RAPID COMMUNICATION

Reduced Sialylation of Podocalyxin—The Major Sialoprotein of the Rat Kidney Glomerulus—in Aminonucleoside Nephrosis

DONTSCHO KERJASCHKI, MD,
ANTHONY T. VERNILLO, PhD, and
MARILYN G. FARQUHAR, PhD

From the Department of Cell Biology, Yale University School of Medicine, New Haven, Connecticut; and the Department of Pathological Anatomy, University of Vienna School of Medicine, Vienna, Austria

In this study the sugar composition of podocalyxin was determined in puromycin aminonucleoside-treated (PAN) rats and controls. Podocalyxin from both control and PAN rats bound ^{125}I-WGA and ^{125}I-peanut lectin (the latter only after neuraminidase treatment) on nitrocellulose transfers. Purified podocalyxin from both control and PAN rats was found to contain sialic acid, Gal, GlcNac, and Man but lacked Fuc and GalNac by gas–liquid chromatography. In PAN rats the sialic acid content of podocalyxin was reduced from 4.5% to 1.5%, whereas the concentration of the other sugars (with the possible exception of Gal) was similar to that of controls. The density of podocalyxin on the epithelial cell surface was estimated after immunogold labeling with anti-podocalyxin IgG, and no differences were found between PAN rats and controls. These data indicate that the reduced total glomerular sialic acid content found in PAN is due to the combined effects of the decreased podocyte plasmalemmal surface area and the reduced sialic acid content of podocalyxin. (Am J Pathol 1985, 118:343–349)

WE HAVE recently purified and partially characterized podocalyxin—a ~140-kd sialoprotein present on the plasmalemma of rat renal glomerular epithelial cells (podocytes) and have shown[1] that this protein is the biochemical equivalent of the histochemically defined glomerular epithelial "polyanion"[2–6] because it carries most of the glomerular sialic acid and is virtually the only protein present in glomerular extracts which stains strongly with cationic dyes and wheat germ agglutinin (WGA). Epithelial polyanion is believed to play an important role in the maintenance of the characteristic and unique foot process and slit organization of the glomerular epithelium, because a reduction in polyanion staining is detected by light microscopy whenever this organization is disturbed—ie, in diseased[4,7] differentiating,[8,9] and cultured[10] glomeruli. The most striking example of simultaneous foot process loss and loss of stainable polyanion is found in minimal change nephrosis—a disease which can be induced experimentally in rats with puromycin aminonucleoside (PAN).[4] At present it is not clear whether the reduction in polyanion staining and the concomitant reduction in glomerular sialic acid content[11] seen in this disease can be explained simply by the reduction in the podocyte plasmalemmal surface area that accompanies loss of the foot processes[12] or whether it reflects a reduction in the sialic acid content of one or more glomerular proteins. This prompted us to investigate the polysaccharide composition of podocalyxin in the PAN rat in order to determine whether or not its glycosylation pattern was abnormal.

Materials and Methods

Materials

The sources of the reagents were given previously.[1,13] Male Sprague–Dawley or Lewis rats (200–250 g) were

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Address reprint requests to Marilyn Gist Farquhar, Department of Cell Biology, Yale University School of Medicine, Sterling Hall of Medicine, P.O. Box 3333, New Haven, CT 06510-8002.

obtained from Charles River or the Tierzucht Institute of the University of Vienna.

Induction of Nephrosis

Rats were given subcutaneous injections of 1.6 mg PAN (Sigma) in 150 mM NaCl for 9 or 11 days[14] and sacrificed on the 10th or 12th day. Proteinuria was determined with Labsticks (Ames).

Isolation and Extraction of Glomeruli

Kidneys of normal and PAN rats were perfused with ice-cold Tris-buffered saline containing protease inhibitors.[13] Glomeruli were isolated and suspended in "sample buffer" (7.7% SDS, 36 mM dithiothreitol, 9 mM EDTA, and 20% glycerol), and the lysate was boiled for 5 minutes and stored at −20 C.

