·Original Article in English·

Podocin expression in rats with puromycin aminonucleoside nephropathy

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Abstract: Objective To investigate the expression and distribution of podocin and its role in the development of proteinuria in rats with puromycin aminonucleoside (PAN) nephropathy. **Methods** The nephropathy model was established by a single injection of PAN. The rats in the Model group were sacrificed on days 1, 3, 10 and 20 after PAN injection. The renal histological changes were observed under light and electron microscopes. The indirect immunofluorescence staining and semiquantitative reverse transcription PCR were used to detect the expression of podocin. Six rats which received normal saline with the same volume as the Model group served as the Control group. **Results** ① The proteinuria which was gradually increased 3 days after PAN injection reached a peak on day 10 ($P < 0.01$) and decreased on day 20 but was still greater than that of the Control group ($P < 0.05$). ② In the process of development of PAN nephropathy, the foot process effacement was seen between 3 and 10 days following PAN injection. ③ The podocin expression began to decrease on day 1, and prominently decreased on day 3 and day 10 compared with those of the Control group ($P < 0.01$). On day 20, the podocin expression resembled that of day 1, remaining lower than that of the Control group ($P < 0.05$) although it partly retrieved with the decreased proteinuria. The podocin expression was negatively correlated with proteinuria ($r = -0.786$, $P < 0.05$). ④ Podocin staining showed a very fine linear-like pattern along the capillary loop in the Control group. It presented a discontinuous pattern on day 1, a granular pattern on day 3, more coarse granular on day 10 and gradually recovered to a linear-like pattern on day 20. ⑤ Podocin mRNA expression levels were slightly elevated on days 1, 3 and 10 (1.2, 1.5, 1.4 folds as the Control group respectively) and recovered to a normal level on day 20 compared with those of the Control group. **Conclusions** Podocin may be involved in the development of proteinuria and it may be a useful early marker of podocyte injury in nephropathy.

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Key words: Puromycin aminonucleoside; Podocin; Proteinuria; Immunofluorescence; Rat

Podocin 在大鼠氨基核苷嘌呤霉素肾病中的表达与分布

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[摘 要] 目的 观察 podocin 在大鼠氨基核苷嘌呤霉素肾病模型中的表达和分布的改变,探讨其在蛋白尿发生中的可能作用。**方法** 通过一次性腹腔注射氨基核苷嘌呤霉素(PAN)建立大鼠肾病模型,分别于注射后 1,3,10,20 d 处死大鼠,每次 6 只。对照组注射等量的生理盐水。应用光镜、电镜观察肾脏病理改变,应用免疫荧光染色结合图像分析、半定量 RT-PCR 的方法,检测肾组织的 podocin 的表达。**结果** ① PAN 注射后第 3 天,大鼠 24h 尿蛋白的排泄量逐渐增加,第 10 天达高峰,较对照组差异有显著性($P < 0.01$);第 20 天,模型组大鼠 24 h 尿蛋白排泄逐渐恢复,但仍高于对照组($P < 0.05$)。② PAN 肾病模型第 3、10 天,透射电镜显示足细胞足突融合。③与对照组比较,肾小球 podocin 的表达在 PAN 注射后第 1 天出现下调,第 3、10 天显著下调,第 20 天 podocin 的表达逐渐恢复,但仍低于对照组($P < 0.01$)。④Podocin 在正常大鼠肾小球沿毛细血管襻,呈均匀连续的线样分布。肾病模型第 1 天 podocin 的分布变得不均匀,局部呈颗粒状分布;第 3 天 podocin 的分布呈现弥散性的颗粒状;第 10 天 podocin 呈粗大的颗粒状分布。第 20 天 podocin 的分布逐渐恢复为线样。⑤肾病模型第 1、3、10 天 Podocin mRNA 水平较对照组表达略有增强,第 20 天恢复正常。**结论** ①Podocin 表达的改变是足细胞损伤的早期指标之一;②

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Podocin可能参与了病理状态下蛋白尿的发生。

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Nephrotic syndrome, one of the common pediatric kidney diseases, manifests clinically massive proteinuria, hypoalbuminemia, edema and hyperlipidemia. Histologically, it is characterized by retraction and effacement of the glomerular visceral epithelial cell (podocyte) foot processes under electron microscope. Massive proteinuria, the most fundamentally pathophysiological change, results from the dysfunction of the glomerular filtration barrier. This barrier consists of three layers, in which the slit diaphragm (SD) is considered as the most essential unit. In 2000, podocin was identified as a product of gene mutation in patients with autosomal steroid-resistant nephrotic syndrome[1]. It was one of the podocyte molecules located at the glomerular SD[2]. However, the precise role of podocin in the development of proteinuria remains unknown. To address this, a rat puromycin aminonucleoside (PAN) nephropathy model was established which closely resembled the human minimal change nephrotic syndrome and the expression of podocin and its role in the development of proteinuria were investigated in PAN nephropathy model.

