PODOENDIN

A New Cell Surface Protein of the Podocyte and Endothelium

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Glomerular podocytes are invested with a thick layer of cell surface fuzzy coat, presumably a carbohydrate-rich husk, glycocalyx, of cell periphery (1). The podocyte fuzzy coat is particularly rich in anionic sialoproteins (2, 3), which also fill the interpedicellar slits (4, 5) and may be part of the anionic charge barrier of the glomerular filter. Decrease in the colloidal iron–stainable sialoproteins has been documented in experimental animal (6) as well as human (7) nephrotic syndrome, when glomerular capillary permeability is increased. Interactions of polycations with the fuzzy coat polyanions cause rapid and profound alterations of podocyte structure (8), suggesting that binding of ligands to the cell surface sentinel molecules may modulate the structural as well as functional state of podocytes. Interest in the podocyte fuzzy coat is further heightened by the discovery (9) that antibodies against one of the podocyte cell surface glycoproteins, gp330, may be responsible for in situ immune complex formation (10) and the membranous glomerulopathy seen in experimental Heymann's nephritis. Recently, Kerjaschki, Sharkey, and Farquhar (11) further identified a major sialoprotein in the podocyte fuzzy coat, a 140 kilodalton (kD) molecule called podocalyxin. In this report, we describe the identification and characterization of another novel podocyte fuzzy coat protein, podoendin, using a monoclonal antibody (mAb)[1] developed in our laboratory. The 62 kD protein is also found on the endothelial cell surface.

Materials and Methods

Isolation and Purification of Renal Glomeruli and Glomerular Basal Lamina. Kidneys removed from 150–200-g Sprague-Dawleys rats were cut into 2–3-mm slices with a clean razor blade. Renal medulla were dissected and discarded (12). The renal cortex was pressed through a nylon sieve (210 μm) and suspended in phosphate-buffered saline (PBS) at 4°C, and containing the following protease inhibitors: 25 mM EDTA, 1 mM benzamidine HCl, 1 mM alpha-toluenesulfonyl fluoride, and 10 mM N-ethylmaleimide (13). The renal homogenate was sequentially washed through nylon sieves of 149 and 105 μm pore size with the same PBS and protease inhibitors. The materials retained by 62 μm sieve

[1] *Abbreviations used in this paper:* BSA, bovine serum albumin; DTT, dithiothreitol; FITC, fluorescein isothiocyanate; HRP, horseradish peroxidase; mAb, monoclonal antibody; PAS, periodic acid/Schiff reagent; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TBS, Tris-buffered saline.

nylon mesh were judged to be 95% pure glomeruli. The isolated glomeruli were subjected to overnight osmolysis in distilled water containing protease inhibitors. The glomerular suspensions were subsequently washed 10 times in distilled water with the aid of a Sorvall GLC-2 table top centrifuge at 2,000 rpm for 20 min, lyophilized, and stored at $-65°C$.

Hybridoma G4. The glomerular basal lamina, 0.5 mg, was suspended in 0.5 ml of sterile normal saline and homogenized with 0.5 ml of complete Freund's adjuvant. The mixture was injected into a 3-mo-old BALB/c mouse subcutaneously at four sites. 4 wk after the primary immunization, the mouse was boosted intraperitoneally with the same dose of glomerular basal lamina in incomplete Freund's adjuvant. Subsequently, at 3-d intervals, we injected antigen suspension intravenously, without adjuvant, into the tail veins. Mice were killed by cervical dislocation. A day before sacrifice, a drop of blood was obtained, and the serum was serially diluted with PBS. The sera were tested with indirect immunofluorescence for antibodies against glomeruli. Only mice with antiglomerular antibody titer >1:16 were used, and their spleen cells were harvested for fusion with NS-1 myeloma cells (14). Hybrid cells secreting antibodies of interest were selected by screening culture media by an indirect immunofluorescence method (vide infra). The selected hybrid cells were cloned twice (14). Monoclonal hybridoma G4 was selected based on its diffuse decoration of glomerular capillary wall and endothelial surface.

Purification of mAb for Immunochemistry. 2-mo-old BALB/c mice were each injected intraperitoneally with 0.5 ml of 96% 2,6,10,14-tetramethylpentadecane (Prestane). 10–14 d later, each mouse received intraperitoneal injection of 5×10^6 of cloned hybridoma G4 cells in 0.5 ml of RPMI medium 1640 (Gibco Laboratories, Grand Island, NY). Ascites were aspirated when the abdomen was distended with peritoneal fluid. Ascites were pooled and centrifuged at 2,000 rpm for 20 minutes to remove cellular debris. The cleared ascites were stored at $-65°C$.

mAb in 100 ml of pooled ascites of mice bearing hybridoma G4 was purified by salting with saturated ammonium sulfate solution according to the methods of Staehlin et al. (15). The precipitated antibody was dissolved in and dialyzed against PBS at 4°C. It was then diluted to 5 mg/ml, based on absorbance at 280 nm. An extinction coefficient of 1.4 ml/mg × cm was used in calculation. Aliquots of 0.4 ml were stored at $-65°C$. This antibody preparation had specificity identical to that of antibody harvested from the culture media of hybridoma G4, as determined by light and electron immunocytochemistry. Therefore, it was used for immunocytochemistry and immune-overlay in immune blotting throughout the experiments.

