The differential expression of nephrin according to glomerular size in early diabetic kidney disease

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The differential expression of nephrin according to glomerular size in early diabetic kidney disease

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Doctoral Dissertation
submitted to the Department of Medicine,
the Graduate School of Yonsei University
in partial fulfillment of the requirements for the degree
of Doctor of Philosophy

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June 2006

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June 2006

ACKNOWLEDGEMENTS

This paper would not have been possible without the support of many people. A special thanks to my supervisor, professor Shin-Wook Kang, who had guided me in every step of the thesis. Also thanks to Professor Soon Jung Park, Professor Bong Soo Cha, Professor Ho Geun Yoon, Professor Hyeong Cheon Park who generously offered guidance and supports

I also thank my colleagues Dong-Ryeol Ryu, Tae-Hyun Yoo, Hoon Young Choi, Joo Sung Kim for their support and many advise on the thesis. Also, I thank Jin-Ju Kim, Dong-Sub Jung, Seung-Jae Kwak for actually carrying out the experiments and taking care of every little details.

I like to thank my father, who is always there when I needed him and also praying for me everyday, and mother who is in heaven looking over me. Finally, to my wife, who endured and understood me during this process and was the biggest supporter to me, I love you and I could not have done it without you. I would like to dedicate this paper to my family, friends, colleagues, and mentor and everyone that I love and care.

Author

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ABSTRACT

The differential expression of nephrin according to glomerular size in early diabetic kidney disease

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Background: Diabetic nephropathy (DN) is clinically characterized by proteinuria. 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. Traditionally, the GB*M* has been considered a coarse filter that restricts large molecules, while the slit diaphragm was thought to function as a fine filter that contributes to ultimate size-selectivity, permitting permeability only to molecules smaller than albumin. 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 diaphragm. Nephrin, a product of the NPHS1 gene was the first protein demonstrated to comprise the slit diaphragm. *M*any 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 is a characteristic finding of DN, but it does not seem to develop in all glomeruli

concurrently. I 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, I investigated the differences in nephrin expression between small and large (hypertrophied) glomeruli isolated from diabetic rats. I focused on the expression of nephrin, the most important filtration barrier-associated molecule, due to the conflicting reports on its expression patterns in early diabetic nephropathy.

Methods: To investigate the differences in nephrin expression according to glomerular size, glomeruli were isolated from 10 control (C) and 10 streptozotocin-induced diabetic rats (D*M*) at 6-weeks after the induction of diabetes by a sieving technique using sieves with pore sizes of 250 ㎛, 150 ㎛, 125 ㎛, and 75 ㎛. We then classified glomeruli into large glomeruli (on the 125 ㎛ sieve, LG) and small glomeruli (on the 75 ㎛ sieves, SG) groups. Glomerular volume was determined using an image analyzer, and mRNA and protein expression by real time-PCR and Western blot, respectively.

Results: The mean volumes of the D*M*-LG ($1.68 \pm 0.08 \times 10^6$ ㎛^{3}) and C-LG ($1.47 \pm 0.09 \times 10^6$ ㎛^{3}) were significantly higher than those of the D*M*-SG ($1.08 \pm 0.04 \times 10^6$ ㎛^{3}) and C-SG ($0.97 \pm 0.03 \times 10^6$ ㎛^{3}) ($p<0.01$). Nephrin mRNA expression was significantly reduced in D*M*-LG (0.49 ± 0.10) compared to D*M*-SG (1.71 ± 0.21) and C glomeruli (C-SG, reference value 1; C-LG, 1.05 ± 0.16) ($p<0.05$), whereas its expression was significantly higher in D*M*-SG compared to D*M*-LG and C glomeruli ($p<0.05$). In contrast, there was no

statistically significant difference in glomerular 18s mRNA expression among the four groups. Glomerular nephrin, both extracellular and intracellular, protein expression was increased in D*M*-SG, whereas there was a significant reduction in nephrin protein expression in D*M*-LG compared to C-SG and C-LG, as shown by nephrin mRNA expression. Densitometric quantitation revealed 56% and 92% increases in extracellular and intracellular nephrin protein expression, respectively, in D*M*-SG compared to C-SG ($p<0.05$). In contrast, extracellular and intracellular nephrin protein expression was decreased by 77% and 86%, respectively, in D*M*-LG versus C-SG ($p<0.05$). There was no significant difference in nephrin protein expression between C-LG and C-SG. WT-1 and β-actin protein expression were similar among the four groups.

Conclusion: These data suggest that the nephrin gene is differentially expressed according to glomerular size. Furthermore, hypertrophied glomeruli with lesser nephrin expression may be responsible for albuminuria in the early stage of DN.

