

Formation of Basement Membranes in the Embryonic Kidney: An Immunohistological Study

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ABSTRACT Specific antibodies to laminin, type IV collagen, basement-membrane proteoglycan, and fibronectin have been used in immunofluorescence microscopy to study the development of basement membranes of the embryonic kidney. Kidney tubules are known to form from the nephrogenic mesenchyme as a result of an inductive tissue interaction. This involves a change in the composition of the extracellular matrix. The undifferentiated mesenchyme expresses fibronectin but no detectable laminin, type IV collagen, or basement-membrane proteoglycan. During the inductive interaction, basement-membrane specific components (laminin, type IV collagen, basement membrane proteoglycan) become detectable in the induced area, whereas fibronectin is lost. While the differentiation to epithelial cells of the kidney requires an inductive interaction, the development of the vasculature seems to involve an ingrowth of cells which throughout development deposits basement-membrane specific components, as well as fibronectin. These cells form the endothelium and possibly also the mesangium of the glomerulus, and contribute to the formation of the glomerular basement membrane. An analysis of differentiation of the kidney mesenchyme *in vitro* in the absence of circulation supports these conclusions. Because a continuity with vasculature is required for glomerular endothelial cell differentiation, it is possible that these cells are derived from outside vasculature.

Basement membranes are rigid extracellular matrices which, by forming a scaffold, may be involved in the regulation of organogenesis (18, 30, 50). They contain collagenous proteins (30), noncollagenous glycoproteins (30, 53), and glycosaminoglycans presumably linked to proteins as proteoglycans (3, 6, 19, 26, 27). These components are apparently produced by the cells resting upon the basement membrane, the epithelial cells (2, 20, 36), and the endothelial cells (16, 30).

The tubular elements of the kidney are lined by basement membranes located at the basal surface. The formation of these tubular basement membranes during embryogenesis requires an inductive tissue interaction (17, 18, 44). This interaction leads to a conversion of the mesenchymal cells to epithelial tubules and to a change in the extracellular matrix composition into a basement membrane type (8, 9). The development of the visceral basement membrane of the glomerulus (GBM) may, however, involve other types of control mechanisms not related to epithelial cell differentiation. The GBM is lined not only by epithelial cells but also by endothelial cells, and both cells may contribute to the formation of the GBM. We have therefore examined whether the development of the GBM is related to epithelial podocyte differentiation or is dependent on endothelial ingrowth into the glomerulus.

We did this by studying the appearance of basement-membrane components during normal mouse glomerular and tubular development at the light microscopy level using antibodies to laminin, type IV collagen, and basement-membrane proteoglycan (19, 49, 52, 53). The distribution of these components was compared to that of fibronectin, a matrix protein found in the kidney in the interstitial tissue, in the mesangium (48, 54), and possibly also in basement membranes. The podocyte sialic acid-rich surface was visualized with fluorochrome-coupled wheat germ agglutinin (WGA), a lectin known to bind to sialic acid (4). We analyzed nephrogenic mesenchyme induced to differentiate *in vitro* in the absence of circulation in a similar way.

MATERIALS AND METHODS

In Vivo Studies

Hybrid mouse embryos BALBc/CBA were used. For *in vivo* studies, kidneys were removed from 11- to 18-d-old embryos. Different stages of developing glomeruli can be found in the cortex of the same kidney, and the nominal age will therefore not always be mentioned in Results.

Tissue Culture

Two types of tissue cultures were performed. Nephrogenic mesenchyme was cultured both in the presence of the normal inductor, the ureteric bud, and with

a heterologous inductor, the embryonic spinal cord (17). Whole kidney anlagen of 11-d embryos were cultured for 4 d and then fixed for immunofluorescence. In the second type of experiment, a transfilter culture method (17, 44, 45) was used. We dissected nephrogenic mesenchyme free from ureteric buds and placed four to five mesenchymes on a Nuclepore filter (nominal pore size of 0.1 μm ; Nuclepore Corp., Pleasanton, Calif.). Of the many embryonic tissues which can exert an inductive action on the predetermined mesenchyme, embryonic spinal cord, a potent inductor was used (17, 43). With this tissue, induction is completed within 24 h (11, 45). After induction, the mesenchymes were then cultured alone for 3–5 d. Tissues were cultured in Eagle's minimum essential medium supplemented with 10% horse serum and antibiotics (100 IU/ml penicillin G and streptomycin 50 $\mu\text{g}/\text{ml}$).

Preparation and Fixation of Kidneys

To demonstrate the main stages of nephron differentiation, kidney rudiments were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.6, for 60 min in 4°C, and thick Epon sections for light microscopy were stained with 1% toluidine blue in 1% sodium borate.

For fluorescence microscopy, the tissues were fixed in cold alcohol as described (9, 42). Tissues were embedded in paraffin at 60°C and serially sectioned at 4–6 μm . Frozen sections gave identical results but offered no advantages, and the morphology was better preserved in alcohol-fixed tissues.

Antibodies

Antibodies to type IV collagen and laminin were gifts from Drs. R. Timpl (Max-Planck Institut für Biochemie) and J.-M. Foidart (National Institutes of Health). They were prepared in rabbits as described by others previously (15, 52, 53). The antibodies were purified by cross immunoadsorption. Their specificity has been verified by radioimmunoassay, ELISA assay, Ouchterlony immunodiffusion, immunoelectrophoresis, immunofluorescence blocking studies, and binding to agarose beads coated with various antigens (15, 52, 53).

Basement-membrane proteoglycans were extracted and purified from a mouse tumor (the Engelbreth-Holm-Swarm tumor) that produces a matrix of basement membrane. Antibodies to basement-membrane proteoglycan, a gift from Dr. G. R. Martin (National Institutes of Health), were raised in rabbits, purified by passage through a column of laminin covalently attached to Sepharose CL-4B (Pharmacia Fine Chemicals AB, Uppsala, Sweden), and by adsorption to a second column composed of proteoglycan coupled to Sepharose CL-4B, as described (19). Immunofluorescence blocking assays and ELISA assays were used to verify the specificity of the antibodies.

