Apoptosis occurs differentially according to glomerular size in diabetic kidney disease

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Apoptosis occurs differentially according to glomerular size in diabetic kidney disease

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ABSTRACT

Apoptosis occurs differentially according to glomerular size in diabetic kidney disease

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Apoptosis, which is involved in the process of mesangial cell and podocyte loss in diabetic nephropathy, is known to be regulated by protein kinase B/Akt (Akt). A number of studies have therefore investigated the activity of Akt under diabetic conditions, but the results have not been consistent. In this study, it was hypothesized that apoptosis may occur differentially and that Akt may be differentially activated according to glomerular size in diabetic kidney disease.

Thirty male Sprague-Dawley rats were injected intraperitoneally with diluent (C, N=15) or streptozotocin (DM, N=15). After 3 months, glomeruli were isolated using sieves with pore sizes of 250 μm, 150 μm, 125 μm, and 75 μm and then classified into large glomeruli (on the 125 μm sieve, LG) and small glomeruli (on the 75 μm sieve, SG) groups. Western blot analyses for phospho-Akt, apoptosis-related molecules (Bax, Bcl-2, cleaved caspase-3, and phospho-p53), and cyclin-dependent kinase-inhibitors (CKIs) were performed. The numbers of total cells and podocytes in isolated glomeruli were determined using transmission electron microscopy.

Akt phosphorylation was significantly decreased in DM-LG, while it was significantly increased in DM-SG ($p<0.05$). The ratio of Bax/Bcl-2 protein expression, and cleaved caspase-3, phospho-Smad3 and phospho-p53 protein expression were significantly increased in DM-LG compared to DM-SG and C-SG ($p<0.05$ to $p<0.001$). In contrast, the expression of $p27^{Kip1}$ and $p21^{Cip1}$ was significantly increased in DM-SG compared to DM-LG and C-SG ($p<0.05$). The numbers of total glomerular cells and podocytes were significantly decreased in DM-LG ($p<0.05$).

In conclusion, these data show differential expression of Akt activity and apoptosis-related molecules according to glomerular size in diabetic nephropathy, suggesting that apoptosis may be more operative in more hypertrophic glomeruli, resulting in fewer glomerular cells and podocytes in diabetic nephropathy.

Key words: diabetic nephropathy, apoptosis, Akt, glomerular size, cyclin dependent kinase-inhibitors

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I. Introduction

Apoptosis removes damaged or unwanted cells and has been implicated in the pathogenesis of numerous diseases such as malignancy, lupus erythematosus, and Alzheimer's disease[1]. In addition, apoptosis has been documented in the course of various renal diseases including diabetic nephropathy[2-6]. Cell death by apoptosis is believed to be involved in the process of mesangial cell loss in the late stage of diabetic nephropathy[4]. In addition, apoptosis is considered to be one of the underlying causes of podocyte loss, which contributes to the development of albuminuria in

diabetic nephropathy[5, 6].

Besides apoptosis, renal hypertrophy is another hallmark of diabetic nephropathy[7, 8]. Kidney size is typically increased in diabetes, even at the time of diagnosis[9]. This is primarily due to glomerular and tubular hypertrophy, although some low-grade proliferation of glomerular cells is present in the early phase[7, 10]. Glomerular hypertrophy is in part the result of glomerular cells hypertrophy and recent studies have suggested that the diabetic milieu *per se*, hemodynamic changes, and local growth factors such as transforming growth factor-β (TGF-β) and angiotensin II (ANG II) are mediators in the pathogenesis of glomerular cells hypertrophy[11, 12].

Protein kinase B/Akt (Akt) is known to regulate a number of cellular functions including cellular hypertrophy and apoptosis[13], two characteristic findings in diabetic nephropathy. In addition, previous *in vitro* studies have demonstrated that high glucose, TGF-β, and ANG II, mediators of diabetic nephropathy, are closely linked with the Akt pathway[14-18]. Owing to these findings, the activity of Akt under diabetic conditions has been heavily investigated, but the results have not been consistent. Most studies focused on renal hypertrophy in diabetic nephropathy have demonstrated an increase in Akt phosphorylation[15, 19, 20], whereas numerous reports aimed at diabetes-

induced apoptosis of glomerular cells have revealed reduced activity of Akt[21, 22]. The reasons for the divergence of changes in Akt activity under diabetic conditions are not clear, but differences between the duration of diabetes or of high glucose stimulation or the species of animals may contribute to these disparities.

Prior studies have suggested that glomerular hypertrophy in diabetes does not develop in all glomeruli concurrently[23-25]. Moreover, Kim et al[26] have previously shown that nephrin expression is different between relatively small and large glomeruli isolated from early diabetic rats. Based on these findings, it can be surmised that the activity of Akt and the expression of apoptosis-related molecules (caspase-3, Bax, and Bcl-2) may also be differential in diabetic glomeruli. In this study, differences in the expression of phospho-Akt and apoptosis-related molecules were investigated between relatively small and large glomeruli isolated from 3-month diabetic rats. In addition, since accumulating evidence has shown that cyclin-dependent kinase-inhibitors (CKIs) play an important role in the process of apoptosis as well as hypertrophy[27-32], I also examined changes in $p21^{Cip1}$, $p27^{Kip1}$, and $p57^{Kip2}$ expression in less and more hypertrophied glomeruli.

II. Materials and Methods

1. 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. Thirty male Sprague-Dawley rats, weighing 250-280 g, were used. Fifteen rats were injected intraperitoneally with diluent (Control, C) and the other 15 with 65 mg/kg streptozotocin (Diabetes, DM). Blood glucose levels were measured 3 days after streptozotocin injection to confirm the development of diabetes. The rats were given free access to water and standard laboratory chow during the 3-month study period. All rats were sacrificed after 3 months.

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

2. Glomerular isolation

Glomeruli were isolated using sieves with pore sizes of 250 μm, 150 μm,

125 μm, and 75 μm. I classified glomeruli into large glomeruli (on the 125 μm sieve, LG) and small glomeruli (on the 75 μm sieve, SG). The C glomeruli, glomeruli on the sieve with a pore size of 125 μm from 2-3 C rats, were pooled because there were few glomeruli on the 125 μm sieve from the individual samples of the C rats. I also determined the proportion of encapsulated and decapsulated glomeruli on both the 125 μm and 75 μm sieves. Since the juxtamedullary glomeruli are known to be larger than superficial and midcortical glomeruli in C and DM rats, I tried to use only the superficial and midcortical tissues for glomerular isolation. In addition, glomeruli were collected under an inverted microscope to minimize tubular contamination.