Key words : Diabetic nephropathy, Proteinuria, Nephrin, Glomerular hypertrophy, Glomerular size

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I. INTROD*U*CTION

Diabetic nephropathy, the leading cause of end-stage renal disease in the *U*S[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 comprised of three layers: a fenestrated endothelial layer, a glomerular basement membrane (GB*M*), and podocyte foot processes connected by a slit diaphragm. Traditionally, the GB*M* has been considered a coarse filter that restricts large molecules, while the slit diaphragm was thought to function as a fine filter that contributes to ultimate size-selectivity, permitting permeability only to molecules 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 designed to demonstrate a relationship between changes in nephrin

expression and/or localization and proteinuria in various forms of glomerular diseases, including diabetic nephropathy, have been inconsistent[12-19]. A reduction in nephrin expression has been observed in experimental glomerular diseases[12] and in adult patients with primary acquired nephrotic syndrome[15], but not in pediatric patients with glomerular diseases[16]. In cases of experimental diabetic nephropathy, 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 diabetic nephropathy are not clear, but differences between species, duration of diabetes, or accompanying hypertension may contribute to these disparities.

*M*ost studies on the glomerular expression of certain molecules have been performed with glomeruli isolated by either microdissection or a sieving technique. At the time of microdissection, especially in studies with diabetic glomeruli, it is apparent that all the glomeruli are not the same in size. A difference in gene expression according to the size of the 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 small and large (hypertrophied) glomeruli isolated from diabetic rats. We focused on the expression of nephrin, the most important filtration barrier-associated molecule, due to the conflicting reports on its expression patterns in early diabetic nephropathy.

II. *M*ATERIALS AND *M*ETHODS

1. Animals

All animal studies were conducted using a protocol approved by the committee for the care and use of laboratory animals of Yonsei *U*niversity College of *M*edicine. Twenty male Sprague-Dawley rats, weighing 250-280 g were studied. Ten rats were injected with diluent (Control, C) and 10 with 65 mg/kg streptozotocin intraperitoneally (D*M*). Blood glucose levels were measured 3 days after the streptozotocin injection to confirm the development of diabetes. The rats were given free access to water and standard laboratory chow during the 6-weeks study period. All rats were sacrificed after 6 weeks. When we measured the urinary albumin excretion in diabetic rats, statistically significant increase in urinary albumin excretion was observed for the first time at 6 weeks after streptozotocin injection. Since the purpose of this study was to examine the changes of nephrin expression in a point of albuminuria, we used 6-week diabetic rats.

Body weights were checked weekly, and kidney weights were measured at the time of sacrifice. Serum glucose was measured weekly and 24-hour urinary albumin at the time of sacrifice. Blood glucose was measured by glucometer and 24-hour urinary albumin excretion was determined by ELISA (Nephrat II, Exocell, Inc., Philadelphia, PA).

2. Glomerular isolation

Glomeruli were isolated by a sieving technique using sieves with pore sizes of 250 ㎛, 150 ㎛, 125 ㎛, and 75 ㎛. Since the juxtamedullary glomeruli are known to be larger than superficial and midcortical glomeruli in control and streptozotocin-induced diabetic rats[20], we tried to use only the cortical tissues for glomerular isolation. In addition, glomeruli were collected under an inverted microscope to minimize tubular contamination. We classified glomeruli into large glomeruli (on the 125 ㎛ sieve, LG) and small glomeruli (on the 75 ㎛ sieve, SG) groups. For the C glomeruli, glomeruli on the sieve with a pore size of 125 ㎛ from 3-4 rats were pooled, because there were few glomeruli on the 125 ㎛ sieve from the individual samples of the C rats.

3. *M*orphometric measurement of glomerular volume

Glomerular volume (V_G) was calculated according to the method of Weibel[21]. 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 Ver. 2.0 (*M*edia Cybernetics, Silver Spring, *M*D).

V_G was calculated using the equation:

$$V_G=\beta/k \times (Area)^{3/2},$$

where $\beta=1.38$ is the shape coefficient for spheres, and $k=1.1$ is the size distribution coefficient.

4. Reverse transcription polymerase chain reaction

A. Total RNA extraction

Glomeruli on the 125 ㎛ (C-LG and D*M*-LG) and 75 ㎛ sieves (C-SG and D*M*-SG) were put in a solution of vanadyl ribonucleoside complex and three hundred glomeruli were counted at 4℃, rinsed and transferred to three tubes (100 glomeruli per tube) containing RNAse inhibitor. Total RNA was extracted as previously described[22]. Briefly, 100 ㎕ of RNA STAT-60 reagent (Tel-Test, Inc., Friendswood, TX) was added to the glomeruli which were lysed by freezing and thawing three times. Another 700 ㎕ of RNA STAT-60 reagent was then added and the mixture was vortexed and stored for 5 minutes at room temperature. Next, 160 ㎕ of chloroform was added and the mixture was shaken vigorously for 30 seconds. After 3 minutes, the mixture was centrifuged at 12,000 X g for 15 minutes at 4℃ and the upper aqueous phase containing the extracted RNA was transferred to a new tube. RNA was precipitated from the aqueous phase by adding 400 ㎕ of isopropanol and then pelleted by centrifugation at 12,000 X g for 30 minutes at 4℃. The RNA precipitate was washed with 70% ice-cold ethanol, dried using a Speed Vac, and dissolved in DEPC-treated distilled water. Glomerular RNA yield and quality were assessed based on spectrophotometric measurements at the wavelength of 260 and 280 nm.