These works have established that the purified antibodies react with the antigen to which they were prepared but not to the other materials, and have shown that these antibodies are constituents of authentic basement membranes. They have been found exclusively in the basement-membrane area (8, 15, 19, 52, 53), and these components will therefore be referred to as basement-membrane specific components. This does not imply that they necessarily are components of the basal lamina. A distinction between the "basal lamina" seen in electron microscopy and the "basement-membrane" area seen in light microscopy should be made (30).

Fibronectin was localized with specific rabbit antibodies against human fibronectin (cold insoluble globulin), which was a gift from A. Vaheri (University of Helsinki, Finland). The preparation of antifibronectin antibodies has been described previously (21, 54, 56). Interstitial collagen was detected with rabbit antibodies to bovine type III procollagen (34, 51).

The antibodies were reacted for 30 min in the following concentrations: antilaminin antibodies, 25 $\mu\text{g}/\text{ml}$; anti-type IV collagen, 40 $\mu\text{g}/\text{ml}$; antibasement-membrane-proteoglycan, 1:100; antifibronectin, 1:50; and anti-procollagen type III, 15 $\mu\text{g}/\text{ml}$. In control experiments, normal rabbit serum (1:40) or phosphate-buffered saline (PBS) was used. Sections were washed three times in PBS, pH 7.4, and the different proteins were then visualized by the indirect immunofluorescence technique using fluorescein-isothiocyanate-conjugated sheep anti-rabbit IgG as described (10) (Wellcome Laboratories, Beckenham, U. K.).

Factor VIII antigen, known to be expressed by some endothelial cells (24), was localized by similar indirect immunofluorescence techniques using an antiserum against human factor VIII antigen (Behringwerke AG, Marburg, W. Germany; dilutions 1:20–1:200).

Lectin Staining

Tetramethylrhodamine (TRITC)-conjugated WGA was obtained from E-Y Laboratories (San Mateo, Calif.). This lectin, which is known to bind preferentially to sialic acid moieties and N-acetylglucosamine, was used to identify the developing podocytes. The binding of WGA to the podocyte surface could be totally abolished by pretreatment of the tissues with neuraminidase (0.01 U/ml, from *Vibrio cholerae*; Behringwerke, Marburg, W. Germany) but not by hyalu-

ronidase (2 mg/ml, from ovine testis, Serva Feinbiochemica, Heidelberg, W. Germany). The use of WGA to demonstrate the podocyte surface coat in histological sections has been described previously (7). Other methods to demonstrate the podocyte surface coat are available (28, 38). Rhodamine coupled WGA was selected because it was essential to study the same glomerulus for immunofluorescent visualization of the basement-membrane proteins. This could be done reliably and conveniently only with the use of different fluorochromes in fluorescence microscopy.

RESULTS

Morphogenesis

Development of the nephron has been studied previously by several authors (1, 23, 25, 29, 37–39, 46, 61). To aid in understanding the immunofluorescence findings, the principal stages of nephron development are shown (Fig. 1).

As a result of a tissue interaction, a condensate first forms around the branching ureter bud (Fig. 1*a*). The mesenchymal condensates soon form spheroidal masses, variously called vesicles or aggregates. In the lower part an indentation develops which changes the aggregate to a comma-shaped structure (Fig. 1*b*). Another cleft soon appears in the upper third of the mass. Thus, an S-shaped body consisting of three major parts develops (Fig. 1*c* and *d*): the upper portion situated close to the collecting tubules forms distal tubules, whereas the proximal tubules develop from the middle part. The lower part and the lower crevice will become the glomerulus. The widening lower cleft is invaded by cells which will form the mesangium and endothelium. In the lower limb of the S-shaped body, close to the lower cleft, two types of cells can be seen. The flattening epithelial cells below become the capsule of the glomerulus. The taller cells situated between the mesangial/endothelial cleft and the lumen (Bowman's space) are the presumptive podocytes. The widening of the mesangial/endothelial area can be more clearly seen in perpendicular sections of the S-shaped body (Fig. 1*e–f*). Such sections reveal that this area consists of a dense cell mass rather than loose mesenchyme (Fig. 1*f*). Between the endothelial and mesangial cells erythrocytes can be seen (Fig. 1*f–h*), which suggests that there is a continuity with outside vasculature from the beginning of development. The base of the glomerulus gradually becomes more constricted (Fig. 1*g–h*), and this process will eventually separate the vascular pole from the tubular pole (proximal tubule) and brings the afferent and efferent vessels close to each other. During the next stage, the epithelium gradually becomes more folded (Fig. 1*i*).

Factor VIII Antigen

Because Factor VIII antigen has been used as a marker for endothelial cells (24), its presence was analyzed in the embryonic kidney by indirect immunofluorescence. Small spots of fluorescence were detected in larger vessel walls but not in capillaries or in the glomerulus. The pattern did not change during development. In adult human and rat kidneys, Factor VIII antigen is also not detected in the glomerulus (E. Linder, University of Helsinki. Personal communication.). With all concentrations of the serum tested, the results remained the same. Thus, the presence of Factor VIII antigen could not be used as a marker for endothelial ingrowth into the glomerulus.

Basement-Membrane Specific Components and Fibronectin

UNDIFFERENTIATED MESENCHYME: Using indirect immunofluorescence, none of the basement-membrane spe-

