Electron microscopic histochemical and immunochemical analyses of heparan sulfate proteoglycan distribution in renal glomerular basement membranes

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Summary. Renal glomerular basement membranes (GBMs) exhibit a charge-selective barrier, comprised of anionic sites, that restrict the passage of anionic molecules into the urine. These sites are located primarily in the laminae rarae interna (LRI) and externa (LRE) of the GBM and consist of heparan sulfate proteoglycan (HSPG). Previous efforts to localize HSPG core protein within various layers of the GBM have been contradictory. In the present study when rat renal cortex blocks were treated by immersion with the cationic probe, polyethyleneimine (PEI), GBMs exhibited anionic sites concentrated primarily in the LRE and more irregularly within the LRI and lamina densa. All sites were heparitinase sensitive indicating that PEI positive sites represent negatively charged groups associated with heparan sulfate. In order to gain information on the distribution of the HSPG protein core, antibodies to HSPG from the EHS tumor matrix [anti-(EHS) HSPG] and GBMs [anti-(GBM) HSPG] were used together with immunogold to label thin sections of Lowicryl embedded kidney cortex. Depending upon the antisera used, markedly different distributions of HSPG were obtained. Immunolabelling with anti-(GBM) HSPG suggested a distribution of HSPG which was restricted to the laminae rarae, whereas labelling with anti-(EHS) HSPG indicated that the protein core penetrates through all layers of the GBM.

Key words: Heparan sulfate proteoglycan, Glomerular basement membrane, Anionic sites, Polyethyleneimine, Immunogold labelling

Introduction

Under normal conditions glomerular basement membranes (GBMs) exhibit a charge-selective barrier,

consisting of an array of anionic sites which are important for electrostatic retention of anionic plasma proteins in the circulation. Electron microscopic histochemical studies show that the anionic sites are concentrated within the lamina rara interna (LRI) and lamina rara externa (LRE) of the GBM where they are organized as a network of particles approximately 20 nm in diameter, distributed in a 60 nm repeating pattern (Kanwar and Farquhar, 1979a). Since these sites are abolished by pre-incubation with heparitinase, this pattern is widely accepted as the distribution of heparan sulfate proteoglycan (HSPG) within the GBM (Kanwar and Farquhar, 1979b). However, the localization of HSPG or anionic sites within the GBM is not resolved. Several investigators report that in addition to the 20 nm sites found within the laminae rarae, smaller anionic sites are distributed throughout the lamina densa (Vernier et al., 1983; Mahan et al., 1986; Carlson, 1987). Furthermore, while it is generally agreed that HSPG is present in the GBM, attempts to establish the localization of HSPG within the various layers (LRE, LRI, lamina densa) of the GBM have met with conflicting results. Immunolabelling of rat GBM with antibodies to the protein core of glomerular HSPG shows that the antigen is concentrated in the LRE and LRI where it corresponds closely to the distribution of the heparan sulfate side chains as detected with cationic probes (Stow et al., 1985; Kerjaschki et al., 1986; Lelongt et al., 1987). Similarly, antibodies against HSPG extracted from the EHS tumor and administered *in vivo* preferentially label HSPG in the laminae rarae as detected by immunoperoxidase (Mynderse et al., 1983). However, other investigators report that antibodies to the core protein from the EHS tumor localize HSPG predominantly in the lamina densa with less reactivity in the laminae rarae (Laurie et al., 1984; Grant and Leblond, 1988).

Conflicting results in the above mentioned experiments have been attributed to differences among HSPG antigens as well as immunolabelling technique. Furthermore, immunoperoxidase techniques have been criticized

for their unreliable results (Lelongt et al., 1987) and tendency for the diaminobenzidine reactions product to diffuse away from the reaction site and bind secondarily to negatively charged sites (Courtoy et al., 1983). Nonetheless, without properly characterizing the distribution of HSPG within the GBM, attempts have been made to evaluate the status of anionic sites (or epitopes on the HSPG protein core) in several disease states with conflicting results (Caulfield and Farquhar, 1978; Caulfield, 1979; Kanwar et al., 1981a,b; Vernier et al., 1983; Mynderse et al., 1983; Kanwar and Jakubowski, 1984; Furness et al., 1986; Rohrbach, 1986; Groggel et al., 1987; Lelongt et al., 1987). Thus it is the aim of the present study to clarify HSPG distributions within normal GBMs using two approaches: 1) The cationic probe, polyethyleneimine (PEI) is used to label GBM negative charges associated with heparan sulfate side chains because of its known high affinity for anionic sites in a number of tissues (Schurer et al., 1977, 1978) and, 2) Antibodies to HSPG core protein extracted from GBMs as well as the Engelbreth-Holm Swarm (EHS) tumor (Orkin et al., 1977) are used together with immunogold techniques to label thin sections of Lowicryl embedded rat kidney cortex.

The results of the current study suggest some possible molecular arrangements by which HSPG may interact with other glomerular capillary wall components to maintain a charge-selective barrier.

Materials and methods

Tissue preparation

For histochemical studies with PEI, adult male Sprague-Dawley rats (200-300 g) were anesthetized by an intraperitoneal injection of sodium pentobarbitol (0.75cc; 65 mg/ml), and the kidneys were perfused with approximately 20 ml of 0.9% NaCl. Kidneys were then removed, and pieces of cortex excised and minced to 1 mm^3. Tissue blocks were placed either in chilled cacodylate buffer or in 10 mM ethylenediaminetetraacetic acid (EDTA) in 0.1% sodium azide.

For immunohistochemical studies rats were overdosed with sodium pentobarbitol. Kidneys were surgically removed and cortical blocks cut to 0.5 mm^3. These were placed in 1 mM phosphate buffered saline (PBS).

Bovine kidneys were purchased from Federal Beef Processors, Inc., Fargo, ND and transported on ice to the laboratory. Renal tissues were minced to 0.5 mm^3 and placed in PBS.

Antisera and Antibodies

Antibodies to the large, low density HSPG from EHS tumor matrix (generously provided by Dr. John Hassell, Pittsburgh Eye and Ear Institute) were generated in rabbits and the resulting antiserum affinity purified in a column of HSPG bound to protein A sepharose. Bound antibodies were eluted with 4 M guanidine, dialyzed against PBS and lyophilized. These antibodies specifically bind the 400 kDa HSPG protein core and do not react with laminin, type IV collagen, or proteoglycans from

cartilage (Hassell et al., 1980; Ledbetter et al., 1987).

Antibodies to HSPG from rat renal glomeruli (generously provided by Dr. Yashpal Kanwar, Northwestern University) were raised against a high density glomerular HSPG (130 kDa) extracted from detergent purified GBMs with 4 M guanidine. The proteoglycan was further purified on a cesium chloride gradient (Kanwar et al., 1981a, 1984). Rabbits were immunized with the proteoglycan and an IgG fraction prepared. Antibodies to the proteoglycan were shown to bind to HSPG core protein (18 kDa) but not to normal serum, albumin, nor to collagenase, pronase and tryptic digests of the GBM using Ouchterlony double immunodiffusion assays (Makino et al., 1986).