B. Reverse transcription

First strand cDNA was made by using a Boehringer *M*annheim cDNA synthesis kit (Boehringer *M*annheim GmbH, *M*annheim, Germany). Total RNA extracted from 100 glomeruli was reverse transcribed using 10 μ*M* random hexanucleotide primer, 1 m*M* dNTP, 8 m*M* $MgCl_2$, 30 m*M* KCl, 50 m*M* Tris-HCl, pH 8.5, 0.2 m*M* dithiothreithol, 25 *U* RNAse inhibitor, and 40 *U* A*M*V reverse transcriptase. The mixture was incubated at 30℃ for 10 minutes and 42℃ for 1 hour followed by inactivation of the enzyme at 99℃ for 5 minutes.

C. Real time-polymerase chain reaction (RT-PCR)

The primers used for nephrin and 18s amplification were as follows:

nephrin	sense:	5′-CCTGCACCACCAACTGCTTAGC-3′
	antisense:	5′- CCAGTGAGCTTCCCGTTCAGC-3′
18s	sense:	5′-CGTGAGAGTGTCTAACGGG-3′
	antisense:	5′-CGAGTCAGGCATTTGGTCC-3′

cDNAs from 0.5 glomeruli per reaction tube were used for the nephrin and 18s amplification.

*U*sing the ABI PRIS*M* 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, *U*SA), PCR was performed with a total volume of 20 μL in each well, containing 10 μL of SYBR Green® PCR *M*aster *M*ix (Applied Biosystems), 5 μL of cDNA, and 5 pmol 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℃ for 30 sec, annealing at 60℃ for 30 sec, and extension at 72℃ for 1 minute; and for nephrin, 40 cycles of denaturation at 94.5℃ for 30 sec, annealing at 57℃ for 30 sec, and extension at 72℃ for 30 sec. Initial heating at 95℃ for 9 minutes and final extension at 72℃ for 7 minutes were performed for all PCRs.

After RT-PCR, the temperature was increased from 60 to 95℃ at a rate of 2℃/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 $2^{-\triangle\triangle CT}$. The results are given as relative expression of nephrin normalized to the 18s housekeeping gene. Signals from C-SG were assigned a relative value of 1.0. In pilot experiments, PCR products run on agarose gels revealed a single band.

5. Western blot analysis

Counted glomeruli were lysed in sodium dodecyl sulfate (SDS) sample buffer (2% sodium dodecyl sulfate, 10 m*M* Tris-HCl, pH 6.8, 10% [vol/vol] glycerol), treated with Laemmli sample buffer, heated at 100℃ for 5 minutes, and electrophoresed in an 8% acrylamid denaturing SDS-polyacrylamide gel. Proteins were then transferred to a Hybond-ECL membrane using a Hoeffer semidry blotting apparatus (Hoeffer Instruments, San Francisco, CA), and the membrane was then incubated in blocking buffer A (1 x PBS, 0.1% Tween-20, and 8% nonfat milk) for 1 hour at room temperature, followed by an overnight

incubation at 4℃ in a 1:2000 dilution of polyclonal antibodies to extracellular or intracellular domain of nephrin (Progen, Heidelberg, Germany) or polyclonal antibodies to WT-1 or β-actin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The membrane was then washed once for 15 minutes and twice for 5 minutes in 1 x PBS with 0.1% Tween-20. Next, the membrane was incubated in buffer A containing a 1:1000 dilution of horseradish peroxidase-linked goat anti-rabbit IgG (Amersham Life Science, Inc., Arlington Heights, IL). The washes were repeated, and the membrane was developed with a chemiluminescent agent (ECL; Amersham Life Science, Inc.).

6. Statistical analysis

All values are expressed as the mean standard error of the mean (SE*M*). Statistical analysis was performed using the statistical package SPSS for Windows Ver. 11.0 (SPSS, Inc., Chicago, IL). Results were analyzed using the Kruskal-Wallis non-parametric test for multiple comparisons. Significant differences by the Kruskal-Wallis test were further confirmed by the *M*ann-Whitney *U* test. P values less than 0.05 were considered to be statistically significant.

III. RESULTS

1. Animal data (Table 1)

All animals gained weight over the 6-week experimental period, but weight gain was higher in C compared to DM rats ($p<0.01$). The ratio of kidney weight to body weight in DM (1.07 ± 0.04%) was significantly higher than in C rats (0.64 ± 0.02%).

The mean blood glucose levels of C and DM rats were 101.7 ± 1.9 mg/dl and 483.3 ± 7.3 mg/dl, respectively ($p<0.01$). Compared to the C group (0.29 ± 0.04 mg/day), 24-hour urinary albumin excretion was significantly higher in the DM group (1.09 ± 0.17 mg/day) ($p<0.05$).

