Pediatr Nephrol (1993) 7: 1–5

Pediatric Nephrology

Original article

Ultrastructural alteration of glomerular anionic sites in nephrotic patients

Kazuhiko Washizawa[1, 2], Shinji Kasai[1, 2], Tetsuo Mori[1], Atsushi Komiyama[1], and Hidekazu Shigematsu[2]

Departments of [1] Paediatrics and [2] Pathology, Shinshu University School of Medicine, Matsumoto, Japan

Received December 20, 1991; received in revised form September 9, 1992; accepted September 11, 1992

Abstract. The relationship of glomerular anionic sites to proteinuria was examined ultrastructurally in human nephrotic syndrome. The anionic sites were analysed morphometrically in patients with minimal-change nephrotic syndrome (MCNS, 11 patients) and in other glomerulonephritides complicated with nephrotic syndrome (4 patients) by the high-iron diamine-thiocarbohydrazide-silver proteinate method. The anionic sites in MCNS patients in remission (7 patients) were normal. In contrast, the anionic sites in nephrotic patients with MCNS (4 patients) and the other glomerulonephritides were decreased in number. Moreover, smaller and irregularly distributed anionic sites or the greater loss of them from the paramesangial region were observed in the nephrotic patients. The loss of glomerular anionic sites may induce structural alteration of the glomerular basement membrane and mesangial matrix. The loss and structural abnormalities of glomerular anionic sites in nephrotic patients may be one of the mechanisms responsible for massive proteinuria.

Key words: Nephrotic syndrome – Proteinuria – Anionic sites

Introduction

It has become apparent that the charge-selective barrier of glomerular capillaries plays an important role in determining the transglomerular passage of macromolecules [1–4]. The glomerular basement membrane (GBM) contains fixed anionic sites [5] consisting of heparan sulphate proteoglycans (HS-PG) [6], and they mainly constitute the charge-selective barrier. Changes in GBM HS-PG may induce the loss of the charge selectivity and permit negatively charged proteins to pass easily through the capillary walls. Moreover, several investigators have reported that changes in HS-PG initiate the structural alteration of the GBM [7–9].

Physiological studies have led to the conclusion that the selective proteinuria associated with minimal-change nephrotic syndrome (MCNS) probably results from the alteration of the charge-selective barrier in the glomerular capillary wall [10, 11]. However, ultrastructural changes in the anionic sites have not yet been shown in patients with this syndrome. We previously reported that the high-iron diamine-thiocarbohydrazide-silver proteinate (HID-TCH-SP) method is specific for staining HS-PG and is useful for the ultrastructural examination of rat or human glomerular anionic sites [12, 13]. In this article, we report ultrastructural changes in the anionic sites in patients with nephrotic syndrome caused by MCNS or other glomeronephritides.

Patients and methods

Patients. Renal tissue was obtained from 23 patients (Table 1) for evaluation of anionic sites. The control kidneys (group C) were obtained by nephrectomy from 3 patients with renal tumours. Urinalysis showed no proteinuria in these patients. Tissue from the portions of these kidneys unaffected by the tumour and showing normal ultrastructure was used for this study. The remaining 20 specimens were obtained by either open or closed renal biopsy. The histological diagnosis was determined by conventional light, immunofluorescence and electron microscopy.

Five of these patients comprised a second control group (HC). All presented with macroscopic or microscopic haematuria, had little or no proteinuria, had never been nephrotic and their renal histology revealed only minor or no glomerular abnormalities.

Eleven patients had MCNS of whom 7 (group 1) were in remission and 4 (group 2) were nephrotic at the time of study. The group 2 patients were evaluated before beginning treatment with steroids or before increasing the dose of steroids in those who were in relapse. The exception was patient no. 16.

The remaining group (group 3) had glomerulonephritis complicated by nephrotic syndrome: 3 had IgA nephropathy and 1 focal segmental glomerulosclerosis. None of these patients were receiving treatment with either steroids or immunosuppressive medication at the time of biopsy.

