Ultrastructural study on nephrin expression in experimental puromycin aminonucleoside nephrosis

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Abstract

Background. Nephrin is a recently identified protein that is a key component of the slit diaphragm. This protein may play a crucial role in maintaining the glomerular filtration barrier, and mutations in the gene for nephrin reportedly lead to congenital nephrosis. However, the expression of nephrin in acquired glomerular disease has not yet been fully clarified. To address this issue, we analysed the expression and localization of nephrin by morphological analysis based on immunoelectron microscopy in normal glomeruli and in glomeruli from proteinuric experimental models.

Methods. Twenty rats were divided into three experimental groups (n=16 total) and a control group (n=4). Rats in the experimental groups received a single intravenous injection of puromycin aminonucleoside (PAN), and were sacrificed at 1 (n=4), 2 (n=6) and 3 weeks (n=6) post-injection. Nephrin expression was assessed by immunoelectron microscopy using a polyclonal antibody against nephrin and gold particles. It was quantified by counting the gold particles and the slit diaphragms and by measuring the average foot process width in microphotographs.

Results. The average foot process width in the 1 week group (5924.5±1523.9 nm) was far greater than that of controls (1112.9±79.8 nm), but decreased thereafter. The average number of total gold particles per unit length (10 000 nm) of the glomerular basement membrane (GBM) underlying the foot processes was reduced at 1 week (26.0±9.5), compared with controls (335.3±125.5), but increased thereafter. Also, the average number of junctional gold particles per unit length of the GBM was lower than controls (208.4±1.7) at 1 week (10.1±3.5), but increased thereafter. There were no significant differences between the numbers of junctional gold particles per slit diaphragm among the groups, but significant differences were observed in the distributions of gold particles among the groups. Gold particles were more frequently seen in cytoplasm at 1 week.

Conclusions. The present ultrastructural studies showed that nephrin expression and its distribution were altered in PAN-treated rats, and this occurred in parallel with foot process effacement. Nephrin expression returned to normal with improved resolution of the effacement. Nephrin expression was found to be rather preserved in areas without foot process effacement. The significance of the above findings in terms of proteinuria and foot process effacement needs further clarification.

Keywords: nephrin; proteinuria; puromycin aminonucleoside

Introduction

Congenital nephrotic syndrome of the Finnish type (CNF or NPHS1) is characterized by massive proteinuria and the development of nephrotic syndrome shortly after birth or within the first few months of life. It is an autosomal recessive disease that is observed in 1 in 8000 newborns in Finland, but many patients have been identified in other populations [1]. Among CNF, the most remarkable pathological feature revealed by electron microscopy is foot process effacement, whereas light microscopy shows only mild mesangial hypercellularity [2]. It was shown recently that mutations in the NPHS1 gene are responsible for CNF [3]. The NPHS1 gene product, which is called...
nephelometry (Hitachi-747, Hitachi, Osaka, Japan). PAN-developed in all PAN-injected animals, as determined by Washington, PA), in a 0.1 M phosphate buffer.

were fixed in 3.5% paraformaldehyde (Sigma) and 0.01% glutaraldehyde (Electron Microscopy Science, Fort Washington, PA), to determine the distribution of nephrin on podocytes at the ultrastructural level. We investigated the distribution of nephrin on podocytes at the ultrastructural level.

Morphological analysis was performed by immunoelectron microscopy.

Puromycin aminonucleoside (PAN) nephrosis, a model of human minimal change disease, can be induced in experimental rats by injection of PAN [8]. Proteinuria develops from day 4 after a single injection and increases to a maximum at around day 10. Morphological changes in PAN nephrosis include pronounced effacement and fusion of foot processes, which is consistent with the general morphological features of CNF. In this study, experimental animals were sacrificed at 1, 2 and 3 weeks after injection of PAN. We investigated the distribution of nephrin on podocytes at the ultrastructural level.

**Subjects and methods**

**Animal experiments**

Young male Sprague-Dawley rats (n = 20), weighing 100–120 g, were divided into three experimental groups and a control group (n = 4). Sixteen rats in the experimental groups were administered a single intravenous (i.v.) injection of PAN (15 mg/100 g, Sigma). The control group received an equal volume of i.v. 0.9% saline. All animals were fed with standard rat chow and had free access to tap water. Urine samples were collected using metabolic cages (21 x 21 x 21 cm) before sacrifice. Proteinuria developed in all PAN-injected animals, as determined by nephelometry (Hitachi-747, Hitachi, Osaka, Japan). PAN-treated animals were sacrificed on post-injection weeks 1 (n = 4), 2 (n = 6) and 3 (n = 6).

Cortical renal tissues from each animal were immediately isolated for immunoelectron microscopic studies. The tissues were fixed in 3.5% paraformaldehyde (Sigma) and 0.01% glutaraldehyde (Electron Microscopy Science, Fort Washington, PA), in a 0.1 M phosphate buffer.