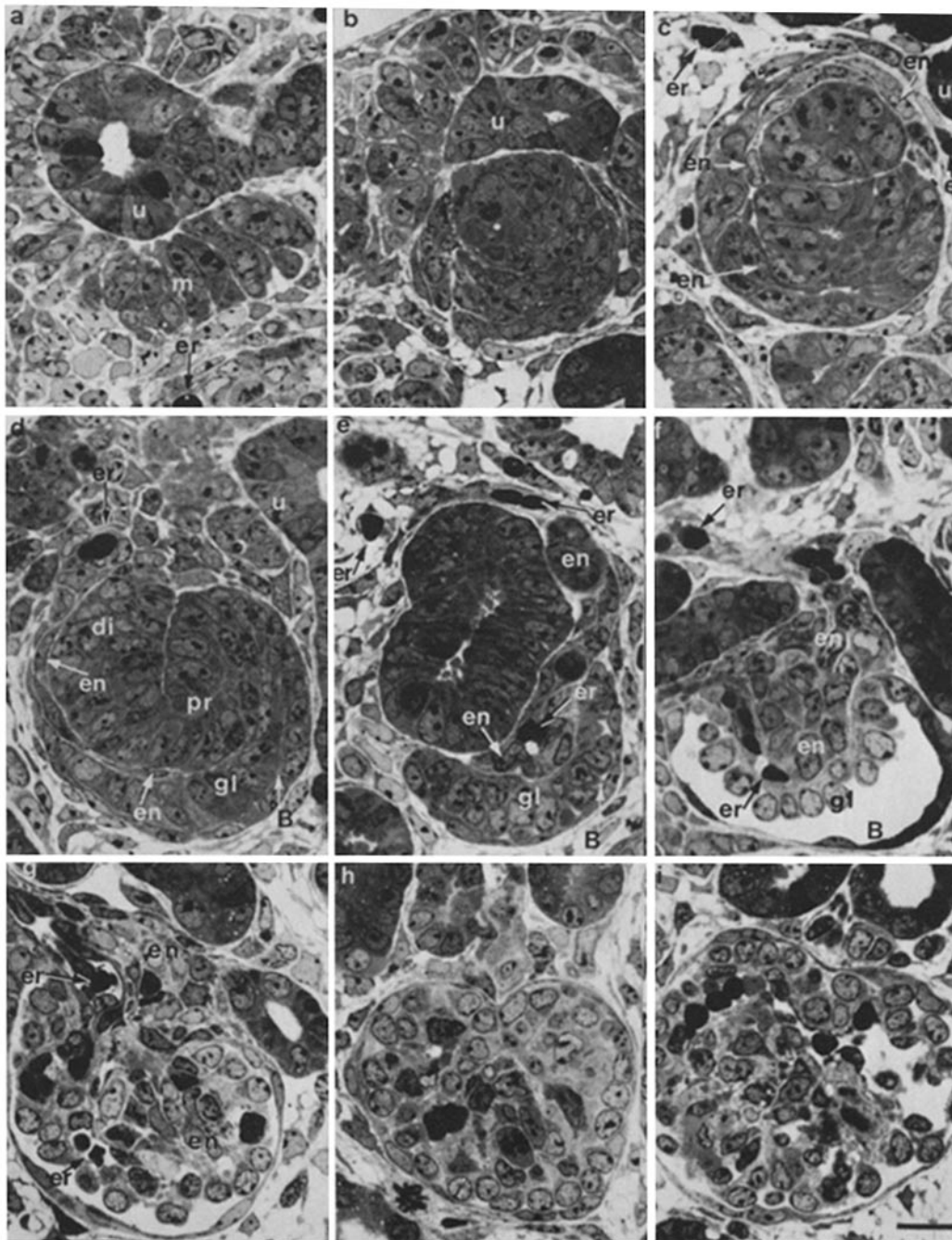


FIGURE 1 Thick plastic sections demonstrating the stages of nephron differentiation in vivo. See Results. Abbreviations: *B*, Bowman's space; *di*, distal tubule; *en*, endothelial cells; *er*, erythrocytes; *gl*, glomerular visceral epithelial cells (podocytes); *m*, cells derived from mesenchyme; *pr*, proximal tubular cells; and *u*, ureter bud. Note that erythrocytes are found close to the epithelial elements of the developing nephron at all stages and are located within vessels. Bar, 15 μm . $\times 570$.

cific components (laminin, type IV collagen, basement-membrane proteoglycan) can be detected in undifferentiated 11-d mesenchymes. These basement-membrane specific components are seen in the aortic anlagen and in the basement membrane of the ureter bud, as shown with antibodies to basement-membrane proteoglycan (Fig. 2*b* and *d*). Fibronectin is expressed by the mesenchymal cells, including those situated close to the ureter bud (Fig. 2*a*).

INDUCTION PERIOD: At day 12, a condensate of mesenchymal cells begins to form around the inducing ureteric bud. With antibodies to the basement-membrane specific components, a reaction is seen in the 12-d kidney in three different

locations, as shown for basement-membrane proteoglycan (Fig. 2*d*). There is fluorescence in the developing vasculature which at this stage has grown close to the induced area, a reaction is seen in the basement membrane of the branching ureter bud, and the first fluorescent spots become detectable in the induced mesenchyme (Fig. 2*d*). Fibronectin is lost from the induced area at day 12, but the other parts of the mesenchyme express fibronectin (Fig. 2*c*).

DEVELOPMENT FROM THE COMMA-SHAPED STAGE TO THE S-SHAPED BODY: The basement-membrane specific components (laminin, type IV collagen, basement-membrane proteoglycan) were now detected along the borders of the

