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A Novel Rat Model with Podocyte Injury in Developing Stage Glomeruli

Tamaki KARASAWA,[1,2] Taeko HASHIMOTO,[1] Naoko MIYAUCHI,[1] Yutaka HARITA,[1] Gi Dong HAN,[1,3] Hiroko KOIKE,[1] Makoto UCHIYAMA,[2] Fujio SHIMIZU[1] and Hiroshi KAWACHI[1]

[1] Department of Cell Biology, Institute of Nephrology; [2] Division of Pediatrics, Graduate School of Medical and Dental Sciences, Niigata University, Niigata, Japan; [3] Department of Food Science and Technology, Yeungnam University, Gyeongsan, Republic of Korea

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Summary. **Recent investigations have revealed that growth *in utero* might be an important determinant of adult disorders. The number of nephrons in humans is determined *in utero*. It is reported that infants who show signs of intrauterine growth retardation (IUGR) at birth have a significant reduction in nephron number and have a high risk for several kidney diseases. The glomerular epithelial cell (podocyte) plays a critical role in maintaining the function and the tuft architecture of glomeruli. In this study, we aimed to establish a rat model with podocyte injury in the developing stage. Five-day-old rats were treated with puromycin aminonucleoside (PAN), which is accepted to have toxicity to podocytes since glomerular maturation lasts after birth in rats, and 5-day-old rats correspond to the late gestational stage in humans. Podocyte injury and its maturity were assessed by the staining of the podocyte functional molecules, nephrin and podocin. In the normal infant rats, nephrin and podocin in podocytes are observed in a continuous linear pattern at the early capillary loop stage. At 24 h after PAN injection, the staining of nephrin and podocin at this stage of glomeruli shifted to a discontinuous pattern, and their staining intensity clearly decreased. Podocyte injury suffered at developing stage of glomeruli resulted in the reduction of podocyte numbers, the compensatory glomerular hypertrophy, and the retardation of any increase in body weight. The model could serve as a mimic of infants who evidence signs of IUGR at birth. The model is suitable to investigate the relation between podocyte injury suffered at the early developing stage and the risk for several kidney diseases in adulthood.**

Key words — podocye, development, intrauterine growth retardation, puromycin aminonucleoside, slit diaphragm.

INTRODUCTION

Recent studies have reported that children who show signs of intrauterine growth retardation (IUGR) at birth have a higher risk of suffering several diseases – including diabetes, hypertension, and cardiovascular diseases– in later life[1-4]. Although the precise mechanisms underlying these associations are poorly understood, it is generally accepted that IUGR results in not only low birth weight but also disturbances of the maturation of several organs.

The number of nephrons in humans is determined *in utero*, and no new nephrons are formed after the 36th week of gestation[5]. It is reported that infants with IUGR have a significant reduction in nephron number [6, 7, 8], as well as a high risk for several kidney diseases[8, 9, 10]. Recently, some studies have focused on the role of the glomerular epithelial cell (podocyte). The podocyte plays a critical role not only in maintaining the barrier function of the glomerular capillary wall to retain plasma protein but also in maintaining the glomerular tuft architecture by covering the outer surface of the

Correspondence: Hiroshi Kawachi, Department of Cell Biology, Institute of Nephrology, Graduate School of Medical and Dental Sciences, Niigata University, 1-757 Asahimachi-dori, Niigata 951-8510, Japan.

Abbreviations– IUGR, intrauterine growth retardation; PAN, puromycin aminonucleoside; SD, slit diaphragm.

basement membrane[11]. The podocyte is understood to be a terminally differentiated cell that does not proliferate in mature glomeruli[11]. Some studies indicate that podocyte loss contributes to the progression of renal diseases[12, 13, 14]. We have previously demonstrated that podocyte injury could be a risk factor in the development of glomerular diseases in rat models[15, 16]. It is supposed that the podocyte injury in the maturing stage could result in a loss of podocyte number and an increase in susceptibility to renal disease in adulthood. However, no report has addressed the influence of the injury of the maturing podocyte on the development of glomerular diseases in later life.

