

Fibronectin Localization in the Rat Glomerulus

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ABSTRACT Fibronectin (FN) has been localized in the rat glomerulus using indirect immunolabeling. It was demonstrated in frozen sections by immunofluorescence, in sections of fixed kidneys by both peroxidase and ferritin-labeled antibodies, and in isolated glomerular basement membranes (GBM) with ferritin-labeled antibodies. Complementary and convergent results were obtained with these approaches. FN was most abundant in the mesangial matrix where it was especially concentrated at the interface between the endothelial and mesangial cells. In the peripheral capillary loop, FN was also detected in the laminae rarae (interna and externa) of the GBM — i.e., between the endothelial and epithelial cells, respectively, and the GBM. These findings indicate that FN is an important constituent of the glomerulus, and they are compatible with the assumption that, in the glomerulus, as in cultured cells, FN is involved in cell-to-cell (mesangial-mesangial, mesangial-endothelial) and cell-to-substrate (mesangial cell-mesangial matrix, epithelium-GBM, endothelium-GBM) attachment.

Fibronectin (FN) (22) is a glycoprotein associated with fibrillar elements on the cell surface (11) and in the extracellular matrix (3, 14, 33) which is involved in cell-to-cell and cell-to-substrate attachment (36). A similar, immunologically indistinguishable protein known as cold-insoluble globulin (CIG) (37), or plasma fibronectin, is found circulating in the blood.

FN appears to be a major connective tissue protein, widely distributed in normal tissues (34). A close alignment or codistribution has been noted between extracellular fibronectin and intracellular actin (15, 33), and the possibility of a transmembrane linkage between the two has been proposed (15). Thus, a complex network¹ involving actin, FN, and probably other components (e.g., proteoglycans [7, 30]) has been envisaged which connects adjacent cells, mediates cell anchorage to the extracellular matrix, and transmits deformation generated by the contractile cells. Because the glomerulus is under a high and variable hydrostatic pressure, it seemed possible that such a network may operate to maintain glomerular integrity and to regulate its blood flow (28). By immunofluorescence, FN has already been localized in the mesangium of newborn (24), adult (5, 29, 31, 32, 34, 38), or diseased glomeruli (25, 31, 32). By electron microscopy, a prominent fibrillar material is observed in the mesangial matrix (MM) (9), the nature of which is unknown. Some investigators described immunoreactivity for fibronectin (5, 29, 31, 32) in the peripheral regions of the capillary loops, whereas others reported that it was absent (25, 34, 38). In these regions, an extensive fibrillar network is also

observed in the laminae rarae of the glomerular basement membrane (GBM), which appears to anchor the epithelial and endothelial cells to the lamina densa (LD). The nature of these fibrils is still unknown, but their attachment to the GBM appears to depend on the presence of sialic acid because removal of sialic acid (by neuraminidase treatment) results in their detachment (19). Based on these findings and the fact that it is a sialoprotein (36), FN appeared to be a good candidate to be involved in attachment of cells to the GBM, and, accordingly, we decided to determine the localization of FN in the normal glomerulus by immunocytochemistry.

MATERIALS AND METHODS

Preparation of Tissues

Male Charles River CD^H rats, weighing 100–150 g, were used. Both kidneys were perfused by the route described previously (19) and flushed with saline at room temperature. One kidney was removed, and the cortex was sliced and immediately frozen in chilled isopentane. The other kidney was fixed *in situ* by perfusion for 3 min with Karnovsky's fixative (3% glutaraldehyde and 1.4% formaldehyde in 0.1 M cacodylate, pH 7.4), followed by perfusion with 0.1 M Tris-HCl, pH 7.4, to end fixation and to quench aldehyde groups. The cortex was then sliced, incubated in Tris-buffered saline containing 10% dimethyl sulfoxide at 4°C for 1 h, and frozen in chilled isopentane. Isolated GBM were prepared as described previously (18).

