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The anionic sites at luminal surface of peritubular capillaries in rats

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The anionic sites at luminal surface of peritubular capillaries in rats. Anionic sites have been demonstrated in the basement membranes of peritubular capillaries. The anionic barrier function of peritubular capillary wall has been ascribed to these sites. Fenestrated capillaries in other organs have anionic sites in the endothelial cell glycocalyx and at the luminal surface of the fenestral diaphragms. The purpose of this study was to map anionic sites at the luminal surface of peritubular capillaries and to assess whether a concentration gradient for albumin exists across the endothelium. Partial chemical characterization of these anionic sites was done by in vivo enzymatic degradation. The difference in distribution of albumin following enzyme digestion was also studied. The binding of cationized ferritin to the luminal surface indicated that the rat peritubular capillaries have anionic sites along the entire luminal surface of the endothelial cell, including the fenestral diaphragms. Partial biochemical characterization of these sites shows that the sites in the glycocalyx are mainly from neuraminic acid, while the fenestral diaphragms have mainly heparan sulfate proteoglycans. Intravascular albumin extended to the endothelial luminal plasmalemma and to the luminal surface of fenestral diaphragms. Digestion with heparitinase was associated with the leakage of albumin outside the capillary wall. These findings suggest that the anionic surface of fenestrae constitutes a charge barrier of the peritubular capillaries.

Transport of water and macromolecules across a capillary wall is controlled by hydraulic and oncotic pressures and the permeability characteristics of the capillary wall. Because of ultrafiltration at the glomerulus, peritubular capillary oncotic pressures are higher than in other capillaries, due to a high concentration of albumin [1]. Peritubular capillary oncotic pressure is one of the factors that influences water and solute reabsorption into the capillary lumen, which in turn has effects on the tubular reabsorption of water and sodium bicarbonate [2–5]. In order to maintain a high intraluminal concentration of albumin, the peritubular capillary wall must restrict the diffusion of albumin into the interstitium. Physiological studies regarding the permeability of peritubular capillaries indicate that the peritubular capillary wall, similar to other capillary walls, functions as a size and a charge selective barrier [6–9]. Anionic sites have been demonstrated in peritubular capillary basement membranes [10] and the anionic barrier function of the peritubular capillary wall has been considered to be due to the anionic constituents of the capillary basement membrane. However, in other organs having capillaries with diaphragmed fenestrae, anionic proteoglycans have been demonstrated lining the luminal surface of diaphragms [11]. We wondered whether the peritubular capillary endothelium too has a similar anionic glycocalyx at its luminal surface.

The initial aim of the present study was to determine whether anionic sites exist at the luminal surface of peritubular capillaries and to map their distribution. In the second part of the study, the composition of the anionic sites in the endothelial glycocalyx and at the fenestral diaphragms was partially characterized by in vivo digestion with specific enzymes directed to degrade the individual proteoglycans. In addition, the anionic barrier function of the anionic sites was examined by the alteration in the distribution of albumin following the digestion of anionic sites with heparitinase.

Methods

Animals

Female Sprague–Dawley rats, weighing 150 to 250 g, from Harlen Sprague–Dawley, Indianapolis, Indiana, USA, were used.

Chemicals

Cationized ferritin (CF, pI 8.4) was used as the probe for anionic sites [12]. CF was obtained from Miles, Naperville, Illinois, and from Sigma Chemical Company, St. Louis, Missouri, USA. Native ferritin (horse spleen) was obtained from Sigma (pI 4.5). IsoGel Agarose and Ampholytes (pH 3.5 to 9.5) and pI markers for isoelectric focusing were obtained from FMC Marine Colloids Division, Rockland, Maine, USA. All the batches of ferritin were dialyzed at 4°C for 48 hours against distilled water and their pI were checked by isoelectric focusing [13]. Dulbecco's phosphate buffered saline and Minimum Essential Medium were purchased from Grand Island Biological Company, Grand Island, New York, USA. The perfusion fluid referred to as DPBS consisted of Dulbecco's phosphate buffered saline (pH 7.2 to 7.4), with 5% Minimum Essential Medium amino acids, 14 mM glucose, and gassed with oxygen.

