Diminution of Podocyte Anionic Sites in Drug-Induced Proteinuric Rats

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Abstract: Controversial results have been reported about the change in anionic charge density on the glomerular basement membrane (GBM) and podocytes in relation to glomerular protein leakage induced by puromycin aminonucleoside (PAN). So we examined the anionic charge on the glomerular wall of rats with PAN-induced proteinuria. PAN, 15 mg/kg, was intraperitoneally injected daily into female Wistar rats for 4 to 14 days. Examinations performed were as follows: Urinary levels of protein and sialic acid, protein fraction analysis of urine by SDS-PAGE, serum chemistry, cytochemistry for assessment of anionic charge by a cationic poly-L-lysine gold (PLG) method (pH 3.3), digestion tests with neuraminidase and heparitinase, immunocytochemistry for the localization of serum proteins and actin, and conventional light and electron microscopy. In proteinuric rats, the anionic sites on podocytes consisting mainly of sialic acids were decreased from the early stage of protein leakage. The urinary sialic acid level was elevated in advanced proteinuria. The density of anionic sites on the GBM remained mostly unchanged even in severe proteinuria. Serum proteins were identified in dense bodies and vacuoles in the podocytes in proteinuric rats. Disarrangement of actin was seen in flattened foot processes. No detachment of foot processes was observed even in severe proteinuric rats. In conclusion, the decrease in anionic charge density on podocytes is attributable to protein leakage through the glomerulus in PAN-induced proteinuric rats. The anionic charge on GBM and the detachment of foot processes seem to be irrelevant to the primary change leading to protein leakage. (*J Toxicol Pathol* 2000; **13**: 97~107)

Key words: anionic charge, podocyte, glomerular basement membrane, sialic acids, actin, proteinuria

Introduction

The process of protein leakage through the glomerular wall has been the matter of controversy for years¹⁻³. As a site attributable to protein leakage, the glomerular basement membrane (GBM) has been energetically studied by many investigators⁴⁻¹². The GBM was demonstrated to possess anionic charge consisting mainly of heparan sulfate proteoglycans, which anions play a role as a charge selective barrier to plasma proteins¹⁻³. This anionic charge has been shown to be decreased concomitantly with proteinuria in several drug-induced glomerular lesions and in immunologically-induced glomerulonephritis^{7,9}. However, opposing results have also been reported^{8,11,12}. The discrepancy among these reports has been suggested to originate mostly from the differences in methods applied for the detection of the anionic sites¹². In spite of extensive studies on the change in anionic charge density in the GBM in relation to protein leakage, no conclusion has been reached yet. On the other hand, the glomerular epithelial cells (podocytes), another fundamental component of the glomerular wall, have also been demonstrated to possess anionic charge, consisting mainly of sialic acids, and to play an important role as a charge selective barrier to plasma proteins^{12,13}. However, the changes in anionic site density on podocytes in relation to proteinuria induced by PAN have not been fully studied.

A structural change observable primarily and principally in relation to protein leakage in the glomerulus has been reported to be flattening of the foot processes of the podocytes^{7,14}. Drenckhahn and Franke¹⁵ suggested that this flattening is caused by the alteration of cytoskeletal proteins in the podocyte, resulting in the alteration of the contractile force of the podocyte and modification of the surface area for filtration. Thus, the importance of the podocytes in glomerular filtration has become emphasized after many studies had been performed on the GBM.

The present study was focused on the primary biological changes in the podocyte at the onset of protein leakage.

Materials and Methods

Animals and induction of proteinuria

A total of 24 female Wistar rats of 6 weeks of age and weighing 150-180 g were used. Daily dose of 15 mg/kg of puromycin aminonucleoside (PAN; Sigma Chemical Co., St. Louis) dissolved at 0.3% in saline was injected intraperitoneally for 4, 6, 8, or 14 days. Three to 8 rats were sacrificed at each administration period. One or 2 rats injected with saline for each administration period served as the control.

Urinalysis and serum chemistry

Urine samples were collected in individual metabolic cages for 24 hr prior to sacrifice and total urine volume was measured. Protein concentration and N-acetylneuraminic acid (NANA) and N-glycolylneuraminic acid (NGNA) levels of the samples were determined by pyrogallol red method and fluorescence method with HPLC¹⁶, respectively. Electrophoresis of the urine was carried out on SDS-polya-

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crylamide gradient gels, and the protein components were determined with silver staining.