3. Western blot analysis

Counted glomeruli were lysed in sodium dodecyl sulfate (SDS) sample buffer (2% SDS, 10 mM Tris-HCl, pH 6.8, 10% [vol/vol] glycerol), treated with Laemmli sample buffer, heated at 100°C for 5 minutes, and then electrophoresed in an 8-12% acrylamide denaturing SDS-polyacrylamide gel. Proteins were then transferred to a Hybond-ECL membrane using a Hoeffer semidry blotting apparatus (Hoeffer Instruments, San Francisco, CA, USA).

The membrane was 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°C in a 1:2000 dilution of polyclonal antibodies to phospho-Akt (Ser473), total Akt, cleaved caspase-3, phospho-p53, phospho-Smad3 (Cell Signaling, Beverly, MA, USA), Bax, Bcl-2, $p21^{Cip1}$, $p27^{Kip1}$, $p57^{Kip2}$, cyclin D1, or β-actin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). 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, USA). The washes were repeated and the membrane was developed with a chemiluminescent agent (ECL; Amersham Life Science, Inc.). Band densities were measured using TINA image software (Raytest, Straubenhardt, Germany).

4. Morphometric measurement of glomerular volume

Glomerular volumes (V_G) of isolated glomeruli were calculated as previously described[33]. Photographs of 50 decapsulated 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, Media Cybernetics, Silver Spring, MD, USA). V_G was calculated using the equation: $V_G = 4/3\pi(\text{Area}/\pi)^{3/2}$.

5. Double immunofluorescence staining

Freshly sieved glomeruli were fixed in paraformaldehyde at 4°C, washed with Hanks' Balanced Salt Solution (HBSS) for 5 min and treated with 0.5% Triton-X solution for 15 min at room temperature. Thereafter, glomeruli were washed with HBSS for 5 min, incubated in HBSS solution containing 0.3% hydrogen peroxide and 0.1% sodium azide for 20 min at room temperature, washed again with HBSS for 5 min and blocked with 10% donkey serum for 1 h at room temperature. Primary polyclonal antibodies to cleaved caspase-3 (Cell Signaling), $p27^{Kip1}$, or $p21^{Cip1}$ (Santa Cruz Biotechnology) were diluted in 1:100 with antibody diluent (DAKO, Glostrup, Denmark) and was applied overnight at 4°C. After washing, Cy3 (red)-conjugated anti-donkey IgG antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) was added for 1 h at room temperature. A 1:200 dilution of polyclonal Wilms' tumor (WT)-1 antibody (Santa Cruz Biotechnology) was then applied for 3 h at room temperature, followed by Cy2 (green)-conjugated anti-donkey IgG antibody

(Jackson ImmunoResearch Laboratories).

6. Determination of total glomerular cells and podocyte numbers

Total glomerular cells and podocyte numbers in isolated glomeruli were determined using the Exhaustive Count method as previously described[34]. Briefly, isolated glomeruli were fixed in 50 mM sodium cacodylate buffer (pH 7.4) containing 2% glutaraldehyde in paraformaldehyde for 30 minutes at 32°C, post-fixed in 1% OsO_4 for 2 hours at 4°C, and dehydrated by treatment with a graded series of ethanol (5 minutes each in 50%, 60%, 70%, 80%, 90%, 95%, and twice in 100%). Next, isolated glomeruli were treated with propylene oxide and embedded in Epon according to standard procedures. The glomeruli were cut into sections with a thickness of 3 μm using an Ultracut R ultratome (Leica) and then stained with toluidine blue. Two adjacent toluidine blue-stained sections were observed in pairs under transmission electron microscopy at a magnification of x 2,000 and the nuclei present in the top section but not in the bottom section were counted and summed. Ten glomeruli in 5 rats from each group and 13-15 semithin sections from the midglomerular area were examined.

7. Statistical analysis

All values are expressed as the mean ± standard error of the mean (SEM). Statistical analysis was performed using the statistical package SPSS for Windows version 11.0 (SPSS, Inc., Chicago, IL, USA). 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 Mann-Whitney U test. P values of less than 0.05 were considered to be statistically significant.

III. RESULTS

1. Animal data

All animals gained weight over the experimental period, but weight gain was higher in C compared to DM rats ($p<0.001$). The ratio of kidney weight to body weight in DM rats (1.07±0.06%) was significantly higher than in C rats (0.49±0.03%) ($p<0.01$). The mean blood glucose levels of C and DM rats were 114±5 mg/dl and 475±15 mg/dl, respectively ($p<0.001$). Compared to the C group (0.42±0.04 mg/day), 24-hour urinary albumin excretion was significantly higher in the DM group (2.34±0.22 mg/day) ($p<0.01$) (Table 1).

Table 1. Animal data

	C (n=15)	**DM (n=15)**
Body weight (Bwt)	616±11	319±8*
Kidney Wt	3.01±0.06	3.42±0.08$^{\#}$
Kidney Wt/Bwt (%)	0.49±0.03	1.07±0.06†
Glucose (mg/dL)	114±5	475±15*
24-h urine albumin (mg/day)	0.42±0.04	2.34±0.22†

* $p<0.001$ vs. C group; $^{\#}$ $p<0.05$ vs. C group; † $p<0.01$ vs. C group.

2. Glomerular volume

First, the volume of glomeruli found on the 125 μm (large glomeruli, LG) and 75 μm sieves (small glomeruli, SG) was examined. The mean volumes of DM-LG ($1.68\pm0.06\times10^6$ μm^3) and C-LG ($1.51\pm0.08\times10^6$ μm^3) were significantly higher than those of the corresponding DM-SG ($0.98\pm0.04\times10^6$ μm^3) and C-SG ($0.91\pm0.03\times10^6$ μm^3) ($p<0.01$) (Figure 1). The proportions of decapsulated glomeruli on the 125 μm sieve in C and DM rats were 89.3±4.5% and 90.7±5.2%, respectively, and on the 75 μm sieve were 91.1±3.2% and 91.9±2.9%, respectively.