Antibodies

Antisera specific for FN were prepared against SDS-gel-purified FN from hamster cells (27). For preparation of absorbed antiserum and of affinity-purified anti-FN, hamster cell FN purified by gelatin affinity chromatography (8) was conjugated to CNBr-activated Sepharose. Anti-FN serum was passed through

¹ This network has been referred to as a "fibronexus" (33).

the column to remove all antibodies against FN. Immunoglobulins specific for FN were then eluted with 5 M NaI plus 10 mM sodium thiosulphate. Immunofluorescence testing on NIL8 hamster cells confirmed removal of anti-FN antibodies from the absorbed serum and recovery of affinity purified antibodies (15).

Antisera were used at a dilution of 1:50 in phosphate-buffered saline containing 5% bovine serum albumin (PBSA). Affinity-purified antibodies were used at 20 $\mu\text{g}/\text{ml}$ in PBSA. Before use, these reagents were centrifuged at 234,000 g for 2 h.

As second reagent, either Fab² fragments of goat anti-rabbit IgG conjugated to rhodamine or ferritin (Cappel Laboratories, Inc., Cochranville, Penn.) or Fab fragments of sheep anti-rabbit IgG conjugated to horseradish peroxidase (HRP) (Institut Pasteur, Marnes La Coquette, France), was used. All were applied at a dilution of 1:50 in PBSA after a 2-min centrifugation in a microfuge (8,000 g).

Immunocytochemistry

For immunofluorescence, 2 μM cryostat sections of unfixed kidneys were incubated as described (27), mounted in buffered glycerol, and examined with a Zeiss photomicroscope II equipped with epifluorescence optics.

For immunoperoxidase, cryostat sections (8–10 μm) of fixed kidneys were cut and quickly thawed in PBSA. Subsequent incubations of sections were performed in suspension to improve antibody penetration. Sections were transferred to the first antiserum and incubated for 1–2 h at room temperature on a rotating wheel after which they were washed three times in a large volume of phosphate-buffered saline containing 0.1% bovine serum albumin. They were then incubated in the peroxidase conjugate for 1–2 h, washed three times, incubated in diaminobenzidine (DAB)-containing medium for 1–15 min (12), rinsed, postfixed in OsO_4 , and flat embedded in Epon. After polymerization, areas rich in glomeruli were selected under a dissecting microscope, and cut out and glued upon prepolymerized Beem capsules. Silver sections were cut, stained with lead citrate, and examined in a Philips 301 electron microscope.

For immunoferritin, both cryostat sections and isolated GBM were used. Incubations were carried out first in the anti-FN antisera and then in the ferritin conjugate for 1–2 h at room temperature in suspension with extensive rinsing after each step. Sections were subsequently fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer, postfixed in OsO_4 , dehydrated, and flat embedded. Isolated GBM were fixed in suspension with glutaraldehyde, pelleted in a microfuge, and processed as described previously (17).

Controls

IMMUNOLOGICAL CONTROLS: Controls consisted of incubations in which (a) the first antiserum was omitted or replaced by either normal rabbit serum (or IgG) or immune serum previously absorbed with pure FN; or (b) the second reagent was replaced by Fab fragments of sheep antibodies against mouse IgG coupled to HRP (Institut Pasteur) or by HRP alone; or (c) the second reagent was replaced by goat anti-mouse IgG coupled to ferritin (Cappel Laboratories, Inc.) or by ferritin alone. In addition, incubations were carried out with affinity purified antibodies to FN (27) (instead of whole serum).

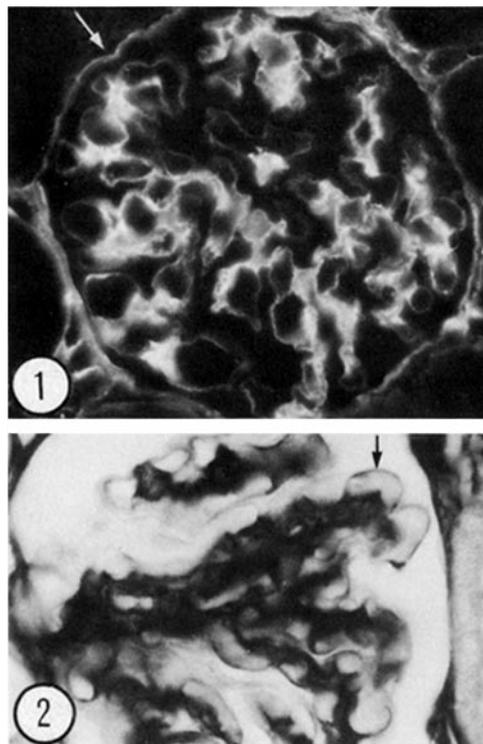
ELECTROSTATIC CONTROLS: To exclude the possible interaction between positively charged immunoglobulins and the anionic sites of the glomerulus (17), we incubated some sections with antibodies over a broad range of pH values (from 6 to 8.5), or in a high ionic strength environment (up to 1 M NaCl).