Correspondence to: K. Washizawa, Department of Paediatrics, Nagano Red Cross Hospital, 1512-1 Wakasato, Nagano 380, Japan

Table 1. Clinical data and number of glomerular anionic sites within the lamina rara externa in the patients studied[a]

Patient no.	Age (years)	Sex	Weight (kg)	Haematuria	Proteinuria (g/24 h)	No. of anionic sites (per 300 nm)	P/C ratio[b]
Group C: control kidneys							
1	1	F	9	–	0.0	20.1±2.1	0.97
2	41	M	75	–	0.0	16.3±1.9	1.04
3	45	M	65	–	0.0	20.8±3.7	0.95
Group HC: haematuria control kidneys							
4	7	M	21	+	0.0	15.4±2.7	1.02
5	9	M	34	+	0.0	21.0±4.5	1.05
6	13	M	45	+++	0.0	15.9±3.7	0.75
7	13	M	57	+	0.1	14.6±3.4	1.09
8	16	M	55	++	0.1	17.4±2.9	1.13
Group 1: minimal-change nephrotic syndrome in remission							
9	3	M	18	–	0.0	16.7±1.6	1.01
10	8	M	26	–	0.0	19.5±2.5	0.95
11	10	M	29	–	0.0	18.5±2.0	0.87
12	13	M	55	–	0.0	19.1±3.3	1.03
13	13	F	44	–	0.0	21.6±2.2	1.27
14	14	M	55	–	0.0	16.4±1.9	0.96
15	16	F	50	–	0.0	17.7±2.0	1.11
Group 2: minimal-change nephrotic syndrome at nephrotic stage							
16	12	M	65	–	9.0	18.2±3.6	0.99
17	13	M	48	–	5.0	14.1±2.5	0.77
18	20	M	60	–	3.5	15.1±2.8	1.02
19	62	M	70	–	21.0	11.5±2.2	0.81
Group 3: other glomerulonephritis at nephrotic stage							
(IgA nephropathy)							
20	6	F	21	+++	2.5	12.1±4.1	0.67
21	4	M	20	++	9.0	11.8±4.3	0.80
22	8	F	27	+++	2.0	10.8±2.0	0.84
(Focal glomerular sclerosis)							
23	9	M	23	+++	2.5	12.1±2.1	0.79

[a] Values are means ± SD

[b] Ratio of the mean number of the anionic sites in the paramesangial region to that in the capillary walls

Tissue processing to determine anionic sites. Renal biopsy specimens were fixed immediately in 2% paraformaldehyde/2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, overnight at 4° C, and washed with 7% sucrose. Specimens were quickly frozen and sliced into 20-μm sections with a cryostat, washed with 7% sucrose and then immersed in HID solution for 18 h at 23° C. The HID solution was prepared by dissolving 120 mg of *N,N*-dimethyl-meta-phenylenediamine-$(HCl)_2$ (Kanto, Tokyo, Japan) and 20 mg of *N,N*-dimethyl-para-phenylenediamine-HCl (Sigma, St. Louis, Mo., USA) in 50 ml of distilled water and then adding immediately to 1.6 ml of 40% *ferric chloride* solution. The sections were washed, dehydrated in a graded ethanol series and embedded in Quetol-812 (Nissin, Tokyo, Japan). Gold-coloured ultrathin sections were cut and then treated with 2% thiocarbohydrazide (Polysciences, Warrington, Pa., USA) dissolved in 10% acetic acid for 40 min and then reacted with 1% aqueous silver proteinate (Merck, Darmstadt, Germany) for 30 min in the dark. Vigorously washed and dried sections were viewed with a HU-11A transmission electron microscope (Hitachi, Japan) at 75 kV.

Enzyme treatment. The biological nature of the anionic sites revealed by the HID-TCH-SP method in control kidneys was evaluated by prior incubation of slices of fixed-frozen kidney in enzyme solutions. Slices of tissue from a control kidney were treated with each enzyme or their respective buffers as controls at 37° C for 12 h; the enzymes used were: (1) heparitinase from *Flavobacterium heparinum* (Seikagaku Kogyo, Tokyo, Japan) 0.1 unit/ml in 0.1 M acetate buffer (pH 7.0); (2) chondroitinase ABC from *Proteus vulgaris* (Seikagaku Kogyo) 5 units/ml in 0.05 M TRIS-HCl buffer (pH 8.0); (3) neuraminidase from *Arthrobacter ureafaciens* (Nakarai, Kyoto, Japan) 1 unit/ml in 0.01 M phosphate buffer (pH 6.8); (4) hyaluronidase from bovine testis (Nakarai) 0.1% in 0.1 M phosphate buffer (pH 5.5).