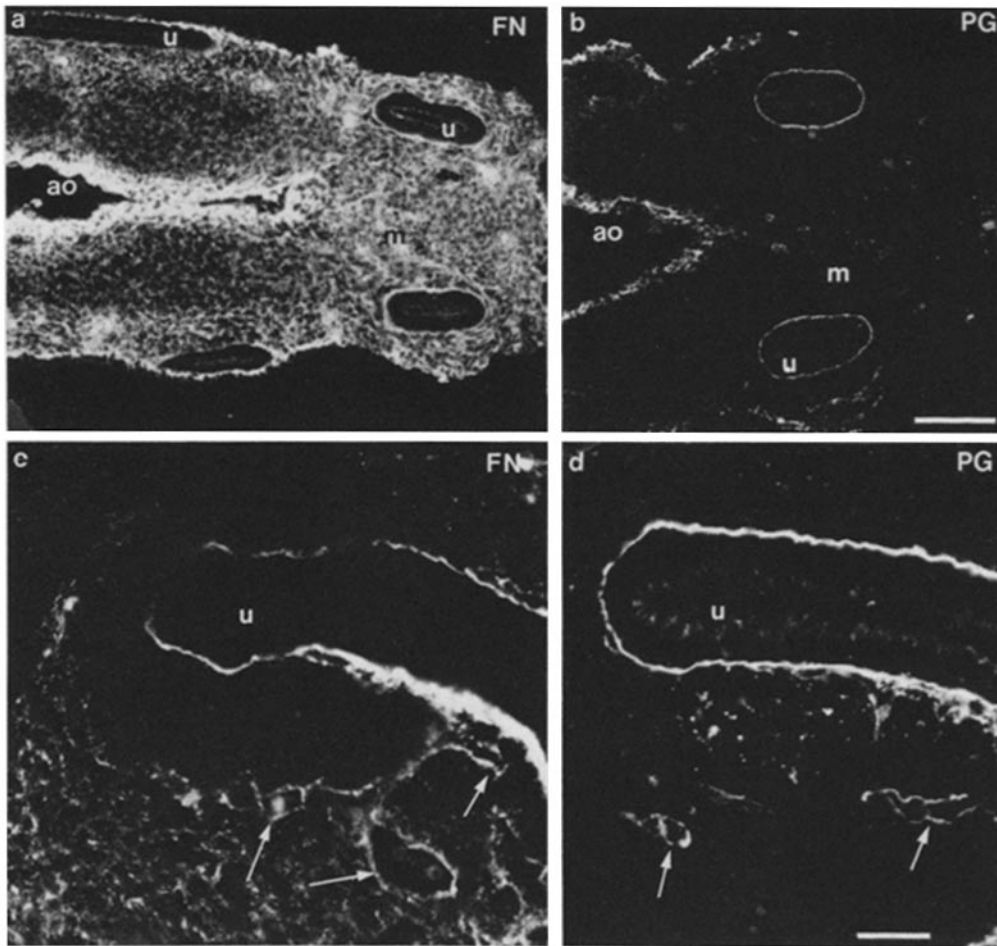


FIGURE 2 Immunofluorescence micrographs of 11- and 12-d-old kidney mesenchyme stained with the indirect immunofluorescence procedure for the localization of fibronectin (FN) and BM proteoglycan (PG). *a* and *b*, 11-d mesenchyme shown together with the Wolffian duct, the ureter bud (*u*), and the aortic anlagen (*ao*). Mesenchyme expresses fibronectin but no detectable proteoglycan. The aorta and cells close to it as well as the basement membrane of the ureter express fibronectin and proteoglycan. Bar, 70 μ m. \times 160. *c*, 12-d kidney shows loss of fibronectin in induced area of mesenchyme. Note that fibronectin remains associated with the basement membrane of the branched ureter. Fibronectin is also found in loose mesenchyme and vascular walls (arrows). \times 350. *d*, 12-d kidney shows appearance of basement-membrane proteoglycan. Note fluorescence in vascular walls (arrows). Bar, 35 μ m. \times 300.

comma-shaped body, as shown for type IV collagen (Fig. 3 *a*). In the S-shaped body, this basement-membrane fluorescence is detected in the lower parts whereas the upper limb expresses prominent spots, as shown for laminin (Fig. 3 *b*). In sections of more elongated S-shaped tubules, the spots are no longer seen in the developing proximal tubules but the presumptive distal tubule still shows this pattern (Fig. 3 *c*).

The basement-membrane specific components were also detected around cells within the lower crevice of the S-shaped body, in the vessel walls outside the S-shaped body, but not in the mesenchymal stroma, as shown for type IV collagen (Fig. 3 *a*) and laminin (Fig. 3 *b* and *c*).

Fibronectin did not codistribute with the basement-membrane specific components during the comma-shaped stage or the S-shaped stage. Fibronectin was detected in the stroma close to the developing tubular structures but not in association with the basement membrane of the comma-shaped body (Fig. 3 *d*). In more advanced stages seen in the same section, fibronectin fluorescence becomes detectable in the basement membrane (Fig. 3 *e*). In the lower crevice of the S-shaped body, fibronectin was detected around all cells (Fig. 3 *e*). Antifibronectin antibodies uniformly stain the mesenchymal stroma

around the epithelial cells (Fig. 3 *e*).

CONSTRICTION OF GLOMERULAR BASE: The base of the mesangiocapillary area, which forms from the cells of the lower cleft of the S-shaped body, is first seen as a wide area. All cells found in this area express type IV collagen (Fig. 4 *a*). The peripheral epithelial cell layer expresses WGA-binding sites as revealed by double staining of the same sections using TRITC-WGA (Fig. 4 *b*). The base of the glomerulus gradually becomes more constricted. During this event, all cells which become entrapped in the mesangial/capillary area express type IV collagen (Fig. 4 *c* and *e*). The endothelial cells of the vessels found outside the glomerulus also express type IV collagen (Fig. 4 *c*). The cells expressing WGA-binding sites remain located peripherally during the constriction of the glomerular base (Fig. 4 *d* and *f*). Basement-membrane proteoglycan and laminin codistributed with type IV collagen. Staining for laminin was, however, weak in the mesangial/capillary area (Fig. 5).

Antibodies to fibronectin also stain cells of the mesangial/endothelial area (Fig. 6 *a*), but none of the mesangial or endothelial cells express interstitial collagens at any stage (Fig. 6 *b*). With the antibodies used, no separate mesangial area