SDS-PAGE, Lectin, and Immunoblots

Glomerular lysates were separated on 5–10% gradient SDS-gels (stained either by 0.1% Coomassie blue or silver) and transferred onto nitrocellulose for 12 hours at 4 C at 30 v as described previously.[1] Podocalyxin was identified on the transfers by overlaying with rabbit anti-podocalyxin IgG (10 mg/ml)[1] and ^{125}I-protein A. Overlays with ^{125}I-WGA and ^{125}I-peanut agglutinin on nitrocellulose transfers and digestion of nitrocellulose strips with neuraminidase were performed as described.[1]

Digestion With Endoglycosidase F (Endo F)

Ten microliters glomerular extract (containing 100 μg protein) in SDS sample buffer were mixed with Nonidet P40 to give a ×10 excess of the latter over SDS. The sample was then mixed with an equal volume of ×2 Endo-F buffer,[15] containing 200 mM Na-phosphate buffer, pH 6.1, 100 mM EDTA, and 1% Nonidet P40 and aliquoted; and increasing amounts of Endo-F (a gift from Dr. Stephen Rosenzweig, Yale University), ranging from 0.05 to 15 U, were added to individual aliquots. Digestion was carried out for 24 hours at 37 C with shaking; then the digested samples were readjusted to the conditions of the SDS sample buffer by addition of solid SDS (to exceed the concentration of Nonidet P40 by 10 times) and by the addition of an equal volume of sample buffer. The samples (final volume, 60–80 μl) were then boiled for 5 minutes and loaded onto the gels.

Isolation of Podocalyxin by Preparative SDS-PAGE

Glomerular extracts from 4–6 kidneys were loaded without a comb onto a 2.5-mm-thick, 5–10% SDS gradient gel. Podocalyxin was localized in the gel as follows. Lanes were cut from the center and sides of the gel, transferred onto nitrocellulose, overlayed with ^{125}I-WGA, and exposed for autoradiography. The autoradiogram was then aligned with the residual gel, and the region coinciding with podocalyxin on the x-ray was excised from the gel.

Chemical Analysis of Podocalyxin

The concentration of protein in the eluted samples was determined by a fluorescamine assay[16] with bovine serum albumin and egg albumin as standards. Sialic acid was determined by the Warren assay or by a more sensitive modification of this assay[17] before and after treatment with 0.1 N NaOH to unmask O-acetylated sialic acids which are not detectable by the standard Warren assay.[18]

Gas–Liquid Chromatography

Normal and PAN–podocalyxin (isolated by preparative SDS–PAGE) were subjected to acid methanolysis, followed by trimethylsilylation of the released methyl glycosides. Analysis (performed by Dr. Rosalind Kornfeld, Washington University) was carried out on a 3% SE-30 column in a Varian 3700G gas chromatograph.[19] Samples were run in duplicate with glucose, mannose, galactose, GlcNac, GalNac, and fucose standards. Alternatively, podocalyxin was subjected to methanolysis for 16 hours at 75 C, the derivatized samples were treated with 33% N-methyl-bis-trifluoroacetamide in pyridine, and the analysis (performed by Dr. Robert Yu, Yale University) was carried out on a gas–liquid chromatograph.[20]

Immunocytochemistry

Podocalyxin was localized in the kidney by an indirect immunogold procedure,[1] which involves incubation of cryostat sections prepared from aldehyde-fixed kidney tissues in anti-podocalyxin IgG (~20 μg/ml for 15 hours) and protein A–gold (10–15 nm) conjugate (diluted 1:5 for 48 hours), then postfixed in OsO_4 and embedded in Epon 812.

For quantitation of the gold particles on the plasmalemma of podocytes, 50-nm ultrathin sections were prepared, and glomeruli were photographed at ×28,500 and enlarged to ×85,000. A total of 110 μ membrane and 1500 gold particles were counted on epithelial cells facing Bowman's space (and therefore freely accessible to the immunocytochemical reagents). Similarly, 400 μ epithelial membrane and 4000 gold particles were counted in PAN glomeruli.