Post-embedding labelling with Immunogold

Renal cortical tissue was processed for Lowicryl embedding and immunolabelling as described by Abrahamson (1986). Briefly, rat renal cortex blocks were fixed 2 hrs in 4% formaldehyde (0-4°C) and washed in 0.5 M NH$_4$Cl in PBS. Tissue was dehydrated in N, N-dimethylformamide and infiltrated at room temperature with Lowicryl K4M. Polymerization was carried out in the presence of UV light within a foil lined ice chest for 45 min - 1 hr at 4°C.

Thick sections were cut from Lowicryl blocks for selection of glomeruli for immunolabelling and ultrathin sections of these areas were picked up on uncoated 400 mesh nickel grids. At room temperature, grids with sections were incubated in 1 M NH$_4$Cl for 1 hr, rinsed with PBS, incubated in 0.1% BSA in PBS for 1.5 - 2 hrs, and washed again with PBS. Individual grids were then placed in BEEM capsules containing either anti-(EHS) HSPG (diluted 1:20 with 0.1% BSA in PBS), anti-(GBM) HSPG (diluted 1:80 with 0.1% BSA in PBS) or non-immune rabbit serum diluted 1:10 with 0.1% BSA in PBS and incubated 12 - 24 hrs at 4°C. Grids were washed with PBS and then incubated 2-3 hrs with goat anti-rabbit IgG-colloidal gold (10 nm gold, Janssen) diluted 1:3 with PBS at room temperature. Grids were washed thoroughly with PBS and finally with distilled, filtered water. Sections labelled with immunogold were counterstained 5 min with saturated aqueous uranyl acetate, washed and stained with lead citrate (Venable and Coggeshall, 1965) for 2 min. Grids were washed again in distilled water and examined at 80 kv with a JEOL 100S transmission electron microscope (TEM).

Indirect Immunofluorescence

Rat renal cortex blocks were snap-frozen in isopentane chilled with liquid nitrogen. Cryostat sections were incubated with anti-(EHS) HSPG, anti-(GBM) HSPG or non-immune serum at 37°C for 30 min. Following several rinses with PBS, the sections were reincubated with FITC-conjugated antirabbit IgG for another 30 min. The sections were again rinsed with PBS, covered with a drop of buffered glycerol, cover slipped, and examined with a microscope equipped for epifluorescence.

Staining with Polyethyleneimine (PEI)

The method of PEI staining was modified from that of Schurer et al. (1978). Tissues were washed in 0.1 M sodium cacodylate/HCl buffer, pH 7.3 (osmolality adjusted to 300 mOsm with sucrose), then immersed for 30 min in a 0.5% solution of PEI (1800 m.w., Polysciences) in 0.9% NaCl, pH 7.3. These were washed twice in cacodylate buffer and immersed 1 hr in 2% phosphotungstic acid (PTA) in cacodylate buffer (pH 7.3). Tissues were postfixed 2 hrs in 1% osmium tetroxide in cacodylate buffer, dehydrated through a series of graded ethanols and propylene oxide, and embedded in Epon 812/Araldite. Thick sections were cut with a glass knife and stained with toluidine blue for selection of areas for further study; thin sections were cut from these areas on a Dupont-Sorval MT2-B Ultramicrotome, stained with uranyl acetate and lead citrate as described above and examined in a JEOL 100S TEM at 80kv.

Enzymatic Techniques

Kidney cortical blocks (approximately 1 mm^3) were pre-incubated in either of the following enzyme solutions under the following conditions: Heparitinase (ICN Immunobiologicals, 0.1 unit/ml) in 0.1 M sodium acetate buffer, pH 7.0, at 43°C for 2 hrs (Linker and Hovingh, 1972) or Chondroitinase ABC (Sigma, 2 units/ml) in HBSS, pH 8.0, at 37°C for 2 hrs (Yamagata et al., 1968). These were washed with 0.1 M cacodylate buffer, pH 7.3, and incubated in PEI-1800 by the method described above. Controls consisted of cortical blocks simultaneously incubated in appropriate enzyme buffer but without enzyme, for the same length of time, followed by incubation in PEI.

Preparation of Acellular Tissue Blocks

Renal cortex blocks (1 mm^3) were made acellular by sequential detergent treatment as previously described (Carlson and Kenney, 1982). In brief, tissue was osmotically lysed with 10 mM EDTA (8-12 hrs, 4°C), followed by 3% Triton X-100 (6-8 hrs, 4°C), 0.025% deoxyribonuclease (DNAse; Sigma) in 1 M NaCl containing 1 mM phenylmethylsulfonyl fluoride (PMSF; 6 hrs, 4° C), and 4% deoxycholate (DEOC; 8 hrs, room temperature). All solutions except DNAse contained 0.1% sodium azide. Extensive distilled water rinses were carried out following each step and all treatments were carried out with continuous shaking.

Morphometry

Random glomerular capillary loops were photographed by TEM at 15,000X magnification and the number of gold particles over the GBM were counted on electron micrographic prints (X 38,000, final magnification). Guidelines for selecting sections of the GBM to be examined for each antigen were as follows:
1. Oblique sections through the GBM were excluded.

2. Only solid, single electron dense 10 nm gold particles were counted.
3. Overlapping particles or «doublets» were not included in counting.
4. Only straight lengths of GBM were examined. Curves in the GBM were considered uncountable.

Lengths of bovine GBM (10^3 nm) meeting the above lengtas criteria were divided lengthwise into fourths. The outermost fourth (subepithelial) was designated «LRE», the middle two fourths designated «lamina densa», and the innermost (subendothelial) fourth, «LRI». The average number of gold particles per 10^3 nm of GBM lengths was calculated in each region and defined as the absolute number of gold particles. The number of particles in the LRE and the LRI were summed and compared to the number of particles counted in the lamina densa. A single classification, Model I analysis of variance (ANOVA) was utilized to test for significant differences between different regions of GBMs. Any cited difference was significant at $p < 0.05$ or less.

Results

When fresh renal cortex blocks were stained by immersion in PEI followed by PTA, a pattern of particulate staining was seen associated with the GBM. Large sites (20 nm in diameter) were distributed along both laminae rarae, with heaviest concentration in the LRE in a 60 nm repeating pattern (Figs. 1, 2). In addition, a poorly defined array of smaller sites (5-10 nm) was detected within the lamina densa. When tissues were incubated in PTA alone, no anionic sites were detected in the laminae rarae, although glomerular epithelial and endothelial plasma membranes were uniformly stained (Fig. 3).

Ultrastructural preservation in these specimens was not exceptional, but adequate. This was not unexpected since aldehydes were omitted in the fixation steps. Despite this treatment, however, GBM structure was maintained and PEI staining was preserved. Occasionally PEI staining was poor or absent on sections taken near the surface of renal cortex blocks. Thus deeper sections were cut to reach areas where satisfactory PEI stain was retained.

In order to characterize PEI positive sites, tissues were digested with specific enzymes. In these studies, the pattern of PEI labelling was consistent for superficial and deep sections of tissue digested with either heparitinase of chondroitinase. When tissues were incubated in heparitinase followed by PEI, however, anionic sites were markedly decreased within the laminae rarae and lamina densa (Fig. 4). In contrast, numbers and distributions of anionic sites were unchanged following pre-incubation with chondrotinase ABC (Fig. 5) or in buffer without enzyme (Fig. 6).