Immunofluorescence Studies. Direct and indirect immunofluorescence, as described in a previous report (16), were performed on 4-μm-thick sections of fresh tissue that had been snap frozen in liquid nitrogen. Some frozen sections, mounted on the slides, were pretreated with 100% ethanol or 0.25% (vol/vol) Triton X-100 in 25 mM Tris buffer, pH 7.2, for 30 min, and rinsed in PBS before application of the first antibody. FITC (fluorescence isothiocyanate)-conjugated rabbit anti–mouse IgG antibody (Miles Laboratories, Inc., Elkhart, IN), diluted 1:10, was used as the second antibody to detect mouse IgG, unless otherwise specified. Appropriate controls were run concurrently, as described in a previous publication (16).

Immunoperoxidase Reaction with Avidin/biotinylated Horseradish Peroxidase (HRP) Complex. Rat kidneys were sliced into 2-mm-thick slices and immediately fixed in Carnoy's fixative (60% ethanol, 30% chloroform, 10% glacial acetic acid) overnight. The tissues were processed for paraffin embedding. An immunoperoxidase reaction, using the avidin/biotin system (17, 18), was carried out with deparaffinized 4-μm-thick sections following the procedures previously published from our department (19). mAb G4 was diluted 1:200. Other reagents were purchased from Vector Laboratories (Burlingame, CA).

To distend capillaries and facilitate visualization of endothelial surface, Carnoy's fixative, ~30 ml, was also injected over a 2-min period into the inferior vena cava via an opening in the abdominal cavity. Organs (lungs and kidneys), which were only partially and irregularly perfused, were excised immediately, sliced into 2-mm sections, and further immersed in Carnoy's fixative overnight before processing for paraffin embedding.

Perfusion Fixation of Kidneys and Lungs. The procedures of Kerjaschki and Farquhar

(9) were followed. Rats, 150–250 gm, were anaesthesized with Penthrane (Methoxyfluorane, USP). The abdominal aorta was cannulated, and the kidneys were perfused with PBS at 100 mm Hg for 3 min to flush out the blood. Perfusion fixation with 3% glutaraldehyde in PBS for 10 min followed. The aldehyde was quenched with PBS containing 50 mM glycine for 20 min. Kidneys were removed and sliced into 2-mm slices, and further rinsed several times with the same saline-glycine solution. Tissues were then immersed in PBS containing 10% dimethylsulfoxide, and snap frozen in an isopentane–dry ice bath. 5-μm-thick sections were cut with a cryostat and kept in suspension in cold saline-glycine solution. For lung tissues, perfusion fixation of lungs was performed, using identical protocol, by cannulating the main pulmonary artery. The perfusion pressure was 30 cm of water. Slices of lung tissues, 2-mm-thick, were infiltrated with Tissue-Tek O.T.C. compound (Miles Laboratories, Inc.) at 1:1 dilution with PBS, under low pressure. Tissues were then frozen and sectioned at 5 μm thickness.

Some of the kidney sections were mounted on slide glass and subjected to proteolytic digestion by 0.05% trypsin in 0.1 M Tris buffer, pH 8.4, at 37°C for 3 h. The control sections were incubated in the same buffer without trypsin. After the incubation, we rinsed the slides in PBS and proceeded with immunoperoxidase reaction.

Electron Immunocytochemistry. The sections, rinsed three times in Tris (50 mM)-buffered saline, were incubated with mAb G4, 2 mg in 100 ml TBS containing 0.1% bovine serum albumin (BSA) for 2 h at 20°C with gentle shaking. Control sections, incubated in the same solution without mAb, were run concurrently. Sections in suspension were then rinsed with TBS for 10 min three times with the aid of a Sorvall GLC-2 centrifuge of 2,000 rpm. This was followed by incubation in suspension in 1:100-diluted HRP-conjugated goat anti–mouse IgG (Miles Laboratories, Inc.) in TBS containing 0.1% BSA for 2 h. After a second antibody reaction, sections were rinsed three times in TBS for 10 min, each and suspended in 10 ml of Tris HCl (50 mM), pH 7.2, containing 0.1% diaminobenzidine. After 10 min, 0.1 ml of freshly prepared 1.0% hydrogen peroxide was added and mixed well. The reaction was allowed to proceed for 3–15 min, until the positive reaction appeared as brownish discoloration on checking random sections under a microscope. The sections were then rinsed three times in distilled water, osmicated in 2% osmium tetroxide in double distilled water for 1 h, and finally centrifuged to a tissue pellet, to which a drop or two of 2% agar solution was added at 45–48°C. After cooling and solidifying, the agar holding the tissue pellet was fixed in 3% glutaraldehyde in PBS for 1 h, and diced into 1-mm cubes. The cubes were then processed for electron microscopy as previously described (12). Thin sections were examined with an AEI 801 electron microscope. Appropriate control sections without uranium or lead staining were also examined.