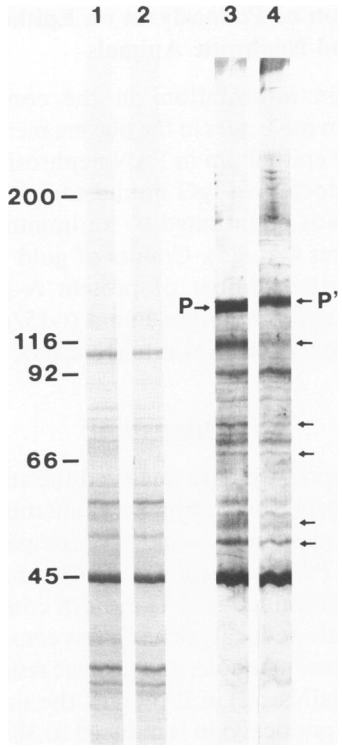


Figure 1—Extracts of isolated glomeruli solubilized in SDS sample buffer and separated on 5–10% gradient SDS-gels. Lanes 1 and 3 are extracts from controls, and Lanes 2 and 4 are from 10-day PAN rats. In Coomassie blue-stained preparations (Lanes 1 and 2), podocalyxin is not seen, and no change in the staining pattern of proteins is evident. After silver staining podocalyxin (Mr ~140,000) represents one of the two (the other is at Mr 45,000) most heavily stained proteins in controls (*P*, Lane 3). In PAN rats it is replaced by a similar broad band with a reduced silver staining intensity which migrates slightly slower (*P'*, Lane 4) than in controls. Several other bands (*arrows*) show a slightly reduced staining intensity but have the same mobility as in controls.

Results

Reduced Mobility of Podocalyxin From PAN Rats in SDS-Gels

When identical amounts of glomerular extracts from control and 10-day PAN-nephrotic rats were compared on Coomassie blue-stained gradient gels, in which podocalyxin is not visible,[1] no differences were detected in the protein pattern (Figure 1, Lanes 1 and 2). By contrast, in comparable silver-stained gels, in which podocalyxin is visible, the podocalyxin band from PAN glomeruli was reduced in staining intensity and slightly retarded in electrophoretic mobility (Figure 1, Lanes 3 and 4). In addition, several other bands (with apparent Mr of 48,000–120,000), the nature of which is unknown, also showed a slightly reduced staining intensity.

The changes in the mobility of podocalyxin from PAN rats was even more obvious in lectin- and immunoblots (Figure 2), where podocalyxin could be selectively visualized. In overlays with anti-podocalyxin IgG (Figure 2, Lanes 1 and 2), ^{125}I-WGA (Figure 2, lanes 3 and 4), or ^{125}I-peanut agglutinin after digestion with neuraminidase (Figure 2, Lanes 5 and 6), an estimated increase (~3000) in the apparent Mr of podocalyxin was seen. In WGA overlays a second band (Mr ~150,000) which was not detectable in normal glomeruli also bound the lectin (Figure 2, Lane 4) but did not bind anti-podocalyxin IgG. Its origin is unknown.

Reduced Sialic Acid Content of Podocalyxin From PAN Rats

The fact that partially desialylated glycophorin shows an anomalous decreased mobility on SDS-gels[21] led us to suspect that the sialic acid content of podocalyxin might be decreased in PAN rats. When the sialic acid content of podocalyxin purified from 10 day PAN rats was determined either by the Warren assay or by gas–liquid chromatography, it was found to contain ~1.3% sialic acid, as compared with 4.5% in controls. These percentages translate into 5 molecules of sialic acid per molecule of PAN podocalyxin versus 16–20 molecules of sialic acid per molecule of normal podocalyxin (Table 1).

The Composition of Other Sugars Is Not Significantly Changed

By gas–liquid chromatography podocalyxin from control and PAN rats was found to contain considerable mannose and GlcNac residues and to lack Fuc or