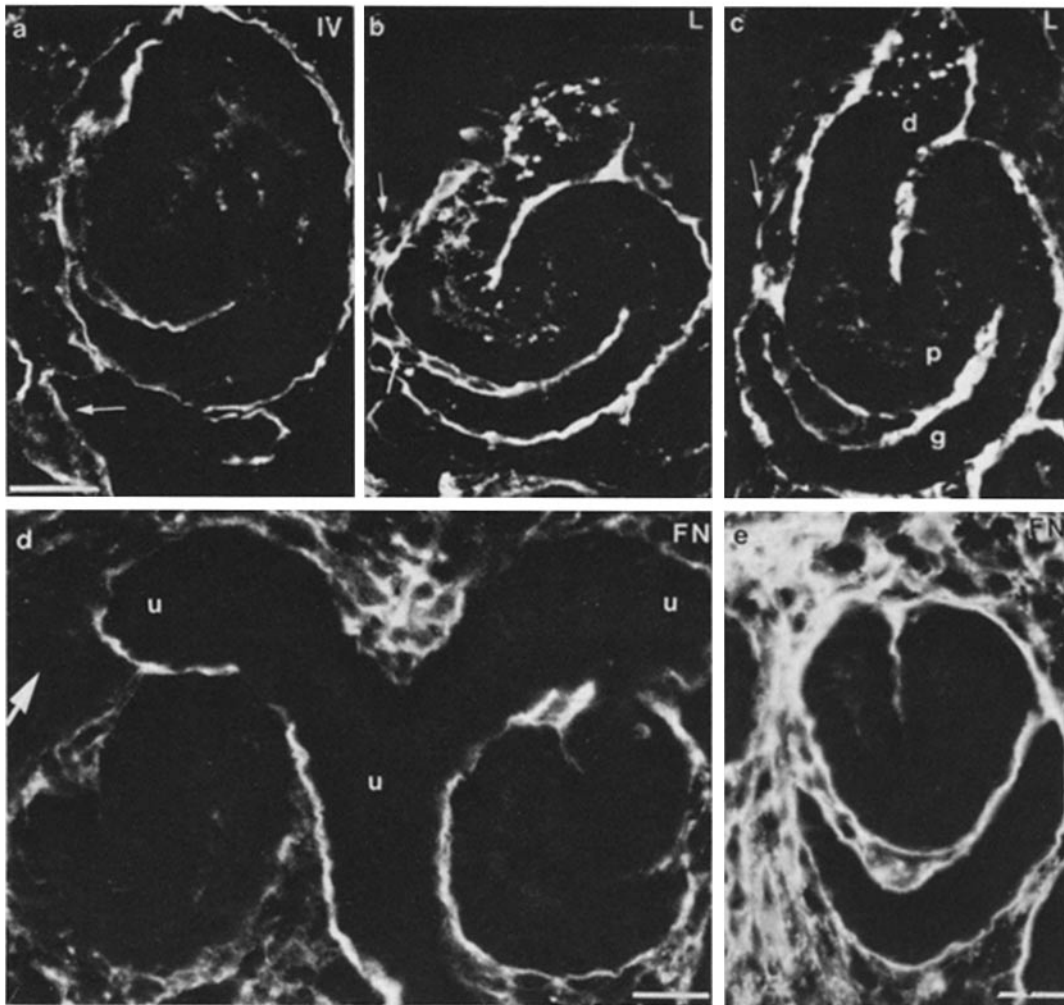


FIGURE 3 Immunofluorescence micrograph of type IV collagen (IV), laminin (L), and fibronectin (FN) expression during development from the comma-shaped body to the S-shaped body. Small arrows in (a-c) denote laminin and type IV collagen expression in vasculature. Type IV collagen is also expressed in the basement membrane of the comma-shaped body (a). Note spots of laminin within the developing tubules (b and c), as compared with the location of fibronectin (d and e). Note that fibronectin is not detected in the basement membrane of the comma-shaped stage (e). In d there is also a new mesenchymal condensate around the tip of the branching ureteric tree (u). Fibronectin is lost from this area (large arrow in upper left corner). Abbreviations: d, distal tubule; g, glomerular podocyte; p, proximal tubule; and u, ureteric tree. Bar, 15 μ m. a, \times 720. b-e, \times 600.

distinct from an endothelial area was found.

FOLDING OF THE GLOMERULAR EPITHELIUM: After constriction of the glomerular base, WGA-binding sites gradually become detectable as deeper grooves between the mesangiocapillary area. This staining pattern was not seen before constriction of the glomerular base. The cell periphery of the epithelial cells express WGA-binding sites, but the base of these cells shows a stronger FITC-WGA fluorescence at this stage (Fig. 7a). Laminin fluorescence was seen in the mesangial area and in the GBM (Fig. 7b). Antibodies to type IV collagen, basement-membrane proteoglycan (Fig. 7c), and fibronectin also stained both the mesangium and the GBM.

IN VITRO DEVELOPMENT: Nephrogenic mesenchymes were induced to differentiate by a 24-h transferrin contact with inductor tissue, and subsequently cultured for several days. Tubules differentiate normally under these conditions (10, 11). Glomerularlike bodies also form, but it was found that only the epithelial parts can be obtained. Morphologically, all cells within the glomerularlike bodies appeared the same (Fig. 8a). The cells of the glomerularlike bodies express the glomerular epithelial polyanion, as judged by their WGA binding. In

explants examined on the fourth day, the WGA-binding cells have formed large bodies (Fig. 8b and c). Several such bodies are seen in one section. In serial sections, several cell layers of the same glomerular body bind WGA uniformly. When these explants are stained with antilaminin antibodies (Fig. 8d), reaction is seen in the tubular basement membranes, but no reaction is detected within the glomerulus. Results were similar with anti-type IV collagen antibodies, antibasement-membrane proteoglycan antibodies and antifibronectin antibodies.

Glomeruli not expressing basement-membrane antigens were also obtained when 11-d whole kidney were cultured in vitro. Thus, the results are not related to the absence of the normal inductor (ureter bud) but seem to be related to the absence of vascularization.

DISCUSSION

The development of basement membranes of the kidney was studied with antibodies against defined extracellular matrix components, laminin (53), type IV collagen (52), basement-membrane proteoglycan (19), and fibronectin (21, 54, 56). It is suggested that the development of tubular and glomerular