CONTROLS FOR PLASMA PROTEINS: Adjacent sections were incubated (as first reagent) with a monospecific antiserum against rat albumin (courtesy of Dr. C. Lombart).

RESULTS

By light microscopy, sections incubated with anti-FN antiserum followed by either rhodamine (Fig. 1) or peroxidase (Fig. 2) conjugates produced similar results; however, localization was more precise with the peroxidase procedure. In both cases, the mesangium was heavily labeled, especially where it faces the endothelium. The peripheral capillary loop showed a weaker but definitive linear labeling. Bowman's capsule was also discontinuously labeled (Fig. 1).²

² The same results were also obtained by immunofluorescence when affinity-purified antibodies prepared by others against FN (K. Yamada) or plasma CIG (R. Timpl; H. Furthmayr) were used. The two latter have also been shown not to cross-react with laminin or type IV collagen.



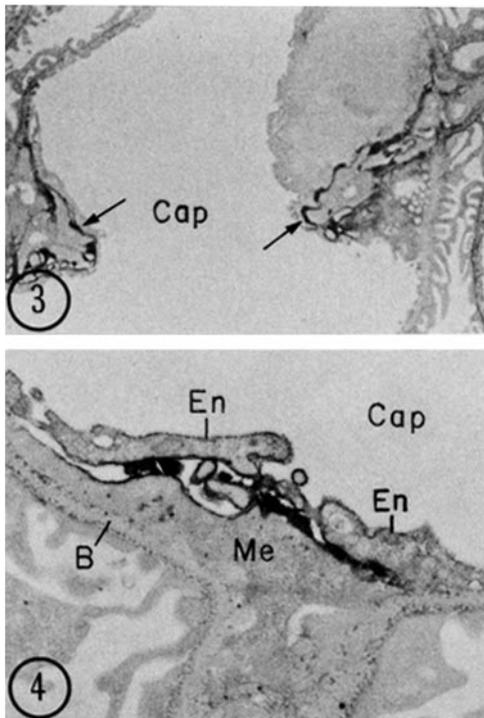
FIGURES 1 and 2 Localization of fibronectin in glomeruli as seen by indirect immunofluorescence (Fig. 1) and immunoperoxidase (Fig. 2). In Fig. 1, the mesangium is brightly fluorescent, and a linear deposit is seen along the peripheral capillary wall. Bowman's capsule is also discontinuously stained (arrow). In Fig. 2, the pattern of staining is identical, the mesangium is heavily stained and the peripheral capillary wall is lightly stained (arrow). $\times 800$.

By electron microscopy, the staining obtained with the immunoperoxidase procedure (Figs. 3–8) was irregular, presumably because of variability in access of the immunoreagents. In areas where antibody penetration was satisfactory, the following pattern was typically observed: deposits of reaction product were mainly confined to the extracellular matrix. The MM was heavily stained, with maximal staining found between endothelial and mesangial cells (Figs. 3–5) in the axial regions and at the branching sites of the glomerular capillaries (Fig. 3). Within the MM, the dense deposits sometimes took on a fibrillar pattern (Fig. 7). Staining was also occasionally detected within Golgi cisternae of some mesangial cells (Fig. 8).

In the peripheral capillary loops, reaction product was observed along those portions of the epithelial and endothelial cell membranes that face the GBM and in the adjacent fibrillar material within the laminae rarae interna (LRI) and the laminae rarae externa (LRE) of the GBM (Figs. 5 and 6). The remaining plasmalemmal domains of the epithelial and endothelial cells, the slit membranes, and the lamina densa (LD) were not noticeably stained.