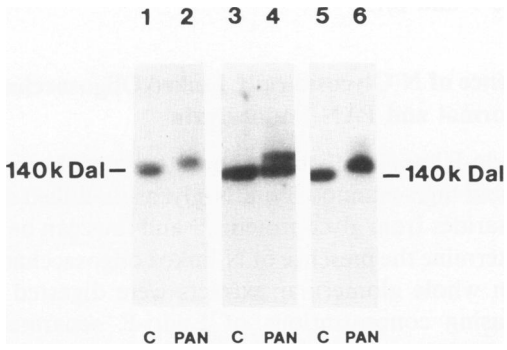


Figure 2—Autoradiograms of overlays of podocalyxin from control and PAN kidneys. Glomerular extracts were separated on 5–10% SDS-gradient gels and transferred onto nitrocellulose. Paper strips (containing the whole pattern of lysates as shown in Figure 1) were incubated with anti-podocalyxin IgG followed by ^{125}I-protein A (Lanes 1 and 2), ^{125}I-WGA (Lanes 3 and 4), or ^{125}I-peanut agglutinin (Lanes 5 and 6), the latter after digestion of the paper strip with neuraminidase. Control (Lanes 1, 3, and 5) and PAN glomerular extracts (Lanes 2, 4, and 6) were run side by side for facilitation of direct comparison of the mobility of podocalyxin. All the probes selectively label podocalyxin from control rats in a Mr 140,000 position. By contrast, podocalyxin from PAN rats has a reduced mobility. In addition, a second band (Mr ~150,000) is labeled by ^{125}I-WGA (Lane 4), which is not present in controls (Lane 3) and is not recognized by the anti-podocalyxin IgG (Lane 2).

Table 1—Sugar Composition of Podocalyxin From Control and PAN-Nephrotic Rats

	Control rats	Nephrotic rats
Mannose	16* (4)†	15.5 (4)
N-Acetylglucosamine	29 (7.3)	28.6 (7.2)
Galactose	16 (3.9)	10.9 (2.8)
Fucose	0	0
N-Acetylgalactosamine	0	0
Sialic acid	16.3 (7.7)	5.0 (0.7)

* Expressed as moles of sugar per mole of podocalyxin.
† Values in parentheses are standard deviations from two determinations performed in duplicate.

GalNac. The Man and GlcNac composition of podocalyxin from normal and diseased animals was similar, and the amount of Gal was only slightly reduced.

The Lectin Binding Properties of Normal and PAN Podocalyxin Are Similar

When transfers of glomerular extracts from PAN rats were overlayed with ^{125}I-WGA, podocalyxin bound this lectin as in controls (Figure 2, Lanes 3 and 4). Binding was prevented by prior neuraminidase digestion (not shown), indicating that WGA binds to sialic acid residues. ^{125}I-peanut agglutinin, which recognizes terminal Gal and GalNac,[22] did not bind to native (undigested) podocalyxin from either control or PAN rats, which indicates that these groups are not exposed; however, the lectin does bind after removal terminal sialic acid residues by neuraminidase digestion (Figure 2, Lanes 5 and 6).

Presence of N Glycosidically Linked Oligosaccharides in Normal and PAN Podocalyxin

Endo-F has recently been shown to remove both complex and high-mannose type, N-glycoside-linked oligosaccharides from glycoproteins,[15] and thus can be used to determine the presence of N-linked oligosaccharides. When whole glomerular extracts were digested with increasing concentrations of Endo-F, separated by SDS–PAGE, and transferred onto nitrocellulose, it was evident in immunoblots that podocalyxin from both normal and PAN rats was degraded in several steps (Figure 3). Ovalbumin, which contains a single N-linked oligosaccharide chain, was degraded in a single step (not shown). Nonglycosylated proteins (myosin, β-galactosidase) were not degraded under the same conditions, which indicates that the Endo-F preparation was not contaminated by proteases.

Quantitation of Podocalyxin on Epithelial From Normal and Nephrotic Animals

To obtain information on the concentration of podocalyxin molecules in the plasma membranes of the glomerular epithelium in PAN nephrosis, the amount of anti-podocalyxin IgG bound to normal and PAN glomeruli was quantitated by an immunogold procedure (Figures 4 and 5). Counts of gold particles indicated that the number of protein A-gold particles bound per micron plasmalemma (~15/μ) was similar in the normal and PAN rats (12 days).