The present study was designed to establish a rat model with podocyte injury in the developing stage. Puromycin aminonucleoside (PAN) was used to cause podocyte injury since 1) it is widely accepted that PAN has toxicity to podocytes, and 2) PAN is considered to have lower toxicity to tubular epithelial cells than other reagents causing podocyte injury such as adriamycin and daunomycin. Podocyte maturation progresses to the final stages of glomerular development. Five-day-old rats were treated with PAN since glomerular maturation lasts after birth in rats and 5-day-old rats correspond to the late gestational stage in humans. Podocyte injury and its maturity were assessed by its morphology and staining of the podocyte functional molecules, nephrin and podocin. We demonstrated here that PAN had toxicity to the developing podocyte and caused a disturbance in podocyte maturation. Low dose PAN treatment to infant rats did not cause proteinuria but rather minor podocyte injury, consequent glomerular number loss, and compensatory glomerular hypertrophy. We believe the model established here could be a useful one to analyze the relation between injury to the developing podocyte and the progression of kidney disease in adulthood.

MATERIALS AND METHODS

Animals

Male and female Wistar rats (6-8 weeks old) were purchased from Charles River Japan (Atsugi, Kanagawa), and mated. All animal experiments were conducted in accord with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Procedures

Infant rats were intra-peritoneally injected with 10 mg/100 g body weight (BW) PAN or phosphate buffered saline (PBS) as a control on day 5 after birth, a time when nephrogenesis has not been completed[17, 18]. Following this, they were returned to their mothers until weaning at 21 days. The mothers and offspring were fed standard rat chow and water *ad libitum*. Rats were sacrificed 24 h, 10 days, three and six weeks after injection. Kidneys were removed, weighed, and used for assessment by light microscopy (LM) and immunofluorescence microscopy (IF). The protein concentration of urine collected three and six weeks after treatment was measured by colorimetric assay with a Bio-Rad Protein Assay Reagent (Bio-Rad, Hercules, CA, USA) using bovine serum *albumin* (BSA) as a standard.

Morphological study

For LM, small pieces of the kidneys were fixed with 10% neutral formalin and embedded in paraffin. Sections (2–3-μm-thick) were stained with periodic acid–Schiff (PAS) and observed with a microscope (BX50; Olympus, Tokyo). The number of glomeruli/high power field (0.2 mm^2) in the cortex was counted in 50 fields. The diameter was measured in 100 full-sized randomly selected glomeruli. The study was carried out in a blinded manner.

IF

IF studies were performed basically according to the method previously reported[19]. Renal tissue was quickly frozen in N-hexane cooled at −70℃. The 3-μm-thick frozen section were cut with a cryostat and fixed with acetone for one min. The sections were incubated with the primary antibodies described below, then stained with secondary antibodies, and observed with a BX50 microscope. The images were further processed using Adobe Photoshop 6.0.

Antibodies

Mouse monoclonal antibody to rat nephrin (mAb 516), and rabbit polyclonal antibody to rat podocin were prepared as described previously[20, 21]. As a secondary antibody, FITC-conjugated anti-mouse IgG1 (SouthernBiotech, Birmingham, AL, UK) for the mAb 516 antibody, and anti-rabbit Igs (DacoCytomation, Glostrup, Denmark) for the anti-podocin antibody were used.

Statistical analysis

All values are expressed as means ± SD. The statistical significance (defined as $p < 0.05$) was evaluated using

