Depletion of Glomerular Anionic Sites and Proteinuria in Nephrotic Syndrome of Children

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Summary
The number and distribution of glomerular anionic sites using polyethyleneimine (PEI) and the ultrastructural changes in the adjacent glomerular basement membrane (GBM) of 33 children with nephrotic syndrome were studied. Compared to the number of PEI-labelled anionic sites in the lamina rara externa per 1000 nm length of the GBM in eight controls (mean ± SD, 25.0 ± 1.49); there was a significant decrease in four patients with minimal change nephrotic syndrome (MCNS; 15.25 ± 2.98, P < 0.05); 10 patients with focal glomerulosclerosis (16.0 ± 5.1, P < 0.014); 14 patients with membranous nephropathy (14.1 ± 3.83, P < 0.009), and five patients with membranoproliferative glomerulonephritis (20.04 ± 1.69, P < 0.036). A moderate inverse correlation between anionic site numbers and proteinuria (estimated by urinary protein creatinine ratio) was found in MCNS only (r = —0.6). These findings suggest that a reduction in the glomerular anionic sites may be only partly responsible for proteinuria in the different types of childhood nephrosis, except for minimal change nephrotic syndrome, where it probably plays a major role.

Introduction
Altered electrostatic charge, and distortions to size and shape of the slits in the glomerular basement membrane (GBM) have been shown to be the most likely reasons for proteinuria although the relative contributions of each of these abnormalities in different renal diseases is not known.1,2 Fixed anionic sites are responsible for the charge-selective property of the GBM and loss of these sites may allow negatively charged proteins to pass easily through the capillary wall.1-4

Polyethyleneimine (PEI) which detects this loss of anionic sites along the glomerular capillary5-7 has been used to study proteinuria in some kidney diseases; there are few such studies and these have included only small numbers of children. The present study was therefore undertaken to address unresolved issues by analysing quantitatively the presence of anionic sites on the GBM in different types of NS in children using PEI, by comparing the magnitude of change in the electrostatic charge barrier in various histological groups, and by seeking an inverse correlation between the loss of anionic sites and the degree of proteinuria.

Patients and Methods

Patients
Thirty-three children with NS (31 African and two Indian) aged 2–12 years, presenting at King Edward VIII Hospital were selected. All patients were in relapse. The following groups of children were studied: six children with minimal change disease, 10 with focal glomerulosclerosis, 14 with membranous nephropathy, 12 HbsAg positive, and five with membranoproliferative glomerulonephritis Type I. The diagnostic criteria for these conditions have been previously reported.8

Methods
Tissue samples were obtained by percutaneous renal biopsy. Control biopsies were obtained from eight patients undergoing post-trauma partial nephrectomy. The histological diagnosis was determined by conventional light and electron microscopy and by immunofluorescence and classified according to Heptinstall.9

Tissue processing with PEI
Unfixed renal biopsy specimens were minced and placed in 0.5 per cent aqueous PEI solution (MW 3000–4000, pH 7.3, 400 m Osm) containing 8.5 per cent sucrose according to the method of Schurer et al.5 The blocks were washed three times for 10 min in cacodylate buffer (pH 7.3, 400 m Osm) containing 8.5 per cent sucrose and then fixed by immersion for 1 h in 2 per cent phosphotungstic acid/0.1 per cent glutaraldehyde (pH 7.3) mixture containing 8.5 per cent sucrose.

All procedures were carried out at 4°C. Thereafter, the tissues were left in 4 per cent glutaraldehyde in
preparation for transmission electron microscopy by conventional techniques and viewed in Joel 100c electron microscope.

Charged density was estimated only in areas cut perpendicularly to GBM. The number of anionic sites in the lamina rara externa was counted over a distance of GBM in the photomicrograph per glomerulus (at least three were counted) and was expressed as mean number \( \pm SD \) of LRE stained anionic sites per 1000-nm length of GBM. Three areas of GBM were counted. Stained sites in the lamina rara internal and the lamina densa were not counted as they were scattered randomly.

