

Original Article

## Unchanged Distribution Density of Anionic Sites on the Glomerular Wall in Rats with Active Heymann Nephritis

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**Abstract:** In various kinds of glomerulonephritis, alteration of anionic charge on the glomerular basement membrane (GBM) and podocytes has been controversial for more than decade. To elucidate the relation between glomerular protein leakage and anionic sites on the glomerular wall, we examined the distribution of anionic sites on the GBM and podocytes of rats with active Heymann nephritis (AHN). Urinalysis for protein levels was conducted, and the kidneys were examined using electron microscopic cytochemistry for the assessment of anionic charge with two cationic probes. The anionic sites on podocytes were decreased in number in the AHN rats; however, the distributions of anionic sites on the GBM were similar in density to those seen in the control animals. From these results, we consider that the decrease in anionic charge density on podocytes might be attributable to protein leakage and that the charge barrier of the GBM is irrelevant to the protein leakage in AHN rats. (DOI: 10.1293/tox.26.11; *J Toxicol Pathol* 2013; 26: 11–17)

**Key words:** glomerular basement membrane (GBM), podocyte, anionic site, Heymann nephritis, proteinuria

### Introduction

The process of protein leakage during proteinuria is unclear, although it has been studied using experimental models of nephropathy by many investigators. Through the glomerular wall, the glomerular basement membrane (GBM), which possesses an anionic charge consisting mainly of heparan sulfate proteoglycans, has been suggested to play an important role in the permeability of plasma proteins as a charge barrier in the glomerular capillary wall<sup>1–3</sup>.

Active Heymann nephritis (AHN) induced by immunization of rats with renal tubular antigen is an experimental model that closely resembles to human membranous glomerulonephritis<sup>4–7</sup>. The formation of subepithelial immune deposits in the GBM has been demonstrated in AHN<sup>8,9</sup>. The anionic charge of the GBM has been shown to decrease during proteinuria in various kinds of nephropathy including AHN<sup>10–14</sup>. However, inconsistent results have been reported, showing that the anionic charge in the GBM was irrelevant to protein leakage during proteinuria<sup>15–18</sup>.

Glomerular epithelial cells (podocytes), another fundamental component of the glomerular permselectivity, have also been demonstrated to possess an anionic charge, con-

sisting mainly of sialic acids, which also acts as a charge barrier<sup>13,19–22</sup>. Several investigators suggested that the decrease in anionic sites of podocytes not only precedes but plays a greater role in causing proteinuria than those of the GBM<sup>16,18,23,24</sup>.

We previously reported showing that the anionic charge in the GBM was irrelevant to protein leakage during proteinuria in puromycin aminonucleoside (PAN)-induced nephrosis and diabetic nephropathy<sup>16,18</sup>. In this study, we reevaluated the anionic charge density on the GBM by using two kinds of cationic markers, poly-L-lysine-gold (PLG) and polyethyleneimine (PEI), in AHN rats. In addition, we also examined the distribution density of anionic sites on the podocytes with respect to the relation between podocytes and proteinuria in AHN.

### Materials and Methods

#### *Animals*

Seven-week-old female Lewis rats were purchased from Charles River Laboratories Japan, Inc. (Atsugi, Kanagawa, Japan). The animals were maintained with a 12-hour light-dark cycle at 23°C and 55% humidity. The rats had free access to a standard laboratory diet and tap water. The animal care and experimental procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals (1996) of the Institute for Laboratory Animal Research (ILAR).

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### *Induction of active Heymann nephritis (AHN) in rats*

The preparation of renal tubular epithelial antigen (RTE), isolated from kidneys of Sprague–Dawley rats, was performed according to the method of Edginton *et al*<sup>6</sup>. After the 7-day quarantine period, twelve rats were immunized by the injection with 2 mg of RTE, emulsified in 0.4 ml of complete Freund's adjuvant (CFA, Chemicon International Inc., Temecula, CA, USA), into each footpad. In addition, 0.05 ml of Bordetella pertussis vaccine (B. pertussis, Wako Pure Chemical Industries, Osaka, Japan) containing  $10^9$  organisms was administered subcutaneously to the dorsum. After 4 weeks, each animal was given a booster injection of 1 mg of RTE. As the control group, five animals were injected with CFA and B. pertussis alone.

### *Urinalysis*

Urine samples were collected for 24 hours before the tissue excision from animals in individual metabolic cages, and the total urine volume was measured. Protein levels were measured by the pyrogallol red method.