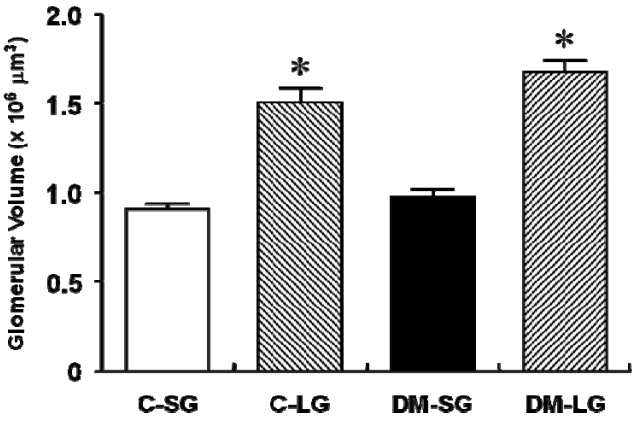

Figure 1. Mean glomerular volume in the C-SG, C-LG, DM-SG, and DM-LG groups. The mean volumes of DM-LG and C-LG were significantly higher than corresponding DM-SG and C-SG volumes. * $p<0.01$ vs. C-SG and DM-SG.

3. Phospho-Akt, Bax, Bcl-2, cleaved caspase-3, phospho-p53, and phospho-Smad3 protein expression in less and more hypertrophied glomeruli

The changes in glomerular phospho-Akt, Bax, Bcl-2, cleaved caspase-3, phospho-p53, and phospho-Smad3 protein expression according to the size of the glomeruli were investigated. Glomerular phospho-Akt protein expression was significantly decreased in DM-LG compared to the C groups, whereas its expression was significantly increased in DM-SG ($p<0.05$). There was no difference in total Akt protein expression among the four groups (Figure 2). Bax, cleaved caspase-3, phospho-p53, and phospho-Smad3 protein expression were also significantly increased in the DM-LG group compared to the DM-SG and C groups. Densitometric quantitation revealed 98, 159, 137, and 115% increases in Bax, cleaved caspase-3, phospho-p53, and phospho-Smad3 protein expression, respectively, in DM-LG compared to C-SG ($p<0.05$ and $p<0.01$). In contrast, the expression of Bcl-2 protein was significantly decreased in DM-LG relative to the C-SG ($p<0.01$) (Figure 3). On the other hand, there were no significant differences in phospho-Akt, Bax, Bcl-2, cleaved caspase-3, phospho-p53, and phospho-Smad3 protein expression between C-SG and C-LG.

To identify in which glomerular cells apoptosis occurred, double immunofluorescence staining with antibodies to WT-1 and cleaved caspase-3 was performed. As seen in Figure 4, apoptosis within DM-LG was significantly increased mainly in podocytes but somewhat in other glomerular cells, maybe mesangial cells.

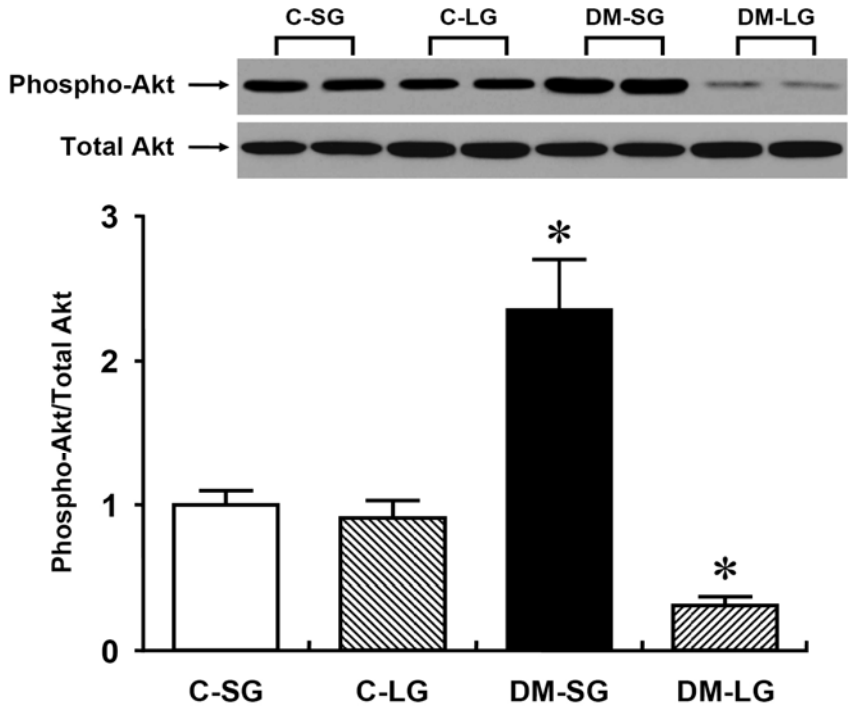

Figure 2. A representative Western blot of glomerular phospho-Akt and total Akt protein expression in the C-SG, C-LG, DM-SG, and DM-LG groups (representative of four blots). Phospho-Akt protein expression was significantly decreased in DM-LG compared to the C groups, whereas its expression was significantly increased in DM-SG. In contrast, there was no difference in total Akt protein expression among the four groups. * $p<0.05$ vs. C-SG and C-LG groups.

