Increased Permeability of the Glomerular Basement Membrane to Ferritin after Removal of Glycosaminoglycans (Heparan Sulfate) by Enzyme Digestion

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ABSTRACT   Glomerular basement membranes (GBM's) were subjected to digestion *in situ* with glycosaminoglycan-degrading enzymes to assess the effect of removing glycosaminoglycans (GAG) on the permeability of the GBM to native ferritin (NF). Kidneys were digested by perfusion with enzyme solutions followed by perfusion with NF. In controls treated with buffer alone, NF was seen in high concentration in the capillary lumina, but the tracer did not penetrate to any extent beyond the lamina rara interna (LRI) of the GBM, and little or no NF reached the urinary spaces. Findings in kidneys perfused with Streptomyces hyaluronidase (removes hyaluronic acid) and chondroitinase-ABC (removes hyaluronic acid, chondroitin 4- and 6-sulfates, and dermatan sulfate, but not heparan sulfate) were the same as in controls. In kidneys digested with heparinase (which removes most GAG including heparan sulfate), NF penetrated the GBM in large amounts and reached the urinary spaces. Increased numbers of tracer molecules were found in the lamina densa (LD) and lamina rara externa (LRE) of the GBM. In control kidneys perfused with cationized ferritin (CF), CF bound to heparan-sulfate rich sites demonstrated previously in the laminae rarae; however, no CF binding was seen in heparinase-digested GBM's, confirming that the sites had been removed by the enzyme treatment. The results demonstrated that removal of heparan sulfate (but not other GAG) leads to a dramatic increase in the permeability of the GBM to NF.

There is now widespread agreement that the glomerular basement membrane (GBM) is the main filtration barrier serving to retain plasma proteins in the circulation, and that it has both size-selective and charge-selective properties (1, 3, 15, 16). Little is known, however, about the role of GBM components in establishing these properties. We have recently presented cytochemical evidence (based on removal with specific enzymes) demonstrating the presence of anionic sites consisting of glycosaminoglycans (GAG) rich in heparan sulfate in the laminae rarae (interna and externa) of the GBM (7). We have subsequently isolated GAG from purified GBM and have confirmed by quantitative biochemical analysis the presence of GAG (heparan sulfate and hyaluronic acid) in the GBM (8). Upon finding the GAG, we speculated that these highly charged polymers might play a role in establishing the permeability properties of the GBM to plasma proteins. We here present data that indicate the removal of GAG (heparan sulfate) from the GBM leads to increased permeability of the GBM to native ferritin (NF), thereby providing direct experimental support for this suggestion.

MATERIALS AND METHODS

Materials

Male Charles River CD^H rats weighing 100–150 g were used. Chondroitinase-ABC (*P. vulgaris*) and Streptomyces hyaluronidase were purchased from Miles Laboratories, Inc. (Elkhart, Ind.). Heparinase was prepared from *F. heparinum* (11). Horse-spleen ferritin (two times crystallized, cadmium free) was obtained from Calbiochem-Behring Corp., American Hoechst Corp. (San Diego, Calif.). Cationized ferritin (CF) (pI = 7.3–7.5) was prepared as described previously (6). Enzymes were tested and found to be active against the appropriate substrate and to be free of proteolytic activity as measured by the Azocoll assay (7, 14).

Enzyme Perfusion

Procedures for perfusion of the kidney with enzyme solutions were given previously (7). Briefly, the kidneys were flushed with Krebs-Ringer's bicarbonate (KRB) at 120–150 mm Hg for 1–2 min and then perfused with 4–5 ml of the enzyme solution. The vessels were clamped (to retain the enzyme), and the kidneys were incubated for 30 min in a 0.15 M NaCl bath as follows: heparinase, 1 mg/ml in 0.05 M NaCl-acetate, pH 7.0, at 30°C; Streptomyces hyaluronidase, 50 U/ml in 0.02 M NaCl-acetate, pH 5.6, at 37°C; and chondroitinase-ABC, 2.5 U/ml in KRB, pH 7.6, at 37°C. Relatively high concentrations of enzymes were used to insure effective digestion of GAG. Control kidneys were perfused and incubated with buffer alone.