Serum levels of total protein and urea nitrogen were determined with the blood collected from the external jugular vein prior to kidney perfusion described below.

Tissue preparation for electron microscopic immunocytochemistry

Rats were anesthetized by intraperitoneal administration of pentobarbital sodium solution (about 50 mg/kg). For the demonstration of anionic charge and actin, the left kidney was cannulated and perfused, first with saline briefly and then with 0.1% glutaraldehyde and 2% paraformaldehyde in 0.15 M sodium cacodylate buffer solution (pH 7.4) for 10 minutes, and was subsequently removed. For the demonstration of serum protein distribution, a small amount of the fixative (0.15–0.2 ml) was injected directly into the cortex of the right kidney with a needle (26G) after clamping the renal artery and vein. After leaving it for about 30 seconds, a discolored portion by fixation was excised out with a razor blade. Small blocks from cortical tissue fixed by both methods were cut and immersed in the fixative for 1 hour.

For cytochemistry and immunocytochemistry, the blocks were immersed in 50 mM NH₄Cl in the cacodylate buffer for 60 min after a brief washing with the buffer in order to block free aldehyde groups. The blocks were then washed with the buffer solution and dehydrated through graded ethanol at –20°C and embedded in LR-White (London Resin Co., UK) at the same temperature. The resin was polymerized by UV light radiation for the first 24 hr at –20°C and then for another 24 hr at room temperature in a box filled with nitrogen gas.

Labeling with PLG

Cationic poly-L-lysine conjugated with colloidal gold (15 nm) (PLG) was purchased from British BioCell (UK). The reaction with PLG was done by a modification of Russo's method¹³. Briefly, thin sections mounted on nickel grids were immersed in 1% bovine serum albumin (BSA) in PBS for blocking nonspecific staining. The sections were then

incubated overnight (around 18–20 hr) at 4°C with PLG complex diluted 1/100 in 0.01 M phosphate-HCl-lysine buffer (pH 3.3) containing 0.2% BSA. After washing with PBS and then with water, the sections were counter-stained with uranyl acetate and lead citrate.

Digestion with heparitinase and neuraminidase

Before the incubation with PLG complex, thin sections were treated with heparitinase or neuraminidase for 1 hr at 37°C. Heparitinase (Kanto Chemicals, Tokyo) and neuraminidase (Sigma, Germany) were adjusted to be 2 mU/ μ l and 5 mU/ μ l, respectively, in 0.1 M phosphate-K buffer of pH 7.0 and 6.5, respectively.

Immunostaining for actin and proteins

Antibodies used were rabbit anti-actin (chicken gizzard, Transformation Research Inc., USA), rabbit anti-rat albumin (Biogenesis, UK), rabbit anti-rat IgG (Nordic Inc., USA), and colloidal gold (10nm)-conjugated goat anti-rabbit IgG (Zymed Lab., USA).

Tissue preparation for conventional light and electron microscopy

The right kidney was examined by conventional light and electron microscopy. For the electron microscopy, sections were stained with uranyl acetate and lead citrate, and examined with a Hitachi HS-8 transmission electron microscope.

Results

Urinalysis and serum chemistry

The data from treated animals were tabulated by classifying the animals into 4 groups, i.e. non-, slight-, moderate-, and severe-proteinuria groups according to the amount of urinary protein per day (mg/day) regardless of the administration period (**Table 1**).

In the non-proteinuria group, no alterations were seen in the urinary protein concentration or in total excretion per

Table 1. Urinalysis and Serum Chemistry of PAN-Treated Rats Classified by the Severity of Proteinuria

Group	No. of Animals	Urinary Volume (ml)	Urinary Protein (mg/dl) (mg/day)		Serum Protein (g/dl)	BUN (mg/dl)
Control	5	18.8 $\pm$ 9.1	6.8 $\pm$ 2.2	1.12 $\pm$ 0.24	5.3 $\pm$ 0.38	14.0 $\pm$ 1.9
Non-Proteinuria	8	12.1 $\pm$ 2.5	8.0 $\pm$ 3.2	0.93 $\pm$ 0.27	5.3 $\pm$ 0.35	15.2 $\pm$ 2.2
Slight Proteinuria	2	12.0 $\pm$ 1.0	15.8 $\pm$ 10.3	1.99 $\pm$ 0.21	5.4 $\pm$ 0.15	14.6 $\pm$ 2.9
Moderate Proteinuria	1	13.0	82	10.7	5.2	17.4
Severe Proteinuria	8	11.3 $\pm$ 4.8	2,302 $\pm$ 669*	245 $\pm$ 74*	3.9 $\pm$ 0.29	78.1 $\pm$ 71.7