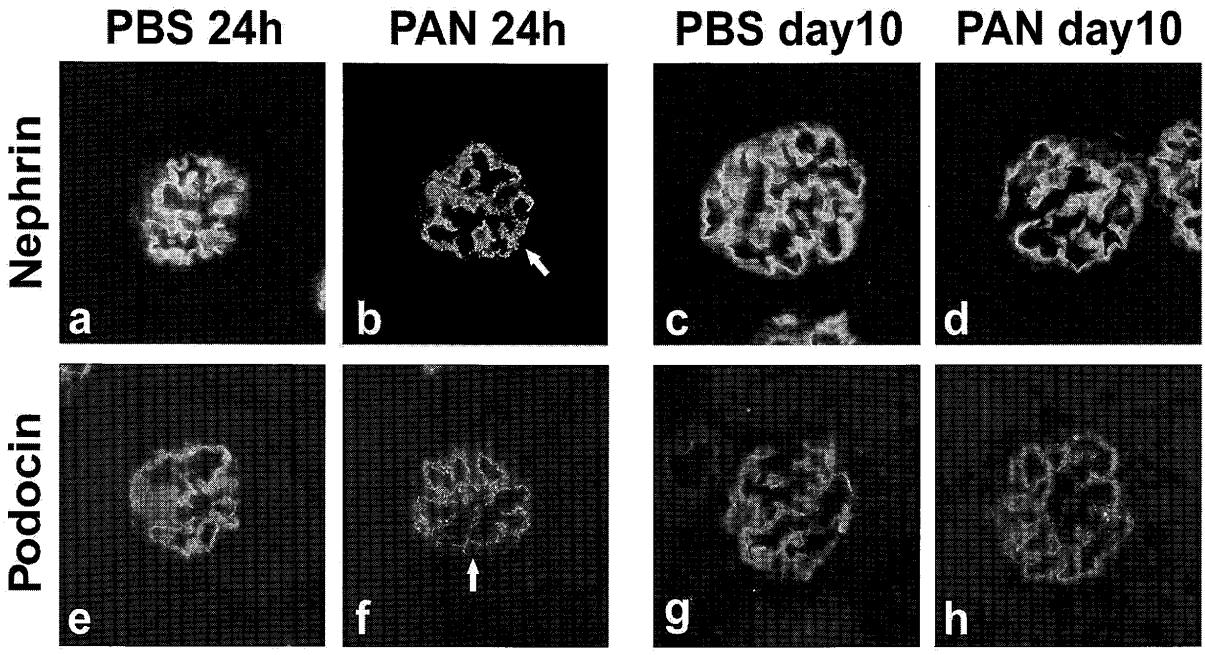

Fig. 1. Immunofluorescence microscopy (IF) findings of nephrin and podocin at 24 h and 10 days after puromycin aminonucleoside (PAN) treatment. In control rats 24 h after PBS injection, nephrin and podocin in podocytes are observed as a continuous linear pattern in early capillary loop stage glomeruli found immediately beneath the glomerular capsule (**a** and **e**). In PAN-injected rats, the intensity of these molecules clearly decreases, and the staining shifts to a discontinuous granular pattern (**b** and **f**, *arrows*). In rats 10 days after injection of PAN, no distinct difference in these stainings is observed between PBS- and PAN-injected groups (**c**, **d**, **g** and **h**). Original magnifications, × 400