At the end of the incubation, the clamp was removed and the kidneys were flushed with 5–7 ml of KRB for 1–2 min followed by perfusion with NF (2.5–10 mg/ml), suspended either in freshly drawn, citrated rat arterial blood or in KRB, containing 7.5% bovine serum albumin (BSA). Perfusion pressures (120–150 mm Hg) and arterial flow rates (2.5–3.0 ml/min) were maintained constant. After half of the perfusate (~10–15 ml) had been utilized, the kidney was fixed *in situ* by subcapsular injection of Karnovsky's fixative (6% glutaraldehyde and 5% paraformaldehyde in 0.15 M cacodylate buffer, pH 7.4) at the hilus while simultaneously dripping fixative on the kidney surface. Subcapsular injection and drip fixation were continued until the remaining perfusate had been consumed. The renal artery and vein were then reclamped, the renal capsule was removed, and the whole kidney was immersed in fixative for an additional hour. Pieces of kidney cortex were then prepared and processed for electron microscopy (6). Experiments with different enzymes were performed in triplicate.

In a separate experiment, the kidney was digested with heparinase and then perfused with CF (0.25 mg/ml) in KRB, pH 7.4, containing 7.5% BSA.

Ferritin Counts

For each experiment 12–15 normal sections through capillaries from at least five to six different glomeruli were photographed and printed at 100,000. The number of ferritin molecules present in the layers of the GBM (lamina rara interna [LRI], lamina densa [LD], lamina rara externa [LRE]) were counted and compared to the number in the capillary lumen. To obtain the latter, the region adjacent to the endothelium and equivalent in width to that of the GBM was counted. The ratio of the number of ferritin molecules present in each GBM layer to that in the lumen was determined, multiplied by 100, and expressed as the percent of the luminal concentration (Table I).

RESULTS

We have shown that heparan sulfate and small amounts of hyaluronic acid are the only GAG detectable upon cellulose acetate electrophoresis of GAG extracted from isolated GBM (8). If other GAG are present, they must be present in relatively small amounts. To assess the affect of removing each type of GAG from the GBM, we employed three different enzymes with different specificities: Streptomyces hyaluronidase which digests only hyaluronic acid; chondroitinase-ABC which digests hyaluronic acid; chondroitin 4- and 6-sulfates and dermatan sulfate; and heparinase which digests all known GAG except keratan sulfate (including heparan sulfate).

As reported previously (7), glomeruli from kidneys treated

TABLE I

*Relative Distribution of Ferritin Particles in the Layers of the GBM as Compared to the Capillary Lumen **

exp	GBM layers	Buffer controls		Enzyme treatment	
		Acetate buffer, pH 7.0		Heparinase	
I	LRI	15.6‡	(±2.7)	19.3	(±4.6)
	LD	3.9	(±1.8)	11.4	(±3.6)
	LRE	0.2	(±0.2)	4.4	(±1.1)
		Krebs buffer, pH 7.6		Chondroitinase-ABC	
II	LRI	10.3	(±2.1)	8.4	(±3.9)
	LD	3.0	(±0.8)	2.4	(±1.3)
	LRE	0.1	(±0.2)	0.3	(±0.1)
		Acetate buffer, pH 5.6		Streptomyces hyaluronidase	
III	LRI	14.8	(±2.9)	15.3	(±5.0)
	LD	3.9	(±0.9)	4.2	(±1.6)
	LRE	0.1	(±0.2)	0.1	(±0.1)

Data are the means ± SD.
* Data represent the results of counts on 12–15 sections from each of three experiments. The average area counted was ~0.3 μm^2/section.
‡ Expressed as the percent of the luminal concentration. The actual luminal concentration of NF varied in different experiments from 400–1,300 molecules/μm^2.

with these enzymes or with the appropriate buffers alone appeared quite intact with no recognizable structural alterations (Figs. 2–5).

Distribution of NF in Buffer Controls

When control kidneys incubated with various buffers were perfused with NF the findings were the same as obtained previously in vivo (5) and in vitro (15) in that the NF molecules did not penetrate to any great extent beyond the LRI, and little or no NF reached the LRE or urinary spaces. NF was seen in high concentration in the capillary lumen and in the endothelial fenestrae and LRI (Fig. 1). Counts of ferritin molecules revealed the existence of two concentration gradients: one between the lumen and the LRI, and the other between the LRI and the LD. As shown in Table I, the concentration of NF in the LRI was 10–15% of the luminal concentration, and the concentration in the LD was 3–4% of that in the lumen. Results were the same whether NF was perfused in citrate or in KRB-BSA.