**Antibodies**

We used polyclonal rabbit antibodies against the intracellular domain of nephrin. Preparation of the antibodies is described in detail elsewhere [9]. Briefly, we chose a peptide of 21 amino acids, DRDTR SSTVS TAEVD M, which is located in the cytoplasmic region of the denuded amino acid sequence, as an immunogen. The rabbits were immunized with 0.1 mg of the peptide conjugated with keyhole limpet haemocyanin as a carrier protein, and boosted twice with 0.5 mg of antigen. Rabbits were sacrificed and bled 2 weeks after the last immunization.

**Immunoelectron microscopy**

Immunoelectron microscopy was performed by using the post-embedding immunogold technique. Samples were fixed for 2 h at 4°C, rinsed in 0.1 M phosphate buffer at pH 7.4 for 12 h and dehydrated in a graded ethanol series. They were then infiltrated initially with 50% LR white resin (London Resin, Basingstoke, UK) for 2 h in 100% ethanol and then with LR white resin for 12 h. Tissues were embedded in gelatine capsules (Agar, Essex, UK) filled with LR white resin. After UV polymerization, specimens were cut into ultrathin sections with a Leichert ultracut S (Wein, Austria) and mounted on carbon/formvar nickel grids. To minimize non-specific labelling, ultrathin sections were incubated twice with a protein blocking agent (Immunotech, Glostrup, Denmark) in PGB (1% bovine serum albumin and 0.1 M glycine in 0.1 M phosphate-buffered saline) at room temperature for 10 min. Grids were incubated for 1 h at 37°C with primary antibody (1:100 dilution for rabbit anti-nephrin antibody in PGB). After rinsing, the grids were incubated with a secondary antibody, immunogold conjugate EM goat anti-rabbit IgG (British BioCell International, Cardiff, UK), in PGB at 37°C for 30 min. The grids were then rinsed in PGB and post-fixed in 0.1 M phosphate buffer containing 1% glutaraldehyde for 10 min. The specimens were first stained with uranyl acetate, then with lead citrate, and were then examined under an electron microscope (Hitachi-7100, Hitachi, Osaka, Japan) at 75 kV.

**Morphometric evaluation**

Ten micrographs were taken from each sample. Printed copies with a final magnification of x 15 000 were examined and gold particles in the foot processes were counted. To quantify the amount of nephrin in and around the slits, a circle of 0.5 cm diameter on transparent film was placed centrally on each slit. Gold particles in the defined circles were counted and expressed as gold particles located at the junction. The gold particles other than junctional particles were classified as located at the basal membrane if they were located within ~0.5 cm from the basal cytoplasmic membrane. The gold particles were classified as located at the apical membrane if they were located within ~0.5 cm from a cytoplasmic membrane other than the basal membrane. The gold particles were classified as located within the cytoplasm if they were located within cytoplasm and the distances from the cytoplasmic membrane were > 0.5 cm.

On each photograph, the curved length of the peripheral capillary basement membrane was measured using a flat scanner and a computer-based morphometric system. The number of slit diaphragms overlying the capillary basement

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**Reference**

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**Acknowledgments**

This work was supported by a grant from the Japanese Ministry of Education, Science, Sports and Culture (17590326) and by a grant from the Japanese Ministry of Health, Labor and Welfare (2008-5). We would like to thank Dr. T. I. Ishikawa for his help in the preparation of antibodies. We are also grateful to Dr. T. I. Ishikawa for his help in the preparation of antibodies.
membrane was counted and the average foot process width (Wp) was calculated using the following formula [10]:

\[ W_p = \frac{\pi}{4} \times \frac{\Sigma BML}{\Sigma Slits} \]

The above equation was used to determine the total number of gold particles/10,000 nm Wp, the number of junctional gold particles/10,000 nm Wp, and the number of junctional gold particles/slit diaphragm.

Statistical analysis

Results are expressed as means ± SD. Between-group comparisons were analysed using the Kruskal–Wallis test. Pairwise comparisons were performed using Scheffe’s test. Fisher’s exact test and multinomial logistic analysis were also used to determine the gold particle distributions. A \( P \)-value of <0.05 was considered statistically significant.

Results

24 h urine volume and protein

The 24 h urine volume was not significantly different between experimental animals and normal controls. Significant proteinuria developed in PAN-treated animals at week 1 (401.9 ± 188.4 mg) and week 2 (431.2 ± 65.0 mg) vs controls (20.1 ± 11.2 mg) (\( P = 0.030 \), Table 1). This decreased at week 2 (2117.5 ± 3637.9 nm) and week 3 (1246.1 ± 579.1 nm). The average number of total gold particles per unit length (10,000 nm) of the GBM underlying the foot processes was significantly reduced at week 1 (26.0 ± 9.5) vs controls (335.3 ± 125.5) (\( P = 0.005 \)), but increased at weeks 2 and 3 (230.1 ± 154.3 and 215.1 ± 67.0, respectively). In addition, the average number of junctional gold particles per unit length in the 1 week group (10.1 ± 3.5) was lower than in the control group (208.4 ± 71.7) (\( P = 0.001 \)). The number of junctional gold particles per slit diaphragm was similar among the groups. The groups showed differences in gold particle distribution, with more frequent gold particles seen in the cytoplasm (\( P = 0.004 \)) at 1 week (Table 1).