Materials and methods

Animal models

Thirty male Sprague-Dawley rats (weighing 80-100 g) purchased from the Experimental Animal Center of Southeast University were enrolled in this study. They were housed in individual metabolic cages and were fed with standard chow and had free access to tapwater. PAN nephropathy was induced according to the previously described protocol[3]. Briefly, rats in the Model group were given a single intraperitoneal injection of PAN (15 mg/100 g; Sigma, USA). The Control group received equal normal saline. Six rats in the Model group were sacrificed 1, 3, 10 or 20 days after injection. The specimen of 24-hour urine was collected prior to sacrificing in order to measure 24-hour urinary protein quantity by the Bradford method.

Antibodies

The rabbit polyclonal antibody p35 was kindly provided by Prof. Antignac, Paris, France. The secondary antibody, FITC-conjugated affinity-purified goat anti-rabbit IgG was purchased from Zhongshan Biotechnology Co. LTD (Beijing, China).

Histology

For the light microscopy examination, freshly harvested kidneys were fixed in neutral formaldehyde. H&E, PAS and Masson stainings were performed using standard methodology. For the transmission electron microscopy examination, kidneys were fixed by perfusion of 2% glutaraldehyde and 4% paraformaldehyde in 0.05 M PBS (pH 7.4) through the left ventricle. Small pieces of cortex were fixed overnight at 4℃ in 2.5% glutaraldehyde dissolved in 0.1 M sodium cacodylate buffer (pH 7.4) and washed in the same buffer. Tissue fragments were postfixed in cacodylate-buffered 1% OsO4 for 1.5 hours, dehydrated and embedded in epoxy resin 618. Ultra-thin sections were cut and stained with 4% uranyl acetate for 45 minutes and subsequently with lead citrate for 4 minutes at room temperature. Then they were examined under a JEM1010 electron microscope.

Indirect immunofluorescence

Fresh kidneys were embedded in Tissue-Tek OCT compound at -20℃. Five-micrometer cryostat sections were cut, transferred to slides, fixed in acetone for 10 minutes, air dried and stored at -20℃ until use. The sections were thawed, washed in PBS and incubated at 4℃ overnight with the primary antibody diluted in PBS (rabbit anti-podocin 1∶400). The sections were then washed in PBS and incubated for 30 minutes with FITC-conjugated anti-rabbit IgG antibody (1∶100). They were again rinsed in PBS, mounted in 90% glycerol in 0.1 M PBS and covered with coverslips.

Digital image analysis

The sections were examined by a BH-2 microscope equipped with a single chip color CCD camera,

connected to a computer with Olympus Viewfinder software. In each animal, five images of individual glomeruli were recorded at ×200 magnification while the exposure time was fixed. The intensity of the staining was determined by highlighting the glomeruli and measuring the mean luminosity value of the region with the histogram function of Image J1.3 software, as described in detail in the previous literature[4]. The recording and analysis of the digital images were performed with fixed settings.

Detection of podocin mRNA expression

Total RNA was extracted from 100 mg kidney cortex using the Trizol-method and 23 μl RNA were used to synthesize cDNA in the presence of an oligo dT15-primer, RNase inhibitor and the M-MLV reverse transcriptase (Promega, USA) in a final volume of 40 μl. Sequence-specific oligonucleotide primers were designed according to rat podocin (5′-GCAGCCACGGTAGTGAATGT-3′, 5′-CAGGAAG CAGATGTCCCAGT-3′), GAPDH (5′-ACCACAGT CCATGCCATCAC-3′, 5′-TCCACCACCCTGTTGC TGTA-3′). The PCR products for podocin and GAPDH correspond to 253 bp and 452 bp respectively. PCR was performed in a 20 μl reaction system which contained 1 μl cDNA, 1 × PCR buffer, 1.5 mM $MgCl_2$, 0.1 mM dNTP, 10 pM of each primer, and Taq DNA polymerase 0.15 μl (Promega, Shanghai). Amplification cycles were 95℃ for 5 minutes, followed by 30 cycles at 94℃ for 30 seconds, 58℃ for 30 seconds, and 72℃ for 30 seconds and terminated by a final extension of 72℃ for 5 minutes. The PCR products were subjected to electrophoresis with 1.5% agarose gel and stained with ethidium bromide. The band intensity was determined by gel image analytic system (Gene company, USA). The level of podocin mRNA expression was calculated by dividing the intensity of the internal control, GAPDH.