When detergent purified GBMs were stained with PEI, regular arrays of 25 nm anionic sites could be clearly detected along the LRE with somewhat fewer, smaller sites scattered through the lamina densa and LRI (Fig. 7).

Table 1. Immunolabelling of bovine glomerular basement membrane for heparan sulfate proteoglycan.

	Anti-(EHS) HSPG (gold particles/1000nm GBM length ± SE)	Anti-(GBM) HSPG (gold particles/1000nm GBM length ± SE)
Laminae Rarae	3.67 ± 0.59 (1.93 ± 0.44 LRE 1.73 ± 0.49 LRI) (n = 15)	15.93 ± 2.14** (11.47 ± 1.80 LRE 4.46 ± 1.10 LRI) (n = 15)
Lamina Densa	3.73 ± 0.51 (n = 15)	6.73 ± 1.66 (n = 15)

** The difference between the average number of gold particles in the laminae rarae and lamina densa of the GBM following incubation with anti-(GBM) HSPG was highly significant $(0.01 > P > 0.001)$. LRE, lamina rara externa; LRI, lamina rara interna.

Immunofluorescence

By indirect immunofluorescence, immunolabelling of kidney cortex with anti-(GBM) HSPG resulted in a strong linear pattern of staining of the GBM, while tubular and Bowman's capsule basement membranes stained weakly (Fig. 8). Similar tissues labelled with anti-(EHS) HSPG resulted in a uniform pattern of staining of the GBM, as well as other cortical basement membranes (Fig. 9). No immunofluorescence was detected over renal basement membranes of controls incubated with non-immune serum and FITC-conjugated IgG (Fig. 10).

Immunoelectron Microscopy

Thin sections of formaldehyde fixed, Lowicryl embedded rat renal cortex blocks demonstrated relatively good ultrastructural preservation. Since tissue was embedded in Lowicryl without passage through osmium tetroxide or any *en bloc* stains, contrast was often enhanced by staining with saturated aqueous uranyl acetate followed by lead citrate (Venable and Coggeshall, 1965).

When ultrathin sections of rat kidney cortex were labelled with anti-(GBM) HSPG followed by immunogold, gold particles localized HSPG predominantly to the LRI and LRE of the GBM (Fig. 11), in contrast to minimal staining in controls (Fig. 13).

In contrast to anti-(GBM) HSPG, staining of rat cortex with anti-(EHS) HSPG and immunogold showed gold particles distributed throughout the entire thickness of the GBM (Fig. 12).

Quantitation of Gold Particles

To facilitate quantitation of gold particle distributions in GBMs, bovine kidney cortex was utilized since GBMs in this species are considerably thicker (250-320 nm) than rat (approximately 136 nm). For quantitation purposes, the LRE and LRI were defined as the outer and innermost fourths of the total width of the GBM (60-80 nm) while the middle two fourths of the

GBM (125-160 nm) was designated as lamina densa. The working concentration of anti-(EHS) HSPG remained the same for labelling bovine cortex as for rat tissue (1:20). However, the concentration of anti-(GBM) HSPG was increased from 1:80 to 1:20 in order to appropriately label bovine cortex.

Despite a somewhat higher background labelling with anti-(GBM) HSPG, aggregates of gold particles were preferentially distributed along the LRE and LRI of the bovine GBM in a regular repeating pattern (Fig. 14). When bovine GBM was labelled with anti-(EHS) HSPG followed by immunogold, gold particles were non-specifically localized to all GBM layers (Fig. 15). To confirm this observation, counts of gold particles per 10^3 nm lengths of bovine GBMs showed that on sections treated with anti-(GBM) HSPG the total number of particles in the LRI and LRE (x = 15.93) was highly significantly different from the number of particles in the lamina densa (x = 6.7), p < 0.01 (Table 1). When anti-(EHS) HSPG was used as the primary antibody for the immunogold procedure, the number of particles in the laminae rarae was not significantly different from the number in the lamina densa (x = 3.67 and 3.73, respectively).

Discussion

Characterization of Anionic Sites

Numerous investigators have used electron dense cationic probes to localize anionic sites associated with GBMs (Rennke et al., 1975; Caulfield and Farquhar, 1976; Schurer et al., 1978; Caulfield, 1979; Kanwar and Farquhar, 1979a; Hunsicker et al., 1981). In the current study, immersion staining of whole renal cortical blocks with PEI was carried out to demonstrate characteristic patterns of anionic sites. These were distributed along the laminae rarae in regular repeating patterns similar to those previously demonstrated (Behnke and Zelander, 1970; Caulfield and Farquhar, 1976; Caulfield, 1979; Kanwar and Farquhar, 1979a). Since this staining pattern is abolished with heparitinase pre-incubation, but not with chondroitinase, we conclude that the PEI
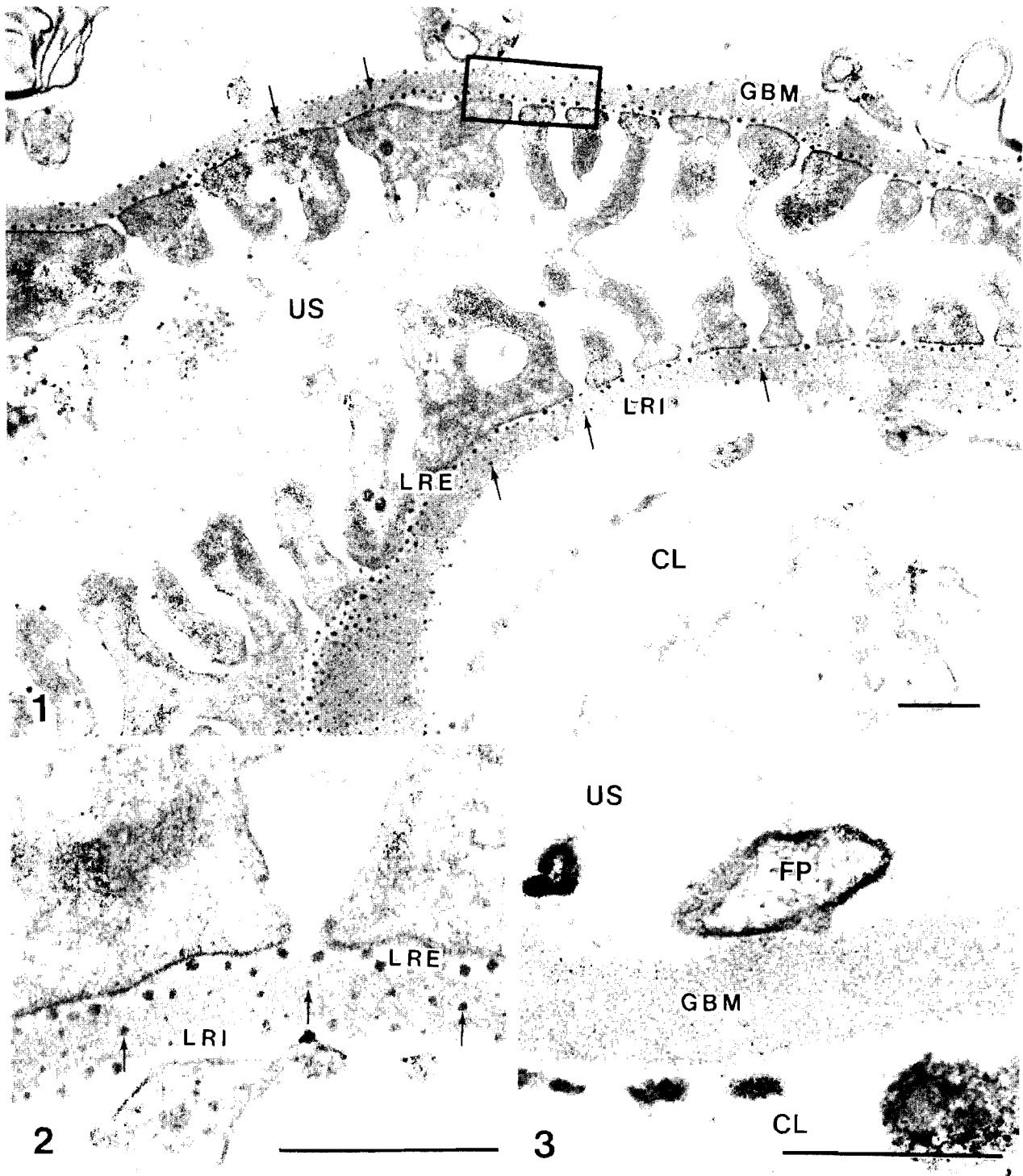
Fig. 1. Portion of glomerulus from rat kidney stained with PEI. Glomerulus basement membrane (GBM) shows pattern of particulate staining indicating charged sites on the lamina rara externa (LRE), lamina rara interna (LRI) and lamina densa (arrows). CL, capillary lumen; US, urinary space. Rectangle indicates area similar to that shown in Fig. 2. Original magnification × 26,400. Bar = 0.5 μm.

