Hypertrophy and Loss of Podocytes in Diabetic Nephropathy

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Abstract

Objective The loss of podocytes has been reported to have a role in the onset and progression of diabetic nephropathy (DN). Although structural changes such as podocyte hypertrophy are considered to be associated with podocyte loss, the relationship has not been thoroughly investigated using human DN renal tissues.

Methods The subjects were 17 patients with DN diagnosed histopathologically by renal biopsy. Immunostaining was performed with antibodies for Wilm’s tumor 1 (WT1) and synaptopodin (SPD), which are markers of podocytes, to determine the number of podocytes and assess podocyte hypertrophy.

Results The number of podocytes was decreased in DN patients compared with the controls. An inverse correlation was observed between the number of podocytes and both the urinary protein excretion and the extent of mesangial expansion. Podocyte hypertrophy was also more marked in DN patients compared with controls.

Conclusion Based on these results, podocyte loss and hypertrophy were suggested to be involved in the development and progression of human DN.

Key words: podocyte loss, podocyte hypertrophy, diabetic nephropathy


Introduction

The number of patients requiring dialysis due to diabetic nephropathy (DN) is increasing year by year in Japan, and it has currently become the number one reason for starting dialysis, followed by chronic glomerulonephritis. The mechanisms leading to the onset and progression of DN should be urgently clarified in order to improve the quality of life of patients with renal failure and to reduce medical costs. The relationship between podocyte damage and renal disease has attracted attention in recent years and reports about involvement of podocyte loss in the progression of DN have been published (1-5). The association of podocyte hypertrophy with podocyte loss has also been reported (6, 7), however, this relationship has not been examined in detail using human DN renal tissues.

Recently, some podocyte specific proteins have been reported. The Wilm’s tumor-1 (WT1) gene encodes the WT1 protein, which is expressed throughout urogenital development and continues to be highly expressed in podocytes. Furthermore, WT1 may play an important role in the maintenance of podocyte function (8, 9). On the other hand, synaptopodin (SPD) is a proline-rich protein, intimately associated with the actin microfilament, and it is exclusively expressed in the foot process of podocytes (10). While SPD is expressed in the foot processes, WT1 is exclusively expressed in the nucleus of the podocyte. A recent study using an experimental diabetic rat model reported down regulation in both WT1 and SPD rats compared with normal condition rats, and these results may be related podocyte loss (11).

In the present study, we aimed to clarify the relationship between podocyte loss and podocyte hypertrophy based on various clinical indexes and immunostaining of human DN
renal tissues for WT1 and SPD, which are different distribution markers of podocytes.

**Subjects and Methods**

**Patients**

The subjects were 17 patients in whom kidney biopsy was performed at Tokai University Hospital and DN was histologically diagnosed based on the results of light microscopy [with periodic-acid-Schiff (PAS), hematoxylin-eosin (HE), and periodic acid methenaminesilver (PAM) staining, fluorescence microscopy, and electron microscopy]. We also examined control samples obtained from 10 subjects using uninvolved portions of surgically removed kidneys afflicted with malignancies (Table 1).

The gender, age, blood pressure, urinary protein excretion (U-Prot), creatinine clearance (Ccr), and angiotensin-converting enzyme inhibitors (ACE-Is) and/or angiotensin II receptor blocker (ARBs) administration were investigated. The extent of mesangial expansion was investigated as a pathological index of the progression of DN. In the present study, we excluded severe mesangial expansion in which Kimmelstiel-Wilson nodules were observed. Mesangial expansion was evaluated by randomly selecting at least 5 glomeruli in which the vascular pole could be identified, and calculating the PAS-positive area in each glomerulus by automated computer analysis with WinRoof software (Mitani Co., Fukui, Japan).

Written informed consent was obtained from each patient for the use of their clinical data and the residual renal tissue after a pathological diagnosis had been made by renal biopsy.

