: $\times 400$ in A; $\times 50,000$ in B through E.

slit pores per 100 μm of GBM was significantly less in diabetic LG (182.9 ± 7.1) versus control SG (260.7 ± 5.6) and diabetic SG (248.2 ± 6.2 ; $P < 0.005$), and the GBM was significantly thicker in diabetic LG compared with diabetic SG ($P < 0.01$) and control glomeruli ($P < 0.001$; Figure 5; Table 2).

Correlation between Nephrin mRNA Expression and FPW

Pearson correlation analysis revealed that there was a significant inverse correlation between the magnitude of nephrin mRNA expression and the mean FPW ($R = -0.571, P < 0.005$; Figure 6).

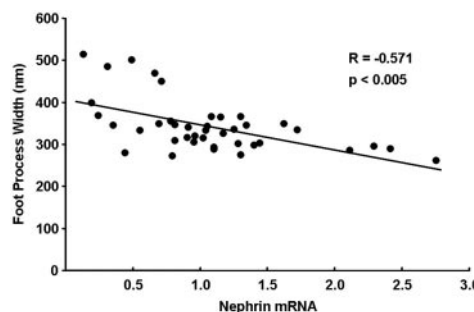


Figure 6. Correlation between nephrin mRNA expression and FPW in C-SG, C-LG, DM-SG, and DM-LG. There was a significant inverse correlation between the magnitude of nephrin mRNA expression and the mean FPW ($R = -0.571, P < 0.005$).

DISCUSSION

In this study, we show that nephrin mRNA and protein expression in diabetic glomeruli varied according to the size of glomeruli. These findings suggest that glomerular isolation by a sieving technique with sieves of various pore sizes or various kidney sizes may lead to diverse results in the expression of certain genes. Finally, on the basis of the finding that nephrin expression is diminished in diabetic LG, we suggest that albu-

minuria may arise first in more hypertrophied glomeruli in the early stage of DN.

The slit diaphragm, which bridges adjacent foot processes derived from different podocytes, functions as the ultimate molecular size filter with $4 \times 14\text{-nm}$ -sized pores.²⁰ The molecular structure of the slit diaphragm is still unclear, but recent studies have revealed a few genes located at the region of the slit

Table 2. Electron microscopic findings in the four groups^a

Parameter	Control		Diabetic	
	SG	LG	SG	LG
Foot process width (nm)	306.2 ± 6.5	323.7 ± 9.4	316.3 ± 10.8	424.8 ± 20.7^b
Slit pore length (nm)	33.4 ± 1.7	34.1 ± 1.2	32.0 ± 1.5	27.0 ± 1.2^c
No. of slit pores (per 100 μm)	260.7 ± 5.6	273.3 ± 6.0	248.2 ± 6.2	182.9 ± 7.1^d
GBM thickness (nm)	197.8 ± 11.7	205.3 ± 9.8	222.3 ± 13.2	268.2 ± 12.9^e

^aGBM, glomerular basement membrane; LG, large glomeruli; SG, small glomeruli.

^b $P < 0.001$ versus other groups.

^c $P < 0.01$ versus other groups.

^d $P < 0.005$ versus other groups.

^e $P < 0.001$ versus control groups; $P < 0.01$ versus diabetic SG.

diaphragm. Nephritin, a product of the *NPHS1* gene that is mutated in patients with congenital nephrotic syndrome of the Finnish type,⁹ was the first protein demonstrated to comprise the slit diaphragm^{10,11} and has been suggested to play a critical role in the pathogenesis of proteinuria in glomerular disease, including DN. Because proteinuria is a cardinal feature of DN, the contribution of changes in nephritin expression to proteinuria in DN has also been studied, but the results were not consistent. Bonnet *et al.*¹⁴ demonstrated a reduction in nephritin mRNA and protein expression in streptozotocin-induced diabetic spontaneously hypertensive rats at 32 wk after the induction of diabetes. A recent study also demonstrated diminished nephritin expression and altered nephritin localization in biopsies of patients with nephropathy from both type 1 and type 2 diabetes.¹⁷ In contrast, Aaltonen *et al.*¹³ observed an increase in nephritin mRNA levels in streptozotocin-induced diabetic rats and in nonobese diabetic mice even before the development of significant albuminuria. In another study, angiotensin II, an important mediator in the pathogenesis of DN, infused by minipump in rats increased nephritin mRNA expression as assessed by real-time PCR and quantitative *in situ* hybridization.²¹ The reasons for the divergence of changes in nephritin expression in DN may be due to a variety of underlying causes, including but not limited to differences in species, duration of diabetes, and accompanying hypertension. However, we submit that the previously observed differences in nephritin expression could be attributed to the variation in the methods used for glomerular isolation. When the sieving technique was used for the isolation of diabetic glomeruli, many of the hypertrophied diabetic glomeruli could not pass through the second to the last sieve, thus leading to data that reflected changes in only the less hypertrophied but not the more hypertrophied glomeruli.