Enzymes having activity against the specific glycosaminoglycans were perfused in the renal vasculature with the aim of removing the individual proteoglycans. The lack of CF binding following the in vivo enzymatic digestion was taken to indicate the presence of the particular proteoglycan. Neuraminidase

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Table 1. Number of experimental and control rats and the sequence of intrarenal perfusion

No. of rats exp/cont	DPBS wash *min*	Enzyme in exp. diluent in cont.	DPBS wash *min*	Tracer	DPBS *min*	Fixative *ml*
3	4	None	None	NF	4	30
4	4–6	None	None	CF	4–6	30
2/2	4–5	Hyalurinidase	1–2	CF	2–4	30
4/2	2–5	Neuraminidase	1	CF	1–4	30
2/1	4–8	Heparitinase	1	CF	2–3	30
4/2	2	Chondroitinase ABC	1	CF	1–2	30
5/1	4–7	Papain	0–1	CF	0–2	30

Abbreviations are: NF, native ferritin (pI 4 · 5); CF, cationized ferritin (pI 8 · 4).

Table 2. Sources of enzymes and conditions of enzyme perfusion

Enzyme[a]	Source	Concentration	pH	Temp.	Retained in vasculature, *min*
Neuraminidase type X	clostridium perfringens (Sigma)	1–2 units/ml	5 · 4	37°C	5–10
Chondroitinase ABC	proteus vulgaris (Sigma)	2 units/ml	7 · 4	37°C	5–10
Hyaluronidase	streptomyces hyalurolyticus (Miles)	50 units/ml	5 · 6	37°C	10
Heparitinase	Flavobacterium heparinum (Miles)	50 units/ml	7 · 0	43°C	10
Papain type IV	Papaya latex (Sigma)	5–10 mg/ml	7 · 0	37°C	5–20

[a] Enzymes were dissolved in 0 · 1 M Na-acetate buffer

(type X, from C. perfringens), chondroitinase ABC (from Proteus vulgaris), and Papain (type IV, from Papaya latex) were purchased from Sigma, while hyaluronidase (Strep. hyalurolyticus) and heparitinase (Fl. heparinum) were purchased from Miles. By manufacturer's specifications, neuraminidase, hyaluronidase, and heparitinase acted specifically on sialic acid, hyaluronic acid and heparitin sulfate respectively, while chondroitinase ABC degraded hyaluronic acid in addition to chondroitin sulfates A, B, and C [14 to 16]. Papain was used to assess whether glycolipids impart the anionic sites in the peritubular endothelial cell glycocalyx.

Anti-rat albumin antibody conjugated with horseradish peroxidase and lyophilized rat whole serum were obtained from Cappel (Cooper Biomedical, Malvern, Pennsylvania), and Diamine benzadine tetrahydrochloride and hydrogen peroxide from Sigma.

Experimental protocol

Intra-renal infusion. Rats were anaesthetized with Ketamine-HCL (8.7 mg/100 g body wt) and Xylazine (1.3 mg/100 g body wt) given intramuscularly [17]. The abdomen was opened and the aorta catheterized with an 18 to 22 gauge polyethylene catheter. The catheter tip was positioned near the origin of the left renal artery. A ligature above and one below isolated this segment of the aorta. A third ligature on the cannulated part of the aorta stabilized the catheter. A hole was made in the left renal vein to achieve open drainage.

Perfusions were done at a flow rate of 3 to 5 ml/min, using an occlusive roller pump (Holter pump, International Medical Corporation, Englewood, Colorado, USA). A calibrated Statham gauge was used to monitor perfusion pressure through a side arm in the infusion line. Perfusion pressures varied between 60 and 90 mm of Hg for the most part, except during fixation when pressures invariably rose above 200 mm Hg. Perfusion steps were as follows: a) two to seven minutes of DPBS to wash out the blood; b) CF 7 mg/100 g body weight, made up to 2 ml in normal saline; c) DPBS one to two minutes to wash out excess CF; d) 30 ml of Karnovsky's fixative pH 7.4. In three rats native ferritin was used instead of CF. Details of perfusion are given in Table 1. For the enzyme digestion experiments, freshly dissolved enzyme in 0.1 M NaCl-acetate buffer was perfused in the renal vasculature following the removal of blood by the DPBS wash. After allowing the time for the action of enzyme, the vasculature was perfused with DPBS again. Following enzyme removal, the perfusion steps were similar to those for rats given CF (Table 1). Table 2 gives the concentration of enzyme used, their source, pH, temperature and the retention time for each enzyme in the renal vasculature. Controls were run in parallel and were exactly similar to the enzyme perfused rats, except that only the buffer instead of the enzyme was perfused in the controls.