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

	Control (n=10)	D*M* (n=10)
Body weight after 6 weeks (g)	418 ± 5	295 ± 4*
Kidney weight (g)	2.69 ± 0.05	3.15 ± 0.07#
Kidney Wt/Body Wt (%)	0.64 ± 0.02	1.07 ± 0.04#
Blood glucose (mg/dL)	101.7 ± 1.9	483.3 ± 7.3*
24-hour urinary albumin excretion (mg/day)	0.29 ± 0.04	1.09 ± 0.17#

Data are expressed as mean ± SE*M*

* ; $p<0.01$, vs. Control

; $p<0.05$ vs. Control

2. Glomerular volume

We first examined the volume of glomeruli found on the 125 ㎛ and 75 ㎛ sieves. The mean volumes of D*M*-LG (1.68 ± 0.08 x 10^6 ㎛^{3}) and C-LG (1.47 ± 0.09 x 10^6 ㎛^{3}) were significantly higher than those of D*M*-SG (1.08 ± 0.04 x 10^6 ㎛^{3}) and C-SG (0.97 ± 0.03 x 10^6 ㎛^{3}) ($p<0.01$) (Fig. 1, 2).

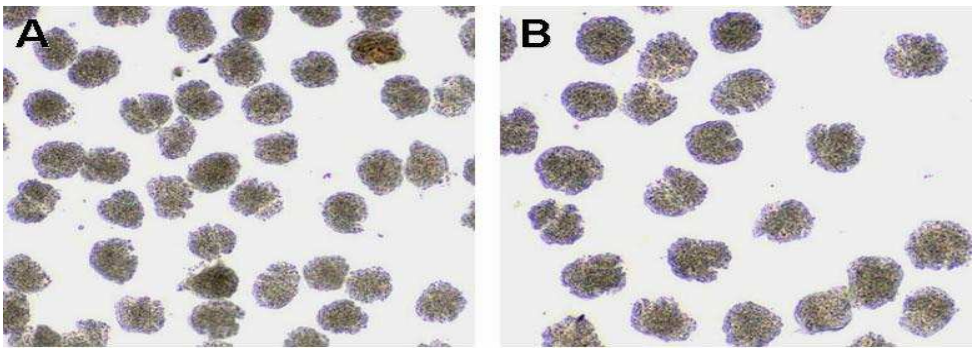

Figure 1. A representative photograph of sieved glomeruli from a diabetic rat. D*M*-LG (on the 125 ㎛ sieve) (B) were significantly larger than D*M*-SG (on the 75 ㎛ sieve) (A).

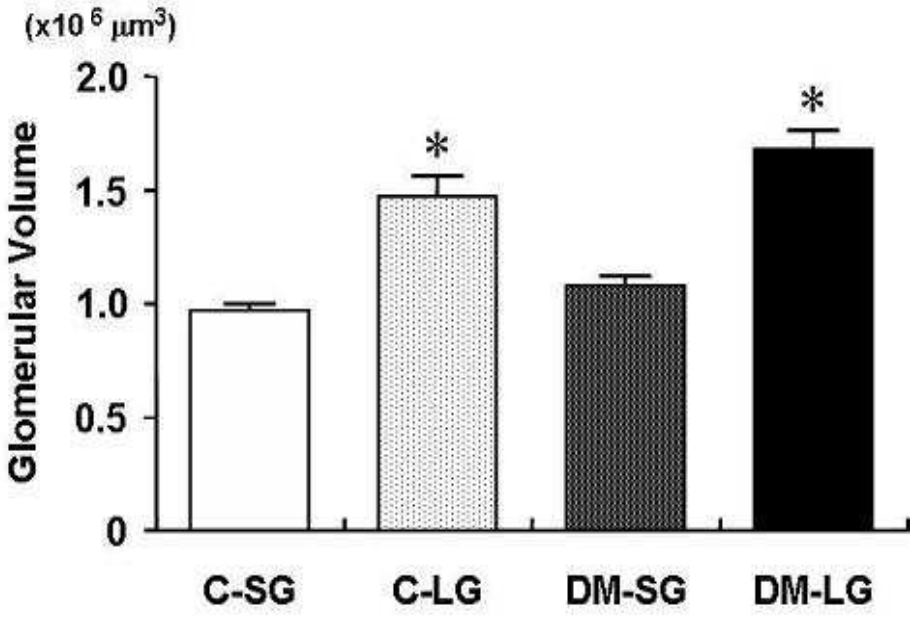

Figure 2. *M*ean glomerular volume in C-SG, C-LG, D*M*-SG, and D*M*-LG groups. The mean volumes of D*M*-LG (1.68 ± 0.08 x 10^6 μm^3) and C-LG (1.47 ± 0.09 x 10^6 μm^3) were significantly higher than those of D*M*-SG (1.08 ± 0.04 x 10^6 μm^3) and C-SG (0.97 ± 0.03 x 10^6 μm^3). *; $p<0.01$ vs. C-SG and D*M*-SG.

3. 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 D*M*-LG (0.49 ± 0.10) compared to D*M*-SG (1.71 ± 0.21) and C glomeruli (C-SG, reference value 1; C-LG, 1.05 ± 0.16) ($p<0.05$), whereas its expression was significantly higher in D*M*-SG compared to D*M*-LG and C glomeruli ($p<0.05$) (Fig. 3). In contrast, there was no statistically significant difference in glomerular 18s mRNA expression among the four groups (Fig. 4). Even after correcting for 18s mRNA, the differences in nephrin mRNA expression between groups remained significant ($p<0.05$) (data not shown).

