Characteristics of podocyte injury in malignant hypertensive nephropathy of rats (MSHRSP/Kpo strain)

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(Received 2 July 2015; and accepted 6 August 2015)

ABSTRACT
Proteinuria is not only a hallmark of renal complication in malignant hypertension, but is also a major deteriorating factor for the progression to end-stage renal disease. Podocyte injury plays a crucial role in the renal damage associated with hypertensive nephropathy, but the underlying mechanism remains unclear. Malignant stroke-prone spontaneously hypertensive rats (MSHRSP/Kpo) represent an original and useful model of human malignant hypertension. In this study, we disclosed the glomerular injuries in the MSHRSP/Kpo. MSHRSP/Kpo exhibited elevated blood pressure at 6 weeks along with renal dysfunction and proteinuria. Histological analysis of the MSHRSP/Kpo glomeruli revealed a severe atrophy, but no change was found in the podocyte number. The expression levels of podocyte-specific proteins, nephrin, podocin, and synaptopodin were decreased in the MSHRSP/Kpo glomeruli, though another podocyte-specific protein, CD2AP, in the MSHRSP/Kpo glomeruli exhibited a similar extent of staining as in normotensive WKY/Kpo rats. Furthermore, desmin was not markedly detected in the WKY/Kpo glomeruli, but was strongly positive in MSHRSP/Kpo. By electron microscopy, well-formed foot processes (FP) were replaced by effacement in MSHRSP/Kpo. An original malignant hypertension strain MSHRSP/Kpo exhibits podocyte injuries associated with the decrease of some podocyte-specific proteins and the upregulation of desmin, along with FP effacement and proteinuria.

Malignant hypertension is critical for various kinds of vascular event and subsequent organ damage, such as stroke, cardiac hypertrophy, nephrosis, and atherosclerosis. Among these, hypertensive nephrosclerosis from long-standing uncontrolled hypertension is a major cause of chronic kidney disease and accounts for significant mortality (3). The histological lesions of hypertensive nephrosclerosis are well recognized and characterized by hyalinization and sclerosis of interlobular and afferent arterioles, together with tubulointerstitial fibrosis, glomerulosclerosis, and tubular cell atrophy (26). A number of factors including a defective renal autoregulatory response to hypertension, renal susceptibility genes, and environmental factors such as salt, smoking, and lead exposure have been suggested to contribute to the development of hypertensive nephrosclerosis (17). In particular, proteinuria is not only a hallmark of renal complication in hypertension, but is also a major deteriorating factor for the progression to end-stage renal diseases (23, 24).

Glomerular epithelial cells, podocytes, which are located on the outside of the glomerulus and cover the capillary wall, play an important role in the glomerular filtration of the kidney. The podocytes do not proliferate after birth under normal conditions and are terminally differentiated to maintain an intricate and polarized cellular organization consisting of a cell body, major processes, and foot process
sections were used. For synaptopodin, desmin, CD2AP, and nestin staining, 10% formalin-fixed sections were used, as reported previously (11, 12). Antigen retrieval was performed by using 1 mM EDTA at pH 8.0 in microwave for 20 min for these staining except PECAM-1 staining. Immunostaining assays were performed as described previously (13). WT-1-positive and total cells in the glomerulus were counted as described previously (11).

**Electron microscopy.** Deeply anesthetized animals were transcardially perfused with PBS followed by ice-cold 1% glutaraldehyde (GA)/1% paraformaldehyde (PFA) in PBS. For transmission electron microscopy (TEM) analysis, the extracted kidneys were fixed in the same solution at 4°C overnight. Tissue specimens were post-fixed in osmium tetroxide (OsO₄) for 2 h at 4°C, dehydrated through an ethanol gradient, and embedded in Epon. Ultrathin sections (90 nm) were cut with an ultra-microtome, stained with 4% uranyl acetate and 1% lead citrate (Pb), and then examined by TEM (HT7700; Hitachi, Tokyo, Japan). For scanning electron microscopy (SEM) analysis, the specimens were fixed in 2.5% GA overnight at 4°C. After washing with 0.1 M phosphate buffer, the specimens were post-fixed in OsO₄ for 1 h at 4°C. The specimens were subsequently dehydrated in a graded series of ethanol and t-butyl alcohol, and then freeze-dried. The dried specimens were finally mounted on stubs, coated with gold palladium, and examined by SEM (SU3500, Hitachi).