Distribution of NF after Enzyme Perfusion

In kidneys digested with chondroitinase-ABC or hyaluronidase, the ferritin concentration in each of the layers of the GBM was similar or identical to that in the corresponding buffer controls (Figs. 4 and 5; Table I).

In kidneys digested with heparinase, however, there was a dramatic increase in the permeability of the GBM to ferritin: NF penetrated the GBM in large amounts, escaped into the urinary spaces, and appeared in the tubule lumina. The amount found in the urinary spaces varied from one capillary to another, depending on the effectiveness of the fixation-gelation of the contents of the urinary spaces, in some (~15%) it reached nearly 50% of the concentration in the capillary lumen (Fig. 2). This is in striking contrast to the situation in buffer controls (Fig. 1) and in kidneys digested with chondroitinase-ABC (Fig. 4) or hyaluronidase (Fig. 5) in which ferritin was seldom found in the urinary spaces.

Increased amounts of NF were seen in all layers of the GBM (Fig. 3) after heparinase treatment. Results of ferritin counts indicated that the concentration of NF in the LRI was only slightly increased (3–4%), whereas in the LD there was three times and in the LRE there was >20 times the amount seen in controls. Particularly notable was the fact that the concentration gradient between the LRI and the LD was greatly reduced (Fig. 3).