Kidney size is typically increased in diabetes, even at the time of diagnosis.²² This is primarily due to glomerular and tubular hypertrophy. The increase in the number of glomerular cells, extracellular matrix accumulation, and the increase in capillary number and size all contribute to glomerular hypertrophy. In this study, we also observed a significant increase in total glomerular cell number in diabetic glomeruli compared with control glomeruli despite of comparable podocyte numbers, suggesting that the increase in glomerular cell number was mainly attributed to the increase in the number of mesangial cells and endothelial cells, partly contributing to glomerular hypertrophy. A recent study used x-ray microcomputed tomography to measure the glomerular volume of the Otsuka Long-Evans Tokushima Fatty (OLETF) rat.²³ This study revealed that glomerular volume was significantly larger in the OLETF rats compared with the age-matched controls of the Long Evans Tokushima Lean (LETO) rats. However, when glomerular volume was normalized to kidney weight or body weight, the two groups were comparable. It is interesting that scattering of glomerular volume, expressed as a coefficient variation (SD/mean), was significantly larger in OLETF rats compared with LETO rats even after normalization, suggesting

more heterogeneity in the glomerular volume distribution. Many other investigators also observed that patients with type 2 diabetes showed much variability in glomerulopathy and tubulointerstitial lesions.^{24,25} Such variation has resulted in difficulty in determining a sensitive histologic parameter for ascertaining the early stage of DN. In contrast, glomerular abnormalities in type 1 diabetes are known to be more uniform compared with type 2 diabetes.²⁵ In this study, the proportion of glomeruli on the 125- μ m sieve to total glomeruli was significantly higher in 6-wk diabetic rats compared with control rats (3.50 ± 0.31 versus $0.64 \pm 0.14\%$; $P < 0.001$); therefore, we surmised that the majority of the glomeruli in the diabetic LG group were more hypertrophied diabetic glomeruli, which may be more affected by the diabetic milieu compared with diabetic SG. An increase in total cell number, albeit statistically insignificant, and a significant increase in GBM thickness in diabetic LG compared with diabetic SG also suggest that more hypertrophied glomeruli are included in the diabetic LG group. These results suggest that the same inconsistent glomerular changes occur in type 1 diabetes as in type 2 diabetes. A recent study found that variation in nephritin expression in biopsies of patients with nephropathy from both type 1 and type 2 diabetes was larger compared with control patients.¹⁷ This finding partly supports our results describing changes in nephritin expression. Because a previous study by Sanden *et al.*²⁶ demonstrated that glomeruli in the inner cortex were statistically larger than glomeruli from the outer cortex and Artachoperula *et al.*²⁷ showed that degree of hypertrophy was comparable among superficial, midcortical, and juxtamedullary glomeruli, there is a possibility that glomeruli in the diabetic LG are located more deeply in the renal cortex, but further study will be necessary to confirm the location within the renal cortex of more hypertrophied glomeruli as well as the factors that dictate this change.