After perfusion the left kidney was harvested. Two longitudinal slices of the kidney were taken. A linear segment of cortex was obtained from each kidney by cutting along the corticomedullary junction, which was well visualized in the washed-out fixed kidney. A less than 1 mm slice of subcapsular tissue was removed from the outer surface to get rid of the capsule and immediate subcapsular tissue. Next, the remaining tissue was cut into 1 mm cubes. The 1 mm cubes of cortex were further fixed overnight in the same fixative, then transferred to sodium cacodylate buffer pH 7.2. These cubes were post-fixed in osmium tetroxide, dehydrated, embedded in Epon, sectioned at 60 nm (gray sections), stained with lead citrate and examined using a Hitachi 11C electron microscope. At least two blocks from each kidney and at least five peritubular capillaries in each section were examined.

Systemic infusion. In three rats, CF was injected directly into the exposed aorta. In these experiments, the dosage of CF was increased to 20 mg/100 g body weight. The CF was injected into the aorta, above the origin of the renal vessels, using a 25 gauge needle. No other manipulations were done to avoid further changes in hemodynamics. Following the injection, left renal vessels were immediately clamped and the left kidney was harvested. One mm cubes of the cortex were again collected

and immersion fixed in Karnovsky's fixative. Further processing and examinations were as for other tissues.

Assessment of the anionic barrier function. The question was whether the anionic layer at the luminal surface of fenestral diaphragm served as a barrier to anionic serum proteins. To answer this, anti-rat albumin antibody labelled with horseradish peroxidase was used to assess the distribution of albumin with and without digestion of this surface layer with heparitinase.

Rats were catheterized as previously described. Blood was washed out of the kidney with 5 ml of DPBS. Four ml of heparitinase (50 U/ml, in 0.1 M NaCl-acetate buffer, pH 7.0, temperature 43°C) was infused and held for 10 minutes. The enzyme was removed by perfusing 10 ml of DPBS. Next, 1 ml of reconstituted rat serum was perfused and held for one minute. Fixation was done by the injection of 1.5 ml of a mixture of rat serum and Karnovsky's fixative (0.5 ml of double strength serum and 1 ml of fixative). Fixative was also dripped on the surface of the kidney for five minutes. The kidney was removed, sliced, and held overnight in the same fixative.

The same procedure was followed in control rats, except that only the buffer was perfused and held, instead of the enzyme. Forty micron thick cryostat sections of the fixed kidney cortex were incubated with the anti-rat albumin antibody labelled with horseradish peroxidase. For each specimen, a set of sections were incubated with diluent instead of antibody as a negative control. All sections were briefly fixed in 2.4% glutaraldehyde and then incubated with diamine benzidene hydrochloride and hydrogen peroxide. Further processing, including osmium post-fixation, were as previously described.

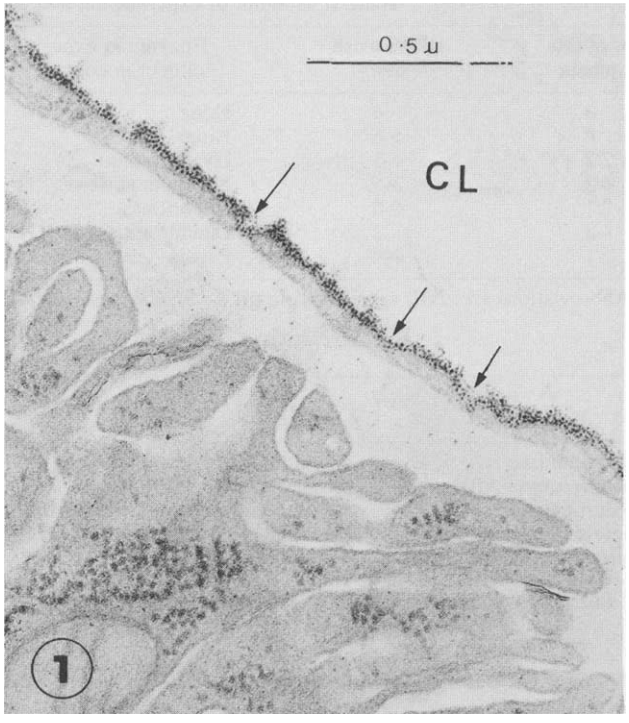

Fig. 1. *Peritubular capillary wall from a rat perfused with cationized ferritin.* Ferritin is seen attached to the endothelial glycocalyx and to the luminal surface of fenestrae (arrows). Capillary lumen (CL) (×57,000).