Discussion

In this report we have analyzed the sugar composition of podocalyxin—the major glomerular sialoprotein of the renal glomerulus—and have compared the results obtained in PAN-nephrotic rats with those in controls. We have determined that 1) in both controls and diseased animals podocalyxin contains considerable sialic acid, galactose, mannose, and GlcNac residues but lacks fucose or GalNac; 2) in PAN rats, the sialic acid composition of podocalyxin is reduced to 30% of the normal level; and 3) no significant differences were detected in the other sugar residues. Thus, we have established that the loss of the epithelial foot process and slit organization—the morphologic hallmark of minimal-change nephrosis—is accompanied by a biochemical defect in the sialylation of this epithelial membrane component.

Spreading or "fusion" of the epithelial foot processes has been used as an aid in the diagnosis of lipoid nephrosis or minimal-change nephrosis in renal pathology since it was first described in renal biopsy specimens about 30 years ago,[23] but the molecular basis for

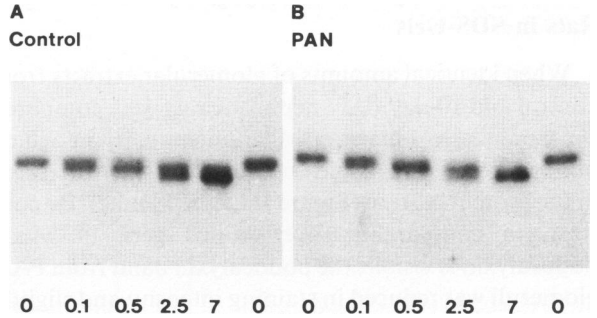
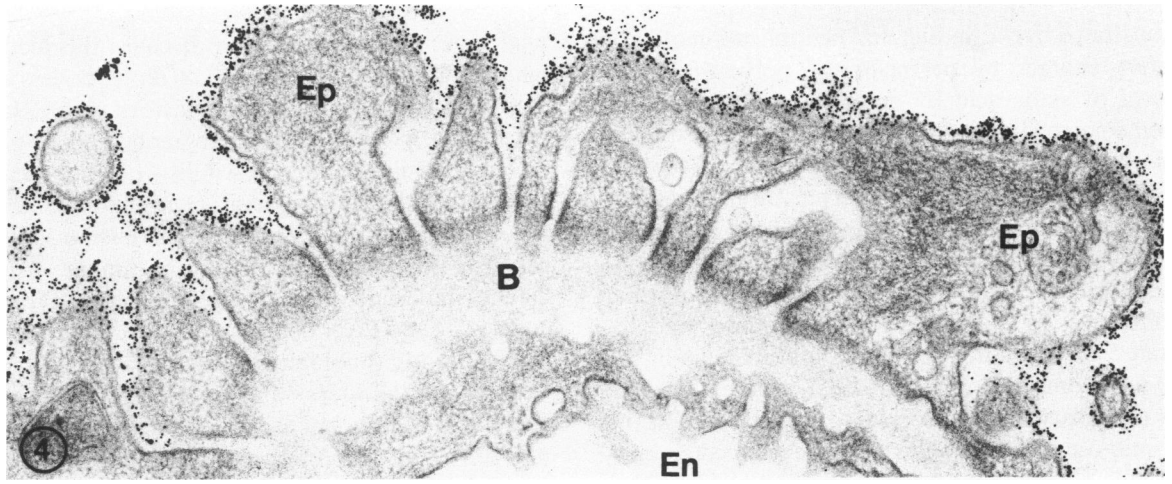
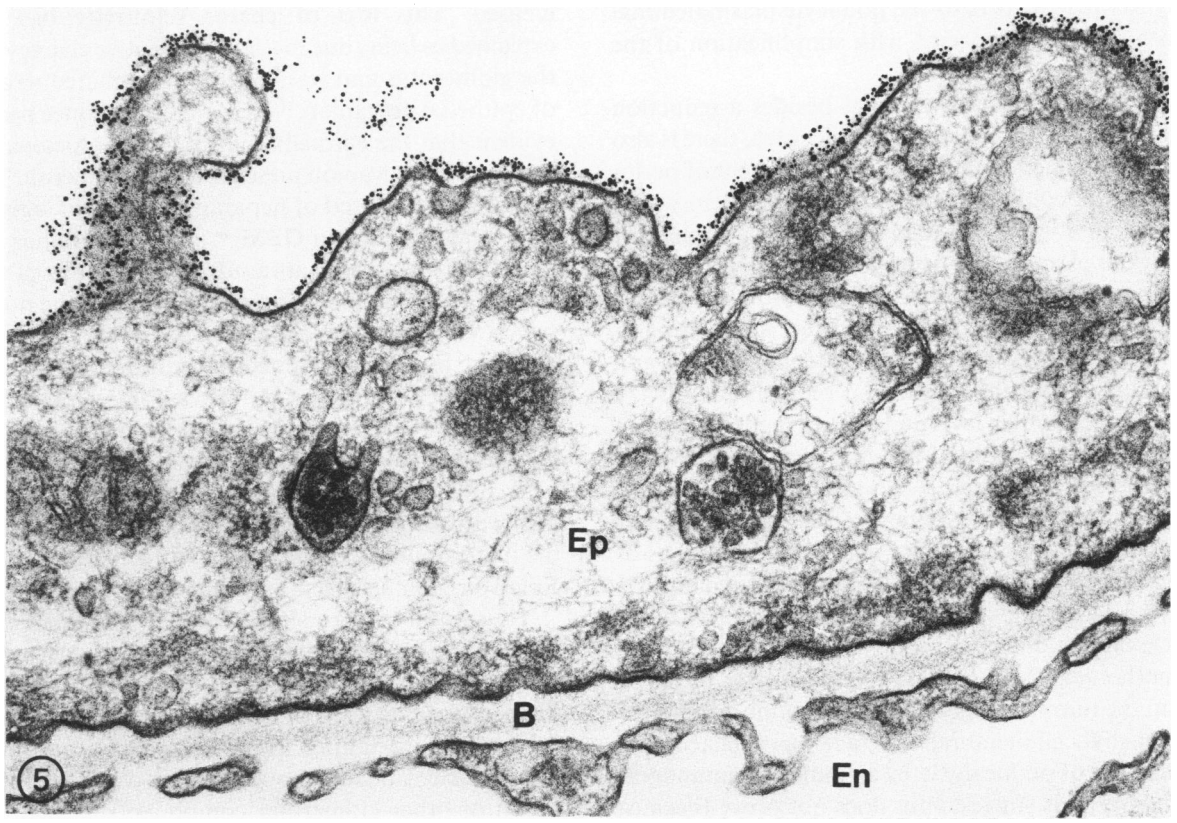