### *Tissue preparation for immunocytochemistry*

Eight to twenty-four weeks after the first induction, rats were anesthetized by intraperitoneal administration of sodium pentobarbital (50 mg/kg). After making an incision in the abdomen, the left renal artery and vein were clamped. A small amount (0.15–0.2 ml) of fixative consisting of 0.1% glutaraldehyde and 2% paraformaldehyde in 0.15 M cacodylate buffer solution (pH 7.4) was injected directly into the cortex of the left kidney with a thin needle (26 G). After leaving it for about 30 seconds, the kidney was removed, and a portion discolored by the fixation was excised out with a razor blade. Small blocks from the cortical tissue were cut and immersed in the fixative for 1 hour. Then the blocks were washed with the cacodylate buffer and immersed in 50 mM  $\text{NH}_4\text{Cl}$  in the cacodylate buffer for 1 hour in order to remove free aldehyde group. After washing with the buffer, the blocks were dehydrated through graded ethanol at  $-20^\circ\text{C}$  and embedded in LR-White (London Resin Company, London, UK) at the same temperature. The resin was polymerized by UV light radiation for the first 24 hours at  $-20^\circ\text{C}$  and then for another 24 hours at room temperature in a box filled with nitrogen gas.

### *Tissue preparation for conventional electron microscopy*

For conventional electron microscopy, several pieces of the right kidney were fixed with 2% glutaraldehyde and 1% osmium tetroxide solutions. The blocks were dehydrated through ethanol and embedded in Epon. The sections were then stained with uranyl acetate and lead citrate.

### *Tissue preparation for PEI staining*

Polyethyleneimine (PEI, MW 45,000; Sigma, St. Louis, MO, USA) was used as a cationic probe for staining anionic sites of the GBM. A dose of 0.08 ml/100 g body weight of 0.5% PEI in saline adjusted to pH 7.4 with 1N HCl and to

400 mOsm with sucrose was injected into the jugular vein under anesthesia with sodium pentobarbital. After 10 minutes, the animals were euthanized by exsanguination, and the right kidney was removed. Small blocks from cortical tissue were immersed in a fixative containing 2% phosphotungstic acid and 0.1% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 15 minutes. Then, the blocks were fixed in a fixative of 2% phosphotungstic acid, 1% glutaraldehyde, and 0.1 M cacodylate buffer for 3 hours. After washing overnight in 0.1 M cacodylate buffer containing 7% sucrose, the blocks were postfixed with 1% osmium tetroxide solutions, dehydrated through ethanol, and embedded in Epon.

### *Labeling with PLG*

Cationic poly-L-lysine conjugated with colloidal gold (15 nm, PLG) was purchased from British BioCell International (Cardiff, UK). The reaction method with PLG was described previously<sup>16</sup>. Briefly, thin sections mounted on nickel grids were immersed in 1% bovine serum albumin (BSA) in 0.01 M phosphate buffered saline (PBS) for blocking nonspecific staining. The sections were then incubated overnight with PLG complex diluted 1/50 in 0.01 M phosphate-HCl-glycine buffer (pH 3.3) containing 0.2% BSA. After washing with PBS and then with water, the sections were counterstained with uranyl acetate and lead citrate.

### *Immunostaining for proteins*

Thin sections mounted on nickel grids were incubated overnight at  $4^\circ\text{C}$  with the first antibodies, rabbit anti-rat albumin (diluted 1:500) (Biogenesis, Poole, UK), and then with colloidal gold (15 nm)-conjugated goat anti-rabbit IgG (EY Laboratories, San Mateo, CA, USA) diluted 1:20 in 0.05 M Tris-HCl buffer containing 0.1% Tween 20 for 60 minutes at room temperature. After washing with PBS and then with water, the sections were counterstained with uranyl acetate and lead citrate.

### *Morphometry in the PEI method*

We chose the PEI method to count the anionic sites on the GBM since there were no differences between the PLG and PEI methods in terms of the morphometry of GBM anionic sites<sup>18</sup>. At least 2 photographs from each animal were taken at  $\times 10,000$ . In order to examine anionic sites in various sites, 9 photographs were taken from one animal each in the control and severe groups. The labeled anionic sites located within the lamina rara externa were counted in 41 sites (1,000 nm lengths/site) of the GBM on photographs. For the counting, only the straight lengths of the GBM were examined excluding oblique sections through the GBM. Since the anionic sites on the lamina densa and lamina rara interna were irregularly distributed, counting was not conducted in these areas. Anionic sites were counted on photographs enlarged at a final magnification of  $\times 40,000$ .