**Statistical analysis.** Student’s t-test was used to determine statistical significance.

**RESULTS**

**Characterization of malignant SHRSP/Kpo**

We first examined the blood pressure of MSHRSP/Kpo compared with that of normotensive WKY/Kpo rats. SHR/Kpo and SHRSP/Kpo strains had almost the same level as the WKY/Kpo at 6 weeks of age (12). However, MSHRSP/Kpo exhibited an increase of blood pressure at 6 weeks (Fig. 1A). MSHRSP/ Kpo further reached to 280 mmHg at 12 weeks (Fig. 1A). We next investigated the urinary and serum biochemical parameters in the MSHRSP/Kpo. MSHRSP/Kpo exhibited proteinuria at 15 weeks by SDS-PAGE analysis (Fig. 1B). Moreover, BUN and serum creatinine levels at 15-week-age MSHRSP/Kpo were 3.0- and 1.6-fold higher than in the age-matched WKY/Kpo, respectively (Fig. 1C and 1D).
Expression of podocyte-specific proteins in MSHRSP/Kpo glomerulus

Since we could not find any changes in the number of podocytes, we focused on podocyte morphology. Podocytes form FPs, highly dynamic cellular extensions that are connected by a slit diaphragm (SD). Nephrin is a transmembrane protein of the immunoglobulin family of cell-adhesion molecules, which is localized to the SD and constitutes the filtration barrier of the kidney (16). In the WKY/Kpo glomeruli, nephrin was strongly detected along the extracapillary areas (Fig. 3A), but was weakly detected in MSHRSP/Kpo at 19 weeks (Fig. 3B). We next examined the expression of podocin, an SD-associated molecule (16). Podocin in the 20-week-old WKY/Kpo glomeruli was strongly detected (Fig. 3C), but in 19-week-old MSHRSP/Kpo, it was faint (Fig. 3D). By determining the nephrin and podocin levels in glomeruli, we found significant differences in these two slit-associated proteins between the two strains (Fig. 3E).

We then examined the change in synaptopodin, a
Expression of intermediate filaments in MSHRSP/Kpo podocyte

In addition to the decrease of podocyte-specific proteins, injured podocytes are reported to exhibit the upregulation of mesenchymal proteins (17, 32). Nestin is a cytoskeleton-associated intermediate filament protein that is reported to be stably expressed in the podocytes of mature glomeruli; it also plays an important role in maintaining the normal morphology and function of podocytes (11, 15). Like 20-week-old WKY/Kpo, 19-week-old MSHRSP/Kpo also strongly expressed the nestin in podocytes (Fig. 4A and 5B). We next examined desmin expression in the MSHRSP/Kpo glomeruli. Up-regulated desmin has been to be suggested as a podocyte injury marker (17). Although desmin signals were not sufficiently detected in the glomeruli of 20-week-old WKY/Kpo,

key player in FP regulation in podocytes (31). Concomitantly with attenuations of nephrin and podocin, the synaptopodin level decreased in the 19-week-old MSHRSP/Kpo glomeruli (Fig. 4B) compared with that in 20-week-old WKY/Kpo (Fig. 4A). We next investigated the glomerular CD2AP expression. CD2AP is a multifunctional adaptor protein localized to the cytoplasm and membrane ruffles in glomerular podocytes, and plays a role in cytoskeletal remodeling, cell survival, and endocytosis (16). Of interest, CD2AP in the 19-week-old MSHRSP/Kpo glomeruli exhibited a strong staining pattern as in the 20-week-old WKY/Kpo glomeruli (Fig. 4C and 4D). Histological quantification analysis revealed significant differences in synaptopodin, but the same levels of CD2AP were found between the two strains (Fig. 4E).

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multiple glomeruli were positive for desmin along the capillary tufts in 19-week-old MSHRSP/Kpo (Fig. 5C and 5D). Actually, statistical evaluation confirmed that the expression of nestin was unchanged between 20-week-old WKY/Kpo and 19-week-old MSHRSP/Kpo glomeruli, but the desmin level was remarkably enhanced in the MSHRSP/Kpo glomeruli (Fig. 5E).