Fig. 2. Higher magnification of glomerular capillary wall in region similar to that shown in rectangle (Fig. 1). PEI positive sites are distributed regularly along the lamina rara externa (LRE) and more irregularly along the lamina rara interna (LRI) and lamina densa (arrows). Original magnification × 73,000. Bar = 0.5 μm.

Fig. 3. Electron micrograph of portion of glomerulus stained with 2% PTA only. There is complete absence of electron densities within the glomerular basement membrane (GBM). FP, foot process; CL, capillary lumen; US, urinary space. Original magnification × 73,000. Bar = 0.5 μm.

Basement membrane proteoglycan ultrastructure
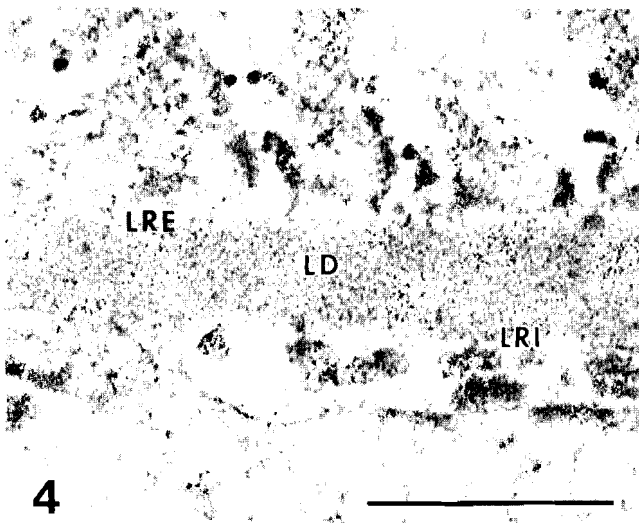

4

5
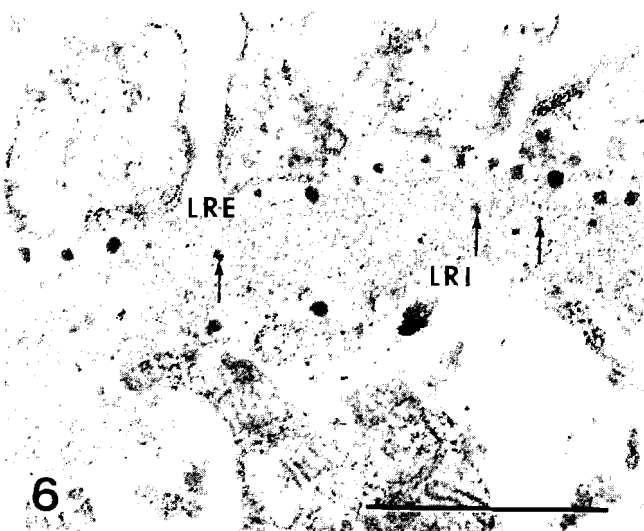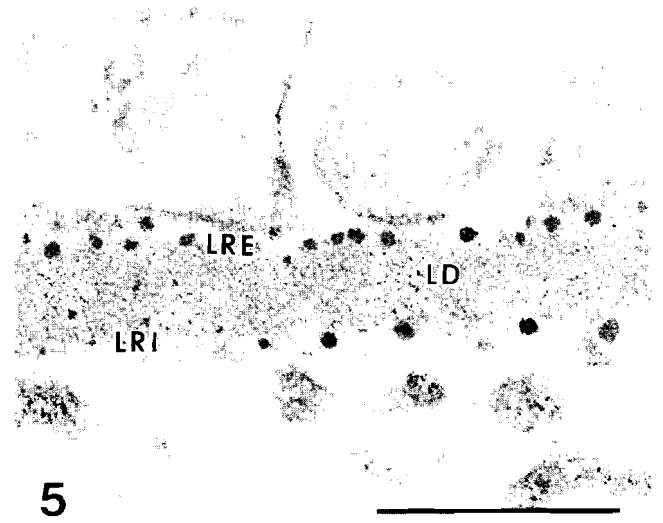
Fig. 4. Portion of renal glomerulus incubated 2 hrs with heparitinase in 0.1 M acetate buffer, followed by incubation in PEI. Numbers of stained anionic sites in the laminae rarae externa (LRE) and interna (LRI), as well as in the lamina densa (LD) are substantially reduced. Original magnification × 75,000. Bar = 0.5 μm.

Fig. 5. Small field of a glomerular capillary incubated 2 hrs with chondroitinase ABC in HBSS followed by incubation in PEI. PEI-stained particles are present in the lamina rara externa (LRE), lamina densa (LD) and lamina rara interna (LRI) of the glomerular basement membrane indicating they were not removed by this treatment. Original magnification × 75,000. Bar = 0.5 μm.

Fig. 6. Portion of control renal glomerulus incubated in 0.1 M sodium acetate buffer, followed by incubation in PEI. PEI-stained sites are seen on laminae rarae externa (LRE) and interna (LRI), as well as lamina densa (arrows). Original magnification × 75,000. Bar = 0.5 μm.