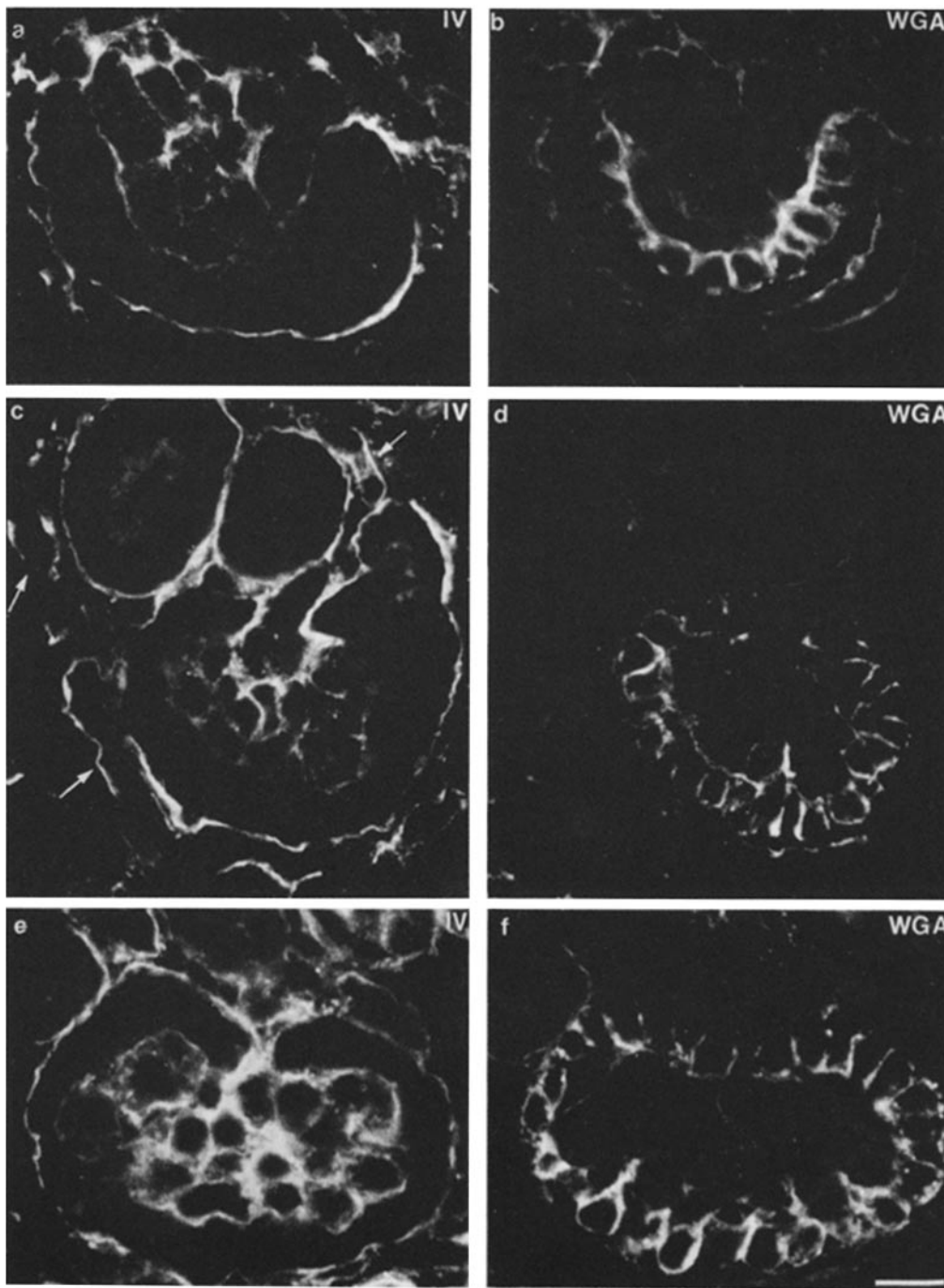


FIGURE 4 Fluorescence micrographs of type IV collagen (IV) expression and glomerular polyanion during the gradual constriction of the glomerular base. After application of anticollagen type IV antibodies, the sections were double-stained with TRITC-WGA and FITC-anti-rabbit -IgG. At all stages, type IV collagen can be found around mesangial and endothelial cells (a, c, and e; FITC-fluorescence). The double-staining procedure shows WGA-TRITC fluorescence in peripherally located cells, showing that podocytes do not move inwards before the constriction of the base (b, d, and f). The arrows in c indicate vascular wall outside glomerulus. Bar, 15 μ m. \times 600.

basement membranes are dependent on two different types of control mechanisms. Whereas the transition of the nephrogenic mesenchyme to epithelial cells producing basement membrane requires an inductive tissue interaction, the development of the GBM cannot be explained by the same phenomenon. It apparently also requires an ingrowth of endothelial cells which produce basement-membrane components. The conclusion is based upon an analysis of embryonic specimens and a comparison of these with nephron differentiation in vitro.

Tubular Basement Membranes

Kidney-tubule differentiation is known to be triggered by an inductive tissue interaction between the ureter bud and the metanephric mesenchyme (17, 18, 44). Recently, it was shown that this interaction leads to a shift in the extracellular matrix composition from an interstitial type to a basement-membrane type (8, 9). It is shown here that fibronectin, a protein found in adult tissues in both the interstitium and basement membranes

(48), is lost from the mesenchyme during the inductive interaction *in vivo*. Thus, it seems to codistribute with the interstitial collagens in this event (9), whereas a newly described proteoglycan, unique for basement membranes (19), is shown to codistribute with laminin and type IV collagen.

The loss of the interstitial collagens and fibronectin may be due to protease activity. Proteases degrading fibronectin are known to stimulate collagenase activity (59). The loss of fibronectin and its absence from the early aggregates has not been clearly documented previously (55, 58). Our results suggest that the basement-membrane specific components, rather than fib-

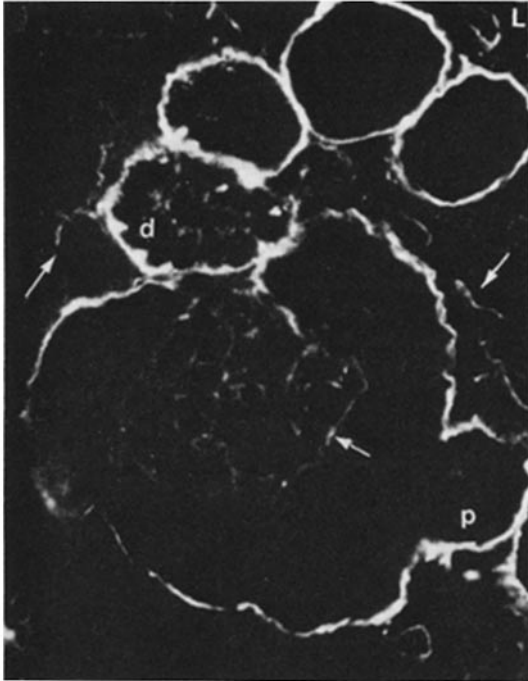


FIGURE 5 Fluorescence micrograph of laminin expression during constriction of glomerular base. The intensity of the fluorescent line between the endothelium and podocytes (the GBM) is weak (arrow) as compared to the intensity of the staining of the tubular basement membranes and Bowman's capsule. The intensity in the GBM is at this stage comparable to that of outside vasculature (arrow) and mesangium. L, laminin. Bars, 15 μ m. \times 600.

ronectin, are involved in the aggregation of the induced nephrogenic cells.