Morphometric analysis of glomerular anionic sites. The number of HID-TCH-SP-stained granules within the lamina rara externa (LRE) was counted in terms of the number of the anionic sites. In order to observe the difference in distribution of the anionic sites, the sites were examined within both the LRE in the capillary walls and in the paramesangial region (epithelial side of mesangial matrix). Four capillary walls and four paramesangial regions in one glomerulus of each patient were photographed at a magnification of ×20,000 and then printed at a final magnification of ×60,000, only in clearly cross-sectioned regions without subepithelial deposits. HID-TCH-SP-stained granules measuring more than 3 nm in diameter were counted and measured.

The mean number of anionic sites per 300-nm length of LRE and the ratio of the mean number of the anionic sites in the paramesangial region to that in capillary walls (P/C ratio) were calculated for each patient.

Statistical analysis. Student's *t*-test for non-paired and paired observations was used for statistical evaluation. All differences were considered significant when the *P* value was less than 0.05.

Results

Control groups

Glomerular anionic sites in the control kidneys were visible as dense granules ranging from 3 to 10 nm in diameter (Fig. 1 a). The granules were densely populated within a subepithelial layer in the GBM. This layer, from 40- to

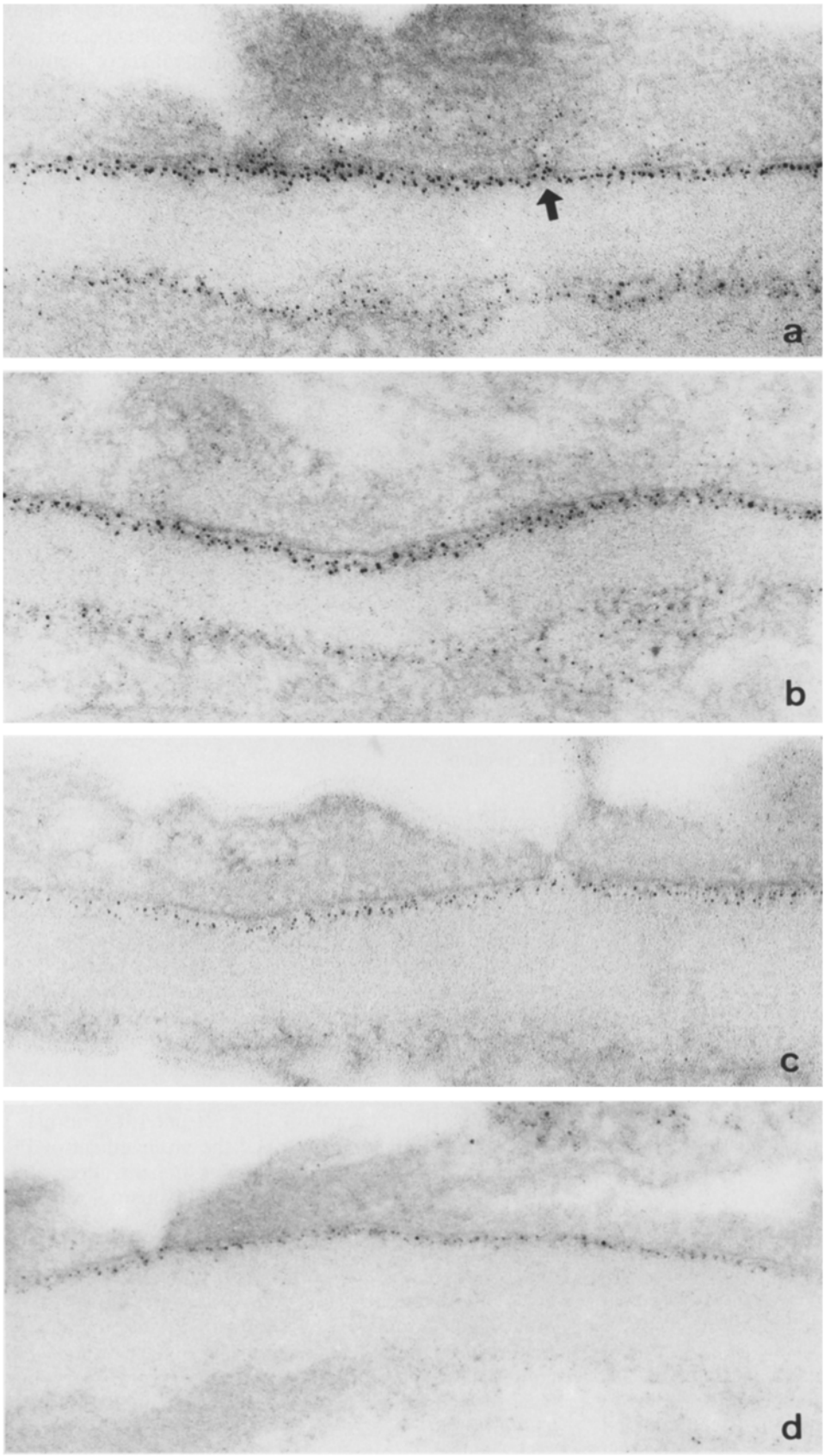

Fig. 1. Electron microscopic localization of glomerular anionic sites. **a** Control kidney (patient no. 3); the anionic sites form a dense band within the lamina rara externa (LRE) and continue into the slit diaphragm (*arrow*). The anionic sites are fewer, smaller and irregularly distributed within the lamina rara interna. **b** Group 1 (patient no. 10); the number, size and distribution of the anionic sites in the patient are similar to those in the control kidneys. **c** Group 2 (patient no. 17); the anionic sites are fewer and smaller than those in the control kidney. **d** Group 3 (patient no. 21); there is a marked loss of anionic sites, (all $\times 60{,}000$)