In cryostat sections incubated with ferritin-conjugates, dense labeling was observed in the MM especially on the fibrils between the endothelial and mesangial cells (Figs. 9 and 10). Ferritin was also seen peripherally in the LRI (Fig. 11), but none was seen in the LRE. Apparently, ferritin conjugates can penetrate endothelial fenestrae to reach the LRI and MM, but they do not penetrate the LD or slit membrane (23) and, therefore, do not have access to the LRE under these conditions.

In isolated loops of GBM incubated with ferritin conjugates, clusters of ferritin with a periodicity of ~120 nm were seen on the epithelial aspect of the LD. These clusters often appeared to decorate wispy filaments emerging from the LRE (Fig. 12).



FIGURES 3 and 4 Immunoperoxidase, electron microscopy. In Fig. 3, two complex mesangial spaces (arrows) are prominently stained at a branching site. Fig. 4 shows staining of the mesangial matrix between two endothelial (*En*) cells and a mesangial (*Me*) cell. *Cap*, capillary lumen; *B*, basement membrane. Fig. 3, $\times 4,800$; Fig. 4, $\times 16,000$.

When the loops were opened, similar clusters were also seen along the residual LRI and along the 10-nm filaments in the MM (Fig. 13).

Controls

The same results were obtained when anti-FN antiserum was replaced by anti-CIG antiserum or by affinity-purified antibodies to FN, or when incubation was carried out at different pHs (from 6 to 8.5) or ionic strengths (up to 1 M NaCl). Immunolabeling was specifically abolished when non-immune first or second reagents, absorbed antiserum, or unconjugated HRP or ferritin were used. In sections from kidneys flushed and incubated with antialbumin antiserum, no labeling was observed in the glomerulus, whereas the endocytic vacuoles in the proximal tubule reacted intensely. This indicates that flushing is effective in removing plasma constituents and suggests that the localization observed with antibodies to FN was not likely to be caused mainly by the presence of a plasma contaminant (CIG).

DISCUSSION

The present results demonstrate the localization of FN predominantly in the MM, especially at the interface between the endothelial and mesangial cells. FN was also detected peripherally at the interface between both the epithelial and endothelial cells and the GBM. These conclusions are based on the convergent findings obtained by a variety of approaches—i.e., with both unfixed and fixed sections and unfixed isolated GBM, with immunofluorescence, immunoperoxidase, and immunoferritin procedures, and including an extensive set of controls.

This multifaceted approach was especially designed to localize FN in the peripheral regions of the capillary loops. By immunofluorescence, FN could be demonstrated in the periph-