Results

State of tracer

Each batch of ferritin, native or cationized, was examined by electron microscopy to ensure monomolecular dispersion of ferritin and to exclude aggregation. Isoelectric points for each batch were tested by isoelectric focusing. Cationized ferritin from both sources gave two major bands at 8.2 and 8.4 pI. Native ferritin had a pI of 4.5 – 5.5.

Distribution of anionic sites. Peritubular capillaries lie between the tubules of renal cortex or medulla. Often a single capillary abutts on two adjacent tubules, while the intervening segment of the capillary wall faces the interstitium. The capillary basement membrane often blends into the basement membrane of the tubular epithelium. The capillary endothelium is a thin cell overlying its basement membrane. Only very rare transendothelial channels and intracytoplasmic vesicles are seen in the endothelial cells of these capillaries.

No ferritin binding to the capillary wall was seen following the perfusion of native ferritin. Rats infused with cationized ferritin showed cationized ferritin attached to the endothelial glycocalyx and attached to the fenestral diaphragms (Fig. 1). Under basal conditions, no CF penetrated the basement membrane, the tubular epithelium or intercellular spaces.

Chemical constituents of the anionic sites. The conditions for enzyme experiments were chosen to optimize enzyme action (Table 2). The alterations in the binding of CF to the capillary wall and other structural alterations, such as those caused by papain, indicated that enzymes reached the lumen of peritubular capillaries and degraded their substrate in the capillary wall. Following digestion by neuraminidase, CF staining was seen predominantly over the fenestral diaphragms. The endothelial glycocalyx now bound only an occasional ferritin molecule (Fig. 2). Conversely, following digestion by the enzyme heparitinase, CF binding over the majority of the diaphragms was nearly abolished, but the endothelial glycocalyx was still stained (Fig. 3). Following perfusion of the enzymes hyaluronidase and chondroitinase, the pattern of staining did not differ from that seen in control rats (Figs. 4 and 5). Papain digestion resulted in a marked loss of binding of CF at the glycocalyx (Fig. 6). Only a few sites in focal areas bound ferritin at the glycocalyx. At the fenestral diaphragms no ferritin was bound following papain digestion. These findings indicate that a few anionic sites in the glycocalyx are from glycolipids (Fig. 6). Endothelial cells were detached from the capillary wall in many places in papain treated kidneys and structural alterations were seen in the tubular cells.

In rats, given CF into the aorta, with no washes before or after, CF was seen attached to fenestral diaphragms, but not to the endothelium (Fig. 7). Aggregates of CF were present in the lumen (Fig. 7).

Anionic barrier function. In the last segment of the study, the distribution of intravascular albumin with or without the enzymatic treatment was assessed (Figs. 8 and 9). In normal rat peritubular capillaries, albumin was seen to extend up to the endothelial cell surface and up to the luminal surface of diaphragms. Albumin was not detected outside the lumen anywhere (8A). Following digestion with heparitinase enzyme, albumin was now seen in the space between endothelial and peritubular cells (9A). Thus, digestion with heparitinase selec-