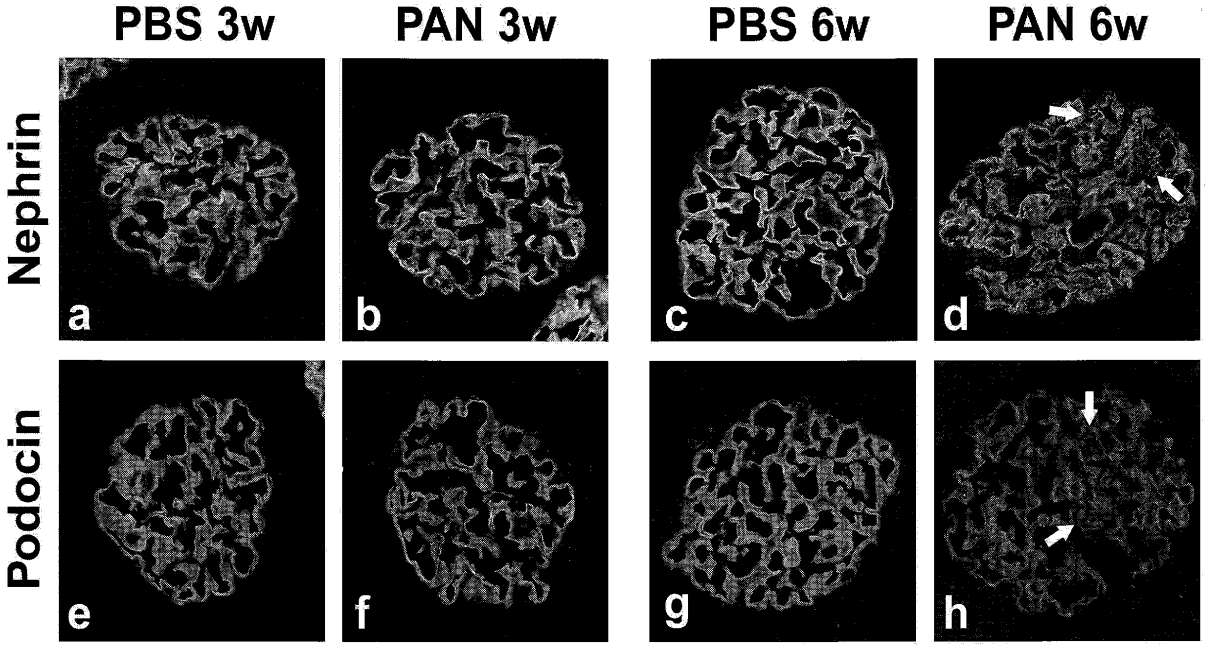

Fig. 2. IF findings of nephrin and podocin at three and six weeks after PAN treatment. Both stainings of nephrin and podocin are observed in a linear pattern along the capillary wall in control rats three weeks after PBS injection (**a** and **e**). No distinct difference in these stainings is detected between PBS- and PAN-injected rats (**b** and **f**). The intensity of nephrin and podocin staining clearly decreases, and their staining pattern changes to a discontinuous one in rats six weeks after PAN treatment (**d** and **h**, *arrows*), although that in control rats is observed as a linear pattern (**c** and **g**). Original magnifications, × 400

Table 1. Morphometric parameters

	3 weeks		6 weeks	
	PAN	Control	PAN	Control
Body weight (g)	64.80 ± 8.29*	83.67 ± 4.27	166.80 ± 16.30*	198.14 ± 36.75
Kidney weight (g)	0.339 ± 0.041*	0.459 ± 0.047	0.782 ± 0.092	0.874 ± 0.143
Kidney weight / body weight ($x10^{-5}$)	524.0 ± 35.3	547.3 ± 34.6	467.9 ± 16.6*	443.4 ± 23.6
Mean glomerular number (/HPF)	3.5 ± 0.2*	4.4 ± 0.3	3.0 ± 0.3*	3.7 ± 0.5
Mean glomerular diameter (μm)	8.38 ± 0.66	7.75 ± 0.22	10.54 ± 0.55*	8.870 ± 0.11

PAN, puromycin aminonucleoside; * $p < 0.05$.

the unpaired *t* test. Data was analyzed using StatView 5.0 (SAS Institute Inc., Cary, NC, USA).

RESULTS

IF findings of nephrin and podocin after PAN treatment

In control rats 24 h after PBS injection, nephrin and podocin staining in podocytes was observed as a continuous linear pattern in early capillary loop stage glomeruli located immediately beneath the glomerular capsule (Fig. 1a and e). In PAN-injected rats, the intensity of these molecules clearly decreased, and their staining shifted to a discontinuous granular pattern (Fig. 1b and f, *arrows*). Ten days after injection of PAN, no distinct difference was observed between PBS- and PAN-injected rats (Fig. 1c, d, g and h).

Both stainings of nephrin and podocin were observed in linear patterns along the capillary wall in control rats three weeks after PBS injection (Fig. 2a and e). No distinct difference in these stainings was detected between PBS- and PAN-injected rats (b and f). The intensity of nephrin and podocin staining clearly decreased, and their staining pattern became discontinuous in rats six weeks after PAN treatment (Fig. 2d and h, *arrows*), although that in control rats was observed as a continuous linear pattern (Fig. 2c and g).