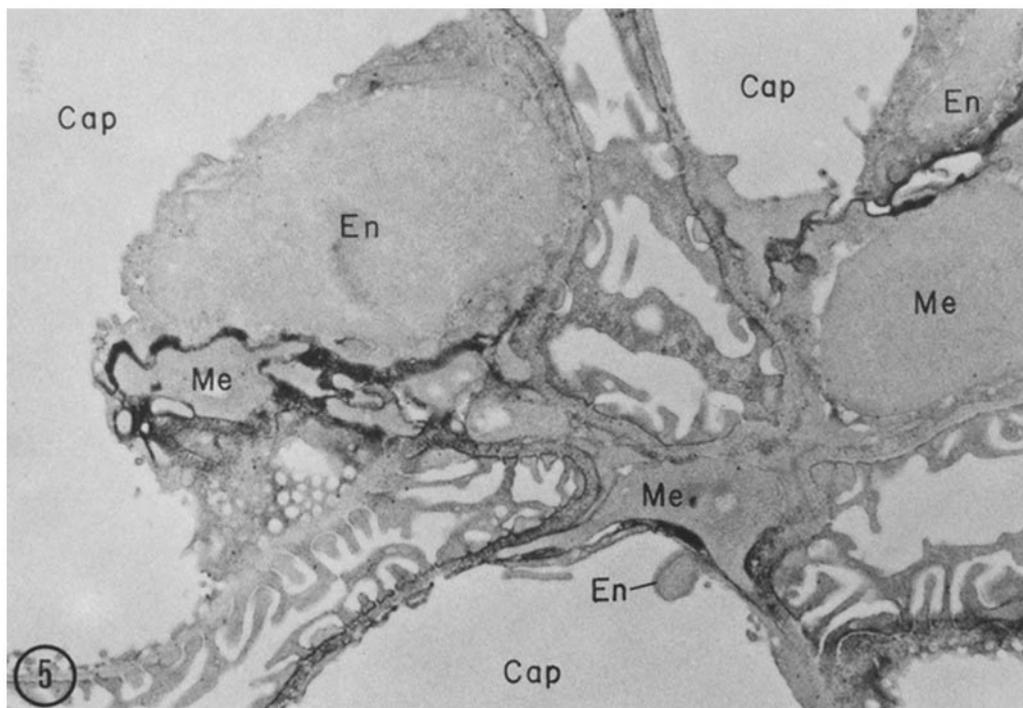
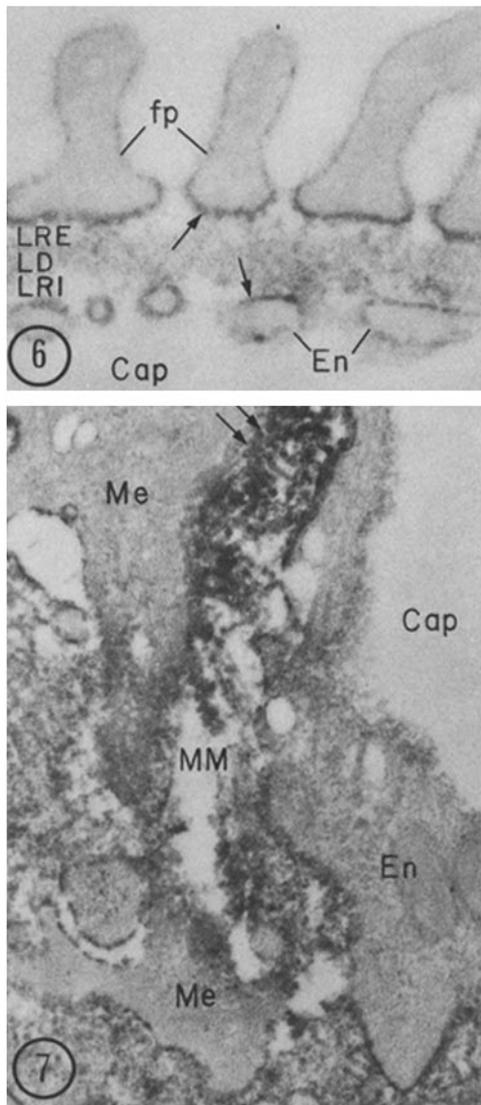


FIGURE 5 Higher magnification view of a portion of the same field as that in Fig. 3, showing fibronectin localization at the endothelial (*En*)-mesangial (*Me*) interface of three glomerular capillaries (*Cap*). $\times 11,500$.



FIGURES 6 and 7 Immunoperoxidase. Fig. 6 shows a portion of the peripheral capillary wall. Staining is mainly observed at the base (arrow) of the foot processes (*fp*) up to the slit membrane, which is not stained. There is also patchy staining in the lamina rara externa (*LRE*) and more discrete staining between the endothelium (arrow) and the lamina rara interna (*LRI*). The lamina densa (*LD*) is not significantly stained. Fig. 7 shows a portion of the mesangial matrix (*MM*) at the interface between an endothelial cell (*En*) and a mesangial cell (*Me*) which shows heavy staining for fibronectin. A fibrillar pattern is discernible (arrows) in the mesangial matrix. *Cap*, capillary lumen. Fig. 6, $\times 56,000$; Fig. 7, $\times 40,000$.