We furthermore investigated the expression levels of nephrin, podocin, synaptopodin, and desmin at earlier age as 10 and 15 weeks to elucidate the time course of podocyte injuries (Table 1). The histological quantification analysis revealed that these proteins displayed significant differences at 15 weeks. Of note, the desmin levels were increased in the MSHRSP/Kpo glomeruli as early as 10 weeks. Thus, we could nominate desmin as a desirable marker for podocyte-injury detection.

**Ultrastructural analysis of podocytes in MSHRSP/Kpo glomeruli**

We finally performed ultrastructural analysis by transmission electron microscopy on 15-week-old...
IZED BY MOLECULAR ALTERATIONS OF THE SD OR BY REORGANIZATION OF THE FP STRUCTURE WITH THE FUSION OF SLITS. IN OUR STUDY, MSHRSP/KPO EXHIBITED HYPERINTENSIVE NEPHROPATHY AND PROTEINURIA, WHICH WERE IN ACCORDANCE WITH PODOCYTE INJURIES. OF NOTE, THE PODOCYTE NUMBER WAS NOT SIGNIFICANTLY DIFFERENT, BUT THE EXPRESSION LEVELS OF NEPHRIN, PODOCIN, AND SYNaptopodin WERE ATTENUATED IN THE MSHRSP/KPO GLOMERULI. SOME REPORTS HAVE REVELED THAT HUMAN AND EXPERIMENTAL HYPERTENSIVE NEPHROSIS GAVE RISE TO A DECREASE OF WT-1-POSITIVE PODOCYTES IN THE GLOMERULUS. THE EXPRESSION LEVELS OF NEPHRIN, PODOCIN, AND SYNaptopodin mRNA WERE DOWN-REGULATED IN PATIENTS WITH HYPERTENSIVE NEPHROSCLEROSIS TOGETHER WITH PODOCYTE LOSS (28). MOREOVER, SALT-LOADED DAHL RATS DEVELOPED SEVERE HYPERTENSION AND PROTEINURIA, AND THE LOSS OF PODOCYTES AND NEPHRIN OCCURRED (21). MEANWHILE, OTHER REPORTS HAVE DESCRIBED THE REDUCTION OF PODOCYTE-SPECIFIC PROTEINS WITHOUT PODOCYTE ATTENUATION. GLOMERULI OF TWO-KIDNEY ONE-CLIP (2K1C) HYPERTENSIVE RATS SHOWED REDUCED LEVELS OF PROTEINS THAT ARE IMPORTANT FOR THE SD, NEPHRIN AND PODOCIN, BUT THE SYNaptopodin LEVEL WAS UN-

**Fig. 5** Immunofluorescence assay of intermediate filament (IF) proteins in the glomeruli of WKY/Kpo (A, C) and MSHRSP/Kpo (B, D). (E) Quantification of the expression levels of IF proteins in the glomerulus. Values are expressed as the mean ± SD. **P < 0.01 (WKY/Kpo, n = 5; MSHRSP/Kpo, n = 3).**

**Table 1** Expression levels of podocyte-specific proteins in the glomerulus

<table>
<thead>
<tr>
<th>Weeks of age</th>
<th>Strain (n = 5)</th>
<th>Nephrin</th>
<th>Podocin</th>
<th>Synaptopodin</th>
<th>Desmin</th>
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<tr>
<td>10</td>
<td>WKY/Kpo</td>
<td>3.42 ± 0.43</td>
<td>3.65 ± 0.20</td>
<td>3.84 ± 0.16</td>
<td>1.01 ± 0.14</td>
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<td></td>
<td>MSHRSP/Kpo</td>
<td>3.27 ± 0.32</td>
<td>3.22 ± 0.63</td>
<td>3.38 ± 0.29</td>
<td>1.30 ± 0.27*</td>
</tr>
<tr>
<td>15</td>
<td>WKY/Kpo</td>
<td>3.54 ± 0.31</td>
<td>3.66 ± 0.38</td>
<td>3.80 ± 0.19</td>
<td>0.56 ± 0.13</td>
</tr>
<tr>
<td></td>
<td>MSHRSP/Kpo</td>
<td>2.41 ± 0.29*</td>
<td>2.18 ± 0.60*</td>
<td>2.52 ± 0.39**</td>
<td>2.09 ± 0.16**</td>
</tr>
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Values are expressed as mean ± SD, *P < 0.05, **P < 0.01 compared with the age-matched WKY/Kpo.
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pression could protect against apoptosis during PAN nephrosis and this process is mediated by maintaining the regular arrangement of the actin cytoskeleton (29). Of interest, CD2AP also inhibits the apoptosis of podocytes in albumin overload-induced endoplasmic reticulum (ER) stress (7). We found that the podocyte number was not significantly decreased, and thus think that nestin and CD2AP might protect podocytes against apoptosis in the MSHRSP/Kpo glomeruli.

