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Blood pressure, renal biochemical parameters and histopathology in an original rat model of essential hypertension (SHRSP/Kpo strain)

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ABSTRACT

Hypertensive nephropathy, a consequence of chronic high blood pressure, is increasingly a cause of end-stage renal diseases and its correct management is very important for clinical outcome. Spontaneously hypertensive rat (SHR/Kpo) and stroke-prone SHR (SHRSP/Kpo) strains represent models of human essential hypertension. However, the kidney injuries in SHR/Kpo and SHRSP/Kpo are not well defined. We therefore characterized the renal pathophysiology of SHR/Kpo and SHRSP/Kpo compared with normotensive control (WKY/Kpo) rats. The SHRSP/Kpo exhibited increased systolic blood pressure at 10 weeks of age, and proteinuria and increased blood urea nitrogen (BUN) and serum creatinine levels at 20 weeks. We simultaneously detected mononuclear cell infiltration, tubular injuries, accumulation of extracellular matrix and marked expression of α-SMA in the tubulointerstitium. Additionally, TGF-β1 and CTGF were up-regulated in the kidney of SHRSP/Kpo. We lastly focused on changes in glomerular cells of SHRSP/Kpo. Nestin, a podocyte marker, was detected but decreased slightly in 20-week-old SHRSP/Kpo. PECAM-1 expression was increased in SHRSP/Kpo glomeruli, indicating the thickening of glomerular endothelial cells. Moreover, we found that α-SMA, a myofibroblast marker, was also upregulated in the glomeruli of SHRSP/Kpo at 20 weeks. These findings suggest that SHRSP/Kpo could be a valuable animal model for human hypertensive nephropathy.

Essential hypertension is a heterogeneous disorder in which both genetics and environmental factors contribute to increased cardiovascular disease and mortality (5, 13). The number of patients with this disease is still increasing despite the development of various treatments to normalize systemic blood pressure (19). Hypertensive nephropathy, a consequence of chronic high blood pressure, is second only to diabetic nephropathy in terms of diagnosis cited as causing end-stage renal diseases (12). Furthermore, the 5-year survival rate of patients undergoing hemodialysis due to hypertensive renal disease is reportedly much lower than the rates of hemodialysis patients with other causes (28). Complications often associated with hypertensive nephropathy include glomerular damage, resulting in inflammatory responses and compromised kidney function that seem to be superimposed on the intrinsic phenotypes of the underlying disease (1). Therefore, the correct management of hypertensive renal disease is very

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Abbreviations: WKY, Wistar-Kyoto; SHR, spontaneously hypertensive rat; SHRSP, stroke-prone SHR; α-SMA, α-smooth muscle actin; PECAM-1, platelet endothelial cell adhesion molecule-1; GEC, glomerular endothelial cells; TGF-β1, transforming growth factor-β1; CTGF, connective tissue growth factor; TEC, tubular epithelial cells; ssDNA, single-stranded DNA; H&E, hematoxylin and eosin
important for the clinical outcome.

Stroke-prone spontaneously hypertensive rats (SHRSP), a substrain of spontaneously hypertensive rats (SHR), represent a model of human essential hypertension and show serious hypertensive renal injury (17, 20). Human hypertension and SHRSP share extremely similar pathological features in the kidney that are characterized by marked medial and intimal thickening, fibrosis and fibrinoid necrosis of arterioles and small arteries, followed by ischemic glomerular changes and tubulointerstitial fibrosis (20). There are some sub-strains of SHR and SHRSP. For example, of the SHR substrains widely used for the study of hypertension, SHR/Izm and SHRSP/Izm and normotensive WKY/Izm rats have been most thoroughly analyzed (12). Although the systolic blood pressure in WKY/Izm rats was stable at 140–150 mmHg from 6–30 weeks of age, blood pressure in the SHR/Izm and SHRSP/Izm was already 170–200 mmHg at 6–7 weeks and continued to rise (6, 27). Other SHR substrains also exhibited hypertension at 5–6 weeks (30). SHR/Kpo, SHRSP/Kpo and WKY/Kpo were established as substrains of SHR, SHRSP and WKY respectively by brother-sister mating more than twenty times in our laboratories. The blood pressure of SHR/Kpo and SHRSP/Kpo was the same level when compared with the WKY/Kpo at 6 weeks of age, and was only elevated from 10 weeks (Fig. 1A). Essential hypertension is typically recognized in human subjects between 25 and 45 years of age, but kidney impairment remains uncommon until patients have experienced at least 10 years of sustained hypertension (15). Therefore, it is important that the blood pressure only begins to increase after adulthood in an animal model for human essential hypertension, and thus SHR/Kpo and SHRSP/Kpo could become valuable for further studies. To understand hypertensive nephropathy in the present study, we characterized the renal pathophysiology in the three stains of model rat.