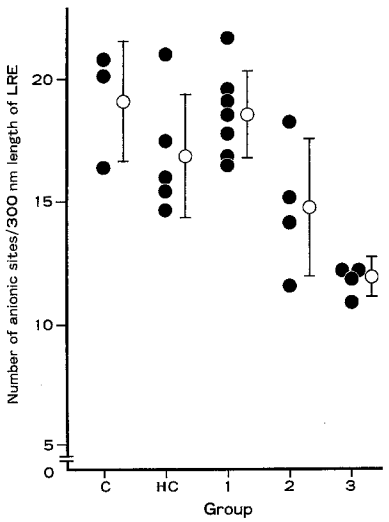

Fig. 2. The mean number of anionic sites per 300-nm length of the LRE in each group; ●, individual values; ○, mean. Group C, control kidneys; group HC, haematuria controls; group 1, minimal-change nephrotic syndrome in remission; group 2, minimal-change nephrotic syndrome when nephrotic; group 3, other glomerulonephritis with nephrotic syndrome. Group C vs. group 3, $P < 0.02$; group HC vs. group 3, $P < 0.02$; group 1 vs. group 3, $P < 0.002$; group 1 vs. group 2, $P < 0.05$

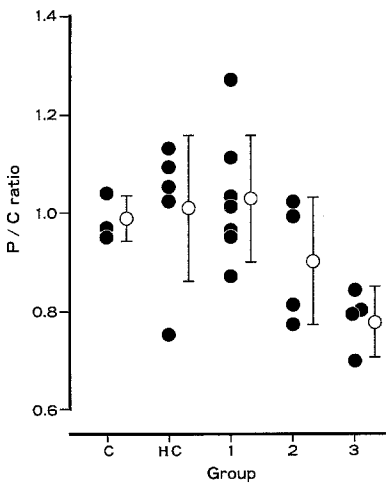

Fig. 3. The ratio of the mean number of anionic sites in the paramesangial region to that in the capillary walls (*P/C*) in each group; ●, individual values; ○, mean. Group C vs. group 3 $P < 0.02$; group 1 vs. group 3, $P < 0.02$

60-nm thick, corresponded to the LRE. The anionic sites numbered about 18 per 300-nm length of the LRE; there was no difference in the number between the capillary wall and paramesangial region. The granules formed a band within the LRE and continued into the slit diaphragm (Fig. 1 a). Fewer, smaller and irregularly distributed dense granules were also visible within a subendothelial layer, corresponding to the lamina rara interna (LRI), mesangial matrix and endoplasmic reticula in glomerular cells. The epithelial cell surface, which has been shown to be covered by sialic acid-rich polyanions [14], was scarcely labelled. The anionic sites in group HC patients with minimal-change disease were similar to those in the control group.

After the control kidney had been exposed to heparitinase treatment, almost all of the granules disappeared from the GBM. In contrast, after incubation with chondroitinase ABC, neuraminidase and hyaluronidase, the granules remained unchanged.

