Ultrastructural background of albuminuria in rats with passive Heymann nephritis

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Abstract

Background. Although it is widely known that proteinuria in rats with passive Heymann nephritis (PHN) is prevented by treatment with cobra venom factor (CVF), the precise mechanisms of complement-dependent proteinuria have not been fully elucidated. The aim of this study was to evaluate morphologically whether the size of subepithelial electron-dense deposits (EDDs) contributes to the onset of albuminuria.

Methods. The size of subepithelial EDDs and anionic sites in the lamina rarae externa (LRE) overlaid with subepithelial EDDs were evaluated by ruthenium red and compared between PHN and PHN treated with CVF in rats.

Results. Overt albuminuria was present on days 3 and 4 after injection of anti-Fx1A. CVF-treatment of rats with PHN prevented albuminuria (PHN + CVF: \( n = 6 \) (53.6 ± 38.8 vs 1.02 ± 0.55 mg/day, \( P < 0.01 \), on day 4). Rat C3 was detected along the glomerular capillary walls on day 4 post-injection in rats with PHN, but not in rats with PHN + CVF. Subepithelial EDDs were observed in both groups. Quantitative morphometric analysis revealed that CVF-treatment decreased the size of subepithelial EDDs as well as the extent of retraction of glomerular epithelial cells. In both groups the density of anionic sites in the LRE overlaid with EDDs was decreased compared with the LRE without subepithelial EDDs. However, no difference was noted quantitatively measured by morphometric analysis.

Conclusion. Depletion of serum complement decreases subepithelial EDDs as well as the number of sites with decreased anionic charge underlying the EDDs. Thus, the size of subepithelial EDDs plays a pivotal role in the onset of albuminuria.

Key words: albuminuria; anionic sites; complement; electron-dense deposit; passive Heymann nephritis

Introduction

Evidence suggests that serum complement factors are important in the generation of proteinuria in rats with PHN (reviewed in [1,2]). Administration of cobra venom factor (CVF) depletes serum complement factors and prevents proteinuria in PHN rats (PHN + CVF). Although it has been demonstrated that the development of proteinuria in this model can be attributed to the formation of the membrane attack complex (MAC), the mechanisms of MAC-induced proteinuria have not been fully elucidated.

In active Heymann nephritis, which is induced by immunizing rats with rat Fx1A, Leenaerts et al. [3] demonstrated that there is a threshold in the size of subepithelial electron-dense deposits (EDDs) necessary to induce proteinuria. However, it remains uncertain if the size of subepithelial EDDs is important in inducing proteinuria in passive Heymann nephritis as well. Treatment of PHN rats with CVF prevented proteinuria although subepithelial EDDs were still noted [4,5]. The absence of any difference in the amount of anti-Fx1A antibodies bound to the glomeruli between PHN and PHN + CVF rats [6] led to the conclusion that there was no intergroup difference in the size of subepithelial EDDs. However, the size of subepithelial EDDs in these studies [4–6] was not quantitatively measured by morphometric analysis.

A reduction in the number of anionic sites in the lamina rarae externa (LRE) in the glomerular basement membrane (GBM) overlaid with subepithelial EDDs has been demonstrated in active and passive Heymann nephritis [7–9]. A reduction in the anionic charge, particularly in the LRE of the GBM, is thought to cause proteinuria [7–9]. Tracer studies using albumin, catalase, or ferritin have demonstrated an increase in permeability in the area of subepithelial EDDs in rats with active Heymann nephritis [10]. However, it remains unknown whether anionic sites in the LRE overlaid with subepithelial EDDs are decreased in PHN rats when CVF is administered.
The aims of this study were to evaluate whether there is a difference in the reduction of anionic sites, as determined by ruthenium red, in the LRE between PHN and PHN + CVF rats, and whether the size of subepithelial EDDs contributes to the induction of albuminuria in PHN rats.

Subjects and methods

Induction of PHN in rats

Rat Fx1A was prepared by the method of Edgington et al. [11]. Anti-rat Fx1A antibody was raised in adult male sheep [6]. The IgG fraction was obtained and concentrated to 20 mg/mL. PHN (n = 12) was induced in male Sprague-Dawley rats (200 g) by a single intraperitoneal injection of anti-Fx1A IgG (40 mg). Rats were kept in metabolic cages and killed on day 4 after the injection of anti-Fx1A IgG. The single radial immunodiffusion method was used to estimate 24-h urinary excretion of albumin using goat IgG against rat albumin (Cappel Laboratories, Durham, NC, USA) [12].