MATERIALS AND METHODS

Animals. Six-, 10-, and 20-week-old, male normotensive WKY/Kpo rats and hypertensive SHR/Kpo and SHRSP/Kpo were used. All rats were kept under a light/dark regimen of 12 h on and 12 h off, and had access to stock chow diet (Funabashi SP; Funabashi farm, Chiba, Japan) and reverse osmotic water ad libitum. All animal experiments were carried out according to the Guidelines for Experimental Animal Care issued by the Prime Minister’s Office of Japan and approved by the Committee on Animal Experimentation of Kinki University.

Blood pressure and biochemical parameters. The blood pressure of conscious rats was measured every two weeks using tail-cuff plethysmography (BP-98A; Softron, Tokyo, Japan). The concentrations of urinary albumin, BUN and serum creatinine were examined as described previously (9). For determining 24-hour urinary albumin, each rat was placed in a metabolic cage for 24-hour urine collection.

Histological analyses. All rats were deeply anesthetized by an intraperitoneal injection of pentobarbital (37.5 mg/kg body weight) and then kidneys were fixed in cold 10% buffered formalin or 70% ethanol for 24 h. Transversally trimmed kidney tissues were submitted to a routine process for paraffin embedding. The renal sections were prepared, deparaffinized, stained with hematoxylin and eosin (H&E) and Masson Trichrome for renal histology. Tubulointerstitial damage was determined as previously reported (9). To detect proliferating and apoptotic TECs, the sections were reacted with primary antibodies against proliferating cell nuclear antigen (PCNA) (1 : 200; DAKO, Glostrup, Denmark) and single-stranded DNA (ssDNA) (IBL Co. Ltd., Gunma, Japan). To detect glomerular proteins, anti-nestin IgG (1 : 200; BD Bioscience, San Jose, CA, USA), anti-PECAM-1 IgG (1 : 200; BD Bioscience), and anti-α-SMA IgG (1 : 200; DAKO) antibodies were used. For PCNA, PECAM-1 and α-SMA staining, 70% ethanol-fixed sections were used. For ssDNA staining, 10% formalin-fixed sections were used. For nestin staining, 10% formalin-fixed sections were used, and antigen retrieval was performed in 10 mM citrate buffer in a microwave at pH 6.0 for 15 min. The sections were dewaxed followed by incubation with 3% bovine serum albumin (BSA) for 1 h. The sections were incubated at 4°C overnight with primary antibodies against proliferating cell nuclear antigen (PCNA) (1 : 200; DAKO, Glostrup, Denmark) and single-stranded DNA (ssDNA) (IBL Co. Ltd., Gunma, Japan). To detect glomerular proteins, anti-nestin IgG (1 : 200; BD Bioscience, San Jose, CA, USA), anti-PECAM-1 IgG (1 : 200; BD Bioscience), and anti-α-SMA IgG (1 : 200; DAKO) antibodies were used. For PCNA, PECAM-1 and α-SMA staining, 70% ethanol-fixed sections were used. For ssDNA staining, 10% formalin-fixed sections were used. For nestin staining, 10% formalin-fixed sections were used, and antigen retrieval was performed in 10 mM citrate buffer in a microwave at pH 6.0 for 15 min. The sections were dewaxed followed by incubation with 3% bovine serum albumin (BSA) for 1 h. The sections were incubated at 4°C overnight with primary antibodies, and then washed with PBS and followed by the secondary antibodies (Histofine; Nichirei, Osaka, Japan) at room temperature for an hour. The antigens were identified through visualization of peroxidase with diaminobenzidine (ImmPACT AEC; Vector, Burlingame, CA, USA). PCNA and ssDNA positive cells were counted as previously described (9). The immunofluorescence assays were performed as previously described (8). The specificity of immunostaining was verified by observing no staining in the absence of each primary antibody.

Western blot. Kidney lysates were prepared from 20-week-old WKY/Kpo, SHR/Kpo and SHRSP/Kpo...
in lysis buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, protease inhibitor cocktail (Roche, Upper Bavarie, Germany)]. The lysates were electrophoresed and immunoblotted as described previously (8). Anti-TGF-β1 IgG (sc-146; Santa Cruz Biotechnology, Dallas, TX, USA) and anti-CTGF IgG (sc-14939; Santa Cruz) antibodies were used.