Group	No. of Animals	Urinary NANA		Urinary NGNA	
		(μ g/dl)	(μ g/day)	(μ g/dl)	(μ g/day)
Control	5	43.1 $\pm$ 14.5	697 $\pm$ 109	8.5 $\pm$ 6.1	126 $\pm$ 68
Non-Proteinuria	8	57.4 $\pm$ 8.2	686 $\pm$ 123	10.9 $\pm$ 3.3	129 $\pm$ 36
Slight Proteinuria	2	54.1 $\pm$ 40.3	651 $\pm$ 15	12.2 $\pm$ 10.5	138 $\pm$ 28
Moderate Proteinuria	1	73.9	961	14.2	185
Severe Proteinuria	8	191 $\pm$ 57	2081 $\pm$ 847	11.2 $\pm$ 5.3	116 $\pm$ 31.3

Mean $\pm$ S.D. NANA: N-acetylneuraminic acid NGNA: N-glycolylneuraminic acid
Significantly difference from control by Student's *t*-test (*, *p* < 0.05).

Fig. 1. SDS-polyacrylamide gradient gel electrophoresis of urine of rats from each group classified by the severity of proteinuria. (a), The urine samples were applied after adjusting the protein concentration to be same for each sample. A band at 200 kDa is seen in each proteinurea group as well as in the non-proteinuria group. (b), The urine samples were applied after adjusting the protein concentration to contain 0.5% of the total amount of protein excreted per day by each animal. Numerous protein bands at 200 kDa and between 116 and 200 kDa as well as around 66 kDa are seen for the severe-proteinuria rat, whereas no protein bands at these positions are seen for the control rat.

day compared with those parameters in the control animals. Total amounts of urinary protein per day in the groups of slight-, moderate-, and severe-proteinuria were 1.8-, 9.6-, and 219-fold, respectively, over the mean value of the control group. Urinary proteins with high molecular weight such as 200 kDa or those between 200 and 116 kDa were detected by SDS-PAGE in all of the treated groups including the non-proteinuria group (Fig 1a). Urinary NANA level was elevated remarkably in the severe-proteinuria group (statistically insignificant due to the large individual variation), but the NGNA level remained within the control level. The elevation of serum urea nitrogen level and decrease in serum albumin level were also seen in this group.

Light microscopy

In the non-proteinuria group, PAS-positive granules or acid fuchsin-positive granules by Masson's trichrome staining were observable in the epithelial cells by careful examination of the glomeruli. The granules were more prominent in the slight-proteinuria group and the moderate-proteinuria animal, and small vacuoles in the glomerular epithelial cells were observable in the latter animal. In the severe-proteinuria group, the lesions varied from animal to animal. The most advanced lesions were the presence of large vacuoles in the epithelial cells and hyaline casts in the Bowman's space and retention of hyaline casts in the tubules.

Electron microscopy

In the non-proteinuria group, the increase in number of coated pits at the sole of podocytes was prominent. Flattening of the foot processes was already seen, and relatively small-sized electron-dense bodies and vacuoles containing flocculent substance were found distributed near the basal region of the foot processes and cell bodies of podocytes (Fig 2-b).

In the slight-proteinuria group, electron-dense bodies and vacuoles of some size were further increased in the foot processes and cell bodies of podocytes. Dilatation of the urinary space between foot processes and the overlaying processes or cell bodies was seen in various degrees. Flattening of foot processes was more abundant than that observed in the non-proteinuria group.

The moderate-proteinuria animal showed numerous electron-dense bodies and vacuoles of various sizes in the cytoplasm of podocytes (Fig 2-c). Flattening of foot processes and dilatation of the urinary space were more prominent than those in the slight-proteinuria group.

In the severe-proteinuria group, the electron-dense bodies of various sizes, many cytoplasmic vacuoles with flocculent or less electron-dense substance were seen in abundance in the foot processes and cytoplasm of podocytes. Cystic dilatation of the urinary space and flattening of foot

Fig. 2. Transmission electron micrographs of glomeruli from a control rat (a) and from rats of the puromycin aminonucleoside-treated non-proteinuria group (b), moderate-proteinuria group (c), and severe-proteinuria group (d). Increase in number of coated pits (arrow) is conspicuous and small-sized vacuoles and dense bodies are seen in the basal region of the podocyte in non-proteinuria group (b). Flattening of foot processes, increased coated pits at the sole, electron dense bodies, and vacuoles with flocculent substance are seen in the podocytes of moderate-proteinuria group (c), and severe-proteinuria group (d). Bar = 1 μ m.