**Immunohistochemistry**

**WT1 and SPD double staining**

Paraffin-embedded tissues were cut into 4 μm sections. After deparaffinization and dehydration in an ethanol series, antigen retrieval was performed by heating in a microwave oven (750 W for 5 min × 5 times in citrate buffer). After cooling, endogenous peroxidase was inhibited by incubation in MeOH-H2O2 (100 : 1) for 15 min. The specimens were rinsed in phosphate-buffered saline (PBS), blocked in the normal goat serum (15 : 1,000) for 10 min, followed by incubation with rabbit anti-WT1 antibody (1:50) (sc-192, Santa Cruz Biotechnology, CA, USA) overnight at 4°C. Then, they were rinsed in PBS, incubated in biotin-anti rabbit IgG (made in goat) for 1 hour, rinsed in PBS, and reacted with Vectastain ABC reagent (Vector Laboratories, Burlingame, CA, USA). After rinsing with PBS, the specimens were stained with Ni-Co-diaminobentidine (DAB) (Nichirei Bioscience, Tokyo, Japan). Then, the specimens were incubated again in MeOH-H2O2 (100 : 1) for 15 min, rinsed in PBS, incubated in avidin solution, and then in biotin solution. After blocking the specimens with normal goat serum, incubation was done overnight at 4°C with mouse anti-synaptopodin (SPD) antibody (1 : 100) (G1D4, Progen, Heidelberg, Germany). After rinsing with PBS, the specimens were reacted with the biotin-anti mouse IgG (made in goat) and Vectastain ABC reagent. After rinsing with PBS, DAB staining was performed for a predetermined duration to avoid any influence of changes in the duration of staining.

At least 5 glomeruli, in which the vascular pole could be identified, were randomly selected from each patient. WT1-positive cells located in the SPD-positive area or proximal to the urinary space (WT1-positive/SPD-positive cells) were defined as podocytes (Fig. 1). The number of these cells in each glomerulus was counted. The SPD-positive area of each glomerulus was calculated by automated computer analysis with WinRoof software (Fig. 2). The ratio of the SPD-positive area to the number of WT1-positive/SPD-positive cells were calculated as indexes of podocyte hypertrophy.

**Statistical analysis**

Results are shown as the mean ± SD, and the Mann-Whitney U-test was used for comparison of 2 groups. The Kruskal-Wallis test was used for comparison of 3 or more groups. Spearman’s rank correlation analysis was used to assess the correlation between each group. In all analyses, p< 0.05 was considered statistically significant. Stat view 5.0 software (SAS Institute Inc., Cary, NC, USA) was used for analysis.

**Results**

Podocytes were identified by immunostaining for WT1 and SPD (Fig. 1). The number of WT1-positive/SPD-positive cells (podocyte count) was significantly decreased in DN patients compared with the controls (Figs. 3, 4). An inverse correlation was observed between the number of WT1-positive/SPD-positive cells and U-Prot (Fig. 5A). There was no significant correlation between the number of WT1-positive/SPD-positive cells and Ccr (Fig. 5B). An inverse correlation was observed between the number of WT1-

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**Table 1. Clinical Characteristics between DN and Control**

<table>
<thead>
<tr>
<th></th>
<th>DN (n=17)</th>
<th>control (n=10)</th>
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<tbody>
<tr>
<td>Gender (M/F)</td>
<td>13 / 4</td>
<td>7 / 3</td>
</tr>
<tr>
<td>Age (years old)</td>
<td>42.3 ± 10.8</td>
<td>50.5 ± 10.6</td>
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<tr>
<td>SBP (mmHg)</td>
<td>140.8 ± 19.1</td>
<td>NA</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>82.7 ± 14.2</td>
<td>NA</td>
</tr>
<tr>
<td>U-Prot (g/day)</td>
<td>1.4 ± 1.9</td>
<td>NA</td>
</tr>
<tr>
<td>Ccr (ml/min)</td>
<td>91.8 ± 20.6</td>
<td>NA</td>
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**SBP** : Systolic blood pressure  **DBP** : Diastolic blood pressure  **U-Prot** : urinary protein excretion  **Ccr** : Creatinine clearance
Figure 1. Immunohistochemistry of WT1 and SPD double staining. (A) WT1 staining showed a nuclear pattern and SPD staining showed a linear pattern (original magnification ×100). (B) WT1-positive cells, located in the SPD-positive area or proximal to the urinary space, were defined as podocytes (arrow, ×200).