### *Statistical analysis*

Data were expressed as the mean  $\pm$  SD. Differences were statistically analyzed by the Student's *t*-test.

## Results

### Urinalysis

The data from AHN animals were tabulated by classifying the animals into 3 groups, i.e., slight, moderate, and severe proteinuria groups according to the amount of urinary protein per day (mg/day) regardless of the induced period (Table 1). Total amounts of urinary protein per day in the slight, moderate, and severe proteinuria groups were 7.5-, 18- and 100-fold, respectively, more than the mean value of the control group (age-matched).

### Conventional electron microscopy

In the slight proteinuria group, small subepithelial electron dense deposits were observed in the GBM. Occasionally, foot processes of podocytes were flattened at the portion

with or without subepithelial deposits (photo not shown).

In the moderate and severe proteinuria groups, the subepithelial deposits were greatly enlarged, and humps were observed in the GBM. Most of foot processes of podocytes were flattened in the GBM (Fig. 1b).

Detachment and fusion of foot processes were not observed obviously. There was no evidence of other abnormalities of podocytes.

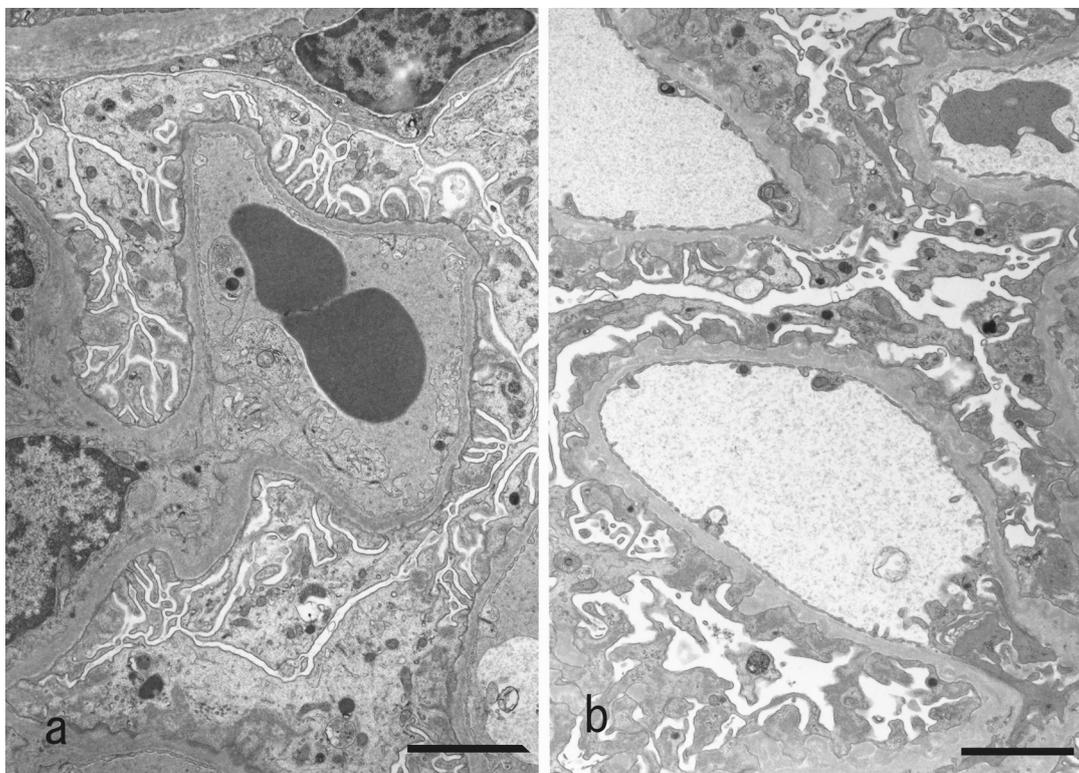
### Anionic site distribution by the PLG method

In the control rats, the distribution of anionic sites detected as gold particles by the PLG reaction method at pH 3.3 in the glomerular wall was highly dense distribution both in the podocytes and the GBM (Fig. 2a). In the podocytes, the gold particles were distributed on the surface of the cell membrane of the cell body and foot processes.