Quantitation of proteinuria

Second morning samples of urine were collected in children for estimation of proteins. Total protein was measured by the biuret method\(^9\) and creatinine was measured by the Jaffe method.\(^10\) Urinary protein/creatinine ratio (Up/c) were then calculated.\(^12\)

Statistical analysis

The mean number of anionic sites in all histological groups were compared using Kruskal–Wallis test for all differences. If the overall result was statistically significant, pairwise comparisons were made using Wilcoxon two-sample test to determine between which groups the differences were.

Results (Table 1)

Control group

Glomerular anionic sites in the control kidneys were visible as a continuous array of particles along the LRE and numbered about 25 ± 1.43 (mean ± SD) per 1000-nm length of the LRE. Fewer irregularly spaced PEI particles were also observed along the LRI and LD.

All patients

There was a significant reduction in the number of GBM anionic sites in patients as a whole \( P < 0.05 \) compared to controls. There was also a significant difference between each group and the controls. No differences were noted between the groups. PEI labelling of anionic sites revealed clearly demarcated electron dense particles (10 nm diameter) within the LRE of the GBM, fewer randomly distributed sites were observed in the LD and the LRI.

MCNS

There was a significant reduction in the number of labelled anionic sites on the LRE in the six children with MCNS. The mean number of anionic sites per 1000-nm length of GBM was 15.25 ± 2.98 \( P < 0.05 \).

The distribution of anionic sites in the LRI and LD appeared to be similar to the controls.

FGS

Indians \( n = 2 \). The mean binding was lower compared to controls \( 19.19 ± 0.15 \). The PEI staining in LRE showed a pattern similar to those in MCNS. However, in some parts of the GBM where the podocytes were not fused, the anionic sites were similar to controls, and seen as a continuous array along the LRE and between the fenestrations. Part of the LRI was not visualized and very few anionic sites were noted in the LRI and the LD.

African \( n = 8 \). The mean number of anionic sites was decreased compared to controls \( 14.19 ± 4.32 \). The GBM appeared to be thinner and complete fusion of the podocytes was noted along the GBM. The labelling was more diffuse, although an array of anionic sites was noted along the LRE. The LRI was not visualized in some samples and very few anionic sites were noted. A few scattered labelling spots were seen in the LD. The two ethnic groups taken together showed a significant difference in the number of anionic sites compared to controls \( P < 0.014 \).

MGN

HbsAg positive. The mean number of sites in the LRE were reduced compared to controls \( 15.98 ± 2.61 \). Scattered labelling was noted in the region of LRI, but very little in the LD. Glomerular anionic sites were absent in the area around the deposits.

HbsAg negative \( n = 2 \). The mean number of anionic sites were reduced compared to controls \( 13.06 ± 2.9 \). The membrane was thickened and the LRE was not clear. No labelling was seen in and around the epithelial deposits, and very few were seen in the LD. In the MGN group taken as a whole there was a significant reduction of PEI binding to anionic sites compared to controls \( 14.18 ± 3.83 \ P < 0.009 \).

Table 1

**PEI-labelled anionic sites in the lamina rara externa of the GBM in different histological groups**

<table>
<thead>
<tr>
<th>Histological group</th>
<th>MCNS ( n = 6 )</th>
<th>FGS ( n = 10 )</th>
<th>MGN ( n = 14 )</th>
<th>MPGN ( n = 5 )</th>
<th>Control ( n = 8 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anionic sites ( \text{mean} ± \text{SD} )</td>
<td>15.25 ± 2.9</td>
<td>16.0 ± 1.9</td>
<td>14.18 ± 3.8</td>
<td>20.04 ± 1.6</td>
<td>25.0 ± 1.49</td>
</tr>
<tr>
<td>( P ) values*</td>
<td>( P &lt; 0.05 )</td>
<td>( P &lt; 0.014 )</td>
<td>( P &lt; 0.005 )</td>
<td>( P &lt; 0.036 )</td>
<td></td>
</tr>
</tbody>
</table>

*No difference between individual groups.

No difference between controls.
MPGN
There was a significant loss in the number of anionic sites compared to controls (20.04 ± 1.69, P < 0.036). The GBM was thinner and the podocytes were not fused. Clear distinct labelling of anionic sites was noted along the LRE and between the fenestrations. Very little labelling was noted in the LRI and LD.