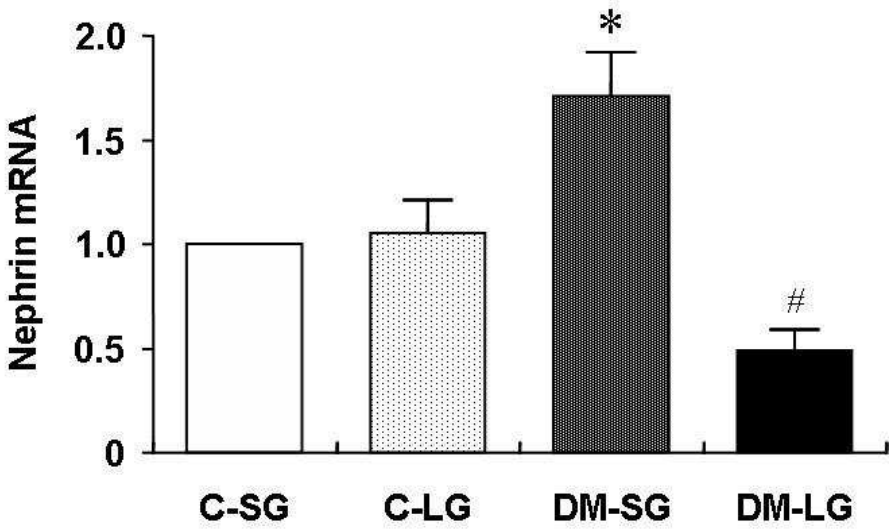

Figure 3. Nephrin mRNA expression in C-SG, C-LG, D*M*-SG, and D*M*-LG groups. Nephrin mRNA expression was significantly reduced in D*M*-LG (0.49 ± 0.10) compared to D*M*-SG (1.71 ± 0.21) and C glomeruli (C-SG, reference value 1; C-LG, 1.05 ± 0.16), whereas its expression was significantly higher in D*M*-SG compared to D*M*-LG and C glomeruli. Data are expressed as relative fold changes of nephrin mRNA expression relative to C-SG. *; $p<0.05$ vs. C glomeruli and D*M*-LG, # $p<0.05$ vs. C glomeruli and D*M*-SG.

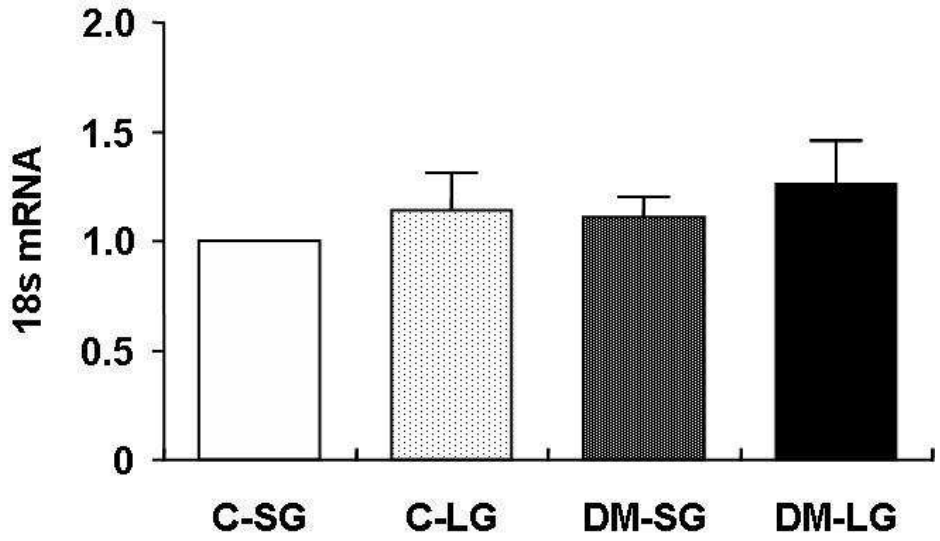

Figure 4. 18s mRNA expression in C-SG, C-LG, D*M*-SG, and D*M*-LG groups. There was no statistically significant difference in glomerular 18s mRNA expression among the four groups. Data are expressed as relative fold changes of 18s mRNA expression relative to C-SG.

4. Glomerular nephrin protein expression

Figure 5 shows a representative Western blot with the lysates of C-SG, C-LG, D*M*-SG, and D*M*-LG at 6-weeks after streptozotocin injection. Glomerular nephrin, both extracellular and intracellular, protein expression was increased in D*M*-SG, whereas there was a significant reduction in nephrin protein expression in D*M*-LG compared to C-SG and C-LG, as shown by nephrin mRNA expression. Densitometric quantitation revealed 56% and 92% increases in extracellular and intracellular nephrin protein expression, respectively, in D*M*-SG compared to C-SG ($p<0.05$). In contrast, extracellular and intracellular nephrin protein expression was decreased by 77% and 86%, respectively, in D*M*-LG versus C-SG ($p<0.05$). There was no significant difference in nephrin protein expression between C-LG and C-SG. WT-1 and β-actin protein expression tended to be higher in both C-LG and D*M*-LG, but did not reach statistical significance.