Immunoperoxidase reactions on fresh, unfixed glomeruli were carried out as follows. Glomeruli were isolated by sieving through nylon mesh, and purified as described previously. Minimum culture medium (Dulbecco's) was used instead of PBS. They were incubated with gentle shaking for 2 h at 4°C in minimum culture media containing mAb G4 at 2 mg/100 ml. After rinsing three times, 10 min each, in the minimum culture medium, they were incubated for 2 h at 4°C in the second antibody, HRP-conjugated goat anti–mouse IgG, diluted 1:100 in the minimum culture medium. Glomeruli were rinsed in cold PBS three times, 10 min each, and fixed in 3% glutaraldehyde in PBS for 1 h at 4°C. They were rinsed in TBS, followed by diaminobenzidine reaction. The rest of the procedure for electron microscopy was identical with that for the tissue section in suspension, as described above. Again, appropriate controls were run concurrently.

Extraction of Antigen from Rat Glomeruli for Immune Blotting. Glomeruli were isolated and purified after rat kidneys were perfused with PBS containing protease inhibitors. The antigen reactive with mAb G4 was extracted from isolated and purified glomeruli in sample buffer (see SDS-PAGE) containing 1% (wt/vol) dithiothreitol (DTT) and 3.6% (wt/vol) sodium dodecyl sulfate (SDS). The mixture was shaken vigorously for 30 min, and heated in a boiling water bath for 5 min, with constant shaking (11). The insoluble materials were removed by centrifugation at 50,000 g for 2 h. The clear supernatant was stored at −65°C. The extract was diluted 1:5, with sample buffer containing 10 mg/ml

DTT, before being used for electrophoresis. The antigen was similarly extracted from purified rat glomeruli with 25 mM Tris buffer, pH 7.2, containing 0.25% (vol/vol) Triton X-100. This extract was only diluted 1:2 before preparation for polyacrylamide gel electrophoresis (PAGE).

Purification of Antigen-Antibody Complexes with a Protein A Column. To obtain podoendin in sufficient quantity for preliminary chemical characterization on polyacrylamide gels, the antigen from rat kidneys and lungs were isolated and copurified with mAb on a protein A–sepharose CL 4 B column, which will be referred to as protein A column. All procedures were performed at 4°C in a cold room.

The antigen from rat kidneys and lungs was extracted with 25 mM Tris buffer, pH 7.2, containing 0.25% (vol/vol) Triton X-100. 20 Sprague-Dawley rats, 200–300 g body weight, were anaesthesized with Penthrane. Lungs and kidneys were perfused with PBS by cannulating pulmonary arteries and abdominal aortas. Because the antigen reactive with mAb G4 was found to be stable in preliminary studies, no protease inhibitors were used in the extraction process. Kidneys (49 g total) and lungs were separately homogenized in 50 ml of 25 mM Tris buffer, pH 7.2, containing 0.25% (vol/vol) Triton X-100. Homogenization was carried out with a Polytron at maximum power setting, five bursts at 10 s each. The containers were kept in an ice bath to maintain ice cold temperature. The homogenates were centrifuged at 4,000 rpm for 30 min. The supernatants were stored at −65°C until use.

Protein A–sepharose CL 4 B (Pharmacia Fine Chemicals, Piscataway, NJ), 1.5 gm, was swollen in PBS and poured into a 10-ml column fitted with a 0.45-μm disc filter (Bio-Rad Laboratories, Richmond, CA), which was prewetted with PBS containing 0.1% nonionic surfactant Nonidet P-40 (Calbiochem-Behring Corp., La Jolla, CA). The total bed volume of the gel was ~5 ml. A Sephadex G-25 (fine) (Pharmacia Fine Chemicals) column was similarly prepared, and coupled to the top of the protein A column. It served as a filter for loaded samples. 10 ml of PBS were applied to wash the two serially coupled columns. The flow rate was adjusted and maintained at one drop per 2 s throughout the experiments.

10–15 ml of thawed ascitic fluid, cleared by centrifuging at 2,000 rpm for 15 min, were applied to the Sephadex G-25 column. After washing the columns with 50–75 ml PBS to remove non-Ig proteins, 25 ml of thawed rat kidney extract was loaded and slowly percolated through columns. The Sephadex G-25 column was then washed with 5 ml of PBS containing 0.25% Triton X-100, and replaced with a 30 ml reservoir. The protein A column was further washed with 50 ml (10× the bed volume) of the same solution. Antigen-antibody complexes bound to protein A were eluted from the column with 25 ml of 0.1 M sodium citrate buffer, pH 4.1. The eluent was collected in 4-ml fractions, in vials containing 2 ml each of 0.6 M sodium phosphate buffer, pH 8.0–8.2, to neutralize the eluent. 0.1 ml aliquots were removed from each vial and mixed with 1.0 ml of PBS. The first three fractions, which had absorbance at 280 nm >0.07, contained a total of 29.2 mg proteins. They were pooled, dialyzed against distilled water, lyophilized, and stored at −65°C until use. The protein A column was regenerated with 10 ml of 0.1 M sodium citrate buffer, pH 3.0, and equilibrated with PBS. 38 ml of the lung extract were purified with the same procedures. 21.5 mg of lung antigen-antibody complexes were obtained.