The number of podocytes is known to decrease in the glomeruli of patients with type 1 diabetes of all ages, with reduced podocyte number even in diabetes of short duration.²⁸ Analysis of kidney biopsies from Pima Indians with type 2 diabetes also demonstrated that individuals with clinical nephropathy exhibited broadening of podocyte foot processes associated with a reduction in the number of podocytes per glomerulus.²⁹ To exclude the possibility that the decrease in nephritin expression in diabetic LG was associated with a reduction in podocyte number, we reevaluated nephritin protein expression by normalizing it with WT-1 protein expression as well as with podocyte number. Even after normalization, nephritin protein expression in diabetic LG remained significantly lower compared with control glomeruli. Encapsulated glomeruli that contain the Bowman's capsule are larger than decapsulated glomeruli. It has been reported that when glomerular isolation was performed using a sieving technique, the proportions of decapsulated and encapsulated glomeruli were 86.0 ± 6.0 and $11.0 \pm 5.0\%$, respectively.³⁰ In this study, we observed nearly the same proportion of decapsulated glomeruli (approximately 90%) from the 125- and 75- μ m sieves. These findings suggest that

the reduction of nephrin mRNA and protein expression in diabetic LG is attributed neither to the decrease in podocyte number nor to the presence of a greater number of encapsulated glomeruli.

Recently, it was suggested that nephrin acts as a signaling molecule that can activate the mitogen-activated protein kinase pathway and may influence podocyte survival.^{31,32} Furthermore, a recent study using transgenic mice demonstrated that nephrin was transcriptionally activated by WT-1.³³ In this study, however, we observed that changes in nephrin and WT-1 expression did not coincide. Even though WT-1 expression was slightly increased in glomeruli on the 125- μm sieve, nephrin expression was significantly reduced in those glomeruli. Further verification is necessary to determine whether the data from transgenic mice are applicable to non-genetically modified animal experiments in the future. Moreover, we also observed an increase in nephrin expression in glomeruli from the 75- μm sieve, which agrees with the results of the study by Aaltonen *et al.*¹³ It was difficult to interpret the meaning of an increase in nephrin expression in diabetic SG, but a compensatory phenomenon for decreased nephrin expression in diabetic LG or increased signaling events could be explanations for this observation.

In addition to the alternations in the expression of podocyte-associated molecules, proteinuria is closely linked to the ultrastructural changes in podocytes in DN. Increased FPW along with reduced slit pore length has been demonstrated in patients with diabetes and in animal model of diabetes.^{34,35} Moreover, Berg *et al.*³⁴ observed a strong correlation between urinary albumin excretion and FPW in patients with type 1 diabetes, whereas Koop *et al.*³⁶ showed that the mean width of the podocyte foot processes was inversely correlated with nephrin expression in various glomerular diseases. In this study, we found a significant increase in the mean FPW and a significant decrease in slit pore length along with a significant decrease in nephrin expression in diabetic LG but not in diabetic SG, supporting our hypothesis that more hypertrophied glomeruli are responsible for albuminuria in the early DN.

Nephrin mRNA and protein expression is decreased in more hypertrophied glomeruli in early experimental diabetes, whereas nephrin expression is increased in the relatively smaller glomeruli. These data suggest that the expression of certain genes is differentiated according to the size of glomeruli and that more hypertrophied glomeruli may be responsible for albuminuria in the early stage of DN.

CONCISE METHODS

Animals

All animal studies were conducted using a protocol approved by the committee for the care and use of laboratory animals of Yonsei University College of Medicine. Twenty male Sprague-Dawley rats that weighed 250 to 280 g were studied. Ten rats were administered an injection of diluent (control) and 10 were administered an injection

of 65 mg/kg streptozotocin intraperitoneally (diabetic). Blood glucose levels were measured 3 d after the streptozotocin injection to confirm the development of diabetes. The rats were given free access to water and standard laboratory diet during the 6-wk study period. All rats were killed after 6 wk. When we measured the urinary albumin excretion in diabetic rats, a statistically significant increase in urinary albumin excretion was observed for the first time at 6 wk after streptozotocin injection. Because the purpose of this study was to examine the changes of nephrin expression in a point of albuminuria, we used 6-wk diabetic rats.

Body weights were checked weekly, and kidney weights were measured when the rats were killed. Serum glucose was measured weekly, and 24-h urinary albumin was measured when the rats were killed. Blood glucose was measured by glucometer, and 24-h urinary albumin excretion was determined by ELISA (Nephtr II; Exocell, Philadelphia, PA).