Body, kidney, and glomerular growth

The average value of kidney weights of PAN injected rats three weeks after the injection was lower than that of PBS-injected rats, but at six weeks after treatment no difference in kidney weight was detected between PBS- and PAN injected rats (Table 1). No difference in kidney index (kidney weight/ BW) at three weeks after treatment was detected in the two groups, but at six weeks after treatment, the kidney index in PAN-treated rats six weeks after injection had clearly increased (Table 1). Mean glomerular number per field of LM was significantly decreased in PAN-injected rats three and six weeks after injection (Table 1). Remarkable morphological changes in neither the glomeruli nor in the tubulo-intersitial area was detected in PAN-injected rats three weeks after treatment (Fig. 3a and b). A significant increase of the mean glomerular diameter was observed in the PAN-injected group six weeks after injection (Fig. 3 and Table 1).

Although the increase in body weight was suppressed for five days after PAN injection, no difference in the rate of increase in body weight from day 10 to six weeks after the injection was observed between the PBS- and PAN-injected groups (Fig. 4).

Functional parameters

Functional measurement of the two groups at three and six weeks after injection is shown in Table 2. Three weeks after PAN injection, a significant decrease in creatinine clearance (Ccr) and a significant increase in blood urea nitrogen (BUN) and creatinine (cre) were observed. Six weeks after PAN injection, no significant differences in BUN, cre, or Ccr were detected between the PBS- and PAN-injected groups. No abnormal proteinuria was observed in either group during the experimental period.

Table 2. Kidney function

	3 weeks		6 weeks	
	PAN	Control	PAN	Control
BUN (mg/dl)	16.82 ± 4.11*	10.07 ± 1.90	24.78 ± 4.61	21.31 ± 3.78
Cre (mg/dl)	0.124 ± 0.017*	0.067 ± 0.024	0.2140 ± .026	0.210 ± 0.014
Ccr (ml/min/100 g BW)	0.346 ± 0.080*	0.953 ± 0.430	0.865 ± 0.630	1.533 ± 0.492

*$p < 0.05$.

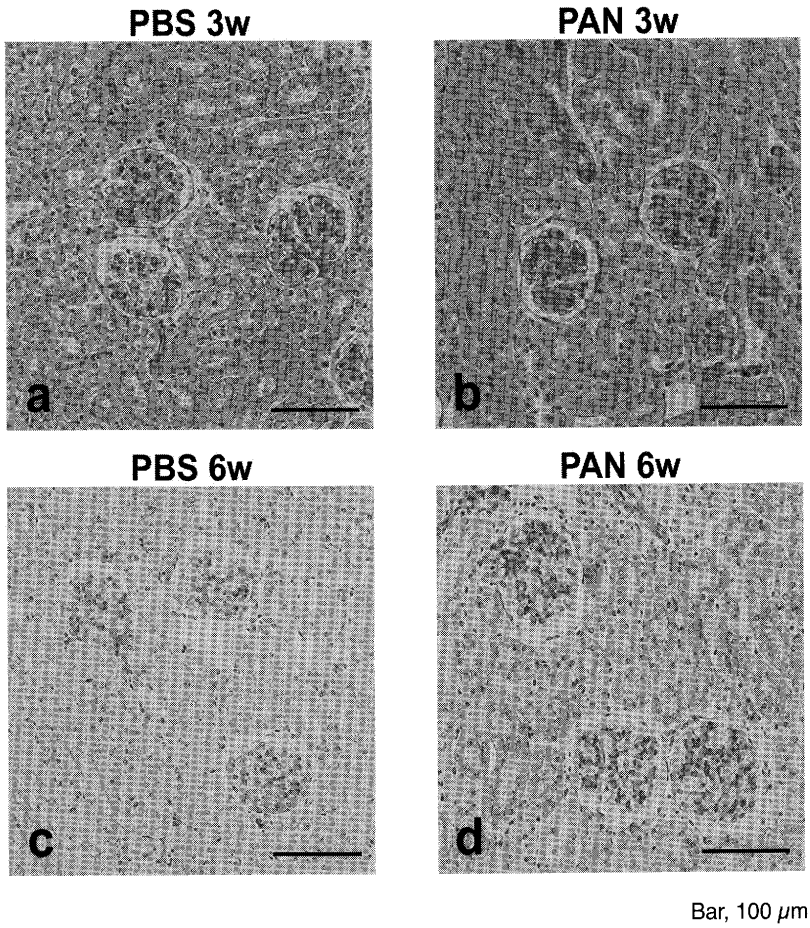

Fig. 3. Light microscopy (LM) findings in rats at three and six weeks after PAN treatment. Representative LM findings of glomeruli of rats three weeks (**a** and **b**) and six weeks (**c** and **d**) after injection. No difference of histological findings is detected between PBS- and PAN-injected rats three weeks after treatment (**a** and **b**). The glomerular size of rats six weeks after PAN injection (**d**) is clearly larger than that of rats injected with PBS (**c**). Original magnification, × 200, *Scale bar,* 100 μm.