Sample Preparation for PAGE. The sample buffer was 10 mM sodium phosphate, 0.1% SDS, 10% glycerol, and 0.01% bromophenol blue at pH 7.2. 1 mg of protein was dissolved in 375 μl of sample buffer. Aliquots of 75 μl were stored at −65°C. Before use, 75 μl of sample buffer containing 20 mg/ml DTT were added. The mixture was heated in a boiling water bath for 5 min. To each sample well of slab gels, 10–15 μl of sample, containing 15–20 μg of proteins, were applied.

Spot Tests for Polyanions. One drop (7 μl) of samples prepared as described for PAGE was applied to nitrocellulose paper, which had been activated for 30 min in TBS, and air dried for 5 min. After sample application, the nitrocellulose paper was air dried for 10 min before immersion in a 3% gelatin solution in TBS for 30 min with gentle shaking. It was then rinsed briefly in distilled water and fixed in 4% paraformaldehyde in PBS.

Aldehyde fixation is not required for successful tests, and tends to increase the background staining. After thorough rinsing with distilled water, the nitrocellulose paper was stained with colloidal iron (20) or 0.1% alcian blue in 3% acetic acid (21).

SDS-PAGE. SDS–polyacrylamide gels were prepared as described previously (22, 23). The gel concentration was 5%. The slab gel dimensions were 13 cm × 13 cm × 0.75 mm. Electrophoresis was performed at a constant current of 40 mA per gel for 4–5 h, when the tracking dye was ¾ of the way through the gel. The protein bands were stained with Coomassie blue (22). After destaining, gels were immersed in 1% glycerol and 10% acetic acid, and dried on a piece of filter paper. Gels were also stained using the periodic acid/Schiff reaction (PAS) for carbohydrate (24), and alcian blue 8 GS (Schmid GmBH and Co., Federal Republic of Germany) (21). Alcian blue stain was performed with and without prior oxidation with periodic acid. For immune blotting, duplicate gels were run concurrently. One was used for transblot, the other for staining with Coomassie blue.

Two molecular weight standards were used. One was obtained from Pharmacia Fine Chemicals (Piscataway, NJ), the other, Bio-Rad Laboratories. Molecular weight was calculated by plotting logarithm of molecular weights vs. relative mobility of proteins (23).

Transblotting and Immune Overlay. Transblotting of protein bands from slab gels to nitrocellulose paper was carried out with a Trans-Blot cell and a Model 250/2.5 power supply (Bio-Rad Laboratories), following the technical information provided. Tris (25 mM) and glycine (195 mM) buffer, pH ~8.3 without adjustment, was used. Transblotting was run at a constant voltage of 30 V (~0.13 A) overnight, followed by 70 V for 2 h. The transblot cell was cooled with an epoxy-coated aluminum supercooling coil connected to running tap water. The temperature was thus maintained at 10–25°C. The buffer system and voltage schedule effectively transferred all protein bands from the gel to the nitrocellulose paper. This was verified by the absence of silver-stainable (Bio-Rad Laboratories) protein bands on the gel after transblotting.

After transblotting, immune overlay (25) was performed with a Bio-Rad Immune Blot (GAM-HRP) assay kit. The protein binding sites of nitrocellulose paper were saturated by incubating in TBS, pH 7.5, containing 3% gelatin. The first antibody, mAb G4, was used at a concentration of 2 mg/100 ml of TBS, and containing 1% gelatin. Incubation time was 2 h at room temperature. After overlay with the first antibody, the nitrocellulose paper was briefly rinsed in double-distilled water, followed by two 10-min rinses in TBS containing 0.05% Tween-20. If the rinsing is cut to only 5 min each, some background nonspecific decoration is noted. We have found this advantageous, because it enables us to compare the banding pattern with Coomassie blue-stained gels, and to identify the decorated band, as in Fig 7. The second antibody, HRP-conjugated goat anti–mouse IgG, was diluted 1:1200, and incubated for 1 h. The nitrocellulose paper was rinsed similarly, as after the first antibody. The peroxidase activity was developed with 4-chloro-1-naphthol. 60 mg of 4-chloro-1-naphthol were dissolved in 20 ml of ice cold methanol. This was mixed with freshly prepared 100 ml TBS containing 60 μl of 30% hydrogen peroxide. Color development was allowed to proceed at room temperature for 15–30 min, followed by thorough rinsing in distilled water and air drying.