Patients groups

There were no differences in the number, size and distribution of glomerular anionic sites between group C, group HC or group 1 (Figs. 1 b, 2). The number (mean ± SD) of anionic sites within the LRE in group 2 was 14.7 ± 7.7, which was less than in group 1 (18.5 ± 1.8, $P < 0.05$) (Figs. 1 c, 2). Glomerular anionic sites were scarcely found within the LRI in group 2. The greatest loss of the anionic sites was observed in group 3: their number was 10.5 ± 3.1 within the LRE (Fig. 2). The granules in the anionic sites in group 3 were smaller and significantly fewer than those in groups HC, C or 1 (Fig. 1 d). The granules were distributed in the narrower band-shaped areas under the flattened epithelial cells.

P/C ratios in group HC, C and 1 were nearly 1.0. The ratio in group 3 was significantly lower than in group 1 and C (Fig. 3), indicating the greater loss of anionic sites from the paramesangial region compared with the capillary walls.

Discussion

Our HID-TCH-SP method demonstrated ultrastructually the glomerular anionic sites, mainly within the LRE, forming a band. The distribution of the anionic sites corresponded to that of HS-PG as demonstrated by immunogold studies using anti-bovine HS-PG antibody [15]. These findings suggest that the anionic sites within the LRE mainly form the glomerular charge-selective barrier.

The mean number of anionic sites within the LRE in control kidneys was about 18 per 300-nm length. This number is approximately 3 times the number of HS-PG anionic sites within the LRE of normal human kidney revealed by studies using polyethyleneimine (PEI) (22 per 1,000 nm) [16] or cuprolinic blue (21 per 1,000 nm) [17]. Yoshimura et al. [18] examined the arrangement of PEI particles within the LRE by the quick-freezing, deep-etching method, and demonstrated many PEI particles around fibrils which connected perpendicularly the podocyte cell membranes with the lamina densa. In contrast, shrinkage of the tissues, destruction of the fibrils and a decreased number of PEI particles within the LRE were shown by the same method followed by contrasting/fixation and post-fixation used in conventional transmission electron microscopy. The structural characteristics of cationic tracers may be artificially disarranged by such chemical treatments. The difference in the number of glomerular anionic sites seen with these methods is probably due to variations in the chemical procedures used to visualize the tracer.

In the present study, the loss of the anionic sites from the GBM was clearly demonstrated in MCNS patients at the nephrotic stage. In contrast, the anionic sites in MCNS

patients in remission were similar to those in control kidneys. The loss of glomerular anionic sites seen in the nephrotic patients probably indicates a dysfunction of the charge-selective barrier, which may permit the leakage of negatively charged proteins into the urinary space at the nephrotic stage. During the recovery process, the anionic sites appear to return to normal, responding to steroids or immunosuppressive medication.

The loss of glomerular anionic sites was also observed in patients with nephrotic syndrome caused by other glomerulonephritides. The loss of the anionic sites was not pathognomonic of MCNS. Moreover, the greater loss of the anionic sites from the paramesangial region than from the capillary walls was observed in the glomerulonephritis patients. Such a change in the anionic sites may be associated with the presence of the mesangial lesions. The glomeruli in the 4 glomerulonephritis patients studied here showed a high degree of mesangial cell proliferation. Mesangial cells in culture can synthesize HS-PG as well as chondroitin/dermatan sulphate glycosaminoglycans [19]. The cell proliferation and matrix expansion in the mesangial area may cause an incomplete synthesis of HS-PG. In contrast, HS-PG can inhibit rat mesangial cell growth [20]. The decreased HS-PG in the mesangial region may induce the proliferation of mesangial cells.

We could not morphometrically analyse the anionic sites within the LRI, since the distribution varied even in the control kidneys. However, the anionic sites within the LRI seemed to have disappeared in patients with nephrotic syndrome. This finding suggests that anionic sites within the LRI are reduced or neutralized as in the LRE in the nephrotic stage.

It has been reported that concomitant alterations can occur in the size-selective barrier with the loss of the charge selectivity of the GBM [8, 9]. Alterations are supposed to occur in the tridimensional molecular structure of the sulphated glycosaminoglycan chains of proteoglycans with reduction or neutralization of anionic sites [21]. In this study, anionic sites in the patients with nephrotic syndrome showed smaller granules and distributed irregularly within a thinner band compared with the control groups. The decrease in the granule size may have occurred as a consequence of the reduction or neutralization of the sulphated glycosaminoglycan chains in the anionic sites. The irregular distribution of the granules in the thinner band may indicate the structural alteration of the GBM and mesangial matrix. The structural alteration associated with the loss of glomerular anionic sites may lead to destruction of the GBM or detachment of the epithelial cells from the GBM. The prolonged loss of the anionic sites could induce irreversible glomerular injuries.

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