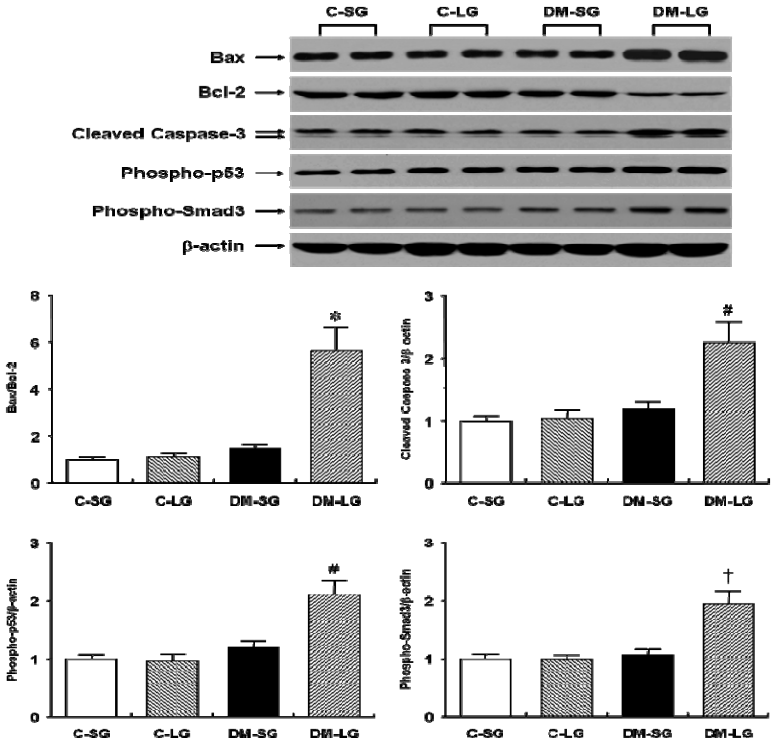

Figure 3. A representative Western blot of glomerular Bax, Bcl-2, cleaved caspase-3, phopho-p53, and phospho-Smad3 protein expression in the C-SG, C-LG, DM-SG, and DM-LG groups (representative of four blots). Bax, cleaved caspase-3, phospho-p53, and phospho-Smad3 protein expression were significantly increased, while Bcl-2 protein expression was significantly decreased in the DM-LG group compared to the DM-SG and C groups. In contrast, there were no significant differences in Bax, Bcl-2, cleaved caspase-3, phospho-p53 and phospho-Smad3 between the C-SG, and C-LG groups. * $p<0.001$ vs. other groups; # $p<0.01$ vs. other groups; † $p<0.05$ vs. other groups.

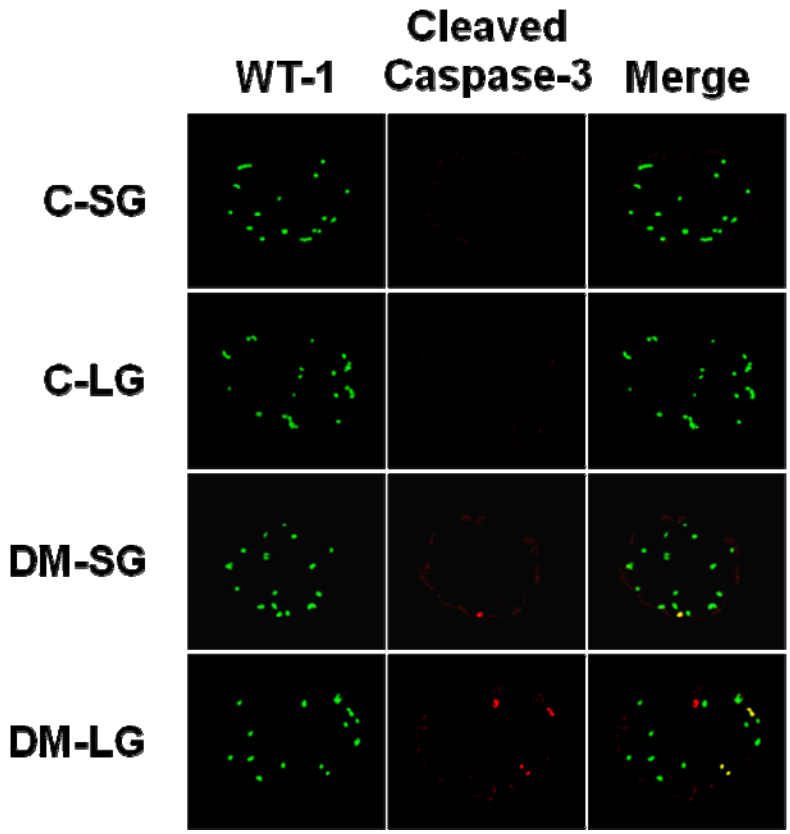

Figure 4. Double immunofluorescence staining for WT-1 and cleaved caspase-3 in the C-SG, C-LG, DM-SG and DM-LG groups. Immunofluorescence staining for cleaved caspase-3 was increased in DM-LG, and double immunofluorescence staining revealed that apoptosis within DM-LG was significantly increased mainly in podocytes but somewhat in other glomerular cells, maybe mesangial cells (x400).

4. CKIs and cyclin D1 protein expression in less and more hypertrophied glomeruli

Figure 5 shows a representative Western blot with the lysates of C-SG, C-LG, DM-SG, and DM-LG at 3 months after streptozotocin injection. Glomerular $p27^{Kip1}$ and $p21^{Cip1}$ protein expression were significantly increased in the DM-SG group compared to the DM-LG and C groups. Densitometric quantitation revealed 118% and 92% increases in $p27^{Kip1}$ and $p21^{Cip1}$ protein expression, respectively, in DM-SG compared to C-SG ($p<0.05$). There was also a significant increase in cyclin D1 protein expression in the DM-SG group compared to the DM-LG and C groups ($p<0.05$). On the other hand, there were no significant differences in $p57^{Kip2}$ and β-actin protein expression among the four groups.

Double immunofluorescence staining for WT-1 and $p27^{Kip1}$ or $p21^{Cip1}$ was performed to clarify the type of glomerular cells with increased $p27^{Kip1}$ or $p21^{Cip1}$ expression and revealed that podocytes were the main cells responsible for the increases in $p27^{Kip1}$ and $p21^{Cip1}$ protein expression in DM-SG (Figure 6).