CVF treatment of PHN rats

PHN rats (n = 6) were administered 50 U cobra venom factor (CVF) (Naja haja, Diamexid, Miami, Florida, USA), dissolved in 0.5 ml of 0.15 M NaCl, by intraperitoneal injection 12 h before, and 48 h after the injection of anti-Fx1A IgG (PHN + CVF). PHN rats that received 0.15 M NaCl without CVF served as controls (n = 6). Blood was sampled via the tail vein 12 h before, immediately, and 48 h after the injection of anti-Fx1A IgG, and collected via the abdominal aorta when the animals were killed. The serum concentration of C3 was determined by the single radial immunodiffusion method using anti-rat C3 (Cappel Laboratories) at a final dilution of 1:100 [13]. Normal pooled rat serum served as the standard. Graded concentrations of the standard serum (5, 10, 25, 50, 75, 100%) were prepared by diluting the normal pooled rat serum with PBS. The serum concentrations of C3 in the experimental groups (PHN and PHN + CVF) were expressed as a percentage of the standard value. If the serum concentration of C3 in the experimental groups exceeded the 100% concentration of the standard value, the serum was diluted with PBS at the ratio of 1:2 and the C3 concentration was determined again.

Perfusion of kidney with ruthenium red solution

On day 4 following the injection of anti-Fx1A IgG, rats were anaesthetized with sodium pentobarbitone. Before the left kidney was perfused with ruthenium red solution, the contralateral kidney was removed for histological evaluation by means of light-, immunofluorescence- and electron-microscopy. The left kidney was perfused through a catheter inserted into the abdominal aorta according to the method of Kanwar and Farquhar [14] with 0.15 M NaCl solution followed by infusion of a 0.2% solution of ruthenium red (Sigma Chemical Co., St Louis, USA) dissolved in Karnovsky’s aldehyde fixative (1% formaldehyde and 3% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, with 4.4 mM CaCl₂). The dye-fixative solution was passed through a millipore filter (pore size: 0.22 μm) to remove large particles of dye. Small pieces of tissue were excised from the

Size of subepithelial EDDs and the retraction of glomerular epithelial cell foot process

Pieces of the right renal cortex were fixed in 2% glutaraldehyde for 3 h, post-fixed in 1% OsO₄ for 2 h, and post-fixed for electron-microscopy (JEOL 100XC, Tokyo, Japan) by the conventional method. Two randomly selected glomerular capillary walls in each kidney (PHN n = 4, PHN + CVF n = 4) were photographed and printed at the final magnification of ×20,000. To evaluate the difference in the size of subepithelial EDDs between PHN and PHN + CVF rats, the surface area of the three largest subepithelial EDDs was measured using a computerized image analyser as described above.

Anionic sites of the GBM

Ultrathin sections (40–60 nm) were prepared and stained with uranyl acetate and lead citrate, and examined by electron-microscopy. Three randomly selected glomeruli from each kidney (PHN n = 3, PHN + CVF n = 3) were evaluated. At least 20 fields, randomly selected in each glomerulus, were photographed and printed at the final magnification of ×50,000. In the area where the sections were cut perpendicular to the long axis of the GBM, the LRE was divided into two regions based on the association with subepithelial EDDs as described above: (1) LRE overlaid with subepithelial EDDs, and (2) LRE without subepithelial EDDs. The total length of the GBM with or without subepithelial EDDs was measured with the computerized image analyser as described above. The number of ruthenium red positive particles in the LRE with or without subepithelial EDDs was counted manually. The number of ruthenium red positive particles per 1 μm GBM length was compared between the two groups (PHN and PHN + CVF).

Light- and immunofluorescence-microscopy

Coronal sections of the right kidney were fixed in freshly prepared 4% formaldehyde in 0.1 M phosphate buffer solution followed by embedding in paraffin. Sections were cut 2 μm in thickness and stained with H & E, periodic acid-Schiff, periodic silver methenamine, and Masson trichrome.

