Alteration of Anionic Sites in Renal Glomerular Basement Membrane of Pigs

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Glomerular anionic sites (ASs) are essential components of the charge-selective barrier in ultrafiltration in the kidney [10]. Rabbits received a single intravenous injection of cationized protein developed a transient interaction of the protein with fixed ASs in the glomerular basement membrane (GBM) resulting in loss of negative charge and concomitant proteinuria [2]. Recent studies have shown that the number of ASs in the GBM decreases in some human glomerulonephropathies [4, 11, 24, 27, 28] and experimental nephropathies [1, 8, 13, 20, 21, 25, 26]. It is interesting to note that in experimentally induced acute serum sickness of rabbits, cationic proteins derived from platelets and neutrophils may bind to glomerular capillary walls and possibly contribute to the neutralization of glomerular polyanions [3]. Various cationic probes have been used to reveal glomerular ASs. Of these, polyethyleneimine (PEI) has the virtues that it reacts with tissues by immersion and ASs can be visualized as electron-dense particles [16].

In a previous study, we revealed a high incidence of spontaneous glomerulopathy characterized by mesangial enlargement with deposition of immunoglobulin and C3 in pigs [18]. Ultrastructural lesions were characterized by a large amount of electron-dense material and clusters of spherical microparticles (SMPs) as well as increased cellular components and extracellular matrix in the mesangium [17, 19]. Histologically glomerulopathy characterized by these pathologic changes in pigs has been classified as mesangial proliferative glomerulonephritis [18]. However, the effects of these morphological abnormalities on the filtration barrier of the glomeruli have not yet been elucidated. In the present study, we attempted to demonstrate an alteration of ASs on the GBM in swine mesangial proliferative glomerulonephritis using a cationic tracer, PEI.

Materials and Methods

Histopathology: Grossly normal kidney samples from 10 slaughtered pigs aged about six months without any significant clinical signs were fixed in 10% neutral buffered formalin and embedded in paraffin. Histologic sections were cut at 4 µm and stained with hematoxylin-eosin, periodic acid-Schiff (PAS), and Heidenhain’s azan (Azan).

Immunofluorescence microscopy: Small pieces of fresh kidneys were frozen in hexane dry-ice-acetone. Frozen sections of 4-µm thickness were cut and washed three times with continuous shaking in chilled phosphate buffered saline for 15 min. They were incubated with rabbit anti-swine IgG (Fc specific, Nordic Immunological Lab., Netherlands), rabbit anti-swine IgM (Fc specific, Nordic Immunological Lab., Netherlands), sheep anti-swine IgA (Fc specific, Nordic Immunological Lab., Netherlands) or FITC-conjugated goat anti-swine C3 (Cappel Lab. Inc., U.S.A.). The secondary antibodies for detection of swine immunoglobulins by the indirect method were FITC-conjugated rabbit anti-sheep IgG (Cappel Lab. Inc., U.S.A.) or FITC-conjugated goat anti-rabbit IgG (Cappel Lab. Inc., U.S.A.).

Ultrastructural Study for ASs: Staining of ASs with PEI (molecular weight = 1,800; Polysciences, Warrington, PA) was performed by a minor modification of the method of Schurer et al. [16]. Fresh specimens of the renal cortex were cut into small cubes and immersed in 0.5% PEI in physiological saline for 30 min at room temperature. After washing in 0.2 M cacodylate buffer (CB), pH 7.4, 400 mOsm, the blocks were reimmersed in a mixture of 2% phosphotungstic acid (PTA) and 0.1% glutaraldehyde (GA), pH 7.4, 400 mOsm at 4°C for 1 hr to obtain insoluble precipitates of PEI bound to glomerular ASs. After washing
three times for 10 min in CB, the blocks were postfixed in 2% osmium tetroxide in CB at 4°C for 2 hr. Then, the blocks were dehydrated in graded ethanol and embedded in epon 812. Ultrathin sections were cut and stained with uranyl acetate, and some were also stained with lead citrate. Examination was carried out with a Hitachi H-300 transmission electron microscope (Hitachi, Ltd., Tokyo) at 75 kV. The PEI solution was adjusted to pH 7.4 with HCl and to 400 mOsm with sucrose. The mixture of 2% PTA and 0.1% GA was prepared within 2 hr before use (osmolarity adjusted to 400 mOsm with sucrose and pH adjusted to 7.4 with concentrated KOH solution).