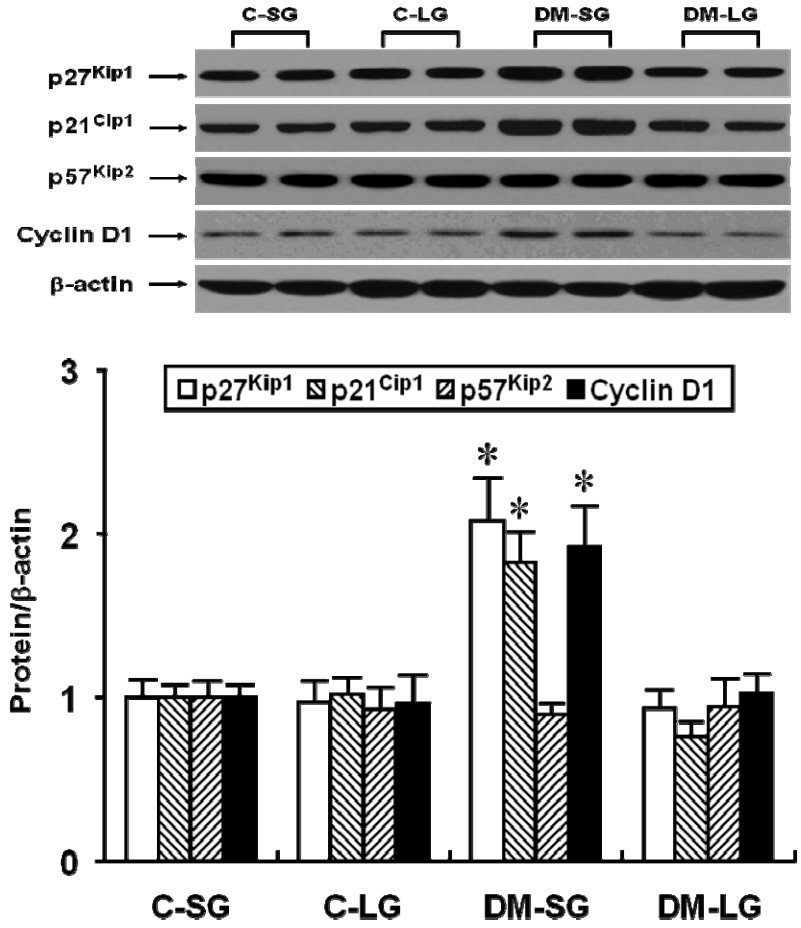

Figure 5. A representative Western blot of CKIs ($p27^{Kip1}$, $p21^{Cip1}$, and $p57^{Kip2}$) protein expression in the C-SG, C-LG, DM-SG, and DM-LG groups (representative of four blots). $p27^{Kip1}$ and $p21^{Cip1}$ protein expression were significantly increased in the DM-SG group compared to the DM-LG and C groups. In contrast, the protein expression of $p57^{Kip2}$ and β-actin was comparable among the four groups. * $p<0.05$ vs. other groups.

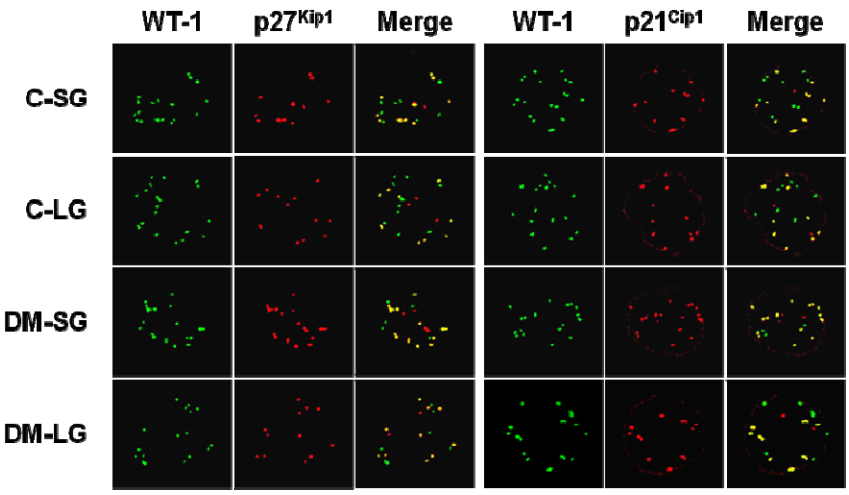

Figure 6. Double immunofluorescence staining for WT-1 and $p27^{Kip1}$ or $p21^{Cip1}$ in the C-SG, C-LG, DM-SG and DM-LG groups. Immunofluorescence staining for $p27^{Kip1}$ and $p21^{Cip1}$ was increased in DM-SG, and double immunofluorescence staining revealed that podocytes were the main cells responsible for the increases in $p27^{Kip1}$ and $p21^{Cip1}$ protein expression in DM-SG (x400).

5. Fibronectin protein expression in less and more hypertrophied glomeruli

Fibronectin protein expression was significantly increased in DM-SG and DM-LG compared to C glomeruli ($p<0.05$). However, there was no difference in fibronectin expression between DM-SG and DM-LG (Figure 7).

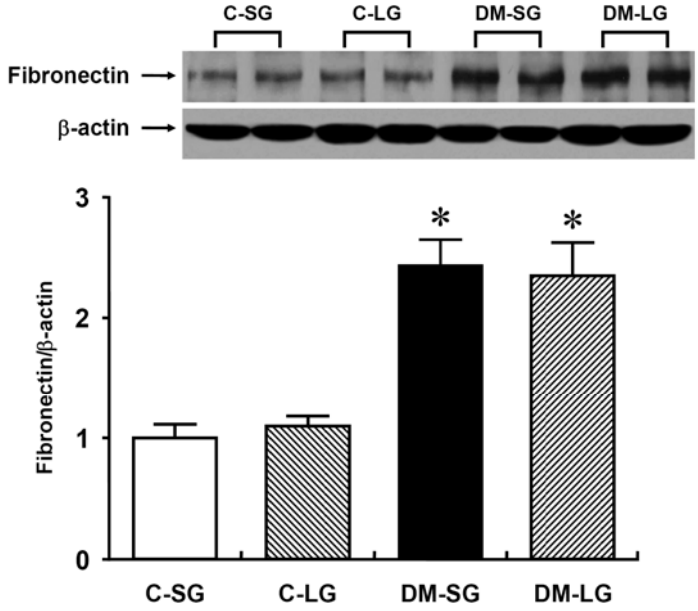

Figure 7. A representative Western blot of fibronectin protein expression in the C-SG, C-LG, DM-SG, and DM-LG groups (representative of four blots). The expression of fibronectin protein was significantly increased in DM-SG and DM-LG compared to C glomeruli. However, there was no difference in fibronectin expression between DM-SG and DM-LG. * $p<0.05$ vs. C-SG and C-LG groups.

6. Total glomerular cells and podocyte numbers in less and more hypertrophied glomeruli

Total glomerular cells and podocyte numbers were determined from the toluidine blue-stained semithin sections. The numbers of total glomerular cells in DM-SG (687.7±15.0/glomerulus) and C-LG (682.0±16.9/glomerulus) tended to be higher than in C-SG (658.8±13.7/glomerulus). In contrast, there were significantly fewer total glomerular cells in the DM-LG group (604.5±14.5/glomerulus) compared to the other groups ($p<0.05$). Podocyte number was also significantly decreased in DM-LG (140.1±5.1/glomerulus) relative to the other groups (C-SG, 172.5±6.9/glomerulus; C-LG 178.3±7.3/glomerulus; DM-SG, 165.9±8.1/glomerulus) ($p<0.05$) (Figure 8).