Distribution of CF after Heparinase Digestion

We have previously shown (6) that CF with a pI of 7.3–7.5 is a useful cationic probe for labeling the heparan sulfate-rich sites in the GBM in vivo because it binds to the GBM sites but does not bind to the adjoining cell membranes. When CF was perfused into the kidney in KRB containing 7.5% BSA the findings were the same as those obtained previously when CF was given intravenously: it bound to the anionic sites in the LRI, LRE, and mesangial matrix where it was found in clusters of 5–10 molecules distributed at regular (60 nm) intervals (Fig. 6). No binding to adjacent cell membranes was observed when CF fractions with this pI range were used (6). However, in kidneys digested with heparinase and perfused thereafter with CF, the distribution of CF was radically changed; it was seen distributed throughout the GBM (compare Figs. 6 and 7). The
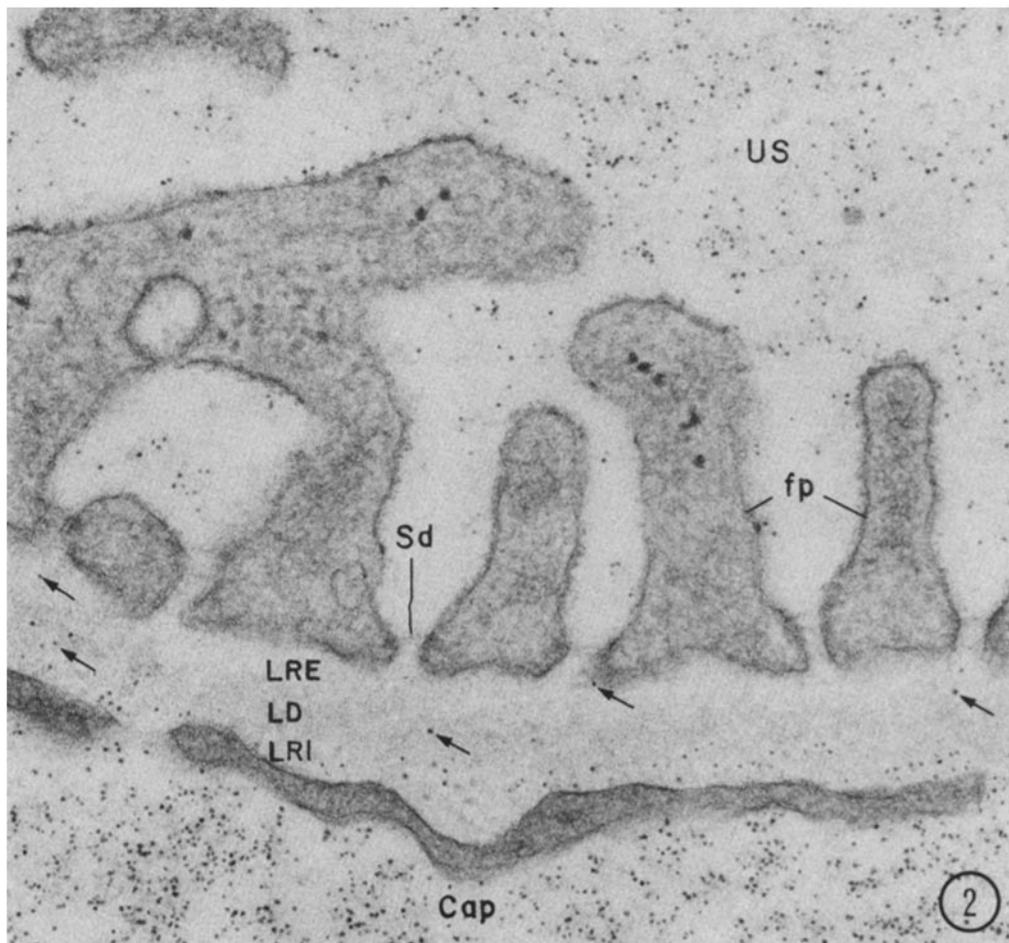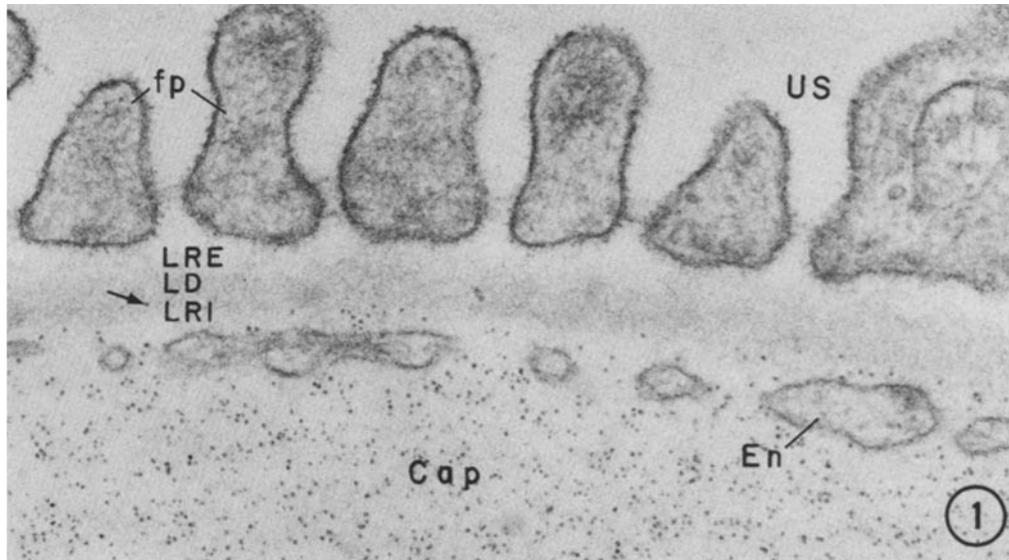