Glomerular Culture. Glomeruli were isolated and purified as described above, but under sterile conditions, from 12 Sprague-Dawley rats, weighing 190–230 g. Glomeruli were cultured (26) in Waymouth media enriched with 20% fetal calf serum containing 125 U penicillin and 125 μg/ml streptomycin. The media were changed every 3–4 d. After 1 wk, outgrowth of monolayer of large polygonal cells was observed. 7 d later, the medium was removed and the cells were rinsed with cold PBS three times, 5 min each. The monolayer cells were processed for indirect immunofluorescence with mAb G4 as the first antibody.

Glomerular Development and Podoendin. Newborn and 5-d-old rats, two each, were killed, their kidneys removed and sliced into 2-mm sections, part of which were snap frozen in an isopentane–dry ice bath and used for immunofluorescence studies. The remainder were fixed in Carnoy's fixative overnight and processed for paraffin embedding. Avidin-biotin–conjugation immunoperoxidase studies were carried out with mAb G4 on 4-μm thick deparaffinized sections, as described above.

Acute Effects of G4 on Rat Kidneys. 12 Sprague-Dawley rats, 200–250 g, were housed individually in a metabolic cage for 2 d before experiments. Urine was collected 24-h before the procedure, and was free of signs of proteinuria or hematuria. mAb G4, 4 mg in 0.4 ml of PBS, was injected into the left ventricle under Penthrane anaesthesia. A 24-gauge needle was inserted through xyphoid notch. Spontaneous rapid back flow of arterial blood into syringe indicated that the needle was in the left ventricle. The antibody was injected as a bolus in a few seconds. Control rats were similarly injected with 0.4 ml of PBS. The animals tolerated the procedure very well and recovered from anaesthesia uneventfully except for one animal, which died of cardiac tamponade within a minute of injection. We used five animals each in the experimental and control groups.

30 min after injection, four rats, two from each group, were killed. The right kidneys were sliced, and portions were fixed in 3% glutaraldehyde in PBS and processed for paraffin embedding and electron microscopy. A slice of 2 mm was also taken from the right kidney and snap frozen for direct immunofluorescence study with FITC-conjugated rabbit anti–mouse IgG (Miles Laboratories, Inc.) to demonstrate renal localization of mAb G4. FITC-conjugated goat anti–rat C3 was used to reveal complement fixation, and was kindly provided by Drs. S. Adler and W. G. Couser, Division of Nephrology, University of Washington. The antibody had been absorbed with rat Ig, and was further shown not to crossreact with mouse IgG. The remainder of the right and the entire left kidneys, still fresh, were rapidly pressed through 210 and 149 μm nylon sieves with PBS. The glomeruli retained by the 62 μm nylon sieve were collected in TBS and pelleted by centrifuging 2,000 rpm for 10 min. The pellet was resuspended and incubated in a 1:100 diluted HRP-conjugated rabbit anti–mouse IgG antibody (Miles-Yeda) for 2 h at 4°C with gentle shaking. The incubation was ended by rinsing three times for 5 min each in cold TBS, followed by fixation in cold 3% glutaraldehyde for 30 min, with constant shaking. The glomeruli were rinsed three times for 10 min each, with TBS containing 50 mM glycine to quench the residual aldehyde of the fixative. The procedures for diaminobenzidine reaction, osmication, and subsequent processing for electron microscopy were identical with the methods given in the section on electron immunocytochemistry.

The remaining six rats, three in each group, were housed individually in a metabolic cage. The 24-h urine specimens were collected for the first 3 d. They were analyzed for blood, proteins, glucose, and ketone bodies with Labstix (Ames Division, Miles Laboratories, Inc.). At the end of the third day, all animals were killed. The right kidneys were fixed in 3% glutaraldehyde in PBS for both light and electron microscopy. The left kidneys were snap frozen for immunofluorescence studies with FITC-conjugated antibodies, as described above.