Statistical analysis

SPSS 10.0 software combined with NOSA 5.0 was used and the data were presented as means ± SD. One-way ANOVA with a post hoc correction was applied for analysis of differences of data between groups. When the conditions for using ANOVA were not met, the Kruskal-Wallis test was used following a multiple comparisons using the Nemenyi test. Correlations were evaluated with the Spearman correlation test. $P<0.05$ was considered statistically significant.

Results

Urinary protein

The single intraperitoneal injection of PAN led to a gradual increase of urinary protein, starting on day 3. The urinary protein reached a peak level on day 10 and increased significantly when compared with the controls ($P<0.01$). The amount of proteinuria decreased on day 20 but was still greater than that of the Control group ($P=0.032$). See Table 1.

Table 1 24-hr urinary protein (mg/d) and podocin expression ($n=6$, $\bar{x} \pm s$)

Day	Urine protein	Staining intensity	Podocin mRNA
0	3.09±1.55	54.95±6.25	90.62±5.53
1	7.30±2.11	45.03±5.10[a]	108.50±8.14[a]
3	14.65±5.57	33.35±6.41[a]	139.09±13.05[a]
10	306.52±90.16[a]	13.58±3.73[a]	133.09±10.42[a]
20	28.21±11.04[b]	42.34±6.65[a]	99.32±9.50

Note: a vs day 0 $P<0.01$; b vs day 0 $P<0.05$

Histology of the kidney

Kidneys from the rats sacrificed on day 10 in the Model group were pale and swollen by visual examination. The H&E, PAS and Masson stainings showed no distinct pathological changes in both the Control and Model groups. However, podocyte foot process effacement was visualized in the kidney cortex from the rats sacrificed on day 3 under transmission electron microscope (Figure 1B). On day 10, the foot-process effacement was more extensive (Figure 1C). Normal podocyte foot process is shown in Figure 1A.

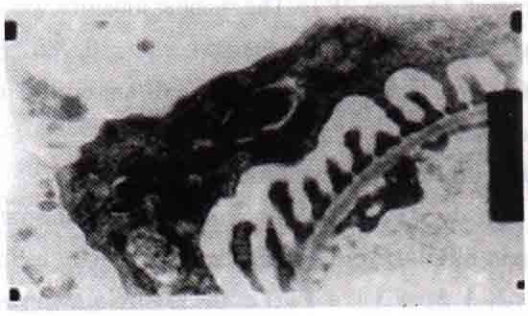

Figure 1A Normal foot process (day 0, 25K)

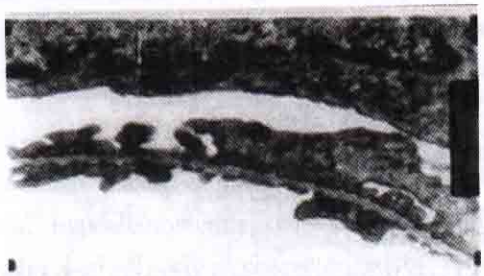

Figure 1B Retracted and effaced foot process (day 3, ×20 K)

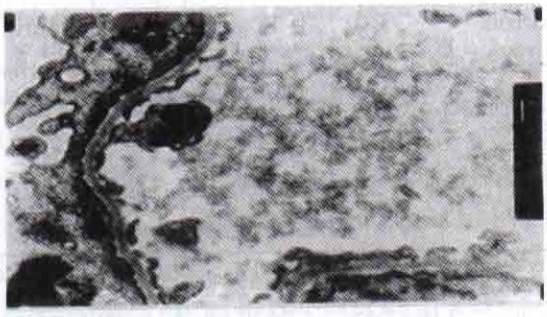

Figure 1C Extensive effaced foot process (day 10, ×20 K)

Figure 1 The images of electron microscope

Podocin protein expression

Podocin staining was strongly detected as the very fine linear-like pattern along capillary loop in the sections of rats in the Control group (Figure 2A). On day 1 after PAN injection, the linear-like pattern became partly discontinuous and granular with decreased intensity of podocin staining compared with the controls (Figure 2B). The staining presented a coarse granular pattern with further decreased intensity on day 3 (Figure 2C). Noteably, the staining pattern become more granular and the intensity was markedly decreased on day 10 (Figure 2D). On day 20, the results resembled that of day 1 (Figure 2E). As a negative control, secondary antibody alone did not stain glomeruli (Figure 2F). The staining intensity was negatively correlated with proteinuria ($r = -0.786$, $P < 0.05$). See Table 1. (Figures 2A－F are on the inside front cover)

Podocin mRNA expression

On days 1, 3 and 10 after PAN administration, podocin mRNA expression in the Model group increased to 1.2, 1.5, and 1.4 folds as the Control group respectively. The mRNA expression regained to normal level on day 20 after injection. See Table 1 and Figure 3.