Fig. 7. Portion of a glomerular capillary incubated with EDTA, Triton, DNAse containing PMSF and 9 hrs with 4% DEOC, followed by staining with PEI. At the end of the detergent procedure, the glomerular basement membrane (GBM) is acellular. However, the PEI positive sites can be seen regularly distributed along the lamina rara externa (LRE) and more irregularly along the lamina rara interna (LRI) and lamina densa. CL, capillary lumen; US, urinary space. Original magnification × 41,250. Bar = 0.5 μm.
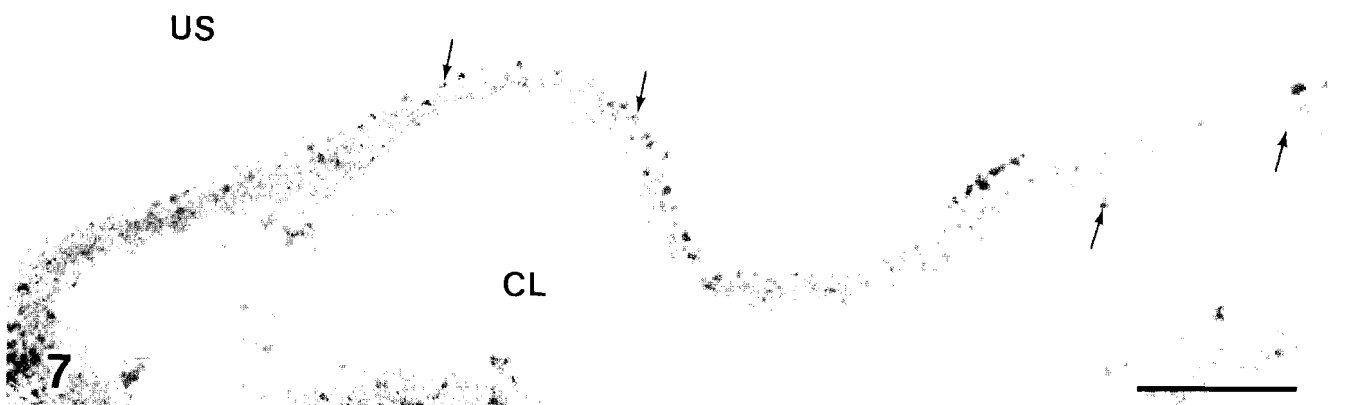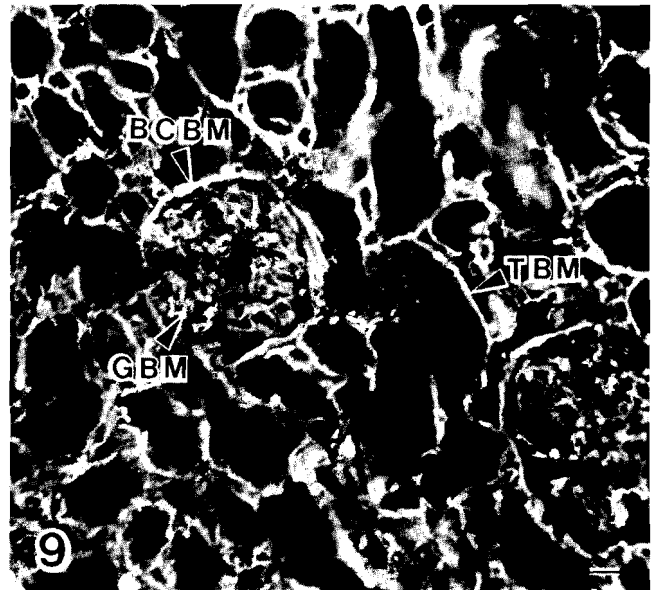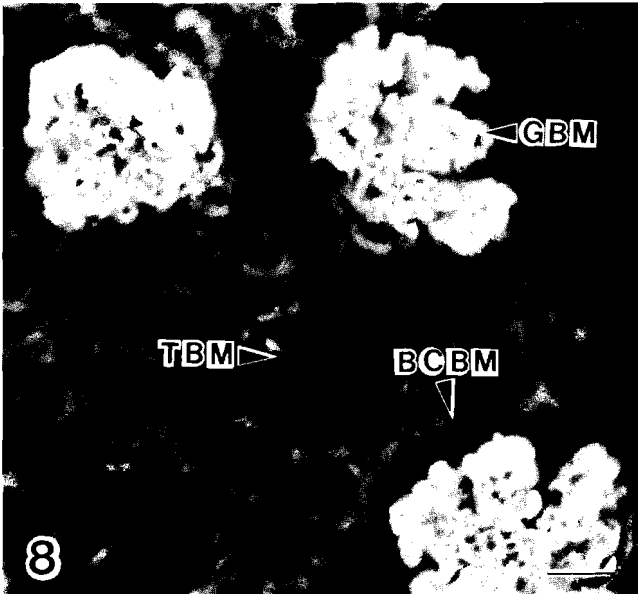
Fig. 8. Indirect immunofluorescent microscopy of rat renal cortex incubated with anti-(GBM) HSPG followed by reincubation with FITC-conjugated anti-rabbit IgG. A linear fluorescence with anti-(GBM) HSPG is predominant over glomerular basement membranes (GBM). A somewhat weaker pattern of staining is present over tubular (TBM) and Bowman's capsule basement membranes (BCBM). Original magnification × 363. Bar = 20 μm.
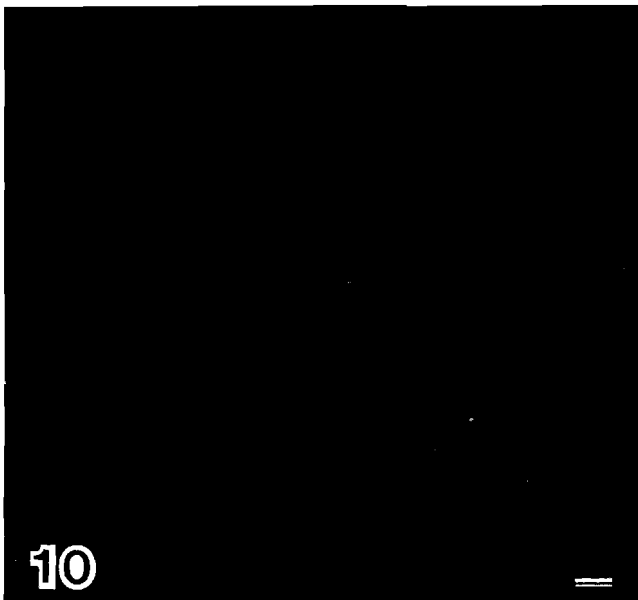
Fig. 9. Indirect immunofluorescent microscopy of rat renal cortex incubated with anti-(EHS) HSPG followed by reincubation with FITC-conjugated anti-rabbit IgG. The fluorescence is uniformly distributed over all renal basement membranes. GBM, glomerular basement membrane; TBM, tubular basement membrane; BCBM, Bowman's capsule basement membrane. Original magnification × 227. Bar = 20 μm.

Fig. 10. Indirect immunofluorescent microscopy of rat renal cortex incubated with normal rabbit serum followed by reincubation with FITC-conjugated anti-rabbit IgG. Original magnification × 227. Bar = 20 μm.