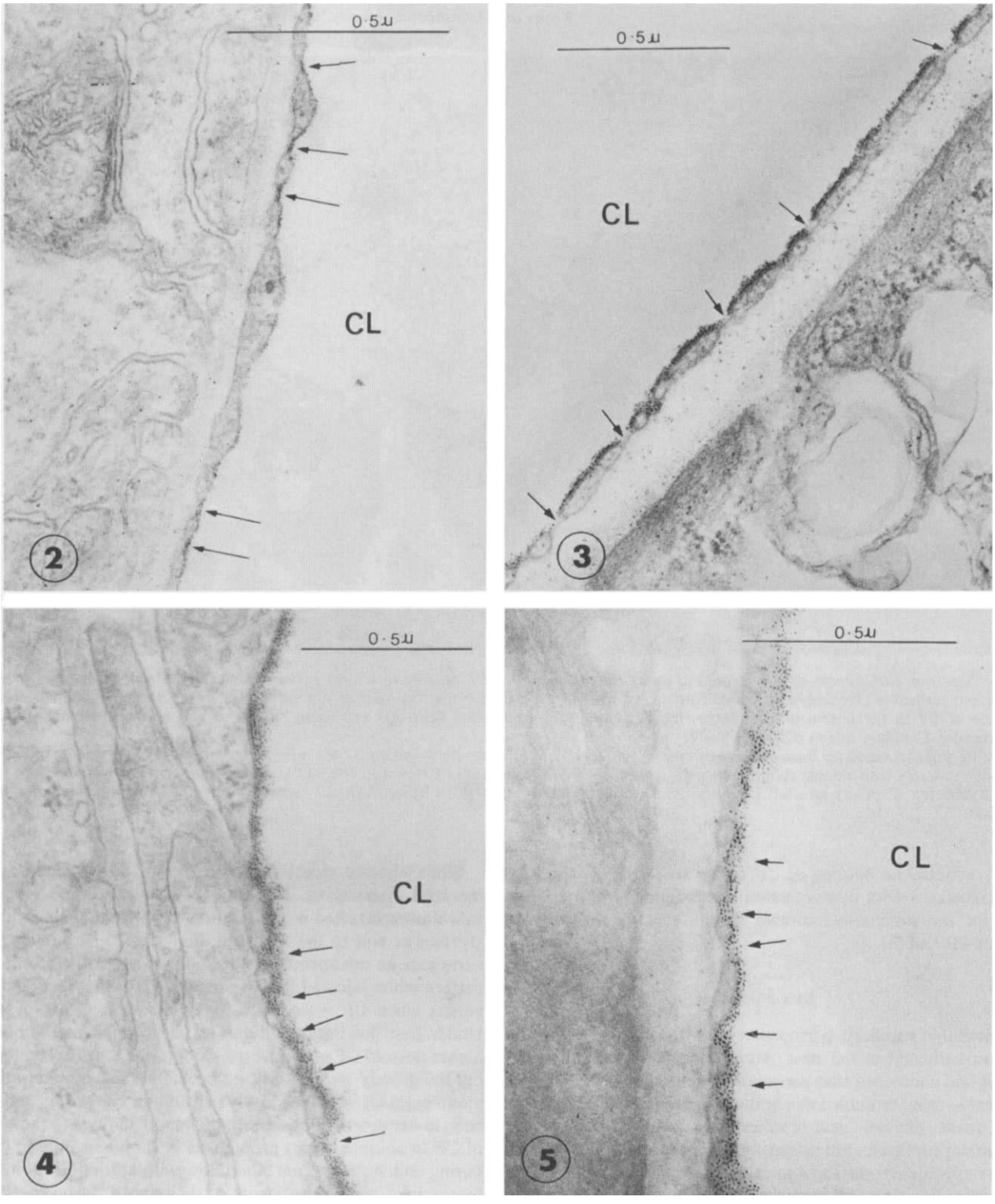

Fig. 2. *Peritubular capillary from a rat subjected to in vivo digestion with neuraminidase.* While the attachment of cationized ferritin (CF) to the glycocalyx is markedly decreased, binding of ferritin to the fenestral diaphragms is present. CF binding in controls was as is illustrated in Figure 1. Capillary lumen (CL) (×74,000).

Fig. 3. *Heparitinase digestion prevented binding of CF mainly at the fenestral diaphragms (arrows).* Glycocalyx still binds CF which appears to be comparable to controls. Capillary lumen (CL). (×59,000).

Fig. 4. *Hyaluronidase digestion did not alter CF binding to the endothelial cell surface in any appreciable manner.* Arrows show fenestral binding of CF. Capillary lumen (CL) (×58,000).

Fig. 5. *Compared to the controls, no alterations were found in the binding of CF following digestion with chondroitinase.* Arrows point to fenestrae. Capillary lumen (CL) (×72,000).