Correlation with proteinuria
PEI labelling to anionic sites and Up/c ratio correlated inversely and moderately only in children with the MCNS (r = −0.6). No such correlation was found in the group as a whole or in the other groups.

Discussion
The main findings in this study are, first, a diminution of anionic sites on the GBM which is common to different histological types of childhood NS, secondly, roughly similar degrees of reduction in anionic sites in MCNS, FGS, MGN and MPGN, and finally, an inverse correlation between anionic site number and the extent of proteinuria in MCNS only. Experimental evidence and work in human subjects suggest that loss of negative charge of the GBM leads to proteinuria. Therefore, the results from the present report can be interpreted to mean that a decrease in the electrostatic charge of the GBM in the diseases studied may account in part for the escape of serum proteins across the glomerular capillary wall into the urine.

This correlation is closest, albeit still modest, for MCNS, indicating that the reduction of anionic charge in the GBM contributes significantly to proteinuria in this disease. The absence of this association in the other histological categories of NS suggests that the decrease in anionic sites is not a major factor in the pathogenesis of proteinuria. However, another alternative explanation for the results obtained is that the methods for detecting changes to the electrostatic properties of the GBM are not sufficiently sensitive to uncover the links with proteinuria. These findings are consistent with other studies which have concluded that the loss of glomerular electrostatic charge is the prime mechanism responsible for proteinuria in MCNS, whereas size selectivity plays a major role in the other varieties of NS. Studies using urinary protein electrophoresis have supported these findings.

We have also recently shown that the anionic charge on red blood cell membranes (using Alcian blue) is markedly reduced and closely associated with proteinuria in MCNS, but not in the other types of nephrosis. The wider implications of the above findings are interesting but remain speculative. The many characteristics which distinguish MCNS from other types of childhood NS are well known and the findings given above on electrostatic charge of the GBM in MCNS may simply be another such feature or may reflect a more fundamental difference. It would be important, for example, to investigate the effect of steroids on restoring anionic balance in basement membranes.

As MCNS is rarely biopsied in most centres, only six such patients were available; findings in these were uniform. In most patients in this study the depletion of anionic sites was pronounced in the LRE; differences were less marked in the LRI and LD. However, in patients with MPGN there was a considerable loss of anionic sites in the LRI and LD; findings which are similar to those reported by Wada et al. The LRI was also markedly abnormal in FGS where it was found to be interrupted and sclerosed with very few anionic sites in seven of the eight patients studied. One hypothesis postulates that the changes detected in the LRI might alter the structure of the GBM thereby impairing its capacity to act as a size and shape barrier to circulating proteins.

The findings in MPGN, by this reasoning, may account for some adverse clinical features of the disease. Our findings in MGN are similar to those reported by Okada et al. The anionic sites were markedly diminished in areas of subepithelial deposits, but were seen where these deposits were absent. Scheeberger et al. suggested that immune complexes are formed in situ by the interaction of antibodies with epithelial surface glycoproteins. These immune complexes accumulate in the LRE and destroy or mask the heparan sulphate anionic sites.

The factors which precipitate relapse or maintain proteinuria in nephrosis are not known. Infections often precede an attack of oedema and proteinuria. There is a gap in our understanding of the connections (if any) between these events and disturbances in the electrostatic charge properties of the GBM. Levin et al. reported the presence of a circulating macromolecule in the plasma of patients with steroid responsive NS responsible for neutralizing the glomerular anionic sites. Tanaka et al. suggested that it is the lymphocyte in MCNS which produces this cationic factor. The initial triggering event could be an interaction between the precipitating agent and immune cells.

This study has provided further evidence that depletion of anionic sites on the GBM is a common feature in the different histological types of NS of childhood and may account to a limited extent for proteinuria in these diseases, except for MCNS where this abnormality probably plays a larger role. The lack of correlation between GBM charge and protein loss in the urine makes it likely (from other lines of evidence) that the major cause of proteinuria in FGS, MGN, and MPGN is the failure of the barrier function of the GBM to circulating proteins on the basis of their size and shape.

References