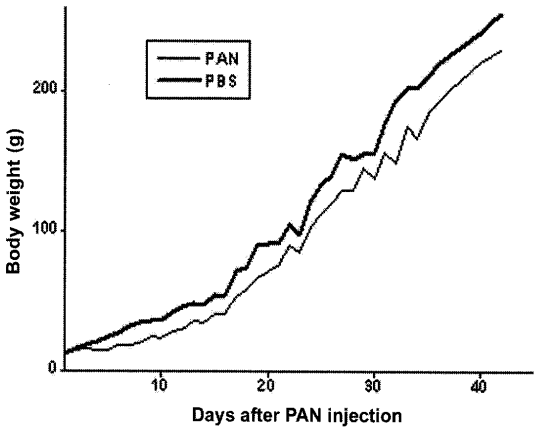

Fig. 4. The growth curve of rat body weight after treatment. The body weight gain of the PAN-injected group (*thin line*) and the control group (*thick line*) after PAN injection is shown. An increase in body weight is clearly inhibited five days after PAN injection, and no difference in the rate of increase in body weight from around days 10 to six weeks after injection is observed between PBS- and PAN-injected groups.

DISCUSSION

The present study, aimed to establish a rat model with podocyte injury suffered in the developing stage. It was demonstrated here that an intra-peritoneal administration of PAN into 5-day-old rats caused minor podocyte injury detected by the altered expression of the differentiated marker of podocytes. Rats treated with PAN showed a loss in glomerular number (three weeks after the treatment) and compensatory glomerular hypertrophy (six weeks). It is conceivable that the model could be a useful one to analyze the mechanism underlying the associations between podocyte injury in developing glomeruli and the susceptibility to several kidney diseases in later life.

In this study, PAN was adopted as a reagent to cause podocyte injury in infant rats because PAN is considered to have toxicity to podocytes in adult rats, and nephropathy caused by PAN is widely used as a mimic of human minimal change type nephrotic syndrome. We have reported that PAN treatment caused the redistribution of functional molecules of the slit diaphragm (SD) such as nephrin and podocin, which clearly showed that PAN had toxicity to the SD[20, 21]. Therefore, it is conceivable that PAN has toxicity to podocytes for which the SD structure is under formation.

Nephrogenesis is bellieved to continue until around day 10 after birth in rats[17, 18]. Glomeruli at several developing stages from the S-shape body stage to the mature stage were observed in a longitudinal section of the 5-day-old rat. Glomeruli of the late S-shaped stage and early capillary loop stage occupied the nephrogenic zone beneath the renal capsule. Late capillary loop stage glomeruli and maturing stage glomeruli were observed at the deeper cortex. At the early S-shaped body stage, a cluster of cells is invaginated by mesenchymal tissues, and then the cells on one side of the mesenchymal cleft become glomerular epithelial cells, or podocytes, while those on the other side of the cleft become proximal tubular cells. At the capillary loop stage, interdigitation of the podocyte foot processes begins. Therefore, the 5-day-old rat was chosen to observe podocyte injury in the developing stage.