FIGURE 1 Portion of a glomerular capillary from a control kidney incubated with acetate buffer followed by perfusion with native ferritin (10 mg/ml) suspended in KRB containing 7.5% BSA. Most of the ferritin molecules are restricted to the capillary lumen (Cap) or the LRI of the GBM. Occasionally ferritin molecules are seen in the LD (arrow) or LRE of the basement membrane, but none are present in the urinary spaces (US). fp, foot processes; En, endothelium. × 100,000.
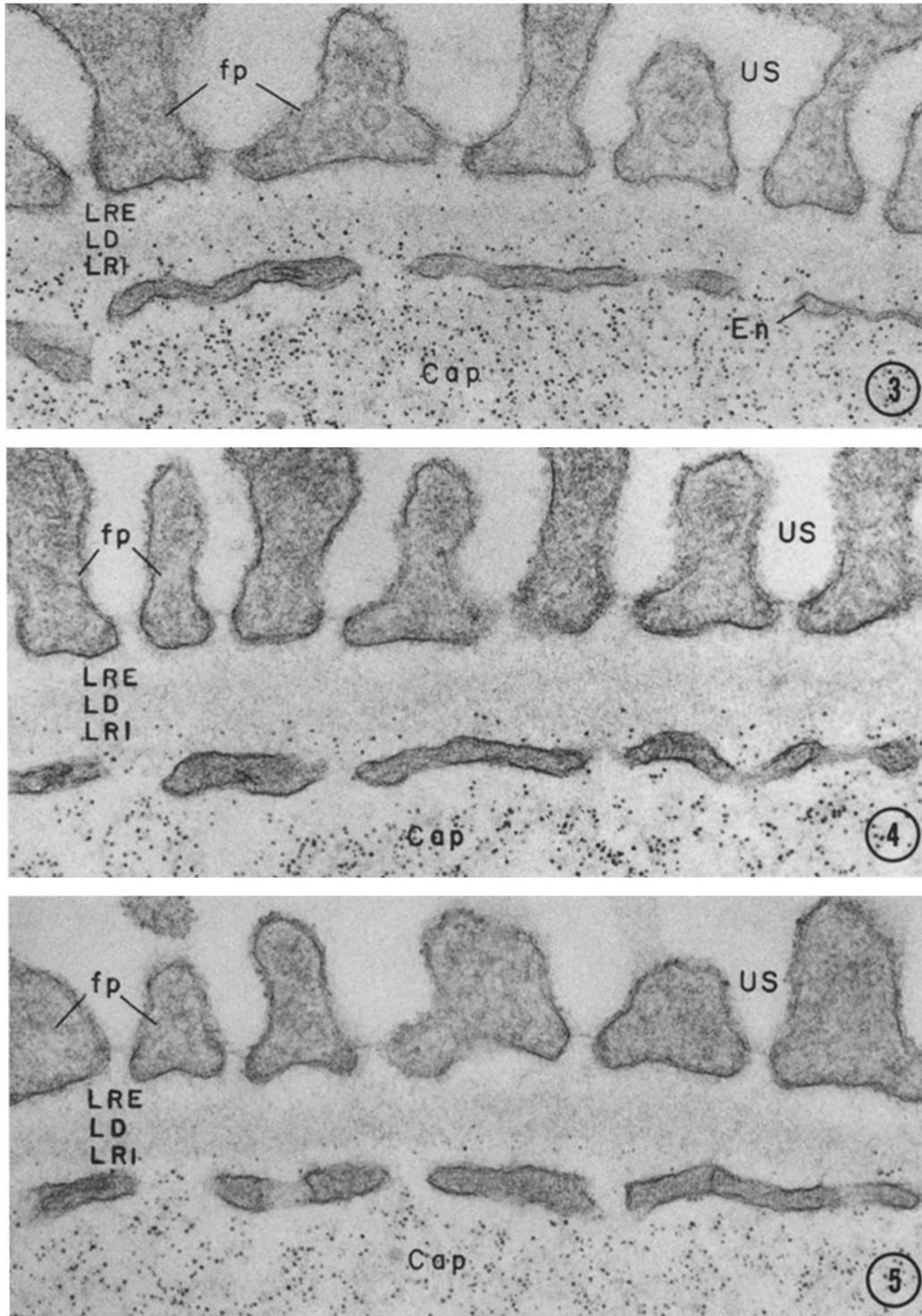
FIGURE 2 Portion of a glomerular capillary from a kidney digested with heparinase followed by perfusion with NF containing 7.5% BSA. Numerous ferritin molecules have penetrated the GBM and are present in the urinary spaces (US). More molecules are seen (arrows) in the LD and in the LRE than in controls. Glomerular structure including that of the slit diaphragms (Sd) is intact. × 100,000.