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

RESULTS
Physiological profiles and urinary and serum parameters
The systolic blood pressure of each strain was approximately 150 mmHg at 6 weeks of age (Fig. 1A). The blood pressure of SHR/Kpo continued to rise to 210 mmHg at 20 weeks, while that of SHRSP/Kpo reached 280 mmHg (Fig. 1A). The body weight of normotensive WKY/Kpo rats increased rapidly from 6 weeks to 20 weeks (Fig. 1B). However, the body weights of SHR/Kpo and SHRSP/Kpo were decreased compared with that of WKY/Kpo rats at 10 and 20 weeks (Fig. 1B). The kidney weights were not different among the SHR/Kpo, SHRSP/Kpo and WKY/Kpo strains at 20 weeks (Fig. 1C).

To investigate proteinuria and renal dysfunction in the SHR/Kpo, SHRSP/Kpo and WKY/Kpo strains, we performed biochemical analyses of urine and serum. The SHRSP/Kpo at 20 weeks excreted higher levels of urinary albumin in 24 h compared with the WKY/Kpo and SHR/Kpo (Fig. 2A), indicating the SHRSP/Kpo exhibited proteinuria at 20 weeks. To assess renal dysfunction, we measured the concentration of blood urea nitrogen (BUN) and serum creatinine. As shown in Fig. 2B and 2C, BUN and serum creatinine levels at 20 weeks of age were 1.8- and 1.5-fold higher respectively than in the age-matched WKY/Kpo. Thus, the SHRSP exhibited renal dysfunction at 20 weeks. From the biochemical analyses of urine and serum, the SHR/Kpo were almost identical to the WKY/Kpo (Fig. 2B, C).

Histological changes in kidneys
To investigate renal lesions in the SHR/Kpo and SHRSP/Kpo, we performed histological examinations after hematoxylin and eosin (H&E) staining. As shown in Fig. 3A and C, mononuclear cell infiltration and tubular injuries were not detected in the WKY/Kpo kidneys. Mononuclear cell infiltration and tubular injuries were also not observed in the SHR/Kpo kidneys or at 6 (Fig. 3B) and 10 weeks

Fig. 1 Blood pressure and body and kidney weights of the SHR/Kpo, SHRSP/Kpo and WKY/Kpo. Blood pressure (A), body weight (B), and kidney weight (C) at 6, 10 and 20 weeks of age. Values are expressed as the mean ± SD. ** P < 0.01, *** P < 0.001. (White column: WKY/Kpo; gray column: SHR/Kpo; black column: SHRSP/Kpo).
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sis using an antibody for α-SMA. In the WKY/Kpo, the immunoreactivity of α-SMA antigen was mostly localized to normal renal blood vessels (Fig. 4C). In contrast, marked expression of α-SMA was found at 20 weeks in the tubulointerstitium of the renal cortex in SHRSP/Kpo (Fig. 4D). We confirmed this result by immunoblot analysis for α-SMA expression, finding that α-SMA protein was increased in the kidney of SHRSP/Kpo at 20 weeks (Fig. 4E).

TGF-β1 is a potent inducer of ECM synthesis and fibrosis, regardless of etiology (4, 25). CTGF, a prominent downstream target of TGF-β1, is the major causative factor in chronic kidney disease etiology and disease progression (24). To examine TGF-β1 and CTGF upregulation in the SHR/Kpo and SHRSP/Kpo kidneys, we performed immunoblot analyses of TGF-β1 and CTGF. TGF-β1 was markedly up-regulated in the kidney of SHRSP/Kpo compared with the WKY/Kpo and SHR/Kpo at 20 weeks (Fig. 5A). CTGF expression was also induced in the kidney of SHRSP/Kpo strain (Fig. 5B).

Proliferation and apoptosis of tubular epithelial cells (TECs)

To quantify the proliferation of TECs in the SHRSP/Kpo kidneys, we performed immunohistochemical analyses using an antibody for α-SMA. In the WKY/Kpo, the immunoreactivity of α-SMA antigen was mostly localized to normal renal blood vessels (Fig. 4C). In contrast, marked expression of α-SMA was found at 20 weeks in the tubulointerstitium of the renal cortex in SHRSP/Kpo (Fig. 4D). We confirmed this result by immunoblot analysis for α-SMA expression, finding that α-SMA protein was increased in the kidney of SHRSP/Kpo at 20 weeks (Fig. 4E).