Figure 3—Digestion of podocalyxin with Endoglycosidase F. Nitrocellulose transfers of glomerular extracts of normal (**A**) and 10-day PAN rats (**B**) were incubated with increasing amounts of Endo-F, indicated at the bottom of the figure (expressed in enzyme units), for 24 hours at 37 C, and podocalyxin was visualized by immune overlaying with anti-podocalyxin IgG and ^{125}I-protein A. In both preparations, Endo-F degrades podocalyxin in several steps (as indicated by the two-step change in its mobility), suggesting the presence of at least two N-glycosidically linked, oligosaccharide chains.





Figures 4 and 5—Localization of anit-podocalyxin IgG on the surfaces of glomerular epithelial cells (*Ep*) as detected by an immunogold technique in a control (**Figure 4**) and a 10-day PAN-nephrotic rat (**Figure 5**). Counts of gold particles bound to the epithelial cell surface indicate that the density of gold particles per micron epithelial plasmalemma is similar (~15/μ) in normal rats to that in PAN rats. The total surface area of the epithelium is greatly reduced in PAN rats because of the loss of the foot process organization. *B*, basement membrane. (**Figure 4**, ×40,000; **Figure 5**, ×30,000)

this morphologic change is still not fully understood. A connection between the changes in the organization of the foot processes and filtration slits and the highly negatively charged, sialic-acid-rich epithelial cell coat was made some time ago when Michael et al[4] noted a reduction in the sialic acid content of nephrotic glomeruli as determined by histochemical staining procedures; and later on, Blau and Michael[12] found a reduction in the sialic acid content in whole glomeruli and in glomerular basement membrane (GBM) and membrane fractions prepared therefrom by the Warren assay. The importance of the negatively charged groups—presumably sialic acid—in the maintenance of the differentiated shape of the epithelial cells was also