Figure 3 RT-PCR findings of podocin mRNA expression in different time points

Lanes 1－5 shows GAPDH mRNA expression on days 0, 1, 3, 10 and 20; Lanes 6－10 shows podocin mRNA expression in the corresponding time point; M stands for 100 bp DNA marker.

Discussion

In the past few years, emerging new molecules such as nephrin, podocin and CD2AP have been identified at the SD area. Nephrin, a major component of the SD, is a transmembrane protein encoded by the NPHS1 gene, which is mutated in congenital nephritic syndrome of the Finnish type[5]. The targeted deletion of CD2AP in mice induced nephritic syndrome[6]. Podocin, encoded by NPHS2, is one of the most important podocyte molecules. It is a new member of the stomatin family, which consists of hairpin-like integral membrane proteins with intracellular NH2－ and COOH-termini. In addition to autosomal recessive steroid-resistant nephrotic syndrome, the spectrum of diseases related to podocin mutations is broadening[7]. Roselli et al[8] generated podocin-deficient mice to investigate the function of podocin and found that the mice developed early glomerular filtration defect. It was proposed that podocin was important for maintaining the integrity of the slit diaphram. Studies have suggested that podocin can interact with nephrin and CD2AP directly and strengthen the signal transduction of nephrin[9,10]. In this study, the expression of podocin in the glomeruli of rats with PAN nephropathy was studied in order to investigate the possible mechanisms underlying proteinuria.

A significantly decreased expression and abnormal distribution of podocin were found in the animal model in this study, which presented with heavy pro-

teinuria and in which foot process effacement in glomeruli was identified by transmission electron microscope. Saleem et al[11] found that disruption of the podocyte cytoskeleton caused the redistribution of podocin. Huber TB et al[12] found that mutations in the NPHS2 gene disrupted nephrin targeting to lipid raft microdomains at slit diaphram. Nephrin can interacts with CD2AP, an adaptor molecule associating with actin fibers[13]. It was also found in this study that podocin distribution became partly discontinuous and granular on the 1st day after PAN injection while effacement of foot process and proteinuria were not developed. Hence, we suppose that the changes of podocin disrupt the connection between SD-associated protein and podocyte cytoskeleton and thus result in the effacement of foot process. Maybe the expression of podocin is a sensitive marker of early podocyte injury. On day 20, the podocin expression resembled that of day 1. This indicated that the podocin expression retrieved partly when proteinuria decreased. Although the proteinuria level was negatively correlated with expression of podocin, the effects of proteinuria on podocin expression and distribution remain unknown. However, this study has shown the dramatically reduced expression and redistribution of podocin preceded the development of proteinuria in PAN nephropathy. This confirmed that it was one of the critical components of the SD for maintaining the barrier function of the glomerula. Interestingly, a slightly elevated level of podocin mRNA was also found and this was not consistent with the declined protein level. This result may indicate that a compensatory reaction of the podocyte resulted from its injury or the alterations in the podocin expression. On day 20, the mRNA level recovered prior to the protein level. The results of this study may reflect a discordant regulation of podocin mRNA and protein levels.

In summary, an early redistribution of podocin and a remarkably reduced expression following heavy proteinuria were found in PAN nephropathy. Podocin may be involved in the development of proteinuria and can be taken as an early marker of podocyte injury. It is not known whether the same kind of changes exist in human proteinuric diseases and the regulation mechanism of podocin expression needs to be studied in detail.

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Podocin expression in rats with puromycin aminonucleoside nephropathy

(These figures refer to the paper on page 161)

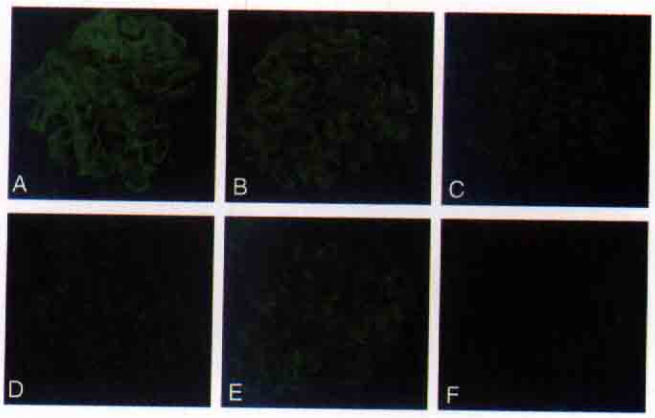


Figure 2 Immunofluorescence staining (magnification, × 200)