eral capillary wall, but its detailed localization could not be resolved. Using immunoperoxidase at the ultrastructural level, FN was detected at the base of both the epithelial and endothelial cells where they face the GBM. The immunoperoxidase procedure has the advantage that it is quite sensitive; however, diffusion-relocation of oxidized DAB can introduce artifactual results. Therefore, we also applied the immunoferritin procedure. Using this technique on sections, we confirmed the subendothelial localization of FN in the LRI (as well as between endothelial and mesangial cells). However, we could not detect FN in the LRE because the ferritin-conjugates do not penetrate the slit membranes (23) or the LD (10, 17). To expose the LRE to the immunoreagents, we applied the im-

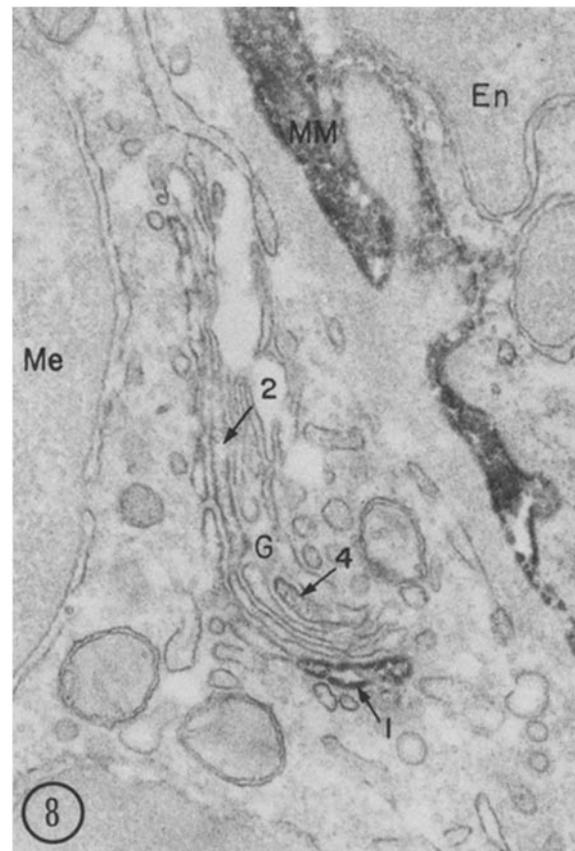


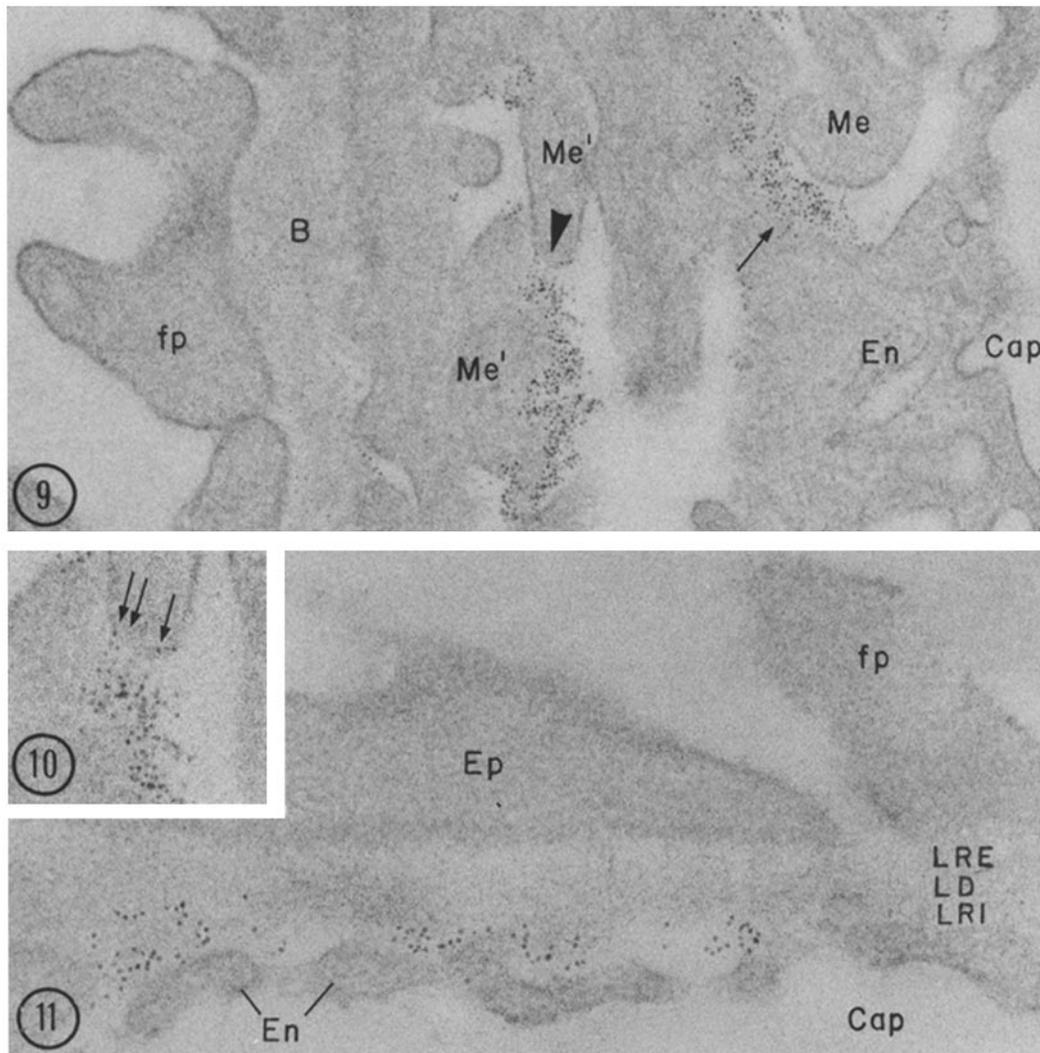
FIGURE 8 Immunoperoxidase localization of fibronectin showing the endothelial-mesangial interface. The mesangial matrix (*MM*) is intensely labeled. Reaction product indicating localization of FN is also detected within several (1, 2, and 4) of the stacked Golgi cisternae (*G*) of the mesangial cell (*Me*). *En*, endothelial cell. $\times 55,000$.