In the early capillary loop stage, nephrin and podocin are observed in a continuous linear pattern at the basal surface of podocytes in normal rats (Fig. 1a and e). At 24 h after PAN injection, the staining of nephrin and podocin in the early capillary loop stage glomeruli shifted to a discontinuous pattern, and their staining intensity clearly decreased (Fig. 1b and f), although no clear morphological alterations were observed in these glomeruli. This observation indicates that PAN has toxicity to podocytes in early stage glomeruli as well as those in mature glomeruli. Although the administration of PAN into the adult rat caused the redistribution of nephrin and podocin staining on day 10, no alterations in their staining were observed at 24 h. These observations suggested that podocytes in the developing glomeruli had a higher susceptibility to PAN treatment than the mature podocytes, and that PAN treatment to 5-day-old rats effectively caused SD injury. However, the diminution of the staining intensity of nephrin and podocin recovered by day 10 after PAN injection, and no differences in their staining patterns were detected between the PBS- and PAN-treated groups (Fig. 1c, d, g and h).

The questions then arise as to whether the podocyte injury completely recovers and whether the disturbance in podocyte maturation is really restored. Another associated question is whether the podocyte injury suffered in the developing stage influences the consequent growth of the glomeruli and the susceptibility to kidney diseases in later life. To address these questions, we followed up on those rats treated with PAN on day 5 after birth.

At three weeks after PAN treatment, no differences in the staining of nephrin and podocin were observed between PAN-injected rats and control rats (Fig. 2a, b, e and f). However, a clear reduction in kidney weight

and glomerular number was detected (Table 1). It is plausible that some glomeruli whose podocytes had been injured were prevented from growing to mature glomeruli and were diminished. The renal function evaluated by the values of BUN, cre, and Ccr clearly was reduced at this time point (three weeks after PAN injection) (Table 2). It is conceivable that the reduction in the number of glomeruli resulted in a diminished renal function. It is widely known that PAN treatment to adult rats causes severe proteinuria on around day 10 after injection, and the proteinuria is recovered by three weeks. It is also known that a PAN injection into adult rats does not cause a reduction in renal function. In this study, we showed that the treatment of PAN into infant rats reduced the renal function although it did not cause an abnormal range of proteinuria. We also showed here that PAN injection into infant rats did not cause any morphological changes in glomeruli or in the tubulo-interstitial area in rats three weeks after injection. It should be noted that no compensatory response such as glomerular hypertrophy was observed in rats three weeks after PAN injection although their renal function remarkably decreased. It is plausible that young rats whose glomeruli are developing could not respond promptly to compensate for the reduced renal function. All of these observations showed that PAN treatment to infant rats influenced the development of the kidney function.

At six weeks after PAN treatment, the renal function was restored, and no differences in the values of BUN, cre, or Ccr were detected between PAN-treated rats and control rats (Table 2). At this time point, a significant increase in the diameter of glomeruli was detected in PAN-treated rats (Table 1 and Fig. 3). It is likely that the remaining glomeruli grew to enlarge the effective filtration area and to compensate for the reduced renal function.

We also demonstrated that the staining intensity of nephrin and podocin clearly decreased in most glomeruli of PAN-injected rats at six weeks after PAN treatment (Fig. 2d and h). The decreased staining of these SD-associated molecules in enlarged glomeruli can be explained by the podocyte extension for covering the increased filtration area because glomeruli with hypertrophy do not accompany the increase in podocyte number[22, 23]. In addition, the decreased staining of these molecules in podocytes in the remaining glomeruli might be explained by the their higher susceptibility to extension because the podocyte had been injured in their developing stage. Whatever the mechanism, it is conceivable that these glomeruli with a decreased expression of the SD associated molecules have a high susceptibility to kidney disease in later life.

It should be mentioned that the intra-peritoneal injection of PAN into 5-day-old rats retarded any increase in body weight just after treatment (Fig. 4). Although the precise mechanism of how the PAN treatment results in growth retardation is uncertain, the following can be considered:1) PAN injection into infant rats immediately causes serious podocyte injury and consequent glomerular alterations; 2) the glomerular alteration severely cripples the renal function; and 3) the reduced renal function is involved in growth retardation.

In conclusion, we established a rat model with injured podocytes at the developing stage. The podocyte injury results in the reduction of podocyte numbers, compensatory glomerular hypertrophy, and retardation of increases in body weight. The model could serve as a mimic of infants who show signs of IUGR at birth. It is also suitable to investigate the relation between the podocyte injury suffered at early stages and the risk for several kidney diseases in adulthood.

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