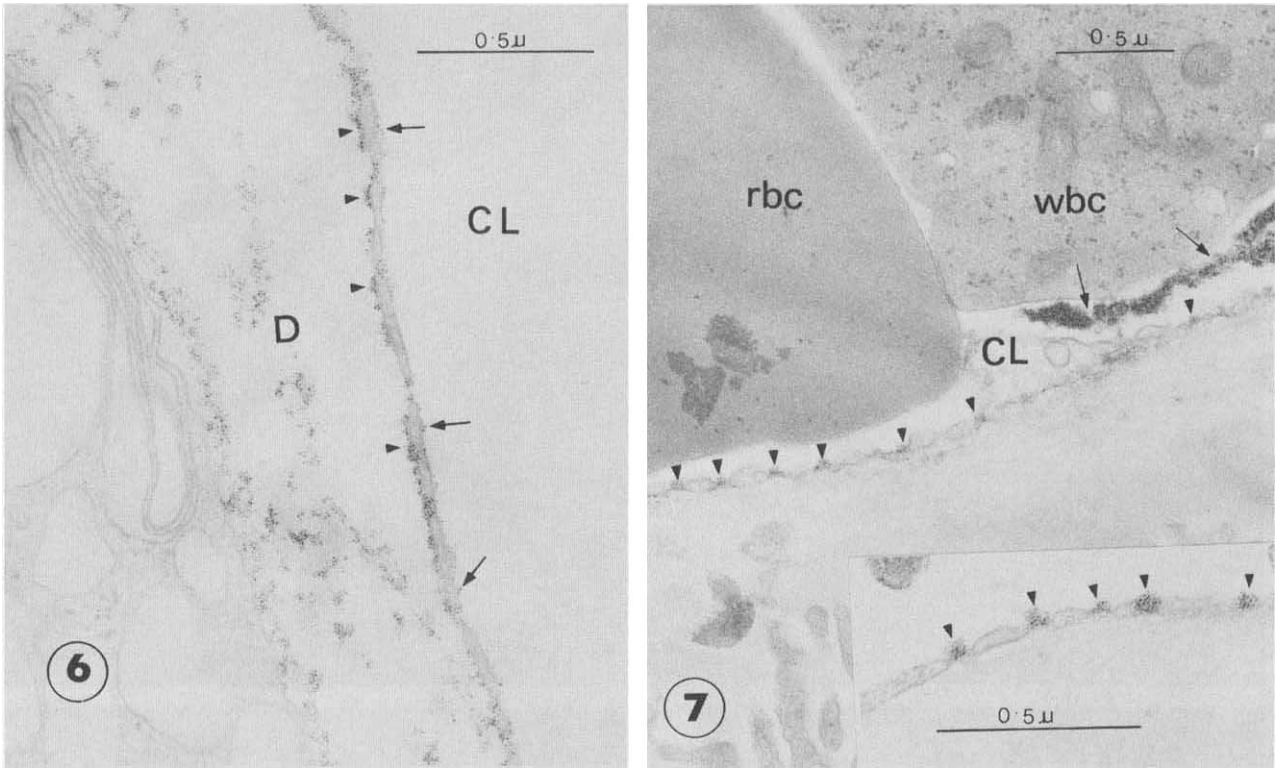

Fig. 6. *Digestion with papain to remove all the glycoproteins prevented CF binding to a large extent.* However, CF still binds to a few sites at the luminal surface in glycocalyx (arrows). Thus, a few sites in the glycocalyx at the luminal surface may be composed of glycolipids. Note the presence of CF in the basement membrane, the detachment of endothelial cells (D) and some binding of CF to the albuminal glycocalyx (arrowheads). Capillary lumen (CL) (×47,000).

Fig. 7. *Peritubular capillary from a rat given cationized ferritin directly into the aorta above the origin of renal artery.* Aggregation of CF within the lumen possibly with anionic serum proteins (arrows) is seen. Attachment of CF is seen only at the fenestral diaphragms (arrowheads) and not at the glycocalyx. Capillary lumen (CL) (×33,000). Inset in right lower part of the figure shows CF attached to fenestral diaphragms (arrowheads). (×55,000).

tively reduced the binding of CF at the fenestral surface and was associated with extravasation of albumin. Negative controls for the immunoperoxidase method are also shown in Figures 8B and 9B.