absence of CF binding demonstrates that the heparan sulfate-rich anionic sites had been digested by the enzyme treatment.

DISCUSSION

In this investigation we have shown that removal of GAG from the GBM by digestion with heparinase causes a dramatic increase in the permeability of the GBM to NF. After heparinase the tracer escaped into the urinary spaces in large amounts, whereas no such leakage occurred under any of the other conditions studied. Furthermore, our results indicate that the permeability increase is specifically caused by removal of heparan sulfate because no increase in the permeability of the

FIGURES 3-5   Portions of glomerular capillaries from kidneys digested with heparinase (Fig. 3), chondroitinase-ABC (Fig. 4), and Streptomyces hyaluronidase (Fig. 5), followed by perfusion with NF suspended in KRB-BSA. After heparinase treatment increased amounts of ferritin are seen in the GBM especially in the LD and LRE, whereas there is no change in the amount of NF found in the GBM after treatment with the other two enzymes. US, urinary spaces; fp, foot processes; En, endothelium; Cap, capillary lumen. × 100,000.
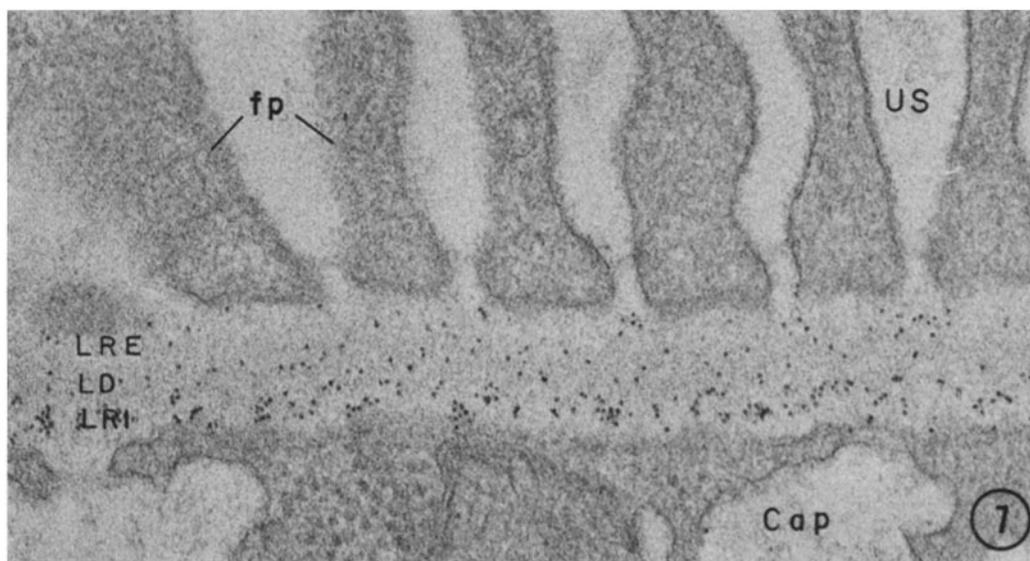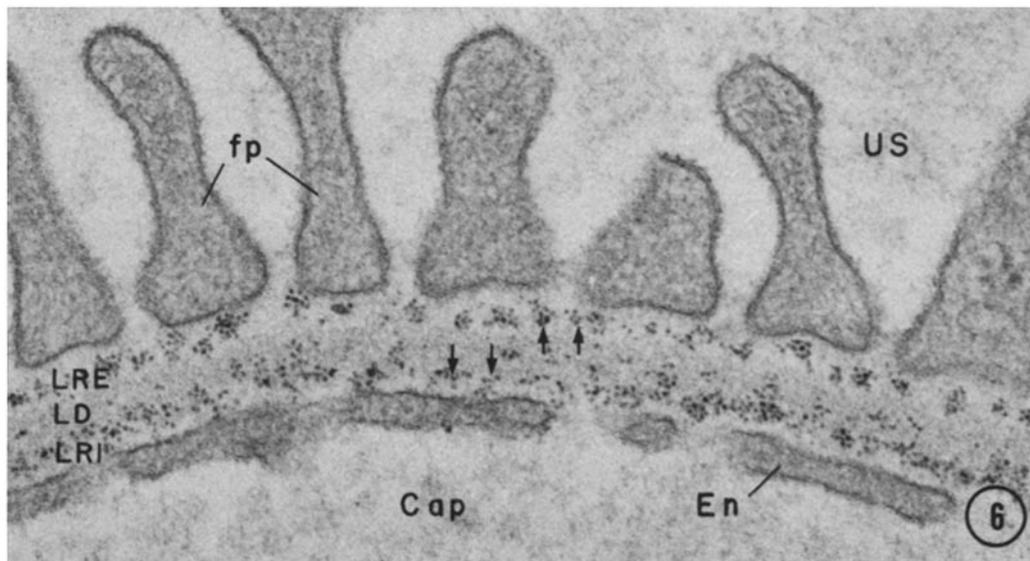
FIGURE 6 Section of a glomerular capillary from a kidney incubated with acetate buffer followed by perfusion with 0.25 mg/ml CF (pI 7.3–7.5) suspended in KRB-BSA. CF binds to heparan sulfate-rich anionic sites at regular (~60 nm) intervals located in the LRI (↓) and LRE (↑) of the GBM. No binding to the adjacent cell membranes is observed. *fp*, foot processes; *US*, urinary spaces; *En*, endothelium; *Cap*, capillary lumen. × 100,000.