Fig. 3. Transmission electron micrographs of normal rat glomerulus treated with poly-L-lysine cationic colloidal gold probe for anionic sites. (a), Incubated with PLG at pH 3.3 for 16 hrs at 4°C. Gold particles (15 nm) are present on the cell membrane of podocytes and on the basement membrane. (b), This section was digested with neuraminidase, 5 mU/ μ l, for 1 hr at 37°C before the incubation with PLG. Dense distribution of gold particles observed in (a) is absent from the cell membrane of the treated podocytes. (c), This section was digested with heparitinase, 2 mU/ μ l, for 1 hr at 37°C before the incubation with PLG. The PLG particles are missing from the basement membrane but remain on the cell membrane of the podocytes. (d), Higher magnification of (c). PLG particles are located on the cell membrane of foot processes at their luminal surface as well as at the soles (arrowheads). Bar=0.5 μ m.

Fig. 4. Transmission electron micrographs of PAN-treated rat glomeruli treated with poly-L-lysine cationic colloidal gold probe for anionic sites. The PLG particles (15 nm) on the foot processes are decreased in number whereas the distribution on the basement membrane is of similar density to that for the control animal (Fig. 3a) in the non-proteinuria group (a), the slight-proteinuria group (b), the moderate-proteinuria group (c), and the severe-proteinuria group (d). Bar = 0.5 μ m.

Fig. 5. Electron microscopic immunocytochemistry with anti-actin antibody. The gold particles (10 nm) demonstrating actin are distributed regularly at the basal part of foot processes in the control rat (a), but the distribution in the slight-proteinuria group (b) is irregular or diffuse. Bar=0.5 μ m.

processes were seen all over the glomerular wall. Regular arrangement of the foot processes was completely lost in most of glomeruli; however, no detachment of the foot processes from the basement membrane was observable (Fig 2-d).

Anionic site distribution by PLG

Control rats :

In the observation of anionic sites detected by the PLG reaction method at pH 3.3 in the glomerular wall, the dense distribution of gold particles, which indicate anionic sites, was seen in both the podocytes and the basement membrane (Fig 3-a). In the podocytes, the gold particles were distributed on the surface of the cell membrane of the cell body and foot processes. The gold particles in the podocytes were not seen when the sections were treated with sialic acid-digesting neuraminidase (Fig 3-b), but remained by the treatment with heparitinase, which digests heparan sulfate (Figs 3-c, d). In the sections digested with heparitinase, the gold particles were also observed at the anchored portions, called the base or sole, of the foot processes (Fig 3-c, d). The distribution at the sole was, however, less dense than that at the other surfaces of the podocytes.

In the basement membrane, on the other hand, the gold particles were distributed diffusely with a higher density in the lamina lara externa. This distribution was lost by the treatment with heparitinase (Fig 3-c, d), but remained by the treatment with neuraminidase (Fig 3-b).

PAN treated animals :

In the non-proteinuria group, the PLG particles in the foot processes and cell bodies of podocytes were decreased in

number; however, in the basement membrane, the particles were distributed densely and evenly, similarly to those of the control animals (Fig 4-a).

In the slight-proteinuria group, an irregular and scarce distribution of PLG in the foot processes and cell bodies of podocytes was seen, whereas the distribution in the basement membrane was regular and similar in density to that of the control animals (Fig 4-b).

In the moderate-proteinuria animal, PLG particles in the cell membrane of podocytes were decreased in number to a similar degree as in the slight-proteinuria group. The distribution in the basement membrane was mostly the same as that seen in the control group (Fig 4-c).

In the severe-proteinuria group, PLG distribution in the cell membrane of the podocytes including the foot processes was apparently low in density. The distribution on the basement membrane showed similar density to that of the control animals in most places (Fig 4-d).

Immunocytochemistry for actin in podocyte

In the control animals, the actin demonstrated by the immunocytochemistry with 10-nm gold particles was distributed mainly at the foot processes regularly and densely near the site adjacent to the sole and rather irregularly and less densely at the cell body of the podocytes (Fig 5-a).