The punctate distribution of the basement-membrane specific components, seen before overt basement-membrane formation, is a distinct feature of early stages of tubulogenesis. It remains to be seen whether the spots represent an intracellular location found only in developing tubules or an extracellular location between tubular cells reflecting an unpolarized deposition of basement-membrane material. The proximal tubules develop their basement membranes before the distal tubules, a finding important for an understanding of the subsequent sequential appearance of the luminal antigens of the different tubules (10, 11).

Vascular Development and GBM Formation

The visceral GBM is currently considered to be the main filtering membrane of the kidney (5, 13, 14, 28), and it is thus of interest to understand its development. Three cell types are found in the glomerular tuft: epithelial podocytes, endothelial, and mesangial cells. The podocytes develop from the same mesenchymal cell aggregates as the tubular cells, whereas the origin of the mesangial and endothelial cells is not fully understood. Presumptive endothelial/mesangial cells as well as erythrocytes are found in the developing glomerulus already at the S-shaped body stage (25, 29). It is currently generally considered that they do not originate as an *in situ* formation from the induced mesenchymal cell aggregate (23, 29, 61), but whether they are formed from the nephrogenic mesenchyme (1, 12, 38, 39) or from outside vasculature is not known. Nor is it known what type of extracellular matrix components these cells produce during their ingrowth into the glomerulus.

We show here that the developing mesangial/endothelial area, either morphologically or by its extracellular matrix composition, does not resemble loose mesenchyme. A dense cell mass invades the glomerulus and there is an apparent continuity with outside vasculature. No interstitial collagen characteristic of uninduced mesenchyme (9, 34) is found within the glomerulus at any stage. Instead, both the developing vessels outside the glomerulus and the mesangial/endothelial area stain with antibodies to type IV collagen, basement-membrane proteoglycan, and fibronectin. Antibodies to laminin initially show a weak reaction in these locations, but during

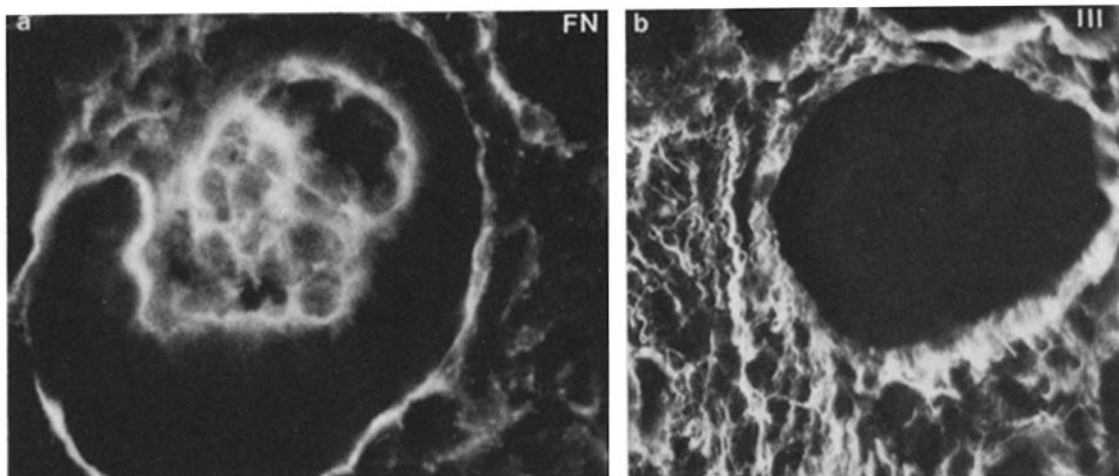


FIGURE 6 Fluorescence micrographs of fibronectin (FN) and type III procollagen (III) during constriction of glomerular base. Fibronectin is detected between mesangial and endothelial cells and appears to be located in the GBM (a). None of the cells within the mesangial/endothelial area express type III procollagen (b). Bar, 15 μ m. \times 720.

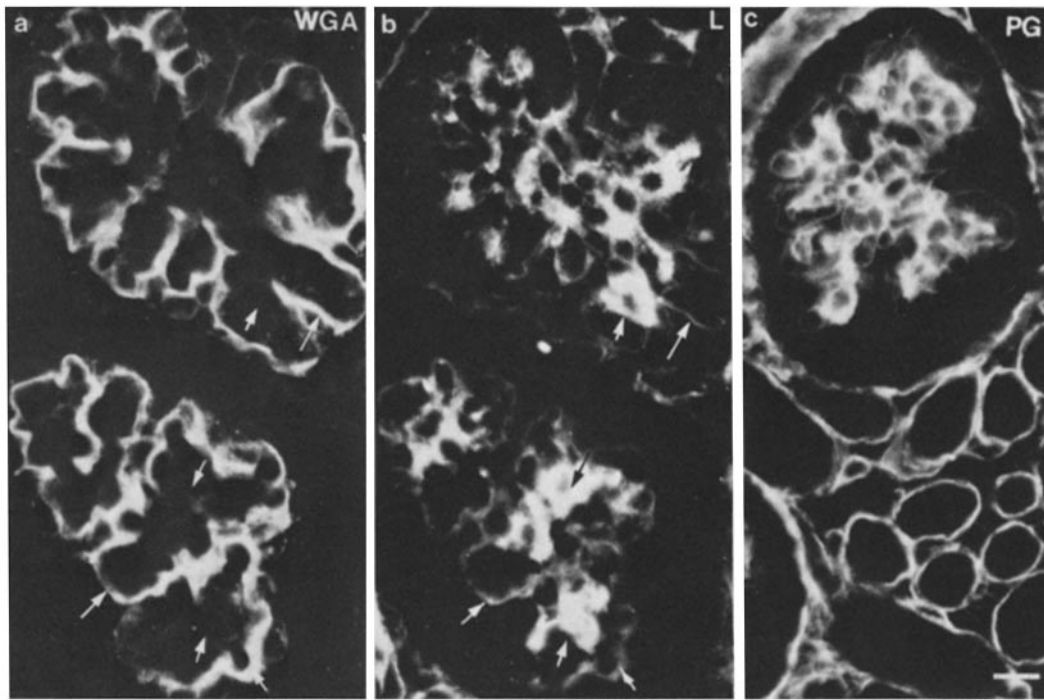


FIGURE 7 Fluorescence micrographs of glomeruli at stages when the epithelium and the GBM have become folded, demonstrating basement membrane material in the GBM and in mesangium. Double-stained glomerulus showing laminin (L) in GBM and prominently in the mesangium (b), and WGA-binding sites at the base of visceral epithelial cells but not in mesangium (a). Arrows denote mesangium and GBM. c, BM proteoglycan (PG) in GBM and in mesangium. Bar, 15 μ m. \times 250.