Glomerular Isolation

Glomeruli were isolated by a sieving technique using sieves with pore sizes of 250, 150, 125, and 75 μm . Because the juxtamedullary glomeruli are known to be larger than superficial and midcortical glomeruli in control and streptozotocin-induced diabetic rats,²⁷ we tried to use only the superficial and midcortical tissues for glomerular isolation. Glomeruli were collected under an inverted microscope to minimize tubular contamination. We classified glomeruli into LG (on the 125- μm sieve) and SG (on the 75- μm sieve) groups. For the control glomeruli, glomeruli on the 125- μm sieve from 3 to 4 control rats were pooled, because there were few glomeruli on the 125- μm sieve from the individual samples of the control rats. In addition, we determined the proportion of encapsulated and decapsulated glomeruli on both the 125- and 75- μm sieves.

Morphometric Measurement of Glomerular Volume

Glomerular volume (V_G) was calculated as described previously.³⁷ Briefly, photographs of 50 glomeruli were taken using a digital camera at the time of sieving, and the surface areas were traced using a computer-assisted color image analyzer Image-Pro (version 2.0; Media Cybernetics, Silver Spring, MD). V_G was calculated using the equation $V_G = 4/3\pi(\text{area}/\pi)^{3/2}$.

Total RNA Extraction and Reverse Transcription

Glomeruli on the 125- μm (control LG and diabetic LG) and 75- μm sieves (control SG and diabetic SG) were put in a solution of vanadyl ribonucleoside complex; and 300 glomeruli were counted at 4°C, rinsed, and transferred to three tubes (100 glomeruli per tube) that contained RNase inhibitor. Total RNA was extracted and reverse-transcribed as described previously.³⁸

Real-Time PCR

The primers used for nephrin, 18s, and WT-1 amplification were as follows: nephrin sense 5'-CCTGCACCACCAACTGCTTAGC-3', antisense 5'-CCAGTGAGCTTCCCGTTCAGC-3'; 18s sense 5'-CGT-GAGAGTGTCTAACGGG-3', antisense 5'-CGAGTCAGGCATTTGGTCC-3'; and WT-1 sense 5'-CGTCCCTTTCATGTGTGCATACC-

3', antisense 5'-GTGCTTCCGGCTATGCATCT-3'. cDNA from 0.5 glomeruli per reaction tube were used for amplification.

With the use of the ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA), PCR was performed with a total volume of 20 μ l in each well, containing 10 μ l of SYBR Green PCR Master Mix (Applied Biosystems), 5 μ l of cDNA, and 5 pmol of sense and antisense primers. Primer concentrations were determined by preliminary experiments that analyzed the optimal concentrations of each primer. Each sample was run in triplicate in separate tubes to permit quantification of the gene normalized to the 18s. The PCR conditions used were as follows: For 18s, 35 cycles of denaturation at 94.5°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 1 min; and for nephrin and WT-1, 40 cycles of denaturation at 94.5°C for 30 s, annealing at 57°C for 30 s, and extension at 72°C for 30 s. Initial heating at 95°C for 9 min and final extension at 72°C for 7 min were performed for all PCR.

After real-time PCR, the temperature was increased from 60 to 95°C at a rate of 2°C/min to construct a melting curve. A control without cDNA was run in parallel with each assay. The cDNA content of each specimen was determined using a comparative C_T method with $2^{-\Delta\Delta C_T}$. The results are given as relative expression of nephrin normalized to the 18s housekeeping gene. Signals from control SG were assigned a relative value of 1.0. In pilot experiments, PCR products that were run on agarose gels revealed a single band.