Fig. 11. Portion of a glomerulus from rat kidney fixed with 4% formaldehyde, embedded in Lowicryl and sequentially labelled with rabbit anti-(GBM) HSPG and colloidal gold-anti-rabbit IgG (immunogold). 10 nm gold particles are bound primarily over lamina rara externa (LRE) and lamina rara interna (LRI) of the glomerular basement membrane (GBM). FP, foot process; CL, capillary lumen. Original magnification × 39,375. Bar = 0.05 μm.
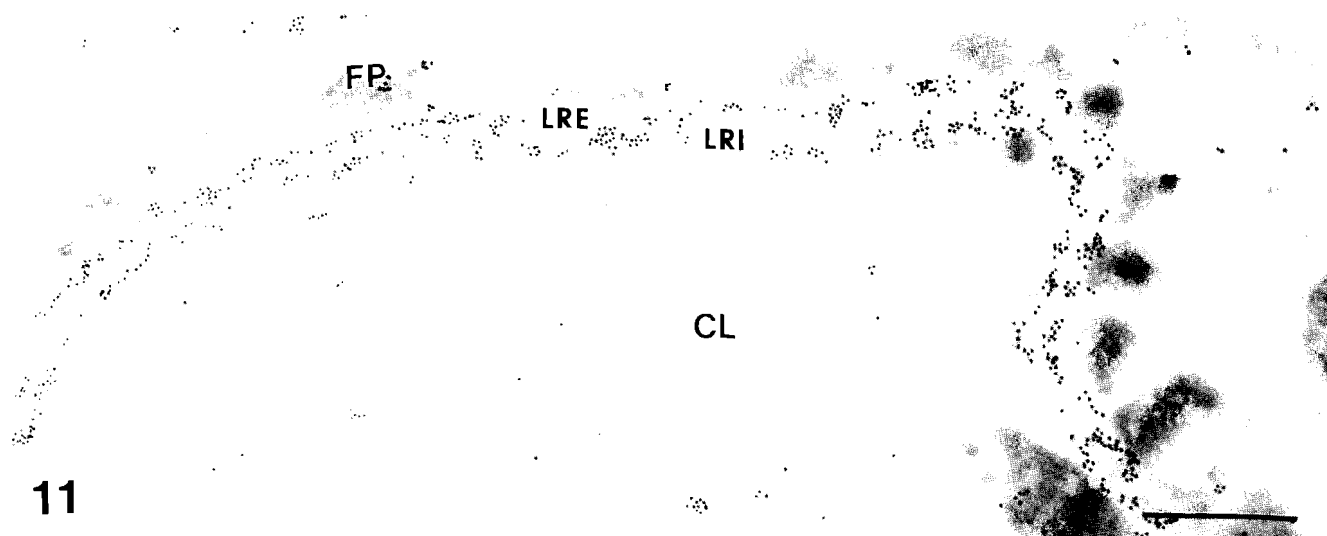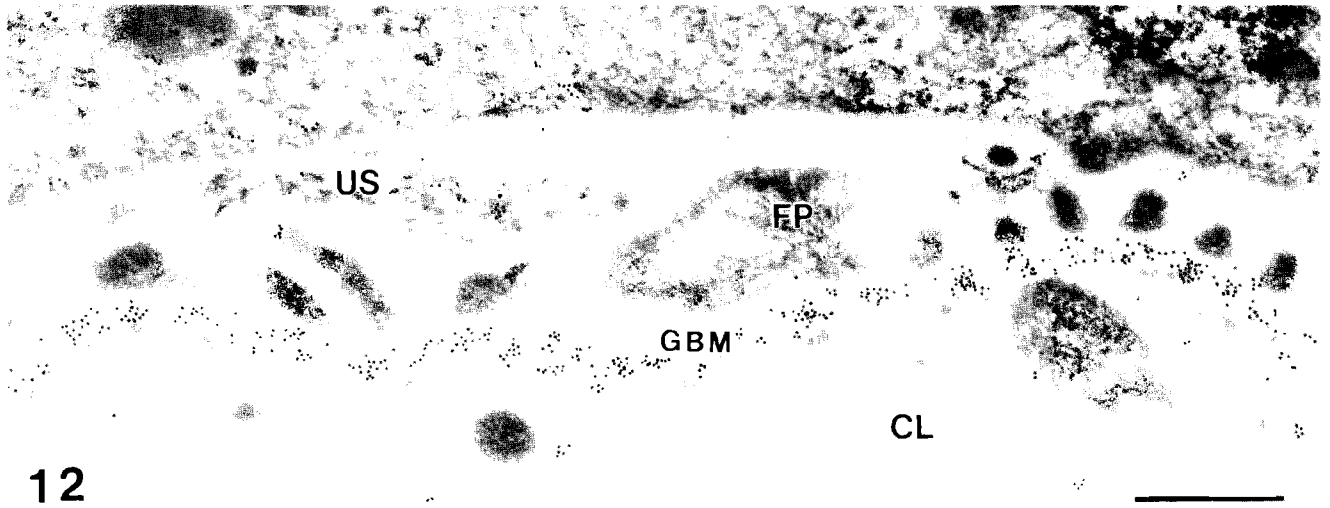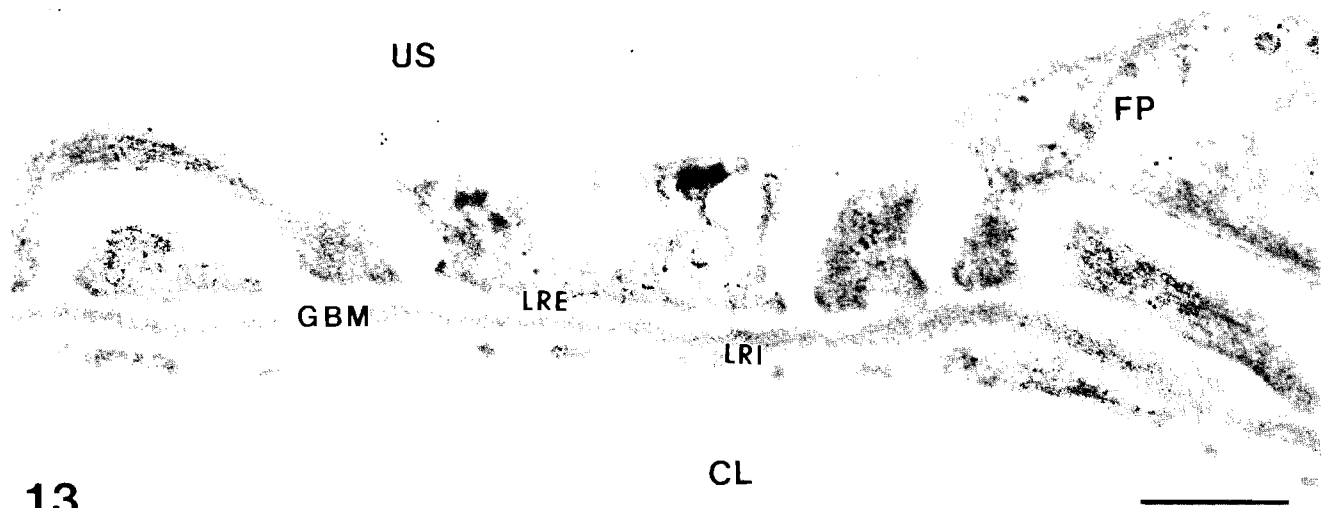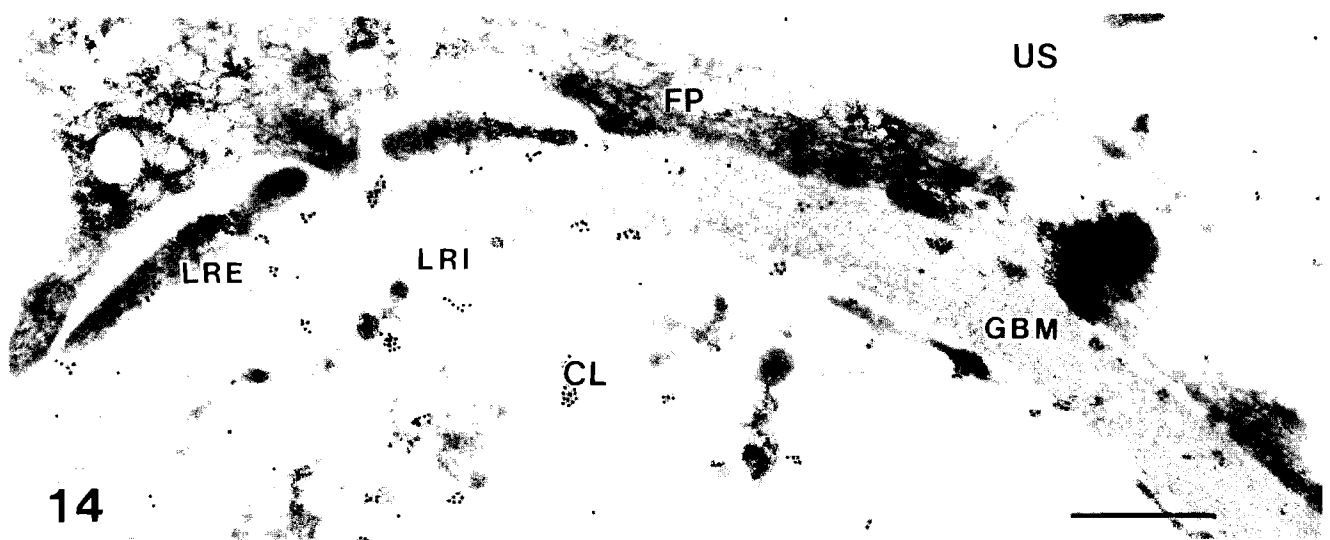
Fig. 12. Electron micrograph of portion of rat renal glomerulus from Lowicryl section sequentially labelled with rabbit anti-(EHS) HSPG and immunogold. Gold paticles are distributed across the entire thickness of the glomerular basement membrane (GBM). FP, foot process; US, urinary space; CL, capillary lumen. Original magnification × 39,375. Bar = 0.5 μm