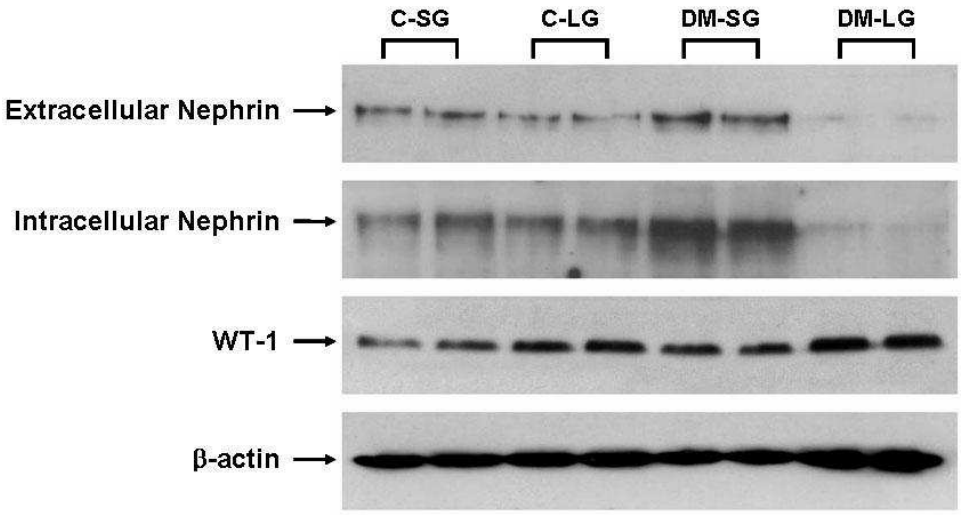

Figure 5. A representative Western blot with the lysates of C-SG, C-LG, D*M*-SG, and D*M*-LG. Glomerular nephrin (both extracellular and intracellular) protein expression was increased in D*M*-SG, whereas there was a significant reduction in nephrin protein expression in D*M*-LG compared to C-SG and C-LG. WT-1 and β-actin protein expression tended to be higher in both C-LG and D*M*-LG, but did not reach statistical significance.

IV. 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 different pore sizes or various kidney sizes may lead to diverse results in the expression of certain genes. Finally, based on the finding that nephrin expression is diminished in hypertrophied diabetic glomeruli, we suggest that albuminuria may arise first in hypertrophied glomeruli in the early stage of diabetic nephropathy.

Diabetic nephropathy 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, which is comprised of the glomerular endothelium, basement membrane, and podocytes connected by a slit diaphragm[3-7]. Podocytes play an important role in the synthesis and maintenance of the subepithelial GBM and in the maintenance of glomerular permselectivity[8]. They lie on the urinary side of the glomerular basement membrane and extend several primary processes that branch into secondary foot processes. The slit diaphragm, which bridges adjacent foot processes derived from different podocytes, functions as the ultimate molecular size filter with 4 x 14 nm-sized pores[23]. 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 diaphragm. 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]. In addition to

nephrin, P-cadherin[24], NEPH1[25], and FAT[26] are also known to exist at the slit diaphragm region, and catenin[24], CD2-associated protein[27], podocin[28], and ZO-1[29] are identified to be associated with the slit diaphragm.

It has been suggested that changes in these glomerular filtration barrier-associated molecules play a critical role in the pathogenesis of proteinuria in glomerular disease. Based on this view, there have been several reports on the changes of these filtration barrier-associated molecules in various kidney diseases, however most of the previous studies were focused on the changes in nephrin[12-19]. Not only have decreases in nephrin mRNA and protein expression been described[12, 14, 17, 19], but also redistribution of nephrin protein has been observed in experimental glomerular diseases induced by puromycin aminonucleoside or mercuric chloride[12]. In the latter, the changes in nephrin mRNA expression preceded the development of proteinuria. Similar changes in nephrin protein expression were also observed in adult patients with primary acquired nephrotic syndrome[15], but not in pediatric patients with glomerular diseases[16]. Since proteinuria is a cardinal feature of diabetic nephropathy, the contribution of changes in nephrin expression to proteinuria in diabetic nephropathy has also been studied, but the results were not consistent. Bonnet et al[14] demonstrated a reduction in nephrin mRNA and protein expression in streptozotocin-induced diabetic spontaneously hypertensive rats at 32 weeks after the induction of diabetes. A recent study also demonstrated diminished nephrin expression and altered nephrin 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 nephrin 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 diabetic nephropathy, infused by mini-pump in rats increased nephrin mRNA expression assessed by real-time PCR and quantitative *in situ* hybridization[30]. The reasons for the divergence of changes in nephrin expression in diabetic nephropathy may be due to a variety of underlying causes, including but not limited to differences in species, duration of diabetes, or accompanying hypertension.