munoferritin technique on isolated GBM. With this approach, we confirmed the presence of FN in the residual LRI and LRE and on the fibrils of the *MM*.

In general, our findings are limited to a determination of the matrix layer (*MM*, *LRI*, *LRE*) in which FN is located, but we could not determine which of the several fibrillar components found in these locations (especially those in the laminae rarae) consist of FN. This will require further refinement of the present methodology. Also, the possibility that low concentrations of FN may be present in the *LD* cannot be ruled out at present.

As far as the source of glomerular FN is concerned, in principal FN could be synthesized locally by any of the three types of glomerular cells (endothelium, epithelium, or mesangium) because endothelial cells (1, 16, 26), epithelial cells (including kidney cells [2, 29]), and certain types of mesenchymal cells (36) have all been shown to be capable of producing FN in culture. Alternatively, plasma CIG could be incorporated (13) and covalently linked (21) to the FN matrix. The demonstration of FN in Golgi cisternae of mesangial cells suggests that these glomerular cells do synthesize FN locally.

Based on the concentration and arrangement of FN found in the axial regions and at the branching sites of the glomerular capillaries, we propose that in the glomerular stalk, there is a "mesangial anchorage system" composed of ~ 10 -nm fibrils (identified at least in part as FN) (see Figs. 7 and 10) and the