The right renal cortex was snap-frozen in dry ice acetone hexane. Sections were cut by a cryostat and studied with fluorescein-conjugated IgG against rat IgG, rat C3, and...
Subepithelial EDDs were observed on day 4 in a rat with PHN. Using a computerized image analyser, the surface area of subepithelial EDDs (encircled areas) was measured as shown in Figure 1A. Bar = 500 nm. The surface length of subepithelial EDDs in the LRE (solid lines) and the length of the LRE free of subepithelial EDDs (dotted lines) were measured as shown in Figure 1B. Bar = 500 nm. EDDs, electron-dense deposits; LRE, lamina rarae externa.
Permeability of the GBM in rats with passive Heymann nephritis

sheep IgG (Cappel, Durham, NC, USA.). More than 20 glomeruli from each rat were evaluated for the immunofluorescence study. The intensity of immunofluorescence staining was graded from 0 to +3.

Statistical analysis

Data are expressed as mean ± SD. Differences were evaluated by the Student’s t test. A level of P < 0.05 was considered statistically significant.

Results

An increase in albuminuria in PHN rats was noted on days 3 and 4 after the injection of anti-Fxa IgG. Treatment of PHN rats with CVF significantly prevented the development of albuminuria (30.0 ± 23.0 vs 0.72 ± 0.46 mg/day, P < 0.05, on day 3; 53.6 ± 38.8 vs 1.02 ± 0.55 mg/day, P < 0.01, on day 4) (Table 1). During the experiment, serum concentrations of C3 in PHN rats ranged between 75 and 150% of the standard value (Table 1). In contrast, administration of CVF to PHN rats reduced the serum concentration of C3 to less than 5% of the standard value (Table 1).

In both groups, sheep IgG was observed along the glomerular capillary walls, while rat IgG was not noted in either group. Rat C3 was observed in the glomeruli in PHN rats (Figure 2A) and the immunofluorescence intensity of rat C3 was 2+ or 3+. In contrast, rat C3 was not detectable in PHN rats administered CVF (Figure 2B).

Subepithelial EDDs were noted in both groups (Figure 3A, B). The surface area of the three largest subepithelial EDDs in PHN rats was significantly greater than that in PHN + CVF rats (0.562 ± 0.267 vs 0.146 ± 0.055 μm², P < 0.05). Furthermore, the length of the GBM overlaid with subepithelial EDDs in PHN rats was greater than that in PHN + CVF rats (0.443 ± 0.059 vs 0.237 ± 0.026 μm/μm GBM, P < 0.01). These results indicate that the size of subepithelial EDDs in PHN rats was greater than that in PHN + CVF rats.

PHN rats exhibited retraction of the glomerular epithelial cell foot processes, which was more extensive in glomerular epithelial cells accompanied by subepithelial EDDs (Figure 3A). Retraction of the glomerular epithelial cell foot processes was also noted in PHN + CVF rats (Figure 3B). Quantitative morphometric analysis revealed that the density of the slit diaphragm per 1μm GBM length in PHN rats was less than that in PHN + CVF rats (0.755 ± 0.104 vs 1.363 ± 0.357 sites/μm GBM, P < 0.05), indicating that glomerular epithelial cell retraction in PHN rats was more extensive than that in PHN + CVF rats.

Particles positive for ruthenium red were arrayed in sections cut perpendicular to the long axis of the GBM in both the LRE and the LRI in PHN rats (Figure 4A). Ruthenium red positive particles in the LRE were more prominent and regular than those in the LRI. A complete loss and/or bizarre staining of the ruthenium-red-positive particles was noted in the LRE overlaid with subepithelial EDDs (Figure 4A). A reduction in the number of anionic sites in the LRE overlaid with subepithelial EDDs was also noted in PHN + CVF rats (Figure 4B). Quantitative analysis of anionic sites (Table 2) revealed no significant difference between the two groups in the density of ruthenium-red-positive particles per 1μm GBM length.

Discussion

In the present study, electron-microscopy was used to elucidate the possible morphological alterations associated with a decrease in albuminuria secondary to the depletion of serum complement by CVF in PHN rats. The density of anionic sites was reduced in the LRE overlaid with subepithelial EDDs compared to the LRE free of subepithelial EDDs in both PHN and PHN + CVF rats; there were no intergroup differences for these two areas of the GBM. The estimated decrease in anionic sites of the LRE overlaid with subepithelial EDDs, together with a size-dependence of subepithelial EDDs for overt albuminuria, raise the possibility that subepithelial immune deposits per se and a reduction in anionic charge of the LRE overlaid with subepithelial EDDs in PHN rats was greater than that in PHN + CVF rats.
Subepithelial EDDs and the retraction of glomerular epithelial cell foot processes were observed on day 4 in both groups: (A) PHN and (B) PHN + CVF. The size of subepithelial EDDs in a rat with PHN was larger than that in a rat with PHN + CVF. A rat with PHN exhibited more extensive retraction of glomerular epithelial cells than a rat with PHN + CVF. Bar = 500 nm. Arrows represent subepithelial EDDs. EDDs, electron-dense deposits; PHN, passive Heymann nephritis; PHN + CVF, passive Heymann nephritis administered cobra venom factor.