Fig. 13. Lowicryl section of a glomerular capillary wall labelled with non-immune rabbit serum followed by immunogold. LRE, lamina rara externa; LRI, lamina rara interna; FP, foot process; US, urinary space; CL, capillary lumen. Original magnification × 39,375. Bar = 0.5 μm

Fig. 14. Portion of glomerulus from bovine kidney fixed with 4% formaldehyde, embedded in Lowicryl and labelled with rabbit anti-(GBM) HSPG followed by immunogold. Aggregates of gold particles are present detected at regular intervals along the lamina rara externa (LRE) and less regularly along the lamina rara interna (LRI) of the glomerular basement membrane (GBM). FP, foot process; US, urinary space; CL, capillary lumen. Original magnification × 39,375. Bar = 0.5 μm.
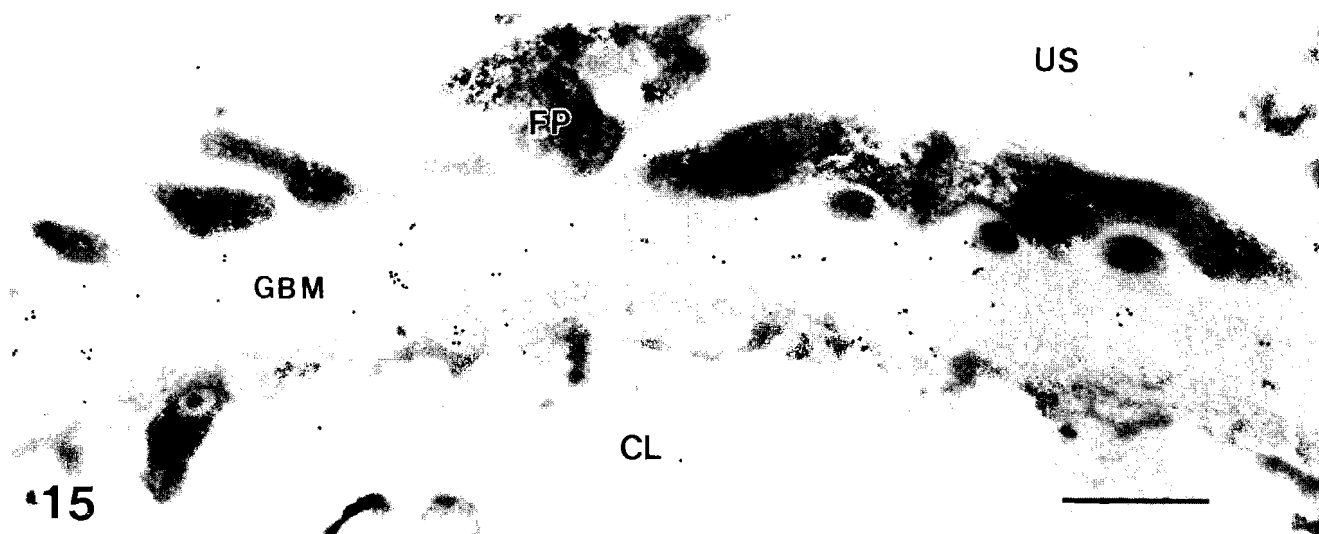
Fig. 15. Lowicryl section of a glomerular capillary wall from bovine kidney treated sequentially with rabbit anti-(EHS) HSPG followed by immunogold. Gold particles are present across the entire thickness of the glomerular basement membrane (GBM). FP, foot process; US, urinary space; CL, capillary lumen. Original magnification ✗ 39,375. Bar = 0.5 µm.
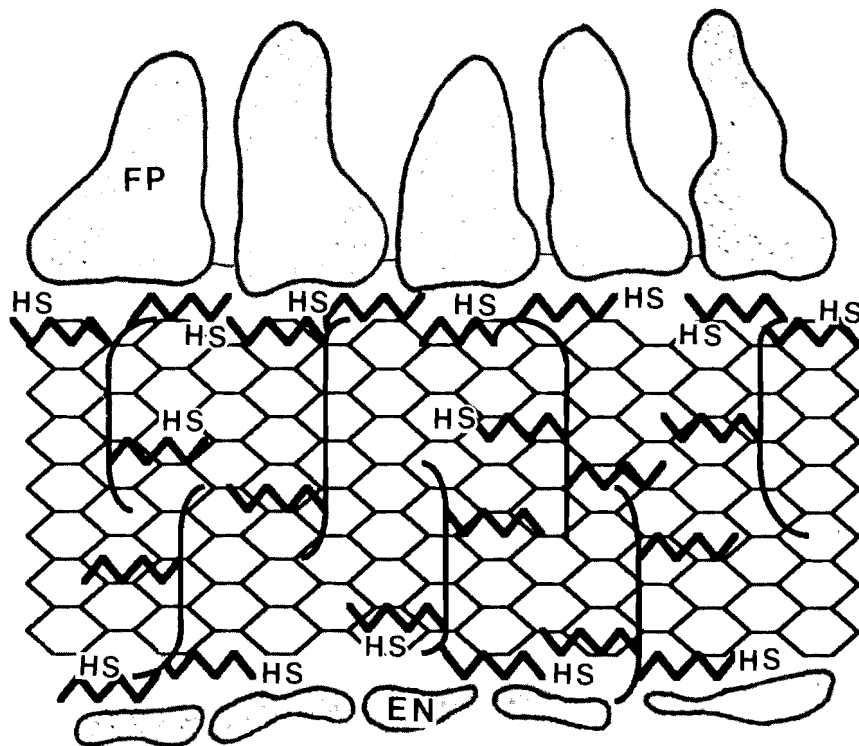