Western Blot Analysis

Counted glomeruli were lysed in SDS sample buffer (2% SDS, 10 mM Tris-HCl [pH 6.8], and 10% [vol/vol] glycerol), treated with Laemmli sample buffer, heated at 100°C for 5 min, and electrophoresed in an 8% acrylamide denaturing SDS-polyacrylamide gel. Proteins were then transferred to a Hybond-ECL membrane using a Hoeffer semi-dry blotting apparatus (Hoeffer Instruments, San Francisco, CA), and the membrane was then incubated in blocking buffer A (1 \times PBS, 0.1% Tween-20, and 8% nonfat milk) for 1 h at room temperature, followed by an overnight incubation at 4°C in a 1:2000 dilution of polyclonal antibodies to extracellular or intracellular domain of nephrin (Progen, Heidelberg, Germany) or polyclonal antibodies to WT-1 (Santa Cruz Biotechnology, Santa Cruz, CA) or β -actin (Santa Cruz Biotechnology). The membrane was then washed once for 15 min and twice for 5 min in 1 \times PBS with 0.1% Tween-20. Next, the membrane was incubated in buffer A that contained a 1:1000 dilution of horseradish peroxidase-linked goat anti-rabbit IgG (Amersham Life Science, Arlington Heights, IL). The washes were repeated, and the membrane was developed with a chemiluminescent agent (ECL; Amersham Life Science).

Electron Microscopy

For transmission electron microscopy, isolated glomeruli were fixed in 50 mM sodium cacodylate buffer (pH 7.4) that contained 2% glutaraldehyde in paraformaldehyde for 30 min at 32°C, postfixed in 1% OsO₄ for 2 h at 4°C, and dehydrated by treatment with a graded series of ethanol (5 min each in 50, 60, 70, 80, 90, and 95% and twice in 100%). After that, isolated glomeruli were treated with propylene oxide and embedded in Epon according to standard procedures, and the glomeruli were localized in the semithin sections stained with tolu-

idine blue. Sections were cut using an Ultracut R ultratome (Leica, Wetzlar, Germany) and counterstained with 8% uranyl acetate and lead citrate. Transmission electron microscopy was performed using a JEOL JSM 1011 microscope (Tokyo, Japan) operating at 80 kV and eight to 10 pictures, covering one or two glomerular cross-sections, were photographed in a random and unbiased manner. The images magnified to 15,000 times were used to measure the GBM length and the number of slit pores, and the images at $\times 50,000$ or $\times 100,000$ magnification were used to determine the FPW, the slit pore length, and the GBM thickness.

A foot process was defined as any connected epithelial segment butting on the basement membrane, between two neighboring filtration pores or slits. From each photograph, the arithmetic mean of the FPW was calculated using the following equation as described previously³⁶: $FPW = \pi/4 \times \Sigma \text{GBM length} / \Sigma \text{slits}$. Σslits is the total number of slit pores counted in each picture, and $\Sigma \text{GBM length}$ is the total GBM length measured in each picture. The correction factor of $\pi/4$ serves to correct for presumed random variation in the angle of section relative to the long axis of the podocyte. The slit pore length was expressed as per 100 μ m of GBM.

GBM thickness was determined at five different sites, where the epithelial and endothelial cytoplasmic membranes were clearly visible, using the technique described by Osawa *et al.*³⁹ Measurements were undertaken perpendicularly from the endothelial cytoplasmic membrane to the outer lining of the lamina rara externa underneath the cytoplasmic membrane of the epithelial foot process. Tangentially sectioned GBM and areas of GBM attached to bars of mesangial matrix were excluded from the analysis.

Total glomerular cells and podocyte numbers were determined by the exhaustive count method as described previously.⁴⁰ Two adjacent toluidine blue-stained semithin sections 3 μ m apart were observed in pairs at a magnification of $\times 2000$, and the nuclei present in the top section but not in the bottom section were counted and summed. Ten glomeruli in five rats from each group and 13 to 15 semithin sections from the midglomerular area were examined.

Statistical Analyses

All values are expressed as the means \pm SEM. Statistical analysis was performed using the statistical package SPSS for Windows (version 11.0; SPSS, Chicago, IL). Results were analyzed using the Kruskal-Wallis nonparametric test for multiple comparisons. Significant differences by the Kruskal-Wallis test were further confirmed by the Mann-Whitney *U* test. The relationship between nephrin mRNA expression and FPW was determined using Pearson correlation test. $P < 0.05$ was considered to be statistically significant.

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DISCLOSURES

None.

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