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analysis of PCNA. In WKY/Kpo, SHR/Kpo and SHRSP/Kpo, the proliferating TEC scores were 33.0 ± 9.0, 27.3 ± 9.5 and 35.5 ± 12.6 at 6 weeks, and then 12.5 ± 5.8, 11.5 ± 5.8 and 15.7 ± 3.3 at 10 weeks, respectively. There were no significant differences between groups. There were very few proliferating TECs at 20 weeks in the WKY/Kpo kidneys (Fig. 6A), but an increased number of proliferating TECs in the SHRSP/Kpo kidneys (Fig. 6B). The proliferating TEC scores at 20 weeks were 1.7 ± 1.9, 2.2 ± 2.1, 10.0 ± 3.0 (P < 0.01, compared with WKY/Kpo). The numbers of proliferating TECs were generally decreased at 10 and 20 weeks compared with 6 weeks in all three strains of rats, since the kidney
Immunofluorescence analyses of glomeruli

As the SHRSP/Kpo strain developed albuminuria at 20 weeks, we next focused on the changes in glomerular cells. Glomeruli consist of podocytes, endothelial cells and mesangial cells. We examined the expression of nestin protein as a histological marker of podocytes (10, 29). In WKY/Kpo kidneys, nestin was found in glomeruli (Fig. 7A). Nestin was also detected, but decreased slightly, in the SHRSP/Kpo glomeruli at 20 weeks (Fig. 7B). We therefore investigated the changes in podocytes, endothelial cells, and mesangial cells in the SHRSP/Kpo glomeruli at 20 weeks.
Model of hypertensive nephropathy

Fibrosis of the tubulointerstitium develops in patients and animals with hypertension, which impairs renal function finally leading to organ failure. Multiple mechanisms have been demonstrated to be involved in hypertensive nephropathy. In addition to elevated blood pressure, numerous local factors including angiotensin II are known to contribute to the development of renal fibrosis. Of note, angiotensin II stimulates TGF-β1 gene expression and protein release (22). TGF-β1 induces renal fibrosis by activating interstitial fibroblasts to become myofibroblasts, which produce large amounts of matrix components (14). Moreover, the effects of TGF-β1 on renal cells appear to be mediated by CTGF, which is markedly increased in human glomerular and tubulointerstitial lesions associated with cellular proliferation. Furthermore, the expression of CTGF mRNA is strongly correlated with the extent of tubulointerstitial fibrosis across different renal diseases (7). As CTGF was increased in the SHRSP/Kpo kidneys in association with TGF-β1 upregulation, our findings are consistent with those of human studies.

The kidney weights of 20-week-old SHRSP/Kpo were not decreased compared with the other strains, although TEC apoptosis occurred, probably due to increased fibrosis and TEC proliferation. The molecular mechanisms behind proliferation of renal cells are not well understood, although several kidney repair mechanisms have been suggested. Using animal models, several soluble factors have been proven to regulate kidney recovery as potential renotrophic factors. These factors include hepatocyte growth factor (HGF), epidermal growth factor (EGF), insulin-like growth factor-1 (IGF-1), heparin-binding epidermal growth factor (HB-EGF), platelet-derived growth factor (PDGF), and bone morphogenetic protein-7 (BMP-7) (16). As an example, HGF is a powerful pleiotropic protein involved in the repair process after acute and chronic kidney injuries, has antiapoptotic properties and is involved in regulating cell proliferation, differentiation and survival (18). We therefore examined the HGF-Met system in the kidney by western blot for tyrosine 1234/1235 phosphorylated Met (p-Met), which indicates HGF intracellular signaling. Compared with WKY/Kpo rats, the kidneys of SHR/Kpo and SHRSP/Kpo rats exhibited down-regulation ofinterep Met activation (unpublished data). Therefore, HGF could not play an important role in TEC proliferation in SHRSP/Kpo rats, and other growth factors may be involved the endogenous repair system in hypertensive nephropathy. Although many renotrophic factors and signaling pathways have been identified, the mechanism by which these growth factors mediate recovery from renal injury is not well understood.

Fig. 7 Glomerular changes of SHRSP/Kpo. Representative images of nestin (A, B), PECAM-1 (C, D) and α-SMA (E, F) staining in the kidneys of WKY/Kpo (A, C, E) and SHRSP/Kpo (B, D, F) at 20 weeks of age.

investigated glomerular endothelial cells (GECs) using immunofluorescence to detect PECAM-1. PECAM-1 was tightly localized to whole glomerular tufts of the WKY/Kpo (Fig. 7C). Notably, PECAM-1 expression was increased in SHRSP/Kpo glomeruli at 20 weeks (Fig. 7D). This increase in expression level could suggest the thickening of GECs. We then investigated the phenotypic changes in mesangial cells by detecting α-SMA protein expression, as α-SMA is a specific marker of the myofibroblast phenotypic transformation of mesangial cells under pathological conditions (11). In the WKY/Kpo, α-SMA was rarely found in glomeruli (Fig. 7E), however α-SMA was markedly upregulated in the SHRSP/Kpo at 20 weeks (Fig. 7F). SHRSP/Kpo kidneys thus exhibit glomerulosclerosis through an increase in myofibroblasts.