Morphometric analysis of ASs: For the quantitative analysis of ASs in peripheral portions of the GBM, the PEI particles within the lamina rara externa (LRE) in a single row were counted. A total of 40 to 60 portions of 1,000 nm of GBM on several capillary loops in one glomerulus were evaluated on each pig. The glomeruli which showed representative changes were selected in toluidin blue-stained semi-thin sections, and portions of peripheral GBM with subepithelial dense deposits or clusters of SMPs were not counted for statistical analysis. The PEI particles were counted only in clearly cross-sectioned regions with a clear view of the epithelial cell membrane. Statistical comparison in the number of ASs in 1,000 nm of GBM between one pig with diffuse global GBM thickening and the remaining pigs without thickening was performed by Mann-Whitney’s U-test.

RESULTS

Light microscopy: Mild to moderate and diffuse global mesangial enlargement in the renal glomeruli was found in the kidneys of all 10 pigs (Fig. 1). Homogeneous proteinaceous deposits of various sizes were frequently seen in the mesangium and occasionally on the glomerular capillary walls in all pigs. Mesangial hyaline droplets were present in some pigs. Diffuse global thickening of the GBM was prominently seen in the kidney of pig 6 (Fig. 2), in which a few renal glomeruli (3.75%, number of glomeruli examined=80) showed segmental sclerosis with or without adhesion to Bowman’s capsules and mild periglomerular fibrosis. In pig 6, hyaline droplet degeneration was rarely seen in the proximal tubules and mild formation of collagen fibers in a few glomeruli was also revealed by Azan staining.

Immunofluorescence microscopy: The renal glomeruli of all pigs exhibited diffuse global, diffuse segmental, or focal segmental deposition of immunoglobulins and C3 in the mesangial areas and capillary walls in various intensities (Table 1). The pattern of the deposition of these substances was lumpy in the mesangium and coarsely or finely granular in the capillary walls.

Electron microscopy: There were many electron-dense deposits and clusters of SMPs in the GBM and mesangium of the renal glomeruli in all pigs. The GBM of peripheral capillary walls showed regular dimensions without thickening in all animals except pig 6. However, attenuation of the GBM was frequently observed accompanying dense deposits or SMPs in the subepithelial regions. In contrast, pig 6 showed irregular thickening of the GBM with small intramembranous and subepithelial dense deposits. Collagen fibrils were frequently present in the mesangium of pig 6.

Mesangial areas were expanded due to increased matrix and mesangial cells as well as dense deposits of various intensities in all pigs.

Ultrastructural studies for ASs: Small electron-dense particles of PEI that were interpreted as ASs were regularly distributed in the LRE of the GBM in areas without subepithelial regions with clearance. The ASs in these normal LRE were observed in a single layer. There were no ASs in the lamina densa (LD) of the normal GBM. On the other hand,
the localization of ASs in the LRE was markedly altered in the renal glomerulus of pig 6, in which ASs were irregularly distributed in the LRE and also frequently present in the thickened LD in the GBM (Figs. 4 and 5). Sometimes PEI particles in pig 6 were smaller than those in the normal GBM of the other nine pigs.

In the LRE of the GBM which had subepithelial electron-dense deposits or clusters of SMPs, PEI particles were markedly fewer or absent in all pigs (Figs. 6 and 7). Smaller PEI particles were occasionally observed in the regions between the LD and electron-dense deposits or clusters of SMPs. Although statistical analysis was not performed, an alteration of ASs in the LRE at portions with subendothelial deposits was rather mild or obscure.