In the PAN-treated groups, the regular distribution pattern of actin was lost and the actin was present diffusely at the basal part of flattened foot processes (Fig 5-b). Little difference was seen among the non-proteinuria group and the proteinuria groups.

Fig. 6. Electron microscopic immunocytochemistry with anti-rat IgG. In a control rat (a), the gold particles demonstrating IgG are distributed only in the flocculent substance in the capillary and the basement membrane but not in the podocyte. In the PAN-treated rats, numerous gold particles are distributed at the small vacuoles and dense bodies near the sole in a non-proteinuria animal (b), and a slight-proteinuria animal (c), and at the large electron-dense bodies in a severe-proteinuria animal (d). Bar=0.5 μ m.

Immunocytochemistry for serum proteins

In the control animals, the gold particles demonstrating of albumin or IgG were distributed only on the flocculent substance in the capillary and the basement membrane, but no gold particles were observed in the podocytes. The density of the gold particle distribution was much higher in the sections stained with anti-albumin (photograph not shown) than those stained with anti-IgG (Fig 6-a).

In the PAN-treated rats, the gold particles demonstrating these serum proteins were distributed abundantly in small vacuoles at basal part as well as in the large electron-dense bodies and the vacuoles containing flocculent substance at apical part of the foot processes and cell bodies of podocytes in the slight-, moderate-, and severe-proteinuria groups (Figs 6-b, c, d).

Discussion

Using cationic poly-L-lysine gold (PLG) as a marker, we studied the anionic charge density of the glomerular wall in relation to protein leakage in rats with various degrees of proteinuria induced by puromycin aminonucleoside (PAN) administration.

In the intact control rats, the polyanionic sites demonstrated by PLG were distributed densely on the cell membrane of podocytes and on the basement membranes of glomeruli. The anionic sites observed in the epithelium are considered to indicate the presence of sialic acid-containing substance(s) since the PLG particles were absent when the sections had been treated, before the reaction with PLG, with neuraminidase, an enzyme that specifically digests sialic acids, but remained after the treatment with heparitinase, which digests heparan sulfate. This result is consistent with that reported by Russo *et al.*¹³.

Using colloidal iron as a marker of sialic acids, Mohas and Skoza¹⁷ were the first to show anionic sites on the epithelial cell membrane including foot processes, where the sialic acid marker was observed both at the free surface, above the slit diaphragm, and at the base or sole. A similar result was obtained by Charest and Roth with *Limax flavus* lectin used as a marker¹⁸. But the distribution was less in the sole. On the other hand, Sawada *et al.*¹⁹ reported that podocalyxin, a 140-kDa sialoprotein, was distributed only at the free surface of the foot processes based on experiments using antibodies that specifically recognized podocalyxin. In our observation, the anionic sites demonstrated by PLG were seen on the free surface of foot processes and at the sole as well, but with a lower density in the latter. Our results are consistent with the report of Mohas and Skoza and with that of Charest and Roth, but in contradiction to the report of Sawada *et al.* These different results concerning the distribution of anionic sites at the sole might be explained by the fact that Mohas and Skoza, Charest and Roth, and we used markers reacting with sialic acids (mol. Wt. 320), whereas Sawada *et al.* used antibodies specific to podocalyxin of 140-kDa as the marker. We consider our finding to indicate the presence of anionic charge consisting of sialic acids other than podocalyxin at the sole of the foot processes and that PLG at pH 3.3 can detect the anionic sites consisting of sialic acids as well as podocalyxin in addition to heparan sulfate.

Since the purpose of this study was to elucidate glomerular biological changes which cause protein leakage into urine, PAN-treated rats were classified into four groups, i.e.,

non-, slight-, moderate-, and severe-proteinuria groups, according to the total amount of urinary protein per day regardless of the duration of the drug administration.

Even in the non-proteinuria animals, the PLG distribution on the cell membrane of podocytes but not on the basement membrane was already decreased concomitantly with the structural changes such as increase in coated pits, flattening of foot processes, increase in electron-dense bodies, and appearance of cytoplasmic vacuoles as well as the appearance of high-molecular-weight proteins in the urine. These structural changes of the podocytes were reported by Charest and Roth¹⁸ to be the primary lesions induced by PAN. In our immunocytochemical study, albumin and IgG were demonstrated in electron-dense bodies and vacuoles of podocytes. Furthermore, the high-molecular-weight proteins with 200 kDa or 160–200 kDa, which are considered to be immunoglobulins in rats²⁰, were detected in the urine of the non-proteinuria animals. Therefore, the electron-dense bodies and vacuoles with proteins distributed in the podocytes are considered to be deeply related to the leakage of serum albumin and also a small amount of the high-molecular-weight protein that was detected by SDS-PAGE but undetected by the determination of total urinary protein concentration. The amounts of protein leaked from the glomeruli in these animals were considered not to exceed the capacity of tubular resorption and resulted in non-proteinuria.