Figure 2A Linear-like pattern of the continuous fine granules in normal glomeruli

Figure 2B Partly discontinuous pattern on day 1

Figure 2C Diffusely granular pattern on day 3

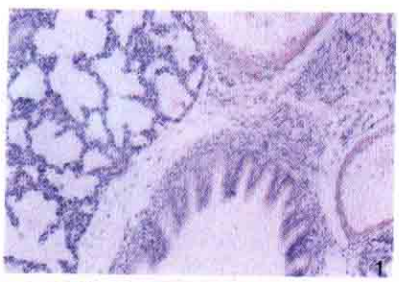
Figure 2D Coarse granular pattern on day 10

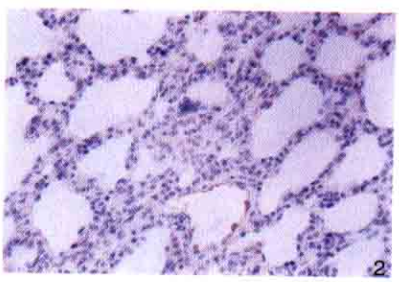
Figure 2E Partly retrieved expression on day 20

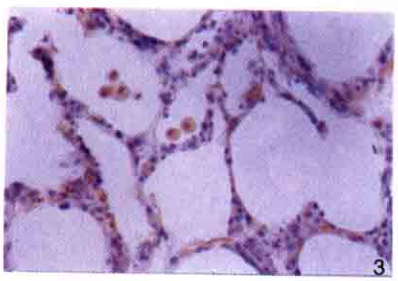
Figure 2F No staining on glomerular (negative control)

Dynamic expression and effect of TGF-β 1 on extracellular matrix in premature rats with CLD

(These figures refer to the paper on page 166)







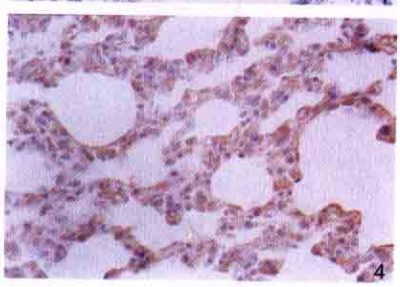


Figure 1 Expression of TGF- β 1 of the normal lung (SABC, × 200)

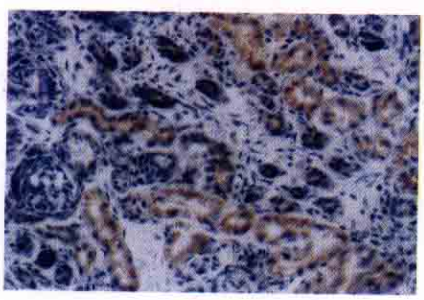
Figure 2 Expression of TGF- β 1 of the model lung on day 7 (SABC, × 200)

Figure 3 Expression of TGF- β 1 of the model lung on day 14 (SABC, × 400)

Figure 4 Expression of TGF- β 1 of the model lung on day 21 (SABC, × 400)

宫内窘迫后胎鼠肾脏细胞间粘附分子-1的表达及意义

（正文见第199页）



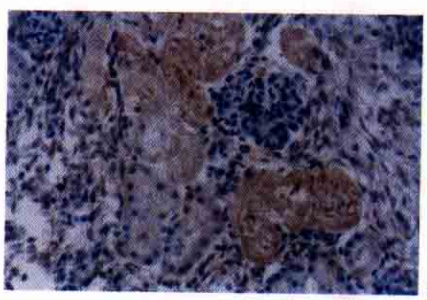


图1 ICAM-1在假手术组胎鼠肾组织即有少量表达，表达部位主要在肾小管上皮细胞胞浆中，近曲小管表达略强于远曲小管，肾小球区则未见表达。（免疫组化染色 × 360）

Figure 1 Small quantity expression of ICAM-1 in the cortical tubuli in the Sham-Operation group (HE × 360)

图2 缺血15 min再灌注15 h时，ICAM-1在近曲小管的表达达到高峰，在坏死和空泡变性明显的小管中其表达最强。（免疫组化染色 × 360）

Figure 2 ICAM-1 expression in the proximal tubuli reached a peak after a 15-minute ischemia and then a 15-hr reperfusion (HE × 360)