However, When the sieving technique was used for the isolation of D*M* glomeruli, many of the hypertrophied D*M* glomeruli could not pass through the second to the last sieve, thus leading to data reflecting changes in only the smaller but not the larger glomeruli.

Recently, it has been suggested that nephrin acts as a signaling molecule which can activate the mitogen-activated protein kinase pathway and may influence podocyte survival[31-33]. Furthermore, a recent study using transgenic mice demonstrated that nephrin was transcriptionally activated by WT-1[34]. In this study, however, we observed that changes in nephrin and WT-1 expression did not coincide. Even though WT-1 expression was increased in glomeruli on 125 ㎛ 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. *M*oreover, we also observed an increase in nephrin expression in glomeruli from the 75 ㎛ 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 glomeruli of smaller size, but a compensatory phenomenon

for decreased nephrin expression in hypertrophied glomeruli or increased signaling events could be explanations for this observation.

Kidney size is typically increased in diabetes, even at the time of diagnosis[35]. This is primarily due to glomerular and tubular hypertrophy. A recent study used X-ray micro-computed tomography to measure the glomerular volume of the Otsuka Long-Evans Tokushima Fatty (OLETF) rat, an animal model of human type 2 D*M*, at 27 to 28 weeks of age[36]. This study revealed that glomerular volume was significantly larger in the OLETF rats compared to 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. Interestingly, scattering of glomerular volume, expressed as a coefficient variation (SD/mean), was significantly larger in OLETF rats compared to LETO rats even after normalization, suggesting more heterogeneity in the glomerular volume distribution. *M*any other investigators also observed that patients with type 2 D*M* showed much variability in renal changes[37,38]. Such variation has resulted in difficulty in determining a sensitive histologic parameter for ascertaining the early stage of diabetic nephropathy. In contrast, glomerular abnormalities in type 1 D*M* are known to be more uniform compared to type 2 D*M*[38]. In this study, however, we demonstrated that early hypertrophied glomeruli, which may be more affected by diabetic conditions, showed different nephrin expression patterns compared to relatively small sized glomeruli. These results suggest that the same inconsistent glomerular changes occur in type 1 D*M* as in type 2 D*M*. A recent study found that variation in nephrin expression in biopsies of patients with nephropathy from both type 1 and type 2 diabetes was larger

compared to control patients[17]. This finding partly supports our results describing changes in nephrin expression. Further study will be necessary to determine the location within the renal cortex of early hypertrophied glomeruli as well as the factors dictating this change.

The numbers of podocytes are known to decrease in the glomeruli of patients with type 1 diabetes of all ages, with reduced podocyte numbers even in diabetes of short duration[39]. Analysis of kidney biopsies from Pima Indians with type 2 diabetes also demonstrated that subjects with clinical nephropathy exhibited broadening of podocyte foot processes associated with a reduction in the number of podocytes per glomerulus[40]. To exclude the possibility that the decrease in nephrin expression in hypertrophied diabetic glomeruli was associated with a reduction in podocyte number, we reevaluated nephrin protein expression by normalizing it with WT-1 protein expression. Even after normalization, nephrin protein expression in hypertrophied diabetic glomeruli remained significantly lower compared to control glomeruli. On the other hand, encapsulated glomeruli containing the Bowman′ capsule are larger than decapsulated glomeruli. It has been reported that when glomerular isolation was performed using the sieving technique, the proportion of decapsulated and encapsulated glomeruli were 86.0 ± 6.0% and 11.0 ± 5.0%, respectively[41]. In this study, we observed nearly the same proportion of decapsulated glomeruli (about 90%) from the 125 ㎛ and 75 ㎛ sieves. These findings suggest that the reduction of nephrin mRNA and protein expression in large D*M* glomeruli is neither attributed to the decrease in podocyte number nor to the presence of a greater number of encapsulated glomeruli.

V. CONCL*U*SION

We hypothesized that differential gene expression might occur depending on glomerular size, and investigated the differences in nephrin expression between small and large (hypertrophied) glomeruli isolated from diabetic rats.

1. The mean volumes of D*M*-LG (1.68 ± 0.08 x 10^6 μm^3) and C-LG (1.47 ± 0.09 x 10^6 μm^3) were significantly higher than those of D*M*-SG (1.08 ± 0.04 x 10^6 μm^3) and C-SG (0.97 ± 0.03 x 10^6 μm^3) ($p<0.01$).

2. Nephrin mRNA expression was significantly reduced in D*M*-LG (0.49 ± 0.10) compared to D*M*-SG (1.71 ± 0.21) and C glomeruli (C-SG, reference value 1; C-LG, 1.05 ± 0.16) ($p<0.05$), whereas its expression was significantly higher in D*M*-SG compared to D*M*-LG and C glomeruli ($p<0.05$). In contrast, there was no statistically significant difference in glomerular 18s mRNA expression among the four groups.