Immunoelectron microscopy

Most of the gold particles, corresponding to nephrin, were characteristically located on the podocyte foot processes at the level of the slit diaphragms in the control kidneys (Figure 1). However, some immuno-gold particles were also seen in the cytoplasm and at the apical plasma membrane and basal portion of podocytes.

In PAN-treated rats, foot processes were widely effaced and nephrin-specific gold particles were almost absent. But in areas with patent foot process, gold particles were observed at the slit diaphragms (Figure 2A). The number and distribution of gold particles in areas with intact foot processes were similar to those in normal kidneys. Gold particles were also found in the cytoplasm and at the apical plasma membrane (Figure 2B).

The average foot process width of the 1 week group (5924.5 ± 1523.9 nm) was much larger than that of controls (1112.9 ± 79.8 nm) (\( P = 0.030 \), Table 1). This decreased at week 2 (2117.5 ± 3637.9 nm) and week 3 (1246.1 ± 579.1 nm). The average number of total gold particles per unit length (10,000 nm) of the GBM underlying the foot processes was significantly reduced at week 1 (26.0 ± 9.5) vs controls (335.3 ± 125.5) (\( P = 0.005 \)), but increased at weeks 2 and 3 (230.1 ± 154.3 and 215.1 ± 67.0, respectively). In addition, the average number of junctional gold particles per unit length in the 1 week group (10.1 ± 3.5) was lower than in the control group (208.4 ± 71.7) (\( P = 0.001 \)). The number of junctional gold particles per slit diaphragm was similar among the groups. The groups showed differences in gold particle distribution, with more frequent gold particles seen in the cytoplasm (\( P = 0.004 \)) at 1 week (Table 1).

![Fig. 1. Expression of nephrin in normal rat kidney. Distinct localization of nephrin at the slit diaphragm is seen (A and B). GBM = glomerular basement membrane (magnification: ×15000).](https://academic.oup.com/ndt/article-abstract/19/12/2981/1807501)

![Fig. 2. Labelling of nephrin in a glomerulus from a PAN nephrosis rat. Nephrin-specific gold particles were markedly reduced. However, nephrin remained at the filtration slits (A). Gold particles were also found at in the cytoplasm and apical plasma membrane (B). GBM = glomerular basement membrane (magnification: ×15000).](https://academic.oup.com/ndt/article-abstract/19/12/2981/1807501)
Nephrin was recently identified during an investigation of the genes that are responsible for NPHS1 [3]. A single gene mutation of nephrin in NPHS1 was found to induce massive proteinuria, and immunoelectron microscopy with anti-nephrin antibody showed that the epitope was localized mainly to the slit membrane region. It was suggested that nephrin is a key component of the slit diaphragm, forming an interdigitating zipper-like isoporous filter structure as presented by Rodewald and Karnovsky [7,11].

Since the slit diaphragm is believed to be the ultimate barrier for the ultrafiltration of proteins such as albumin, and since proteinuria is the hallmark of most if not all glomerular diseases such as glomerulonephritis, diabetic nephropathy and hypertensive glomerulosclerosis, nephrin has been suggested to play a role in acquired proteinuric glomerular disease [5,6].

Because of this, many studies have investigated nephrin expression in acquired human glomerular disease and in experimental animal models. However, the human studies have not been consistent. Several authors found reduced nephrin expression, whereas others reported contradictory findings [6,12–14]. Studies in experimental animal models, especially in puromycin-induced nephrosis models, have more consistently found that nephrin expression is decreased [5,15]. The discrepancies between these studies are difficult to explain, but may be due to different methodologies to detect nephrin expression. Morphological changes in podocytes such as foot process effacement during glomerular disease are not always consistent in the same category of disease and can change even in samples from the same disease. The foot processes may be severely effaced in certain areas of the samples, whereas they may be preserved in other areas. Therefore, when studying nephrin expression, which is localized in the slit diaphragm of the podocyte foot process, ultrastructural methods such as immunoelectron microscopy are better able to detect changes in nephrin expression in association with foot process effacement than methods such as immunohistochemistry or immunofluorescence.