FIGURE 7 Section of a glomerular capillary from a kidney digested with heparinase followed by perfusion with CF suspended in KRB-BSA. CF is seen in high concentration in the LRI and distributed throughout the LD. The regular binding to the anionic sites of the LRI or LRE is lost, indicating that the anionic sites have been effectively digested. × 100,000.

GBM to ferritin was seen after removal of hyaluronic acid (by treatment with Streptomyces hyaluronidase) or after digestion with chondroitinase-ABC, an enzyme that removes most other GAG (hyaluronic acid, chondroitins 4- and 6-sulfates, and dermatan sulfate) but not heparan sulfate. This constitutes direct evidence that the presence of heparan sulfate is necessary for maintaining the normal restrictive permeability properties of the GBM to proteins the size of anionic ferritin (diameter = 11 nm) and smaller.

We have previously shown (6) that the sulfated GAG (heparan sulfate) present in the GBM is concentrated in particles, presumably as proteoglycans (protein-polysaccharide complexes) which are distributed in a regular lattice-like network. It cannot be determined whether the main effect of removing heparan sulfate is on the charge-selective or the size-selective properties of the GBM, or, in molecular terms, whether the

increased permeability is caused by the removal of an electrostatic or a steric exclusion effect. However, because of the large size of the ferritin and the amount of leakage seen, an effect on both seems likely.

The only GAG other than heparan sulfate detected so far in the GBM in any significant quantity is hyaluronic acid. On the basis of the studies of Laurent (10), removal of hyaluronic acid might be expected to affect the permeability (diffusion) of the GBM to proteins. However, removal of hyaluronic acid by digestion with either Streptomyces hyaluronidase or chondroitinase-ABC had no effect on the penetration of NF into the GBM. Of course this does not rule out an effect on its permeability to molecules smaller in size than NF.[1]

[1] We have recently obtained autoradiographic evidence indicating that removal of hyaluronic acid (by digestion with either hyaluronidase or

In the past it has been widely assumed that sialic acid present on the surfaces of endothelial cells and especially on epithelial cells is responsible for the creation and maintenance of the charge-barrier properties of the glomerulus (1, 9, 18). It should be mentioned, however, that this assumption was not based on direct evidence, but rather was based on (a) the fact that, at the time, sialic acid was the only polyanion known to be present in the glomerulus (13), and (b) the finding that sialoproteins are reduced in glomeruli from animals with experimentally induced glomerular diseases associated with proteinuria (12).

The accumulated evidence based on use of electron-dense tracers (reviewed in references 3 and 4) indicates that the main glomerular barrier to penetration of macromolecules lies at the level of the LRI of the GBM because a variety of anionic and neutral macromolecules (NF [5], albumin [16], IgG [17], and dextrans [2]) fail to penetrate beyond this level. The present studies indicate that there are two concentration gradients for NF: one between the lumen and the LRI and the other between the LRI and the LD. It is the latter that is eliminated or greatly reduced after removal of heparan sulfate, and as a consequence, NF penetrates the LD and gains access to all the layers distal to the LD. This constitutes direct evidence that heparan sulfate is essential for maintenance of the normal glomerular permeability barrier, and that maintenance of the heparan sulfate-rich sites in the GBM are essential to the integrity of that barrier.

In summary, we have demonstrated that heparan sulfate plays a role in creating the normal restrictive permeability properties of the GBM to macromolecules. Because, according to our data (4, 6, 7), anionic sites similar to those in the GBM are widely distributed in basement membranes, GAG can be assumed to play a similar role in the permeability of other basement membranes. Finally, the fact that GAG are essential for normal filtration functions and their removal leads to

chondroitinase-ABC) leads to increased permeability of the GBM to ^{125}I-BSA. Leakage of BSA into the urinary spaces of kidneys perfused with heparinase was three times and those perfused with S. hyaluronidase or chondroitinase ABC two times greater than that seen in controls (Kanwar and Farquhar, 1980, Fed. Proc. 39:334).

protein leakage, raises the possibility that alterations in GAG may play a role in the pathogenesis of diseases in which there is proteinuria of glomerular origin.

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