Results

mAb G4 diffusely decorates glomeruli along glomerular capillary walls on indirect immunofluorescence studies of fresh frozen sections (Fig. 1 *a*). The fresh frozen sections do not allow us to identify the decorated components of the glomerular capillary wall. The endothelium of extraglomerular capillaries in the kidney are also decorated, but the decoration is too weak to be properly photographes on the same frame with glomeruli without overexposure of glomerular decoration. Prior treatment of fresh frozen sections in 25 mM Tris HCl buffer, pH 7.2, containing 0.25% Triton X-100 completely abolished both glomerular and endothelial decoration (Fig. 1 *b*), indicating effective extraction of the antigen by 0.25% Triton X-100. Prior treatment of fresh frozen sections in 100% ethanol for 30 min greatly improves the decoration of glomeruli (Fig. 1 *c*). Based on spot-metering photometer readings, the exposure time required to photograph a glomerulus at a film sensitivity of ASA 320 decreases from 17 s to 7 s, on the average, indicating that the fluorescence intensity has more than doubled. Ethanol treatment (fixation) also greatly enhanced endothelial decora-
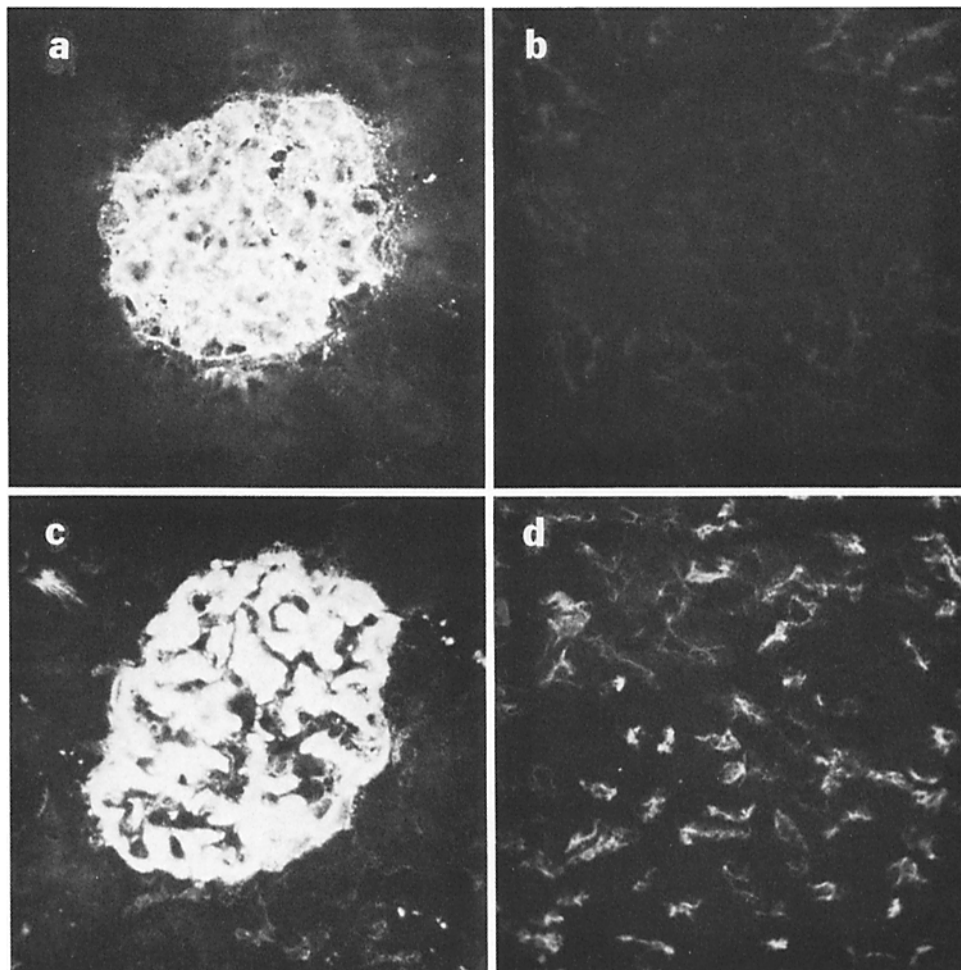
FIGURE 1. Indirect immunfluorescence decorations of fresh frozen sections of rat kidneys using mAb G4 as the first antibody. (a) Glomerulus from fresh frozen sections without prior treatment is diffusely decorated by G4. The decoration of the extraglomerular capillaries are too weak to be properly photographed. (b) Antibody decoration is competely abolished by treating fresh frozen sections in 25 mM Tris HCl, pH 7.2, containing 0.25% (vol/vol) of Triton X-100 for 30 min. There is no decoration of either glomeruli or extraglomerular capillaries. (c) Prior treatment of fresh frozen section in 100% ethanol for 30 min greatly enhances the decoration of glomeruli and extraglomerular capillaries. (d) Enhanced decoration of the endothelium in the capillaries of the renal medulla after treatment with 100% ethanol. 150×.

tion (Fig. 1 d). The endothelium of lungs is also diffusely decorated (results not shown).