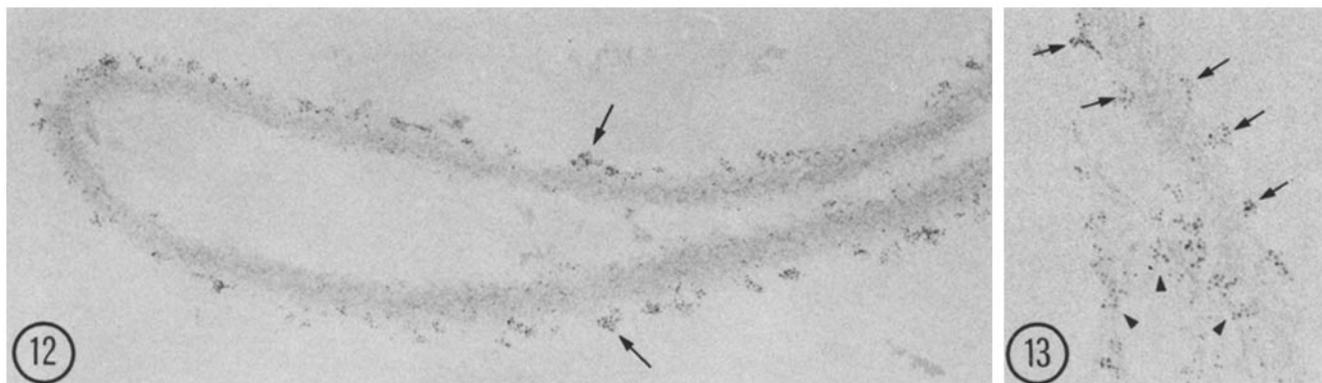
FIGURES 9-11 Figure 9 is a grazing section and Fig. 11 is a normal section through glomeruli from a specimen incubated for fibronectin with the immunoferritin procedure. In Fig. 9, dense labeling with ferritin is seen in the matrix between an endothelial cell (*En*) and a process of a mesangial cell (*Me*) (arrow) as well as between two mesangial cells (*Me'*) (arrowhead). Fig. 10 is a high magnification view of part of Fig. 9 (at arrowhead) showing ferritin molecules distributed in a linear array apparently decorating matrix fibrils (arrows). In Fig. 11, ferritin is seen in the lamina rara interna (*LRI*) between the endothelium (*En*) and the *LD*. The lamina rara externa (*LRE*) is not labeled presumably because neither the slit membranes between the foot processes (*fp*) nor the lamina densa (*LD*) are permeable to ferritin conjugates. Fig. 9, $\times 67,000$; Fig. 10, $\times 114,000$; Fig. 11, $\times 100,000$.

abundant intracellular ~ 6 -nm filaments of the mesangial cells (tentatively identified as actin) (32). In three dimensions, this arrangement of the glomerular stalk could be organized into static rings which could function to maintain the convolutions of the capillaries and prevent capillary aneurysm. In addition, assuming that mesangial cells, which resemble pericytes in other capillaries (10), have contractile properties as has been proposed (28), it could also provide mesangial cells with a means of controlling glomerular blood flow. In this regard, it is of interest that a similar concentration of FN has been detected between endothelial cells and pericytes in other capillaries (4).

FN was also detected in the laminae rae in the peripheral regions of these capillaries where a fibrillar network that is neuraminidase-sensitive appears to anchor the adjacent cells to the *LD* because perfusion with highly purified neuraminidase results in a detachment of both epithelial and endothelial cells (19). In view of this finding and the fact that FN is a sialogly-

coprotein (36), it seems likely that FN constitutes at least one of the elements involved in the attachment of both epithelial and endothelial cells to the *LD*. However, our preliminary results (6) indicate that laminin, another sialoglycoprotein involved in cell attachment (35), is also localized in the laminae rae, rendering it likely that laminin and perhaps other unidentified matrix proteins might also be involved in this anchorage.

FN is known to bind to collagen (8) and to be closely associated with sulfated proteoglycans at the surface of cultured cells (7, 30). Evidence has also been presented which suggests that FN-heparan sulfate complexes are involved in cell-to-substrate adhesion (7). Because the *GBM* is known to contain collagenous proteins (20), sulfated glycosaminoglycans (the main component of which is heparan sulfate [18]), and FN (present work), and laminin (6), these interactions might also be expected to play a role in maintaining the integrity of glomerular capillaries.



FIGURES 12 and 13 Isolated GBM (unfixed) demonstrating fibronectin localized by the immunoferritin technique. In Fig. 12, ferritin molecules are distributed in patches or clusters along the residual LRE (arrows) of this closed GBM loop. The clusters are located at an average distance of ~ 120 nm from each other and ~ 20 nm from the LD. When the loops are open (Fig. 13) clusters of ferritin are seen on the left (arrows) along the residual LRI and along the residual LRE to the right (arrows). Clusters are also seen along residual mesangial fibrils below (arrowheads). Fig. 12, $\times 50,000$; Fig. 13, $\times 65,000$.

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