It is important to elucidate the mechanisms of podocyte injuries in MSHRSP/Kpo. As glomeruli become irreversibly scarred and a decline in the functional nephron mass ensues, the remaining nephrons are subjected to an elevated pressure gradient across the capillary wall, resulting in an exces-

changed (5). In women with preeclampsia, a pregnancy-specific disorder characterized by hypertension and proteinuria, the nephrin level was down-regulated, but that of podocin was unchanged (6). Since the MSHRSP/Kpo strain started to die by spontaneous hemorrhage or ischemic stroke at 15 weeks (manuscript in preparation), we further plan to investigate the podocyte injuries in prone-suppressed MSHRSP/Kpo kidneys.

It is reported that nestin expression of podocytes was significantly reduced in nephrotic patients with proteinuria (9). By contrast, in the puromycin aminonucleoside (PAN)-induced rat nephrosis model, nestin expression was up-regulated in the absence of podocyte apoptosis, even though the FP was significantly effaced (29). Increased nestin expression could protect against apoptosis during PAN nephrosis and this process is mediated by maintaining the regular arrangement of the actin cytoskeleton (29). Of interest, CD2AP also inhibits the apoptosis of podocytes in albumin overload-induced endoplasmic reticulum (ER) stress (7). We found that the podocyte number was not significantly decreased, and thus think that nestin and CD2AP might protect podocytes against apoptosis in the MSHRSP/Kpo glomeruli.

It is important to elucidate the mechanisms of podocyte injuries in MSHRSP/Kpo. As glomeruli become irreversibly scarred and a decline in the functional nephron mass ensues, the remaining nephrons are subjected to an elevated pressure gradient across the capillary wall, resulting in an exces-
sive mechanical stress. Independent of the inciting events, glomerular capillary hypertension ensures further damage to the podocytes and represents a final common pathway to glomerulosclerosis and end-stage renal failure (14). Thus, current therapy for hypertensive patients is aimed at prohibiting the vascular events caused by hypertension rather than decreasing blood pressure (22). It is important to assess the correlation of podocyte injuries with glomerular endothelial cells in hypertensive nephropathy. In addition, the renin-angiotensin system (RAS) is closely correlated with the pathogenesis of hypertension and its sequence. Mechanical stretch has an important role on the increase of angiotensin II (ANG II) production in conditionally immortalized podocytes (4). ANG II promotes podocyte injury through increased calcium influx and the generation of reactive oxygen species, and has been associated with deleterious effects on podocyte structure, apoptosis, and possibly epithelial-mesenchymal transition (18, 30). Of interest, ANG II leads to the reorganization of the actin cytoskeleton with a decrease of SD proteins such as nephrin and actinin-4 (8, 20). It is well known that renin activity is increased in SHRSP/Kpo according to the elevation of blood pressure (27). Angiotensin-converting enzyme inhibitors and ANG II type 1 receptor blocker ameliorate the hypertension and provide better renoprotection than other antihypertensive drugs (22). Therefore, inhibitors of the RAS could not only protect renal function but also lower proteinuria, partially through direct effects on podocytes.

MSHRSP/Kpo exhibited hypertension at six weeks, and started to exhibit renal dysfunction and proteinuria. This is the first report of MSHRSP/Kpo as a new strain for malignant hypertension, and we will disclose its physiological and pathological features. We characterized the podocyte injuries of MSHRSP/Kpo by immunostaining assay. This strain exhibited decreases of nephrin, podocin, and synaptopodin, along with the upregulation of desmin. Together with these molecular changes, MSHRSP/Kpo exhibited FP effacement and proteinuria.

Acknowledgments

We are grateful to Mr. Horiiuchi for technical assistance of electron microscopic analyses. This work was supported by a grant from the Ministry of Education, Culture, Sports, Science and Technology of Japan (No. 14454911 to TK).

CONFLICTS OF INTEREST

The authors have no conflicts of interest to report. Although the study was supported by external grants, there was no direct benefit (commercial/non-commercial) to the sponsors.

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