Discussion

Peritubular capillary permeability for macromolecules has been investigated in the past. Morphological studies utilizing tracers had concluded that peritubular capillary walls are highly permeable to macromolecules in the size range of albumin [18]. Subsequent physiological studies have shown that while peritubular capillaries are increasingly less permeable to neutral macromolecules (from 2 to 4 nm size), anionic macromolecules of comparable size are better retained [9]. Thus, peritubular capillary walls, like the walls of other capillaries, function as a size and a charge selective barrier. In capillaries of other organs with diaphragmed fenestrae, heparan sulfate proteoglycans are found predominantly at the luminal surface of fenestral diaphragms and form the majority of the anionic sites [11]. Our findings show that peritubular capillaries have a similar distribution of anionic sites at the luminal surface of fenestral diaphragms. The glycocalyx at the endothelial cell surface also contains anionic sites. These sites predominantly are from neuraminic acid containing anionic glycoproteins.

When injected directly into the aorta, CF attached only to fenestral diaphragms. Perfusion of CF into blood–free renal vasculature resulted in CF binding to both the endothelial cell glycocalyx and to the fenestral diaphragms. Several explanations can be advanced for the differences in the CF binding pattern when injected into aorta during free flowing conditions versus when CF is perfused following washing out of blood. Under free flowing conditions of aortic injection, although a higher dose of CF was injected (20 mg/100 g into aorta versus 7 mg/100 g body wt perfused intrarenally), the concentration of monomeric CF reaching in the peritubular capillaries may have been lower due to dilution with the blood and due to the binding of CF to anionic serum proteins as is shown in Figure 7. Also during undisturbed renal blood flow with aortic injection, shear forces may have been stronger than those during perfusion. Thus, CF at the anionic sites in endothelial glycocalyx may have been removed if the shear forces exceeded the electrostatic binding forces. These considerations suggest that the anionic sites at the fenestral diaphragms have a higher anionic charge or charge density as compared to the glycocalyx anionic sites. Lastly, capping and extrusion of the ligand has been demonstrated to occur at the endothelial cell surface [19]. All or some of these factors may have contributed to the selective binding of CF to the fenestral diaphragms when CF was given in the aorta.