The decrease in PLG distribution on the cell membrane of podocytes was advanced further concomitantly with the increase in morphological changes in podocytes such as flattening of foot processes, accumulation of electron-dense bodies, cytoplasmic vacuolation, and cystic dilation of the urinary space between foot processes and the cell body. In the animals with these advanced changes, the amount of protein excreted into the urine increased in parallel with the excretion of the high-molecular-weight proteins and excretion of a sialic acid, N-acetylneuraminic acid (NANA), into the urine. These results indicate that the decrease in anionic sites on the cell membrane of podocytes is the primary change causing protein leakage through the glomerular wall. We consider the sialic acid increased in the urine to have been derived from that which had been shed from the cell membrane of podocytes. No elevation of urinary NANA level was observed in moderate-, slight-, or non-proteinuria groups. This result is considered to indicate that the amount of NANA shed from podocytes did not exceed the amount of tubular resorption in these groups.

From the above results we speculate that the decrease or loss of anionic sites on the cell membrane at the sole of foot processes causes the passage of proteins including large molecular size ones such as immunoglobulins by endocytosis with increased coated pits into the cytoplasm of podocytes and that the decrease in such sites on the cell membrane of the free surface of foot processes and of the cell body causes the leakage of proteins into the urinary space. This seems to occur at a very early stage of PAN-induced lesions while the total urinary protein level remains within the normal level. We consider that the localization of serum proteins including IgG in the electron-dense bodies and vacuoles in the podocytes was the result of permeation of the proteins through the basement membrane and the cell membrane at the sole of foot processes rather than due to pinocytosis or phagocytosis of proteins that had been excreted into Bowman's space since the electron-dense bodies and vacuoles with

serum proteins were found in the cytoplasm adjacent to the soles at the early stage of protein leakage, such as non-proteinuria group. The endocytic pathway through pits in the cell membrane at the basal face of podocytes has been demonstrated²¹.

Contradictory results on the hypothesis that a decrease in the anionic site density on the basement membrane is responsible for proteinuria induced by PAN have been reported by investigators using various methods for the demonstration of the anionic charge⁵⁻¹¹. Our results reported here clearly demonstrate that the density of anionic sites on the basement membrane detected with PLG were not altered even in the animals with severe proteinuria induced by PAN. From our findings, we think that it is good inductive reasoning to define the podocyte as the primary site of lesion by PAN. This conclusion is supported by experiments in the literature demonstrating that PAN induces lesions in the podocytes by producing hydroxyl radicals or reactive oxygen species in them^{22,23}.

Many ultrastructural studies have suggested that the detachment of foot processes from the basement membrane is the cause of protein leakage induced by PAN^{6,24,25}. However, no detachment of foot processes was observed even in the animals with severe proteinuria, i.e., with excretion 219-fold over that in the control animals, in this study. Therefore, we consider that the detachment of foot processes is not feasible to be a primary cause of protein leakage induced by PAN. Protein leakage without the detachment of foot process has been reported in mice treated with *Vibrio cholerae* sialidase²⁶.

A decrease in anionic site density and loss of regular arrangement of actin were observed in the animals with proteinuria and flattening of foot processes in this study. The correlation of these two changes might be explained by the speculation introduced by Salant²⁷ that charge loss triggers an intercellular signaling event that alters the relationship among the slit diaphragm, cytoskeleton, integrin, and extracellular matrix. Disarrangement of actin in relation to the flattening of foot process induced by PAN was reported by Whiteside *et al.*²⁸.

We demonstrated a decrease in anionic site density on the cell membrane of podocytes in the early stage of proteinuria induced by PAN and discussed its possible relation to protein leakage through the glomerular wall. This hypothetical mechanism would not be applicable to the protein leakage in immunologically-induced glomerulonephritis, in which the primary lesion has been proved to occur mainly at the subepithelial basement membrane. Further studies will elucidate the mechanism of protein leakage in the various forms of glomerulonephritis.

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