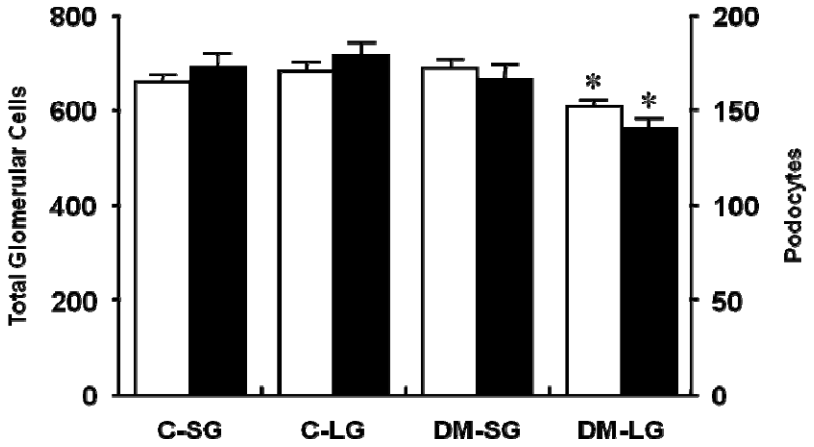

Figure 8. The numbers of total glomerular cells (white bar) and podocytes (black bar) in the C-SG, C-LG, DM-SG, and DM-LG groups. The number of total glomerular cells in DM-SG and C-LG tended to be higher than in C-SG. In contrast, there were significantly fewer total glomerular cells and podocytes in the DM-LG group compared to the other groups. * $p<0.05$ vs. C-SG, C-LG, and DM-SG groups.

7. Effect of insulin on the expression of phospho-Akt, Bax, Bcl-2, cleaved caspase-3 protein, and total glomerular cells and podocyte numbers in less and more hypertrophied glomeruli

In additional experiments using diabetic rats treated with 3–5 U/day of insulin (Ultralente; Eli Lilly, Indianapolis, IN), the increases in cleaved caspase-3 protein expression and the ratios of Bax/Bcl-2 protein expression and the decrease in Akt phosphorylation were significantly abrogated in LG of these rats (Figure 9). In addition, insulin treatment significantly ameliorated the reduction of total glomerular cells (696.3±13.1/glomerulus) and podocyte numbers (174.2±6.2/glomerulus) in DM-LG. These findings suggest that the changes in diabetic glomeruli were not due to STZ *per se*.

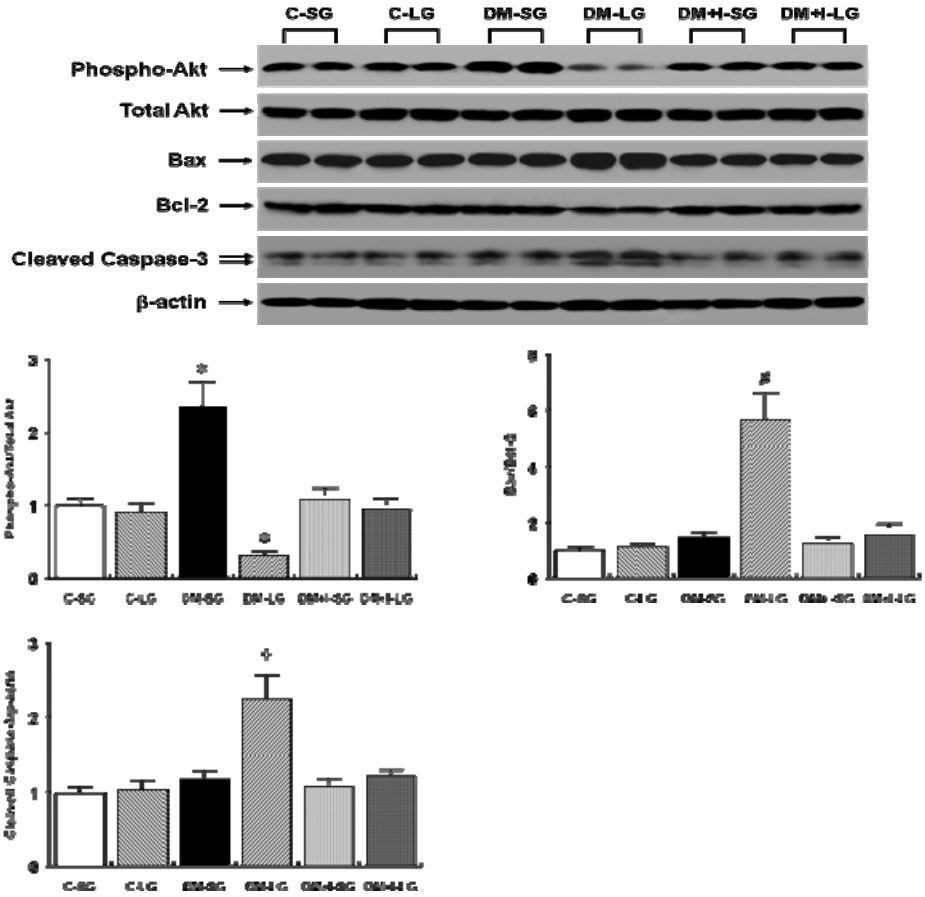

Figure 9. A representative Western blot of glomerular phospho-Akt, total Akt, Bax, Bcl-2, and cleaved caspase-3 protein expression in the C-SG, C-LG, DM-SG, DM-LG, DM+insulin (I)-SG and DM+I-LG groups (representative of four blots). Insulin treatment significantly abrogated the increases in cleaved caspase-3 protein expression and the ratios of Bax/Bcl-2 protein expression and the decrease in Akt phosphorylation in LG of DM rats. * $p<0.05$ vs. other groups; $^{\#}$ $p<0.001$ vs. other groups; † $p<0.01$ vs. other groups.

IV. DISCUSSION

In this study, it was shown that Akt activity is significantly decreased in DM-LG glomeruli along with concomitantly increased protein expression of Bax, cleaved caspase-3, and phospho-p53 and decreased Bcl-2 protein expression. In contrast, Akt phosphorylation is significantly increased in DM-SG. In addition, total glomerular cells and podocyte numbers are significantly lower in relatively large diabetic glomeruli. These findings suggest that apoptosis may occur differentially in diabetic glomeruli. In particular, a more operative process of apoptosis resulting in fewer glomerular cells may occur in more hypertrophied diabetic glomeruli.