Carnoy's solution fixation, followed by paraffin embedding and immunoperoxidase reaction with the avidin-biotin system reveals a similar decoration pattern by mAb G4. In kidneys, it diffusely decorates the constituents of the glomerular capillary wall and extraglomerular endothelium (Fig. 2a). In glomeruli, it decorates the podocyte surface and an intensely decorated zone following the general topography of the epithelial basal lamina (27). The parietal epithelial cell surface
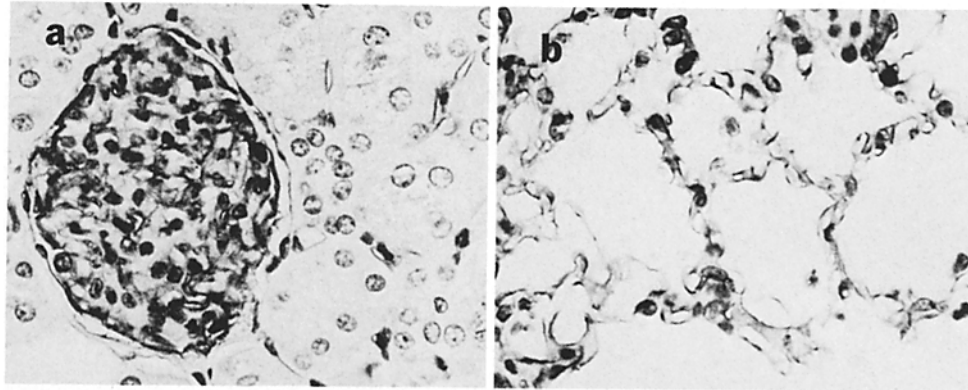
FIGURE 2. Immunoperoxidase reaction with avidin/biotinylated peroxidase complex technique. The tissues are fixed in Carnoy's fixative and embedded in paraffin. (a) Kidney shows decoration of the glomerular capillary wall, parietal epithelial cells of Bowman's capsule, and extraglomerular vascular endothelium. (b) Lung exhibits diffuse decoration of the endothelium. Hematoxylin nuclear counterstain. 400X.
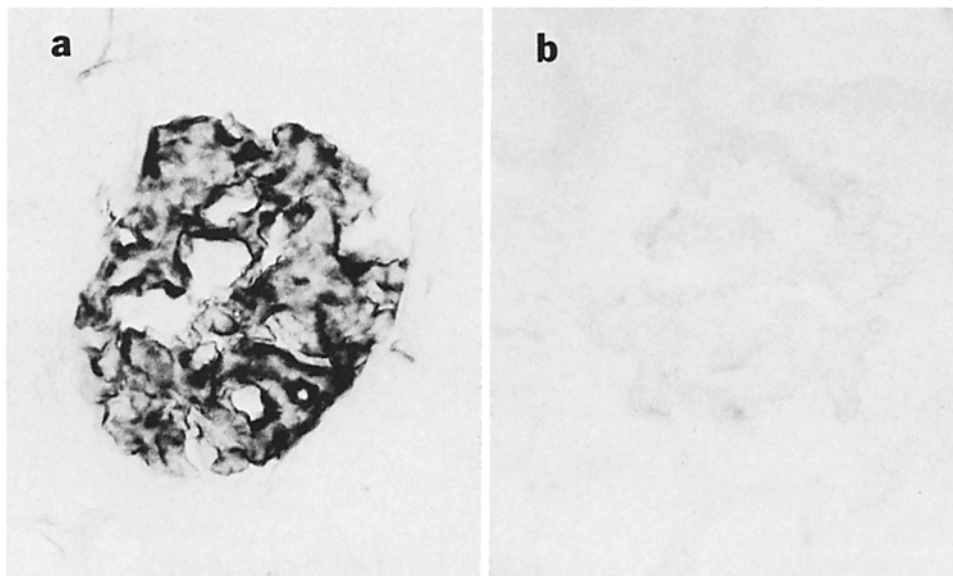

FIGURE 3. Indirect immunoperoxidase reaction of frozen sectioned kidneys, perfusion fixed with 3% glutaraldehyde for 10 min. Sections are incubated in 0.1 M Tris buffer, pH 8.4, at 37°C for 3 h. (a) Without and (b) with 0.05% trypsin. Trypsin completely abolished antibody decoration. 425X.

facing urinary space also shows decoration (Fig. 2a). Decoration of the endothelium of lungs is shown in Fig. 2b.

The antigen decorated by mAb G4 is sensitive to trypsin digestion. After 3 h of incubation at 37°C, it is completely cleaved (Fig. 3).