demonstrated by the finding that neutralization of fixed negative charges by perfusion of polycations[24] or removal of sialic acid by neuraminidase digestion[25] caused a narrowing or collapse of the slits and spreading and simplification of the foot processes. However, some doubt was cast on these interpretations by studies in which the amount of binding of cationic stains to the epithelial surface was analyzed at the electron-microscopic level, and no differences between normal and PAN rats were detected.[12] These findings were interpreted as indicating that there is no change in the sialic acid composition of epithelial cell membrane in PAN nephrosis; and the decreased staining seen at the light-microscopic level,[4] as well as the reduced glomerular sialic acid content,[11] was explained as being due solely to the reduction of the podocyte plasmalemmal surface area, which occurs with simplification of the foot processes.

Our findings demonstrate that besides a reduction in the epithelial plasmalemmal surface area, there is also a dramatic reduction in the sialic acid content of podocalyxin—the epithelial membrane sialoprotein which accounts for >50% of the total sialic acid content of glomerular extracts.[1] To what extent the defect in sialylation applies to other glomerular sialoproteins remains to be determined. The failure to detect a reduction in binding of cationic probes such as colloidal iron and ruthenium red to the epithelium of PAN rats at the electron-microscopic level undoubtedly reflects the fact that the charge density of the epithelial cell surface is not sufficiently reduced to prevent or reduce binding of these highly cationic probes. A reduction in the surface charge of the glomerular epithelium as well as of the anionic sites in the GBM has been detected in PAN rats with lysozyme—another cationic probe of lower surface charge density.[26]

Another fiinding in the present study is that no reduction in the number of gold particles bound per micron of epithelial cell membrane surface was detected after localization of podocalyxin by an indirect immunogold procedure. This suggests but does not prove (because it is possible that the antigenic sites are more accessible in PAN rats) that the concentration of podocalyxin per micron of membrane is similar in control and PAN rats.

The nature of the oligosaccharide chains to which the sialic acid on podocalyxin is attached is currently unknown. The data obtained in this study by digestion with Endo-F, together with the sugar composition of podocalyxin, suggest that both normal and PAN podocalyxin contain at least two N-glycosidically linked oligosaccharide chains. The presence of O-linked Gal-GalNac residues in podocalyxin was previously suggested by the finding[1] that podocalyxin binds peanut agglutinin after desialylation[1] because this lectin has a high affinity for the GalNac-Gal dimer or, less avidly, for Gal.[22] However, in the present work no GalNac was detected by gas–liquid chromatography, a finding which essentially rules out this possibility. Further structural analysis is required for precise determination of the type of oligosaccharide linkage that carries the sialic acid moieties in podocalyxin and establishment of the full extent (sialic acid only or sialic acid plus Gal or other sugars) of the PAN defect.

The pathophysiologic hallmark of minimal-change nephrosis in PAN rats[27] and in humans[28] is proteinuria associated with loss of charge selectivity of the glomerular filter—ie, the filtration of anionic molecules is increased while filtration of neutral molecules is decreased. This loss of charge selectivity has been explained as being due to a loss of negative charges from the glomerulus and was originally attributed to a loss of epithelial polyanion.[27] However, it has since become evident that the epithelial polyanion (podocalyxin) is not the only polyanion present in renal glomeruli.[29] Anionic sites composed of heparan sulfate proteoglycans have been detected in GBM,[30] and evidence has been presented that the heparan sulfate side chains of these molecules are required for maintenance of the normal selective permeability properties of GBM.[31] Moreover, evidence has been obtained indicating that the staining of these heparan-sulfate–rich anionic sites,[26] as well as the turnover of heparan sulfate[29,32] in the glomerulus, are altered in this disease. The latter are now known to include both heparan sulfate proteoglycans associated with basement membranes[29,34] and an antigenically distinct population of heparan sulfate proteoglycans found on endothelial and epithelial cell membranes.[29,33,35]

Thus, rather than a single defect in PAN nephrosis, there appear to be several changes which affect at the same time sialoglycoprotein(s) and proteoglycans. The discovery of the defective glycosylation of podocalyxin raises the intriguing possibility that puromycin aminonucleoside somehow interferes with the glycosylation of other glomerular components as well.

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