3. Glomerular nephrin, both extracellular and intracellular, protein expression was increased in D*M*-SG, whereas there was a significant reduction in nephrin protein expression in D*M*-LG compared to C-SG and C-LG, as shown by nephrin mRNA expression. Densitometric quantitation revealed 56% and 92% increases in extracellular and intracellular nephrin protein expression, respectively, in D*M*-SG compared to C-SG ($p<0.05$). In contrast, extracellular and intracellular nephrin protein expression

was decreased by 77% and 86%, respectively, in D*M*-LG versus C-SG ($p<0.05$). There was no significant difference in nephrin protein expression between C-LG and C-SG. WT-1 and β-actin protein expression tended to be higher in both C-LG and D*M*-LG, but did not reach statistical significance.

Nephrin mRNA and protein expression are decreased in 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 hypertrophied glomeruli may be responsible for albuminuria in the early stage of diabetic nephropathy.

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ABSTRACT(IN KOREAN)

초기 당뇨 백서에서 사구체 크기에 따른 nephrin의 발현 차이

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배경: 당뇨병성 신병증은 투석이나 신이식이 필요한 말기 신부전증의 원인 질환 중 가장 많은 빈도를 차지하는 질환으로, 임상적으로는 단백뇨가 특징적인 소견이다. 최근의 연구에 의하면 당뇨병성 신병증을 포함한 대부분의 사구체 질환에 의한 단백뇨는 사구체 여과 장벽의 이상에 의하여 발생하는 것으로 보고되고 있다. 사구체 여과 장벽 중 알부민보다 크기가 작은 분자에 대한 여과 장벽 역할을 하는 것으로 알려져 있는 세극막은 서로 다른 족세포에서 기원한 족돌기들을 연결시켜 주는 섬유상의 구조로, 4 x 14 nm의 세공을 가지고 있다. 이러한 세극막을 구성하는 대표적인 분자로 nephrin이 있는데, 단백뇨를 동반하는 여러 실험적 동물 모델과 성인 신증후군 환자에서 nephrin의 발현이 감소되었다는 보고들이 있다. 그러나 당뇨병성 신병증의 경우 연구자에 따라 nephrin의 발현이 감소 또는 증가되었다고 보고하여, 서로 상반된 결과가 있는 실정이다. 이에 본 연구자는 당뇨병성 신병증에서 사구체 비후가 모든 사구체에서 동시에 일어나지 않을 것이라는 가정 하에 뇨단백 배설이 증가되기 시작하는 당뇨병성 신병증 초기에 비후된 사구체와 정상 크기의 사구체 사이에 nephrin의 발현에 차이가 있는 지를 알아보고자 하였다.

방법: Streptozotocin으로 당뇨를 유발시킨 백서 (Sprague-Dawley rats) 10 마리 (D*M*)와 대조군 배석 10마리 (C)를 대상으로, 당뇨 유발 6주 후에 체공 크기가 250 ㎛, 150 ㎛, 125 ㎛, 그리고 75 ㎛인 sieve를 이용하여 사구체를 분리하였다. 이 과정을 통하여 분리된 사구체 중 125 ㎛ sieve에 걸린 사구체를 비후된 사구체 (large glomeruli, LG), 그리고 75 ㎛ sieve에 걸린 사구체를 작은 사구체 (small glomeruli, SG)로 분류하여 실험하였다. 사구체 용적은 영상 분석기를 이용하여 산출하였으며, nephrin의 mRNA 발현은 실시간-중합효소 연쇄반응 (real time-PCR)으로, 그리고 단백 발현은 Western blot을 이용하여 분석하였다.

결과:

1. 사구체의 평균 용적은 D*M*-LG (1.68 ± 0.08 x 10^6 ㎛^{3})와 C-LG (1.47 ± 0.09 x 10^6 ㎛^{3})에서 D*M*-SG (1.08 ± 0.04 x 10^6 ㎛^{3})와 C-SG (0.97 ± 0.03 x 10^6 ㎛^{3})에 비하여 유의하게 컸다 ($p<0.01$).

2. C-SG의 nephrin mRNA 발현을 1로 하였을 때, D*M*-LG와 D*M*-SG에서의 nephrin mRNA 발현은 각각 0.49 ± 0.10과 1.71 ± 0.21로, nephrin의 mRNA 발현이 D*M*-LG에서 의미있게 감소되었던 반면에 D*M*-SG에서는 의의있게 증가되었다 ($p<0.05$). 한편, 18s의 mRNA 발현은 모든 군에서 유사하였다.

3. Nephrin의 단백 발현도 mRNA의 발현과 유사하였다. C-SG에 비하여 D*M*-LG에서 세포외 및 세포내 nephrin의 단백 발현이 각각 77%, 86% 감소되었던 반면에 D*M*-SG에서의 발현은 각각 56%, 92% 증가되었다 ($p<0.05$). 한편, Wilm's tumor-1과 β-actin의 단백 발현은 모든 군에서 유사하였다.

결론적으로, 사구체 크기에 따라 nephrin 유전자의 발현에 차이가 있었으며, nephrin의 발현이 감소되어 있는 비후된 사구체가 초기 당뇨병성 신병증에서 동반되는 단백뇨와 관련이 있을 것으로 생각된다.

핵심되는 말 : 당뇨병성 신병증, 단백뇨, nephrin, 사구체 비후, 사구체 크기