Mesangial matrix was also well reacted with PEI, and the epithelial sides of the GBM surrounding the mesangium showed the same staining property as the LRE of the peripheral GBM.

No PEI particles were detected in electron-dense deposits and clusters of SMPs.

The ASs in the lamina rara interna (LRI) of the GBM were distributed irregularly and were much fewer than those seen in the LRE.

Quantitative assessment of ASs in the GBM: The number of ASs in the LRE of 1,000 nm of the GBM in pig 6 was compared with those in the other nine pigs, and the number was significantly low (P<0.001) in pig 6 (Table 1).

DISCUSSION

This study showed that ASs in the renal glomeruli of pigs were clearly stained with PEI, and ASs in LRE in the normal peripheral GBM were distributed regularly in a single layer as described previously [14]. In contrast, ASs in LRI are relatively few and distributed irregularly, as reported in the rat [1, 14, 26], dog [20] and human kidneys [22]. Therefore, we assessed ASs only in the LRE of the GBM in this study.

The ASs of the GBM are essential for the charge-selective barrier in glomerular filtration, and their loss or decrease in the GBM is closely related to proteinuria [10]. The reduction in numbers of ASs in LRE of the GBM was related to the degree of proteinuria in human patients with various types of glomerulonephritis [11, 27]. Histopathologically renal changes in the present pigs were regarded as mesangial proliferative glomerulonephritis. There was no clinical information on urinary protein of the pigs. However, a statistically significant decrease in the number of ASs in the GBM was actually observed in the case showing irregular thickening of the GBM as compared with other cases without thickening of the GBM. Whether the number of
Fig. 6. Electron micrograph. Renal glomerulus; pig 10. Marked decrease in anionic sites in lamina rara externa in an area with subepithelial dense deposit (asterisk). A few polyethyleneimine particles were present at the surface of the dense deposit (arrows). Polyethyleneimine-uranyl acetate-citrate staining. C = capillary lumen. EN = endothelial cell. EP = epithelial cell. U = urinary space. Bar = 0.5 µm.

Fig. 7. Electron micrograph. Renal glomerulus; pig 10. A cluster of spherical microparticles (asterisk) associated with a decrease in number of anionic sites in LRE. A few polyethyleneimine particles were present at the surface of the cluster (arrows). Polyethyleneimine-uranyl acetate-lead citrate staining. C = capillary lumen. EN = endothelial cell. EP = epithelial cell. U = urinary space. Bar = 0.5 µm.

Table 1. Polyethyleneimine-stained anionic sites in the glomerular basement membrane and renal changes

<table>
<thead>
<tr>
<th>Pig No.</th>
<th>No. of anionic sites per 1,000 nm-GBM in the lamina rara externa (No. of sites evaluated)</th>
<th>Distribution of immuno-deposits IgG/IgM/IgA/C3</th>
<th>Renal lesion</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>16.98 ± 2.29 (40)</td>
<td>D-G/D-G/D-S /D-G</td>
<td>Mild mesangial enlargement</td>
</tr>
<tr>
<td>2</td>
<td>14.43 ± 2.06 (40)</td>
<td>D-G/D-G/D-S /D-G</td>
<td>Moderate mesangial enlargement</td>
</tr>
<tr>
<td>3</td>
<td>13.80 ± 2.13 (40)</td>
<td>D-G/D-G/D-G/D-G</td>
<td>Mild mesangial enlargement</td>
</tr>
<tr>
<td>4</td>
<td>16.78 ± 2.67 (49)</td>
<td>D-G/D-G/F-S/D-G</td>
<td>Mild mesangial enlargement</td>
</tr>
<tr>
<td>5</td>
<td>14.02 ± 4.07 (40)</td>
<td>F-S /F-S /F-S /F-S</td>
<td>Mild mesangial enlargement</td>
</tr>
<tr>
<td>6</td>
<td>9.47 ± 2.28 (51)*</td>
<td>D-G/D-G/D-G/D-G</td>
<td>Global thickening of GBM</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Segmental glomerular sclerosis</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(3.75%, n=80)</td>
</tr>
<tr>
<td>7</td>
<td>14.57 ± 2.76 (40)</td>
<td>D-G/D-G/D-D/G/D-G</td>
<td>Mild mesangial enlargement</td>
</tr>
<tr>
<td>8</td>
<td>15.08 ± 1.89 (60)</td>
<td>D-G/D-G/D-G/D-G</td>
<td>Mild mesangial enlargement</td>
</tr>
<tr>
<td>9</td>
<td>15.93 ± 3.48 (40)</td>
<td>D-G/D-G/D-S /D-G</td>
<td>Moderate mesangial enlargement</td>
</tr>
<tr>
<td>10</td>
<td>14.57 ± 2.76 (40)</td>
<td>D-G/D-G/F-S /D-G</td>
<td>Mild mesangial enlargement</td>
</tr>
</tbody>
</table>