DISCUSSION

Fibrosis of the tubulointerstitium develops in patients and animals with hypertension, which impairs renal function finally leading to organ failure. Multiple mechanisms have been demonstrated to be involved in hypertensive nephropathy. In addition to elevated blood pressure, numerous local factors including angiotensin II are known to contribute to the development of renal fibrosis. Of note, angiotensin II stimulates TGF-β1 gene expression and protein release (22). TGF-β1 induces renal fibrosis by activating interstitial fibroblasts to become myofibroblasts, which produce large amounts of matrix components (14). Moreover, the effects of TGF-β1 on renal cells appear to be mediated by CTGF, which is markedly increased in human glomerular and tubulointerstitial lesions associated with cellular proliferation. Furthermore, the expression of CTGF mRNA is strongly correlated with the extent of tubulointerstitial fibrosis across different renal diseases (7). As CTGF was increased in the SHRSP/Kpo kidneys in association with TGF-β1 upregulation, our findings are consistent with those of human studies.

The kidney weights of 20-week-old SHRSP/Kpo were not decreased compared with the other strains, although TEC apoptosis occurred, probably due to increased fibrosis and TEC proliferation. The molecular mechanisms behind proliferation of renal cells are not well understood, although several kidney repair mechanisms have been suggested. Using animal models, several soluble factors have been proven to regulate kidney recovery as potential renotrophic factors. These factors include hepatocyte growth factor (HGF), epidermal growth factor (EGF), insulin-like growth factor-1 (IGF-1), heparin-binding epidermal growth factor (HB-EGF), platelet-derived growth factor (PDGF), and bone morphogenetic protein-7 (BMP-7) (16). As an example, HGF is a powerful pleiotropic protein involved in the repair process after acute and chronic kidney injuries, has antiapoptotic properties and is involved in regulating cell proliferation, differentiation and survival (18). We therefore examined the HGF-Met system in the kidney by western blot for tyrosine 1234/1235 phosphorylated Met (p-Met), which indicates HGF intracellular signaling. Compared with WKY/Kpo rats, the kidneys of SHR/Kpo and SHRSP/Kpo rats exhibited down-regulation of p-Met activation (unpublished data). Therefore, HGF could not play an important role in TEC proliferation in SHRSP/Kpo rats, and other growth factors may be involved the endogenous repair system in hypertensive nephropathy. Although many renotrophic factors and signaling pathways have been identified, the mechanism by which these growth factors mediate recovery from renal injury is not well understood.
The ultrafiltration of plasma in the kidneys occurs through the capillary wall of GECs, the glomerular basement membrane and podocytes. These three layers of glomerular filtration must function to prevent the leakage of protein into urine, with destruction of these layers resulting in proteinuria. Notably, the SHRSP/Kpo exhibited proteinuria at 20 weeks. As we could not find significant changes in nestin expression in this study, we will further investigate podocyte injury in the SHRSP/Kpo glomeruli in future. In essential hypertension, an increase of urinary albumin excretion may be related to a generalized, systemic dysfunction of the endothelium (21). We thus focused on GECs which play a major role in the modulation of factors regulating vascular tone and glomerular filtration. From the immunofluorescence assay, PECAM-1 expression was upregulated in the glomeruli of SHRSP/Kpo compared with the WKY/Kpo at 20 weeks, which suggested GEC hypertrophy. We hypothesize that GEC hypertrophy occurred because of physical pressure from intraglomerular hypertension, but other possibilities also exist. For example, angiotensin II induces its mitogenic effect through the receptor AT1, which is localized mainly on GECs (3), and also further increases expression of vascular endothelial growth factor (VEGF) by mesangial cells and podocytes (2, 23). Therefore, further studies need to evaluate the mechanisms of GEC hypertrophy in greater detail.

In summary, we demonstrated that the SHR/Kpo and SHRSP/Kpo strains of rat are novel and valuable models of spontaneous hypertension, with rats displaying increased blood pressure from 10 weeks of age. We characterized renal injuries by biochemical, histopathological and molecular analysis and found that SHRSP/Kpo began to exhibit renal injuries and interstitial fibrosis at 20 weeks of age. Notably, they also demonstrated hypertrophy of GECs.

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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/noncommercial) to the sponsors.

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