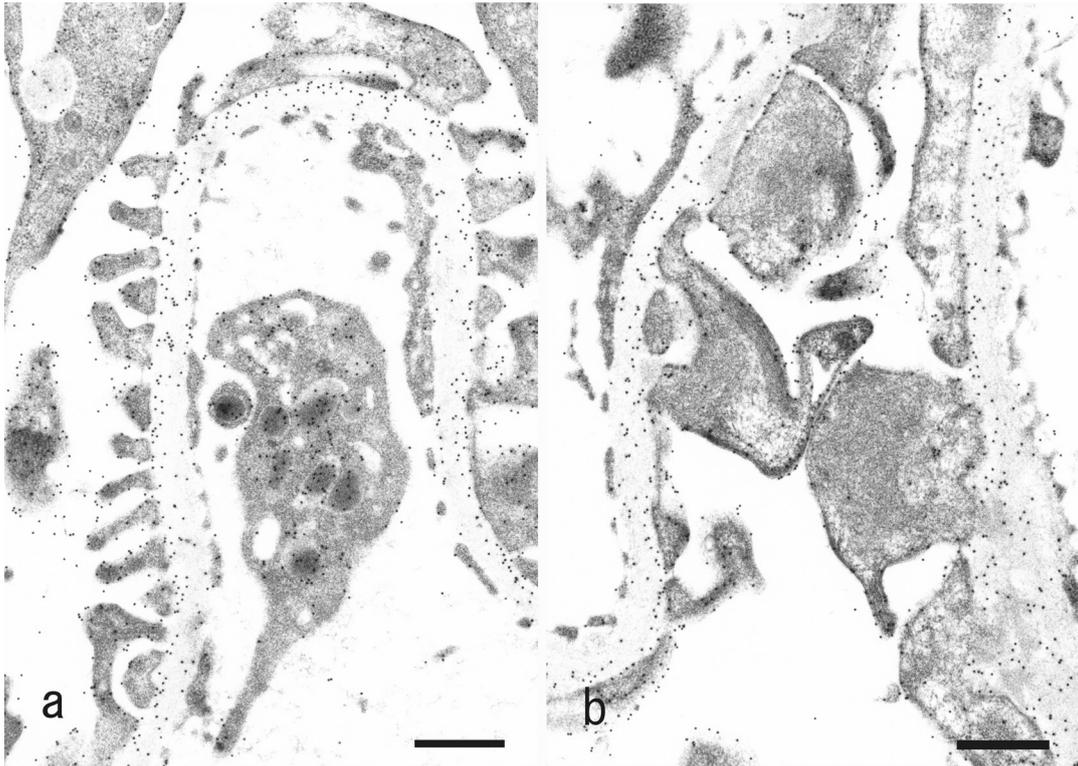
**Table 1.** Urinary Protein of Rats with Heymann Nephritis Classified by Severity of Proteinuria

Group	No. of animals	Urinary volume (ml/day)	Urinary protein (mg/day)
Control	5	8.8 ± 2.1	3.4 ± 0.6
Slight	2	12.9 ± 5.4	25.4 ± 8.1
Moderate	4	10.8 ± 3.9	62.0 ± 7.3**
Severe	6	12.6 ± 1.9*	298.3 ± 150.1**

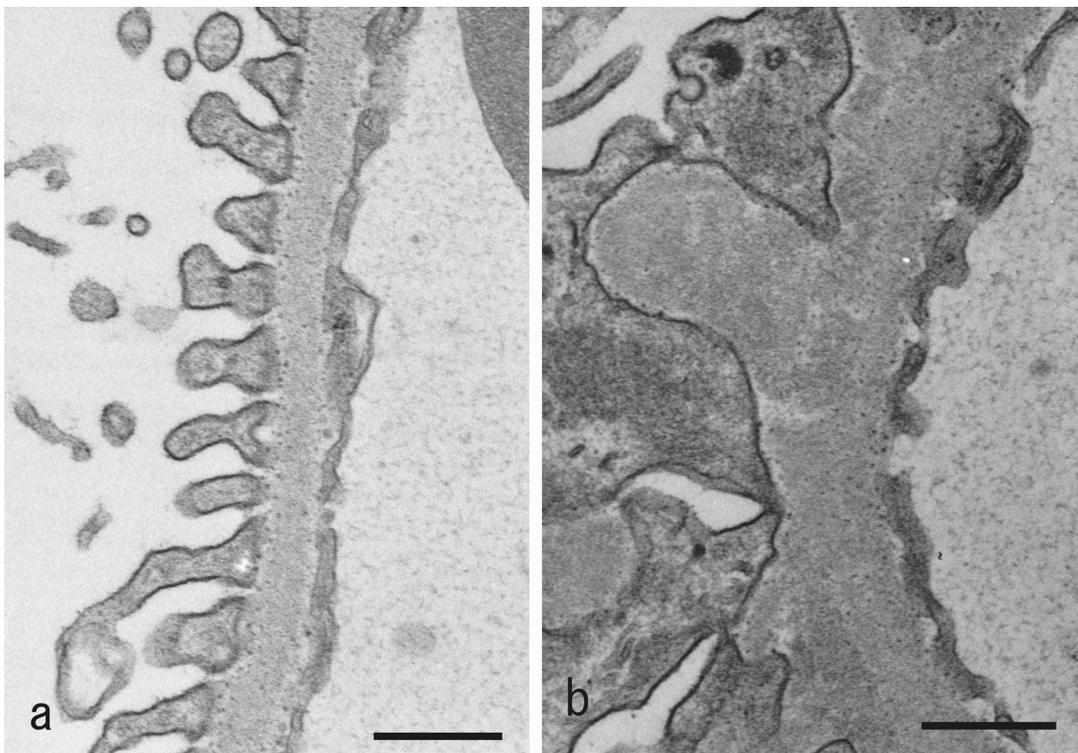
Mean ± SD. Significantly different from the control by the Student's *t*-test (\**p* < 0.05, \*\**p* < 0.01).