Figure 2. The calculation method of the SPD-positive area. The expression level of synaptopodin protein was quantitated by percentage of immunohistochemically-positive area in a glomerulus [(A)/(B)×100, where (A) is the immunohistochemically-positive area in the glomerulus and (B) is the total area of the glomerulus]. Finally, SPD-positive area per the number of WT1-positive/SPD-positive cells (number of podocytes) was calculated as an index of podocyte hypertrophy.

Figure 3. Immunohistochemistry of WT1 and SPD double staining in DN (A) and control (B). The ratio of the SPD-positive area to the number of WT1-positive/SPD-positive cells and the PAS-positive area (Fig. 5C). The ratio of the SPD-positive area to the number of WT1-positive/SPD-positive cells, an index for evaluating hypertrophy of podocytes, was significantly increased in DN.
Discussion

In this study, we identified podocytes by double-staining for WT1 and SPD, and counted the number of such cells. Our results again showed evidence of podocyte loss in patients with DN. Because podocyte loss was correlated with indexes of clinical and pathological progression (i.e., proteinuria and mesangial expansion), it was suggested to also be correlated with the progression of DN. Various reports on the relationship between podocyte loss and progression of renal disease have been published in recent years (12-15). Kritz et al reported that podocyte loss occurs because of lack of proliferation of these terminally differentiated cells in the glomeruli, which leads to denudation of the glomerular basement membrane (GBM) and finally results in glomerulosclerosis after adhesion with Bowman’s capsule (16-19). Progression of DN was reported to be related to podocyte loss by other authors (1-5), in agreement with the results of this study. Podocyte loss was reported to show a stronger relationship with the renal prognosis than GBM thickening or mesangial expansion, which are pathological features of DN (2). Based on such results, it may be important to prospectively investigate the severity of podocyte loss in relation to the renal prognosis of individual patients in the future.

Investigation of the ratio of the SPD-positive area to the number of WT1-positive/SPD-positive cells in this study suggested that podocyte hypertrophy occurs in DN patients. Regarding the mechanism of podocyte hypertrophy, it has been suggested that residual podocytes on the GBM after partial loss may enlarge to cover the denuded areas caused by lack of proliferation of mature podocyte, leading to hypertrophy (1). In contrast, Wiggins et al studied aging rats and suggested that podocyte hypertrophy already occurs before proteinuria and podocyte loss (20). Further assessment of the above reports is necessary to determine whether podocyte loss and podocyte hypertrophy is the cause or result. The patients with DN in this study had overt nephropathy rather than microalbuminuria, and both podocyte loss and...
podocyte hypertrophy were observed. It was therefore difficult to determine which event was the earlier event and this remains to be elucidated in the future. However, the results of this study are useful because detailed evaluation of podocyte loss and podocyte hypertrophy has not been performed in Japanese patients with DN. Future investigation of podocyte loss and podocyte hypertrophy in early DN (including normo-albuminuria) might contribute to the elucidation of the mechanism underlying the onset and progression of DN.

It is important to clarify the detailed mechanism of podocyte hypertrophy for development of treatment. In addition to the report of Pagtalunan et al (1), some authors have reported that mechanical stretching due to increased internal glomerular pressure causes hypertrophy by influencing the cell cycle via the cyclin-dependent kinase inhibitors such as P21 (7, 21), whereas others have suggested that high glucose activates ERK1/2 and Akt/PKB via production of reactive oxygen species (ROS) and induces hypertrophy that is further potentiated by angiotensin II (22). However, all of these results were obtained in animal experiments. A recent clinical study revealed that ACE-Is and ARBs play an important role in remission or regression of diabetic nephropathy (23). However, from our results, both clinical indexes and pathological indexes were not significantly different between ACE-Is and/or ARBs prescribed group and the non prescribed group (Table 2).

Although podocyte hypertrophy was suggested to be targeted by antioxidant therapy, ACE-Is and ARBs, further research into the detailed mechanisms of podocyte loss and podocyte hypertrophy is needed.

**Conclusion**

Double staining for WT1 and SPD suggested the occurrence of podocyte loss and podocyte hypertrophy in patients with DN. It was also suggested that these changes are possibly related to the onset and progression of DN. It is necessary to confirm these results in a large series of patients with early DN.

### References