Glomerular hypertrophy is a hallmark of diabetic nephropathy. Although inflammatory cell infiltration, extracellular matrix (ECM) accumulation, and hemodynamic factors are known to play a role in glomerular hypertrophy[35], the changes in glomerular cells themselves are the main reason for the observed hypertrophy. In this study, the glomeruli were classified into two groups of relatively small and large glomeruli. Since encapsulated glomeruli containing Bowman's capsule are larger than decapsulated glomeruli, the proportions of decapsulated and encapsulated glomeruli were determined. In addition, to rule out the possibility that the difference in ECM accumulation

may affect glomerular size, the protein expression of fibronectin was also examined. As results, I observed comparable proportions of decapsulated glomeruli (~90%) from the 125 and 75 μm sieves and found that there was no difference in fibronectin protein expression between relatively small and large glomeruli, suggesting that the difference in glomerular size may be primarily attributed to the variation in glomerular cell hypertrophy.

Glomerular hypertrophy in diabetes does not develop in all glomeruli concurrently[23-25]. Moreover, Kim et al[26] previously demonstrated that nephrin messenger RNA and protein expression were different between relatively small and large glomeruli isolated from early diabetic rats. Based on these findings, I hypothesized that there would be a wide variation of glomerular size in diabetes and that the expression of multiple genes in addition to nephrin would also differ according to the size of the glomeruli. The results of this study show that the expression of phospho-Akt is decreased in DM-LG, while its expression is increased in DM-SG. This provides another explanation for the inconsistent results regarding the changes in Akt activity under diabetic conditions[15, 19, 20, 21, 22] and suggests differential ongoing events according to glomerular size in diabetic nephropathy.

In addition to hypertrophy, apoptosis has also been implicated in the

pathogenesis of diabetic nephropathy[4-6]. Cell culture experiments using mesangial cells under high glucose conditions and *in vivo* studies using various models of diabetes have suggested that self-limited low-grade proliferation occurs initially, leading to an increase in mesangial cell number[7, 36]. In the late stage of diabetic nephropathy, however, mesangial cell loss is observed and apoptosis is surmised to be involved[4]. These findings suggest that apoptosis may be a homeostatic mechanism regulating the mesangial cell population. On the other hand, the number of podocytes is decreased in the glomeruli of patients with type 1 and type 2 diabetes[37, 38], and a good deal of evidence has shown that apoptosis is also implicated in the process of podocyte loss under diabetic conditions[39]. In the present study, the activity of Akt was significantly decreased in DM-LG along with increased expression of cleaved caspase-3, Bax, and phospho-p53 protein and decreased Bcl-2 protein expression. Since the phosphoinositide-3'-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) pathway is considered a typical pro-survival/antiapoptotic pathway, these findings suggest that apoptosis is more operative in more hypertrophied diabetic glomeruli. The results of this study also showed that the numbers of total glomerular cells and podocytes were significantly decreased in DM-LG. When these data were compared to the

results of the previous study with 6-week C and DM rats[26], total glomerular cells as well as podocyte numbers were significantly lower in 3-month DM-LG relative to 6-week DM-LG. Furthermore, the changes in the expression of apoptosis-related molecules were in significant in 6-week DM-LG (data not shown). Double immunofluorescence staining for WT-1 and cleaved caspase-3 revealed that apoptosis within 3-month DM-LG was significantly increased mainly in podocytes but somewhat in other glomerular cells, maybe mesangial cells. Taken together, I assume that apoptosis occurs principally after 6 weeks of DM induction especially in more hypertrophied diabetic glomeruli, resulting in fewer glomerular cells and podocytes in DM-LG. A recent study by Menini et al.[40] also suggested that glomerular cell apoptosis was not an early feature in the course of experimental diabetic glomerulopathy, since it is preceded by glomerular hypertrophy, supporting my assumption.

CKIs are members of cell cycle regulatory proteins and are known to play an important role in the development of cellular hypertrophy under diabetic conditions. CKIs expression was found to be increased under diabetic conditions, both *in vitro* and *in vivo*, and was closely associated with cellular and glomerular hypertrophy[29, 30, 31, 41, 42]. In addition, recent studies have suggested that CKIs are involved in protecting nonmalignant cells from

apoptosis[32, 43, 44]. Moreover, several recent studies have suggested that there is an interaction between the PI3K/Akt/mTOR pathway and the expression of CKIs[16, 45]. Taken together, the evidence suggests that CKIs are important molecules not only in the development of cellular hypertrophy but also in cell survival pathways by protecting against apoptosis under diabetic conditions via coordination of the cell cycle and cell death programs. Considering these facts, I also determined the expression of CKIs in relatively small and large diabetic glomeruli and found that $p27^{Kip1}$ and $p21^{Cip1}$ expression was increased only in DM-SG. Even though they are difficult to confirm, the results of the present study suggest that the still-ongoing hypertrophic process or the cellular response to apoptotic stress may be linked to the increase in CKIs expression in DM-SG. Contrary to most previous studies, on the other hand, $p27^{Kip1}$ and $p21^{Cip1}$ expression was not increased in DM-LG. As aforementioned, since CKI expression is dependent on the PI3K/Akt/mTOR pathway, the differential expression of Akt activity may partly contribute to the differential expression of CKIs. In addition, the CKI expression in a relative small proportion of LG to total glomeruli ($11.3 \pm 1.2\%$) in 3-month DM rats may be masked by their expression in DM-SG in most previous studies, which used whole glomeruli for experiments[31, 41, 42]. In this study, cyclin D1 expression, which is central to

the regulation of the G1 to S phase transition and has been known to be involved in apoptosis in neuron cells[46, 47], was increased in DM-SG but not in DM-LG. An increase in cyclin D1 expression was also demonstrated in renal cortex of diabetic mice and high-glucose-stimulated mesangial cells and was associated with glomerular and mesangial cell hypertrophy[48]. In addition, Jiang et al.[49] suggested that increased expression of cyclin D1 with a concomitant increase in $p21^{Cip1}$ expression induced G0/G1 progression but a cell cycle arrest at G1 to S transition, leading to cell hypertrophy. Collectively, increased CKIs and cyclin D1 expression only in DM-SG may imply that the hypertrophic process is underway in less hypertrophied diabetic glomeruli, while it is completed and replaced by apoptosis in more hypertrophied diabetic glomeruli.

TGF-β1, an important mediator in the pathogenesis of diabetic nephropathy, is also involved in cell growth, differentiation, ECM production, and apoptosis[11]. TGF-β1 exerts these diverse effects by binding its receptor complex and subsequently activating the same members of the Smad family of transcription factors. Recently, however, in addition to the Smad pathway (canonical pathway), the TGF-β1 receptor complex has been demonstrated to induce non-Smad signals in various cell types, including mitogen-activated

protein kinases, PI3K, Akt, and mTOR (noncanonical pathway)[50]. Ultimately, integration of Smad and non-Smad signaling pathways determines the nature of the cellular response. Consistent with these previous findings, the results of the present study showed that in DM-SG, Akt might be activated in part by TGF-β1 via noncanonical pathway, while in DM-LG, in which Akt activity was not increased and apoptosis was in progress, TGF-β1 might exert its effect via the classical Smad pathway.