a) Figures represent mean ± S.D.
b) Evaluated by immunofluorescence; D-G=diffuse global; D-S=diffuse segmental; F-S=focal segmental.
c) Multifocal.

* Significantly different from the other nine pigs by Mann-Whitney’s U-test; p<0.001.
ASs in the latter cases would be comprised within a normal range remains to be established. However, irregular thickening of the GBM of the pig might be associated with abnormal charge-selective filtration of the GBM. On the other hand, although the reduction in charge density did not correlate with the onset of proteinuria, it correlated with epithelial abnormalities in both adriamycin and puromycin nephrosis [8, 29]. Also, protein leakage from the GBM was not associated with decreased ASs in LRE in certain renal diseases such as congenital nephrotic syndrome of the Finnish type [12].

An age-related increase in extracellular matrices including the GBM was not accompanied with significant alteration in glomerular ASs in ddY mice [6]. In the present study, however, irregular thickening of the GBM was accompanied by reduction of ASs in LRE, suggesting that it may be a pathologic condition. The glomerular ASs are mainly composed of heparan sulphate proteoglycan (HSPG) which is one of the major constituents of the GBM [10]. Immunohistochemical studies have revealed no diminution in glomerular staining of HSPG in human diabetic glomerulosclerosis [30], human glomerulonephritis [7] and nephrotoxic nephritis in dogs [20]. In contrast, a structural alteration of HSPG has been suggested to explain the loss of charge selectivity resulting in proteinuria [9]. In the present study, PEI particles were occasionally localized in irregularly thickened LD of pig 6 which showed a reduction of ASs in LRE of the GBM. Although immunostaining of HSPG were not performed in the present study, an alteration of HSPG’s distribution might be coincident with GBM thickening.

Immunofluorescence microscopy revealed depositions of immunoglobulins and complements in the renal glomeruli of all cases. These immune deposits might be consistent with electron dense deposits in the mesangium and capillary walls. Distinct decrease in ASs was evidenced in LRE of GBM in the portions with subepithelial electron-dense deposits in this study. Experimental studies have suggested that subepithelial immune deposits were closely related to deposits in this study. Experimental studies have suggested that subepithelial immune deposits were closely related to dense deposits containing immunoglobulins and/or complements might mask or neutralize ASs in swine glomerulonephritis.

In a previous study, we showed a high incidence of SMP formation in the renal glomeruli of pigs, but its effects on the GBM were not substantiated [19]. The present study clearly indicated that SMPs were closely associated with a local disturbance of anionic charge in the GBM.

In conclusion, subepithelial dense deposits and clusters of SMPs in the GBM reduce the local anionic charge of LRE, and pathologic GBM thickening may result in an irregular distribution and a decrease in number of ASs in swine glomerulonephritis. The changes of HSPG in the GBM remain to be clarified in swine glomerulonephritis.

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REFERENCES