**Fig. 1.** Transmission electron micrographs of the glomeruli. a: Control rat. No abnormalities are seen. b: AHN rat. The subepithelial deposits are remarkable, and humps are observed between the podocyte and the GBM. Almost all foot processes of podocytes are flattened. Bar = 2 μm.



**Fig. 2.** Transmission electron micrographs of the glomeruli treated with a poly-L-lysine cationic colloidal gold probe for anionic sites. Incubated with PLG at pH 3.3 for 16 hrs at 4°C. a: Control rat. Gold particles (15 nm) are present on the cell membrane of podocytes and on the GBM. b: AHN rat. Gold particles are decreased in number on the cell membrane of podocyte in spite of a distribution similar to that of control rat on the GBM. Bar= 0.5  $\mu$ m.



**Fig. 3.** Transmission electron micrographs of glomeruli treated with polyethyleneimine for anionic sites. a: In the control animals, anionic sites are distributed in a regular linear pattern in the lamina rara externa of the GBM. b: In the AHN rats, the distribution of anionic sites is similar to that of the control animals. Bar= 0.5  $\mu$ m.

In the AHN rats, the densities of the distributions of PLG in the foot processes and cell bodies of podocytes, including the portion of flattening of foot processes, were apparently low. The distribution on the GBM showed similar density to that of the control animals in most places (Fig. 2b).

#### *Anionic site distribution by the PEI method in the GBM*

In the control animals, anionic sites detected by the PEI method were distributed in a regular linear pattern in the lamina rara externa of the GBM (Fig. 3a). In AHN rats, the distribution of anionic sites was similar to that of the control animals (Fig. 3b). The number of anionic sites per 1000 nm in the GBM was not significantly different between the AHN rats and the control animals (Table 2).