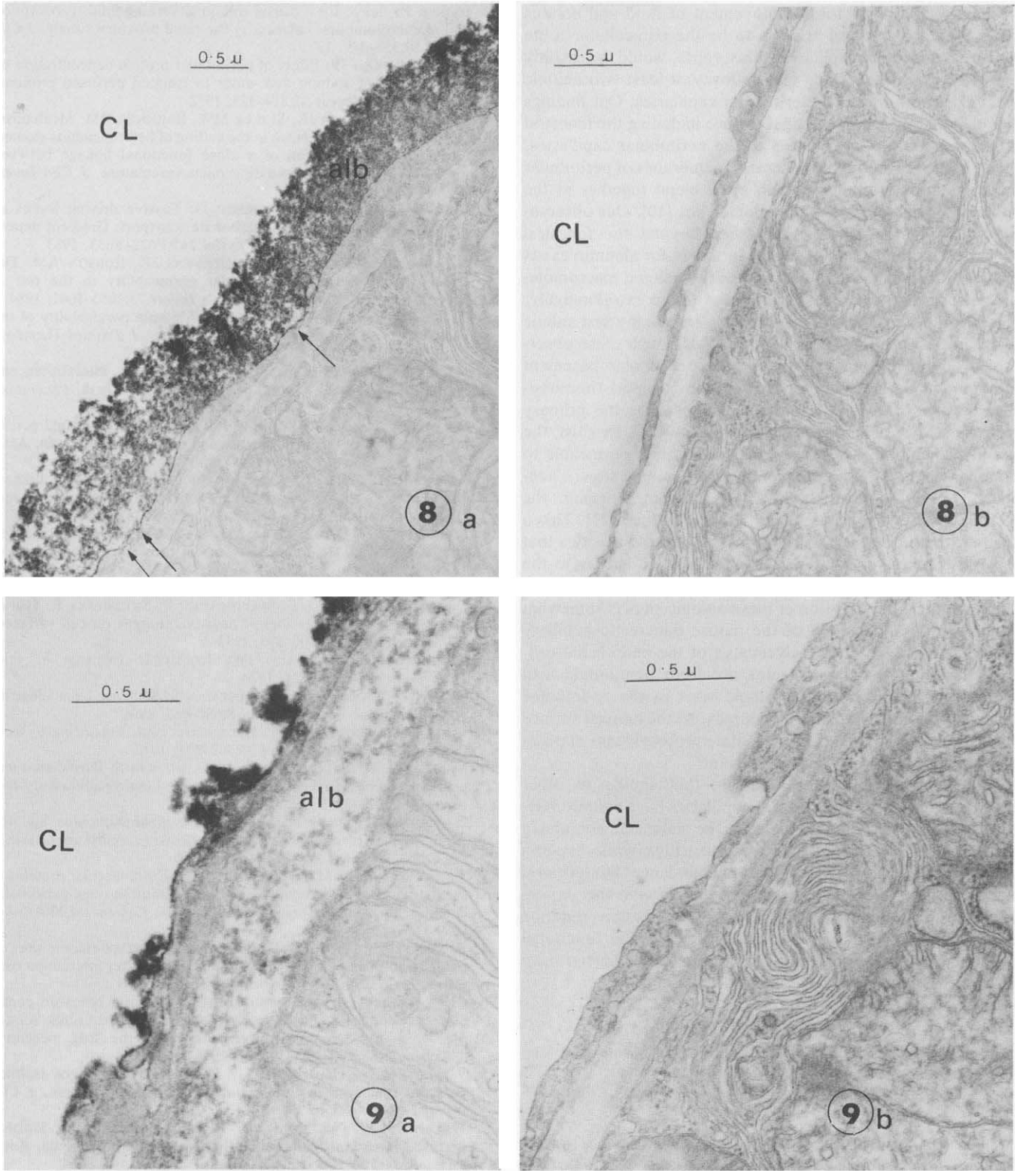

Fig. 8. *Peritubular capillaries showing distribution of albumin in normal rat.* **A.** Normal rat kidney: albumin (alb) is seen along the luminal surface of the endothelium. No reaction product is seen under the endothelium. Fenestral diaphragms indicated by arrows. Capillary lumen (CL) (×20,800). **B.** Normal rat kidney control, processed in parallel but without antibody. No reaction product is seen (×27,500).

Fig. 9. *Peritubular capillary showing distribution of albumin following heparitinase digestion.* **A.** Heparitinase treated kidney: reaction product is seen along the luminal surface. Less dark reaction product is also seen between endothelium and peritubular cells. **B.** Heparitinase treated kidney: control tissue processed without antibody. Note the absence of reaction product. Capillary lumen (CL) (both figures, ×38,000).

The major pathway for the movement of fluid and solutes across the capillary wall appears to be the paracellular route [20]. In fenestrated capillaries, this route would be mainly across the fenestrae. Along this pathway at least two anionic layers exist in the walls of peritubular capillaries. Our findings show that all through the luminal surface including the fenestral surface, an anionic layer exists in the peritubular capillaries. Others have shown that the basement membranes of peritubular capillaries and of tubules (which often blend together at the proximal tubules) also contain anionic sites [10]. Our observations show that albumin is not seen beyond the fenestral diaphragm and a high concentration gradient for albumin exists across the fenestra. Upon digestion of the anionic macromolecule with specific enzyme, albumin was found extraluminally. Thus, in vivo, the fenestral diaphragms serve as the first anonic barrier in the peritubular capillary wall. Although these observations do not exclude a role for the peritubular basement membrane as a barrier, other observations support the possibility that the basement membrane may not be the primary electrical barrier of the peritubular capillary wall. In vitro, the basement membranes of the tubules were quite permeable to serum albumin [21, 22], although they had anionic sites. Theoretical considerations dictate that to prevent clogging, the electrical barrier has to be at the luminal surface [23]. Taken together, these considerations are consistent with the idea that fenestral diaphragms serve as an initial anionic barrier to the intravascular albumin.

The chemical composition of these anionic sites is somewhat similar to the composition of the mouse pancreatic–capillary anonic charges [24]. In the glycocalyx of the endothelial cell, the majority of the anionic sites are from neuraminic acid residues. A minority of the anionic sites in the endothelial glycocalyx appear to be from glycolipids. At the luminal surface of fenestral diaphragms, heparan sulfate proteoglycans appears to be the major anionic macromolecule.

In summary, our findings indicate that similar to other fenestrated capillaries, peritubular capillaries have anionic macromolecules at their luminal surface. The sialic acid containing macromolecules are located in the glycocalyx while heparan sulfate containing macromolecules are abundant at the fenestral luminal surface. Our findings suggest that in vivo the proteoglycans at the luminal surface of fenestral diaphragms function as an electrical barrier of the capillary wall. The functional consequences of a reduction in the charge of this barrier have yet to be examined.

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