the folding of the GBM this staining becomes stronger. Taken together, these findings suggest that no conversion of mesenchyme to endothelium and mesangium occurs within the developing glomerulus, as often postulated (1, 38, 39). The demonstration of the basement-membrane antigens in locations where only mesangial/endothelial cells are found leaves little doubt that these cells produce basement-membrane material. This suggests a dual origin (22, 50) rather than exclusively an epithelial origin of the GBM (57). Reeves et al. (39) show that qualitative changes in the glycosaminoglycan composition occur during the fusion of the two basement membranes.

With the antibodies used in this study, the endothelial cells could not be distinguished from the mesangial cells. Both cells apparently produce type IV collagen, basement-membrane proteoglycan, fibronectin, and laminin. The possibility that these two cell types are derivatives of the same ingrowing cell type should therefore be considered. Being similar types of cells, they may both continuously produce basement membrane material, a postulate that would explain why type IV collagen, laminin, fibronectin, and basement-membrane proteoglycan are found both in the mesangium and in the GBM (19, 31, 32, 35, 40, 41, 47, 48, 60).

The *in vitro* culture method (44) offered an opportunity to study nephron differentiation in the absence of vasculature. Tubular basement membranes formed normally in such cultures. Segment-specific luminal antigens are also expressed (10, 11). Glomerularlike bodies have also been demonstrated in these tissues (10, 11, 43) but it has not been known what types of cells there are in these.

In this study, staining with WGA was used as a marker for podocyte differentiation. This lectin binds to sialic acid moieties and N-acetylglucosamine (4). WGA was found to react

with the polyanionic coat of the podocytes during *in vivo* development exactly as other probes used to detect this coat (28, 33, 38). In the *in vitro* cultures, all cells within the glomerularlike bodies expressed WGA-binding sites. No cells resembling (ingrowing) endothelial and mesangial cells could be found within the glomerularlike bodies. Thus, under the experimental conditions used, glomerularlike bodies consisting only of epithelial podocytes were obtained. These epithelial podocytes formed irregular clusters. Attempts to demonstrate laminin, type IV collagen, basement-membrane proteoglycan, or fibronectin in these bodies have failed. Both the *in vivo* and the *in vitro* findings therefore point to the possibility that normal GBM formation requires the presence of endothelial cells.

The *in vitro* cultures suggest that a continuity with outside vasculature is required for normal glomerular endothelial and mesangial differentiation. This requirement may mean that these cells are derived from outside vasculature.

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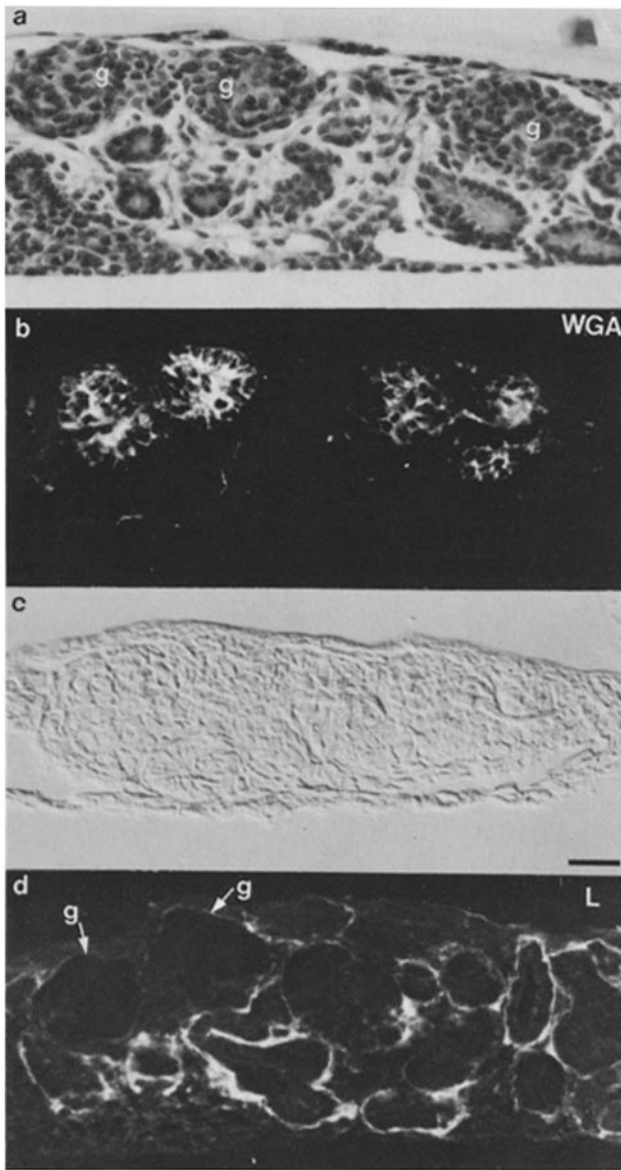


FIGURE 8 Demonstration of glomerularlike bodies in explants cultured in vitro for 120 h. *a*, histological section showing several glomerularlike bodies. Note that the cells within these appear morphologically the same. *b*, staining with WGA showing several large bodies of cells all expressing the glomerular epithelial polyanion. *c*, Nomarski optics micrograph showing explant in (*b*). *d*, staining with antilaminin antibodies showing fluorescence in tubular basement membranes and Bowman's capsule, but not in the glomerularlike bodies. *g*, glomerularlike body; *L*, laminin, WGA, wheat germ agglutinin. Bar, 15 μm . $\times 400$.

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