#### *Immunocytochemistry of plasma proteins in the glomeruli*

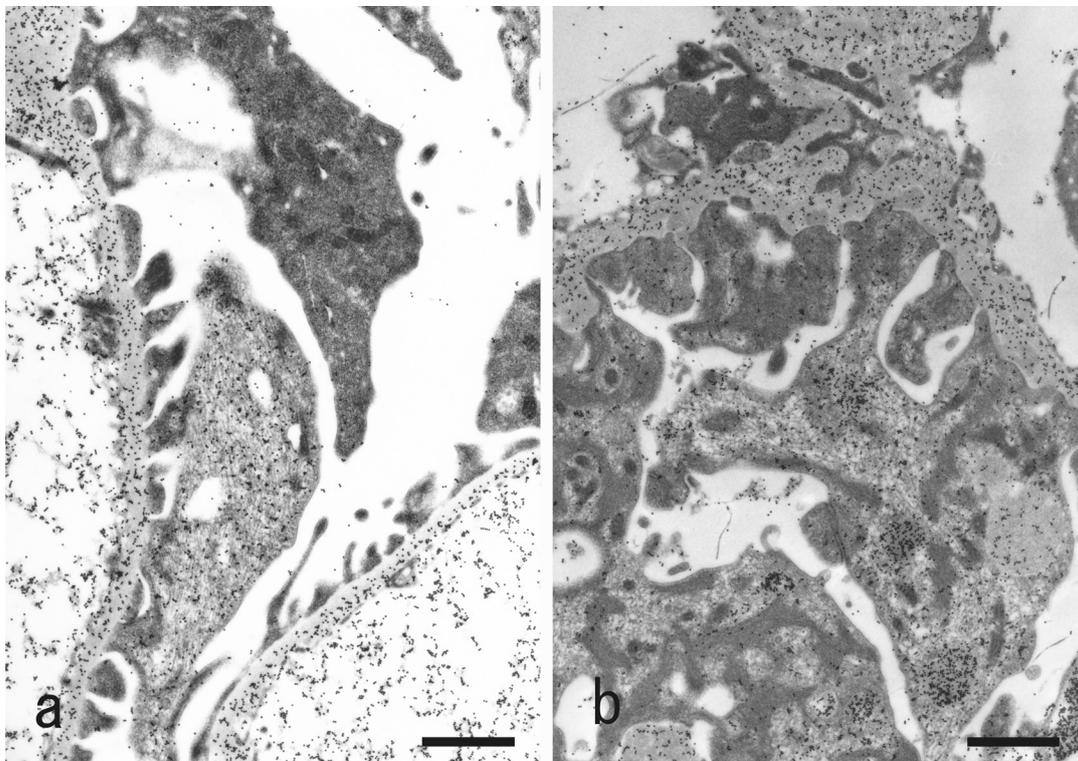
In the control animals, the gold particles demonstrating albumin were distributed densely on the homogenous substance in the capillary luminae and on the GBM. More than a few gold particles were seen in the cytoplasm of major foot processes as well, and only a few particles were distributed on the intact foot processes (Fig. 4a).

In the AHN rats, the gold particles demonstrating albumin were distributed abundantly in small vacuoles at the basal part as well as in the large electron-dense bodies and the vacuoles containing flocculent substance at the apical part of the foot processes and cell bodies of podocytes (Fig. 4b).

**Table 2.** Number of Anionic Sites on the Glomerular Basement Membrane

Method	Group	Total counted sites (1 site: 1000 nm)	Number/ 1000 nm
PEI method	Control	41	24.1 ± 4.8
	Active Heymann nephritis (severe)	41	22.4 ± 6.4

Mean ± SD. Data were analyzed by the Student's *t*-test.



**Fig. 4.** Electron microscopic immunocytochemistry with anti-rat albumin. a: In the control animals, the gold particles are distributed densely on the homogenous substance in the capillary luminae, and on the GBM. More than a few gold particles are seen in the cytoplasm of major foot processes as well, and only a few particles are distributed on the intact foot processes. b: In the AHN rats, numerous gold particles are distributed at the small vacuoles and dense bodies in the podocytes. Bar= 0.5  $\mu$ m.

## Discussion

Several reports have indicated that protein leakage through the glomerular wall is related to the decrease in anionic charge density of the GBM in several kinds of nephropathy including active Heymann nephritis<sup>11,25–29</sup>. However, inconsistent results were obtained by other investigators<sup>15,17</sup>. We examined the distribution of the anionic charge density on the GBM and podocytes in relation to protein leakage in rats with active Heymann nephropathy using two kinds of markers (PLG and PEI). The anionic sites on the GBM were distributed regularly in the AHN rats, and no differences were observed from the control animals. From these findings, it is considered that the anionic charge on the GBM is irrelevant to the protein leakage in AHN rats. This consideration is supported by our previous experiment demonstrating that no changes in the anionic charge density on the GBM were observed in the puromycin-induced nephritic rats and streptozotocin-induced diabetic rats<sup>16,18</sup>.

The flattening of foot processes of podocytes has been discussed in relation to protein leakage in several types of nephropathy<sup>21,22,30</sup>. In this study, flattening of foot processes was also observed in the AHN rats, which may suggest a close relation to proteinuria. Many ultrastructural studies have suggested that the detachment of foot processes from the GBM is the cause of protein leakage in proteinuria<sup>30,31</sup>. However, no detachment of foot processes was observed in this study. Therefore, we consider that the detachment of foot processes is not the trigger to induce protein leakage in AHN. Protein leakage without detachment of foot process has been observed in patients in glomerulonephritis<sup>32</sup> and demonstrated in mice treated with *Vibrio cholerae* sialidase<sup>33</sup>.

In conclusion, we consider that the decrease in anionic charge density on podocytes is attributable to protein leakage and that the charge barrier of the GBM is irrelevant to the protein leakage in AHN rats.

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