Only a small number of studies have attempted a quantitative ultrastructural analysis of nephrin expression in acquired human glomerular disease or in disease models. In previous quantitative analysis based on immunoelectron microscopy, we found that nephrin expression was reduced in glomerulonephritis patients, but not in areas where the podocyte foot processes were well preserved [13]. Wernerson et al. [12] recently reported similar results. By employing a similar quantitative immunoelectron microscopy method, they found that nephrin expression was decreased in minimal change nephrotic syndrome patients, whereas it was preserved in the intact slit area [12].

The present study was designed to examine ultrastructural nephrin expression. PAN nephrosis is a well-established animal model which closely resembles

| Table 1. Immunoelectron microscopy of gold-labelled antibody against nephrin |
|---|---|---|---|
| Group (week) | Foot process width (nm) | Total gold/ fp* | Junctional gold/ fp* |
| 0 | 1112.8 ± 298.8 | 23.3 ± 25.5 | 354.5 ± 323.9 |
| 1 | 5924.5 ± 1523.9* | 26.0 ± 35.4* | 301.5 ± 380.9 |
| 2 | 2117.5 ± 344.8 | 210.1 ± 31.0 | 100.9 ± 81.3 |
| 3 | 1246.1 ± 579.1 | 215.1 ± 67.0 | 97.2 ± 46.9* |

fp* indicates unit length (10000 nm) of foot process.

*Significant difference (P < 0.05).
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human minimal change nephropathy. After administration of a single PAN injection, heavy proteinuria reaches its peak around day 10 and this is accompanied by podocyte foot process effacement. Thereafter, the proteinuria decreases with resolution of foot process effacement [8]. PAN nephrosis is an excellent model to study nephrin expression and its significance in kidney pathology since it induces reversible heavy proteinuria accompanied by reversible foot process effacement. These are the only pathologic changes and there are no changes in mesangial or capillary cells, or in inflammatory cell infiltration or immune complex deposits. Using PAN and immunoelectron microscopy, we observed overall decreases in nephrin expression where there was heavy proteinuria and foot process effacement. However, we also observed normal nephrin expression where the slit area was preserved. These findings are in good agreement with previous studies examining nephrin expression using quantitative ultrastructural analysis [8,12,13]. We found that the distribution of nephrin appeared to be altered at 1 week after PAN administration, which agrees with the report of Doublier et al. [14]. These alterations in subcellular localization of nephrin may be secondary to cytoskeletal alterations caused by puromycin [14]. Wernerson et al. [12] suggested that local reductions in nephrin below a certain level may dissolve the slit membranes causing nephrin to recirculate intracellularly [12]. We observed a slight increase in nephrin expression per slit which was not statistically significant at 1 week after PAN injection. Increased expression of nephrin has been found in the early phase of experimental diabetes, and the authors suggested a role for activated protein kinase C as a mediator of the nephrin increase [16]. Furthermore, our study showed that the altered expression of nephrin was reversed when the proteinuria decreased and foot process effacement resolved.

It is possible that the altered nephrin expression in our PAN-treated rats played a pathogenetic role in proteinuria and foot process effacement. As occurs in CNF, an altered expression of nephrin by PAN can lead to foot process effacement and slit membrane disruption that ultimately results in proteinuria. However, it is also possible that the altered nephrin expression in our PAN-treated rats occurred as a consequence of cytoskeletal alterations and podocyte foot process effacement. Indeed, several examples of this have been reported in the literature. Disruption of a single component of the cytoskeletal structure interconnecting the actin cytoskeleton, the molecules around the slit membrane such as nephrin, or cell surface molecules such as podocalyxin can lead to foot process effacement and proteinuria. Mutations of podocin [17] or α-actinin-4 [18], or disruption of CD2-AP [19] or podocalyxin [20] are well-described examples of this mechanism, although the degree of proteinuria and podocyte effacement and the pace of these changes can be variable. In addition, the podocyte depletion that was reported in PAN nephrosis [21] may be linked to cytoskeletal alterations and podocyte foot process effacement, leading to the altered nephrin expression that we observed in the current experiment. Morphological studies such as ours do not allow detailed mechanistic studies of how PAN causes altered nephrin expression, nor of the pathogenetic role of this altered expression in foot process effacement and proteinuria.

In summary, our quantitative ultrastructural studies showed that nephrin expression in PAN-treated rats was altered in the areas that showed foot process effacement. However, nephrin expression was comparable with that in control animals in areas where the foot process interspaces were well preserved. The altered nephrin expression was reversed when proteinuria was reduced and foot process effacement was resolved. The significance and pathogenetic mechanism of these observations warrant further investigation.

Acknowledgements. We thank Mrs Hee Suk Woo for her skilful technical assistance. The results of this study were presented in abstract form at Korean Society of Nephrology, Seoul, Korea, April 2002 and at ASN, Philadelphia, November 2002.

Conflict of interest statement. None declared.

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Received for publication: 10.7.03
Accepted in revised form: 19.5.04