In conclusion, Akt phosphorylation was significantly decreased in relatively large diabetic glomeruli along with concomitantly increased protein expression of Bax, cleaved caspase-3, and phospho-p53 and decreased Bcl-2 protein expression. In addition, the numbers of total glomerular cells and podocytes were significantly lower in relatively large diabetic glomeruli. These findings suggest that apoptosis may be more operative in more hypertrophic glomeruli, resulting in fewer glomerular cells and podocytes in diabetic kidney disease.

V. CONCLUSION

In this study, I investigated the differences in the expression of phospho-Akt and apoptosis-related molecules between relatively small and large glomeruli isolated from 3-month diabetic rats. Since accumulating evidence has shown that CKIs play an important role in the process of apoptosis as well as hypertrophy, the changes in $p21^{Cip1}$, $p27^{Kip1}$, and $p57^{Kip2}$ expression were also examined in less (SG) and more hypertrophied glomeruli (LG).

1. All animlas gained weight over the experimental period, but weight gain was significantly higher in C compared to DM rats. However compared to the C group, the ratio of kidney weight to body weight, blood glucose levels, and 24-hr urinary albumin excretion were significantly higher in the DM group.
2. The mean volumes of DM-LG and C-LG were significantly higher than those of the corresponding DM-SG and C-SG.
3. Glomerular phospho-Akt protein expression was significantly decreased in DM-LG compared to the C groups, whereas its expression was significantly increased in DM-SG.
4. Bax, cleaved caspase-3, phospho-p53, and phospho-Smad3 protein expression were significantly increased in the DM-LG group compared to

the DM-SG and C groups. In contrast, the expression of Bcl-2 protein was significantly decreased in DM-LG relative to the other groups.

5. Immunofluorescence staining revealed that apoptosis within DM-LG was significantly increased mainly in podocytes but somewhat in other glomerular cells, maybe mesangial cells.
6. Glomerular $p27^{Kip1}$ and $p21^{Cip1}$ protein expression were significantly increased in the DM-SG group compared to the DM-LG and C groups. There was also a significant increase in cyclin D1 protein expression in the DM-SG group than the other groups.
7. Fibronectin protein expression was significantly increased in DM-SG and DM-LG compared to C glomeruli. However, there was no difference in fibronectin expression between DM-SG and DM-LG.
8. The numbers of total glomerular cells and podocytes were significantly decreased in DM-LG compared to the other groups.
9. Insulin treatment significantly ameliorated the changes in Akt phosphorylation and the expression of apoptosis-related molecules, and the reduction of total glomerular cells and podocyte numbers in DM-LG.

In conclusion, these data show differential expression of Akt activity and apoptosis-related molecules according to glomerular size in diabetic rats, suggesting that apoptosis may be more operative in more hypertrophic glomeruli, resulting in fewer glomerular cells and podocytes in diabetic kidney disease.

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ABSTRACT (IN KOREAN)

당뇨병성 신질환에서
사구체 크기에 따른 세포사멸의 차별적 발생

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정동섭

당뇨병성 신병증에서 사구체 내 메산지움 세포와 족세포의 수적 감소와 관련이 있는 세포사멸은 protein kinase B/Akt (Akt)의 영향을 받는 것으로 알려져 있다. 당뇨 조건 하에서 Akt 의 활성에 대한 기존의 연구는 많이 있어 왔으나, 그 결과는 보고자에 따라 서로 달랐다. 이에 본 연구자는 당뇨병성 신질환에서 사구체의 크기에 따라 Akt 의 활성에 차이가 있으며, 이에 따라 세포사멸도 사구체 크기에 따라 차별적으로 발생할 것으로 생각하여 본 연구를 시행하였다.

동물 실험은 30 마리의 Sprague-Dawley 백서를 이용하였으며, 대조군 (C, 15 마리)과 streptozotocin 으로 당뇨를 유발시킨 당뇨군 (DM, 15 마리)으로 나누어 진행하였다. 당뇨 유발 3 개월 후 신장을 적출한 후 250, 150, 125, 그리고 75 μm 크기의 구멍을 가진 체를 이용하여 사구체를 분리한 후, 125 μm 크기의 구멍을 가진 체에 걸린 사구체를 큰 사구체 (large glomeruli, LG)군, 75 μm 크기의 구멍을 가진

체에 걸린 사구체를 작은 사구체 (small glomeruli, SG)군으로 분류하였다. Phospho-Akt, 세포사멸에 관련된 단백 (Bax, Bcl-2, cleaved caspase-3, 그리고 phospho-p53), phospho-Smad3, 그리고 cyclin-dependent kinase inhibitors 의 단백 발현은 western blot 을 이용하여 확인하였으며, 분리한 사구체 내 총 세포 및 족세포의 수는 전자 현미경을 이용하여 산출하였다.

Akt 의 활성화는 DM-LG 군에서 유의하게 감소되었던 반면에, DM-SG 군에서는 의미있게 증가되었다. Bax 와 Bcl-2 단백의 발현 비율, 그리고 cleaved caspase-3, phospho-Smad3 및 phospho-p53 의 단백 발현은 DM-SG 군과 C 군에 비하여 DM-LG 군에서 의의있게 높았다. 이와는 반대로, $p27^{Kip1}$ 과 $p21^{Cip1}$ 의 단백 발현은 DM-LG 군과 C 군에 비하여 DM-SG 군에서 유의하게 증가되었다. 한편, 사구체 내 총 세포 및 족세포의 수는 다른 군에 비하여 DM-LG 군에서 의미있게 적었다.

이상의 결과들로 미루어 보아, 당뇨병성 신질환에서 사구체 크기에 따라 Akt 의 활성 및 세포사멸과 관련된 단백들의 발현에 차이가 있으며, 이러한 차이와 연관되어 더 큰 당뇨 사구체, 즉 더 비후된 당뇨 사구체에서 세포사멸이 더 많이 발생되어 더 큰 당뇨 사구체 내 총 세포 및 족세포 수의 감소가 나타나는 것으로 생각된다.

핵심되는 말: 당뇨병성 신병증, 세포사멸, Akt, 사구체 크기, cyclin-dependent kinase inhibitors

PUBLICATION LIST

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