Table 2. Density of ruthenium-red-positive particles of the GBM

<table>
<thead>
<tr>
<th>Region</th>
<th>Ruthenium-red-positive sites/μm GBM</th>
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<tbody>
<tr>
<td></td>
<td>PHN (n = 3)</td>
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<tr>
<td>LRE with subepithelial EDDs</td>
<td>11.1 ± 0.8</td>
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<tr>
<td>LRE without subepithelial EDDs</td>
<td>19.8 ± 1.0</td>
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Data are expressed as mean ± SD.

Subepithelial EDDs both contribute to the defect of barrier function against macromolecules. This concept is supported by the finding of Schneeberger et al. [10] who found that in active Heymann nephritis, the permeability of three kinds of tracers were increased in the GBM overlaid with subepithelial EDDs, whereas the tracers behaved as in normal rats in other areas of the GBM devoid of subepithelial EDDs, i.e. they were retained in the lamina rarae interna. Therefore permeability of the glomerular capillary wall in a rat with active and passive Heymann nephritis is likely to depend on the extent of subepithelial EDDs.

However, the validity of estimating anionic charge by the morphological method using a single cationic probe is controversial (reviewed in [15]). We have employed ruthenium red as the probe to label the anionic site of the GBM. This probe was initially believed to specifically stain heparan sulphate [16], which was also believed to constitute the main charge-based barrier against anionic protein such as albumin. Subsequently, however, evidence has emerged suggesting that ruthenium red does not specifically stain heparan sulphate [17], and that other negatively charged substances in addition to heparan sulphate form the charge-based barrier in the GBM (reviewed in [15]).

Furthermore, merely enumerating ruthenium-red-positive particles does not allow quantitative conclusions regarding relative changes in anionic charge at each stained site [18]. Accordingly, unaltered density of the particles may not always reflect unaltered anionic charge.
In addition to the GBM, glomerular epithelial cells possibly play a role in increased permeability of the glomerular capillary wall to macromolecules. In our PHN model, no evidence of glomerular epithelial cell detachment was obtained with a meticulous survey by electron-microscopy. Detachment of glomerular epithelial cells occurs as an eventual result of molecular interaction between the GBM and glomerular epithelial cells. Because anti-Fx1A antibody recognizes a β1-integrin on cultured glomerular epithelial cells and inhibits adhesion of glomerular epithelial cells to matrix components of the GBM, such as fibronectin, laminin, collagen I and collagen IV [19], adhesion of glomerular epithelial cells to the GBM in PHN may be loosened, causing functional (not morphologically verified) detachment of glomerular epithelial cells.

MAC has been proposed as playing a role in altering glomerular epithelial cells in PHN rats. Quigg et al. [20] showed that the addition of anti-Fx1A antibody and complement to the medium caused sublytic injury of cultured glomerular epithelial cells. Another in vitro experiment by Savin et al. [21] demonstrated that
immune deposits associated with anti-Fx1A antibody and MAC formation were sufficient to increase the permeability of the glomerular capillary wall independent of complement-induced haemodynamic events or involvement of inflammatory cells. The sublytic alterations in glomerular epithelial cells occurring in vivo may also provide the haemodynamic factors, such as increased hydraulic pressure and/or increased filtration surface area by perturbed adhesion of glomerular epithelial cells to the GBM, which are responsible for increase in albuminuria. Accordingly, we conclude that depletion of serum complement by administering CVF prevented an increase in immune deposits and reduced the extension of decreased anionic sites of the LRE underlying the immune deposits, resulting in the prevention of albuminuria. Amelioration of glomerular epithelial cell injury, indicated by decreased density of the slit diaphragm, which is also related to the depletion of complement, may have additionally contributed to the prevention of albuminuria. However, the precise mechanisms by which the growth of immune deposits relates to the sublytic glomerular epithelial cell injury remains to be elucidated.

References


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