Fig. 16. Schematic representation of the proposed arrangement of HSPG within the glomerular basement membrane (GBM) based on electron microscopic histochemical and immunochemical studies. The core protein penetrates into the lamina densa of the GBM. Extending from one end of the core are the predominant heparan sulfate side chains (HS), located in the laminae rarae. Additional heparan sulfate side chains may extend from portions of the core protein in the lamina densa, as suggested by PEI staining of anionic sites in this region. FP, foot process; EN, endothelium.

study because their use resulted in inconsistent staining of anionic sites. It is possible that this may be due to alteration of charged (amino and carboxyl) groups within tissues as suggested by Furness et al. (1986). It has been suggested that aldehyde fixation may cause GBMs to contract and pull away from cell membranes, leading to condensation of cell surface GAGs and increased density of GBMs (Goldberg and Escaig-Haye, 1986). These factors may partially explain the improved PEI staining of anionic sites observed in the present study in the absence of aldehydes.

In the current study, PEI positive sites could be detected on detergent extracted GBM, similar to the distribution of sites seen on cellular tissue, indicating that PEI labels negative charges on HSPG which are integral to the GBM. However, inclusion of 1 mM PMSF in the endonuclease step was necessary to insure adequate PEI labelling in purified specimens. It is possible,

positive sites localize negative charges associated with heparan sulfate. In addition, an irregular distribution of smaller PEI-positive sites was detected within the lamina densa which was also heparitinase sensitive. These smaller sites have also been reported by other investigators (Vernier et al., 1983; Mahan et al., 1986).

Aldehyde fixatives were omitted in the current

therefore, that negatively charged GBM components may be particularly sensitive to non-specific proteases which are known to contaminate DNAse preparations.

Immunolocalization of HSPG within the GBM

Using immunohistochemical methods, at both the light and electron microscopic levels, HSPG has been shown to be present in the GBM and in other basement membranes. However, attempts to utilize immunoelectron microscopy to establish the localization of HSPG more precisely within the various layers (LRI, LRE, lamina densa) of the GBM have met with disagreement. In an effort to clarify the localization of the HSPG protein core, the current comparative immunohistochemical study was undertaken utilizing antibodies specific for the 18 kDa core protein of HSPG extracted from rat GBM (Makino et al., 1986) and antibodies to the 400 kDa core protein of HSPG from the EHS tumor (Hassell et al., 1980).

The results from this study show that depending upon the source of primary antibody used in the immunolabelling procedure, two distinctly different distributions for HSPG within the GBM can be obtained. When thin sections of Lowicryl embedded rat kidney cortex were incubated in anti-(GBM) HSPG, followed by immunogold, gold particles bound primarily to laminae rarae. This laminae rarae labelling pattern was also evident in bovine GBMs where morphometric analysis demonstrated that the number of gold particles in the laminae rarae was significantly greater than the number in the lamina densa.

On the other hand, immunolabelling with anti-(EHS) HSPG resulted in a pattern of gold particles evenly distributed throughout rat GBM layers (LRE, LRI and lamina densa). Quantitation of gold particles on bovine GBM confirmed this observation, which indicated that the number of gold particles in the laminae rarae was not significantly different from the number in the lamina densa. Previous studies, using antibodies to a 200 kDa portion of the EHS protein core lacking heparan sulfate side chains (P200) localized HSPG primarily in the lamina densa (Laurie et al., 1984; Grant and Leblond, 1988). However, antibodies used in the present study recognized epitopes all along the length of the 400 kDa HSPG core protein.

Glomerular HSPG has been localized previously to the laminae rarae by cationic probes (Kanwar and Farquhar, 1979b) and by several immunohistochemical investigations (Mynderse et al., 1983; Stow et al., 1985; Lelongt et al., 1987). However, as indicated previously, other investigators have identified HSPG within the lamina densa (Laurie et al., 1984; Grant and Leblond, 1988). Furthermore, under optimal conditions, 4.5-5.0 nm paired rods, called «double tracks» which are suspected to represent HSPG can be identified extending indefinitely throughout the thickness of the GBM. These double tracks resemble the arrangement of purified HSPG from the EHS

tumor when incubated at 37° C (Inoue et al., 1987) and have been shown to stain positively with antibodies to EHS HSPG in Reichert's membrane (Inoue and Leblond, 1988) and the GBM (Inoue et al., 1989).

Rotary shadowing of the EHS proteoglycan reveals an 80 nm protein core and two to three side chains of length 146 ± 53 nm attached to one end of the core (Laurie et al., 1988). If oriented so the side chains project in the laminae rarae as suggested by anionic site studies, the protein core might penetrate deep into the lamina densa, possibly extending completely through the GBM. In the present study, data from immunolabelling of GBM with antibodies to the EHS 400 kDa protein core supports this hypothesis (Fig. 16). Immunolabelling with glomerular antibodies, however, suggests that the protein core may be present only in the laminae rarae. Data from our lab and others (Makino et al., 1986) suggest that the HSPG protein core is sensitive to proteolysis during extraction procedures from GBM. Therefore, it seems possible that the high density HSPG fraction collected from GBM primarily contains N-terminal regions of a larger protein core. Accordingly, antibodies to this HSPG fraction would recognize epitopes on the protein core at the N-terminus, close to the site of attachment of the heparan sulfate side chains, which are located primarily along the LRE and LRI. Alternatively, two genetically distinct HSPGs may be present in GBMs: one population located in the laminae rarae cross reacts with anti-(GBM) HSPG, while another population located throughout the entire thickness of the GBM cross reacts with anti-(EHS) HSPG. Further studies on the relationship between the glomerular and EHS precursor proteins are needed to resolve this problem.

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