Electron immunocytochemistry of fresh glomeruli reveals diffuse decoration of podocyte surface by mAb G4 (Fig. 4). Glomerular basal lamina is not decorated. The glomerular endothelial cell cytoplasm, which is normally highly
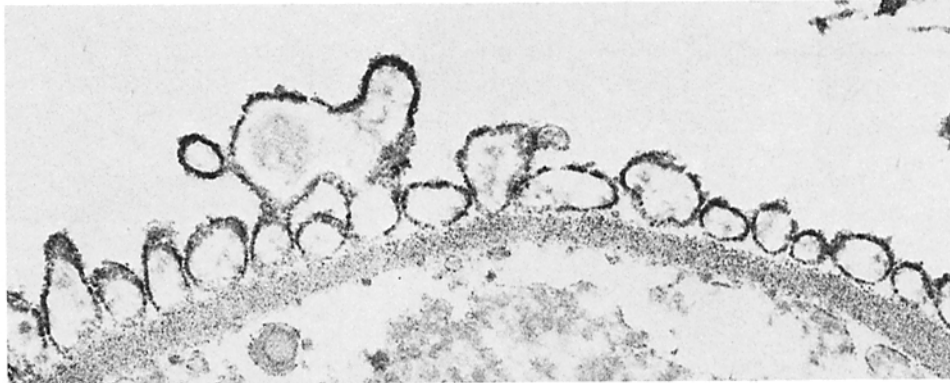
FIGURE 4. Electron immunocytochemistry of freshly isolated glomeruli with indirect immunoperoxidase technique. Diffuse decoration of the podocyte surface by mAb G4 is evident. The basal lamina is not decorated. Structures inside capillary lumens are poorly preserved. 42,500×.
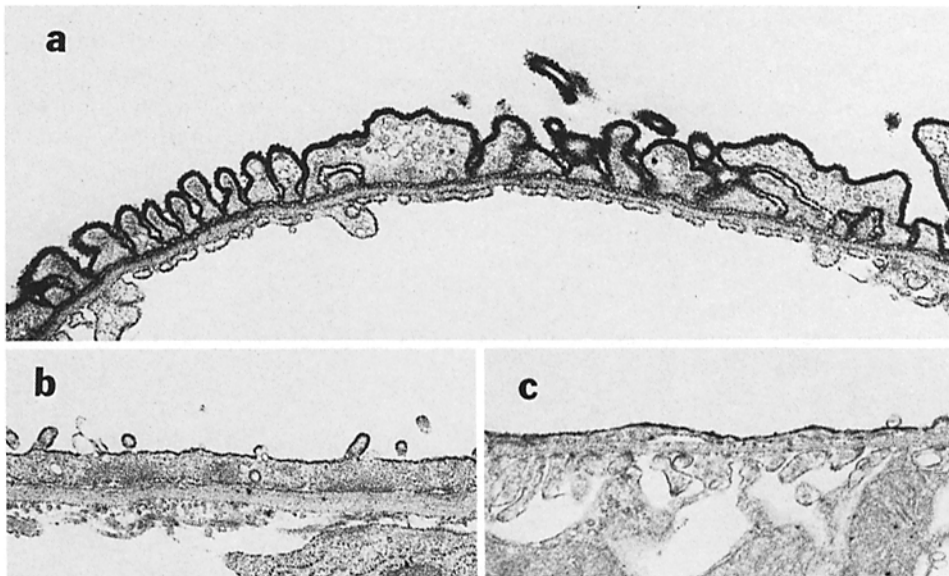

FIGURE 5. Electron immunocytochemistry of rat kidneys fixed in 3% glutaraldehyde for 10 min. (a) Diffuse and intense decoration by mAb G4 of the podocyte fuzzy coat facing urinary space. The part of podocyte membrane that abutts on the lamina rara externa is only weakly decorated. The surface of glomerular capillary endothelium is also weakly decorated on the luminal side. (b) Decoration of the urinary surface of a parietal epithelial cell of a Bowman's capsule. (c) Decoration of the luminal surface of the endothelium of an interstitial capillary. 17,000×.

attenuated and extensively fenestrated, is poorly preserved. No decoration by mAb G4 is discernible within capillary lumens.

Excellent preservation of ultrastructure of glomerular and lung capillaries is obtained by brief perfusion fixation with 3% glutaraldehyde. In renal glomeruli, there was a diffuse, uniform decoration of the cell surface of podocytes (Fig. 5 a). The decoration extends to the surface of pedicels facing interpedicellar slits.

The surface of pedicels abutting on the lamina rara externa of the glomerular epithelial basal lamina, however, is only weakly decorated. The endothelial surface, especially the luminal side, is weakly decorated (Fig. 5a). There is also weak decoration of the urinary surface of the parietal epithelial cell (Fig. 5b). The luminal surface of various extraglomerular endothelial cells in kidneys showed diffuse decoration by mAb G4 (Fig. 5c), as do the endothelia of the pulmonary capillary bed (Fig. 6). In the latter, diffuse decoration of caveolae facing endothelial surface is evident.

The results of SDS-PAGE of glomerular extracts obtained by Triton X-100 and SDS buffer are similar in banding pattern on Coomassie blue stain (Fig. 7, A). One of notable differences is in the 40–65 kD range of the gels, where the SDS extract reveals three discrete bands. The trailing band is estimated to be 62 kD. The Triton X-100 extract shows a broad plateau in this range, and three corresponding bands are barely discernible. Transblot and immune overlay of both extracts identify a solitary, discrete antigen band with an apparent molecular mass of 62 kD (Fig. 7, B). This band is not decorated if the first antibody (G4) is omitted in the control.

Fig. 8 shows the results of slab gel electrophoresis, transblot and immune overlay of mAb G4 and antigen-antibody complex purified by the protein A column. Fig. 8 A is Coomassie blue–stained bands; B, transblot and immune overlay with mAb G4 of a duplicate gel. The mAb purified by the protein A column contains light and heavy chains with apparent molecular masses of 23 and 45 kD respectively (Fig. 8, lane 1 of A). Both bands are decorated on immune overlay (Fig. 8, lane 1 of B). The antigen-antibody complex purified from kidney
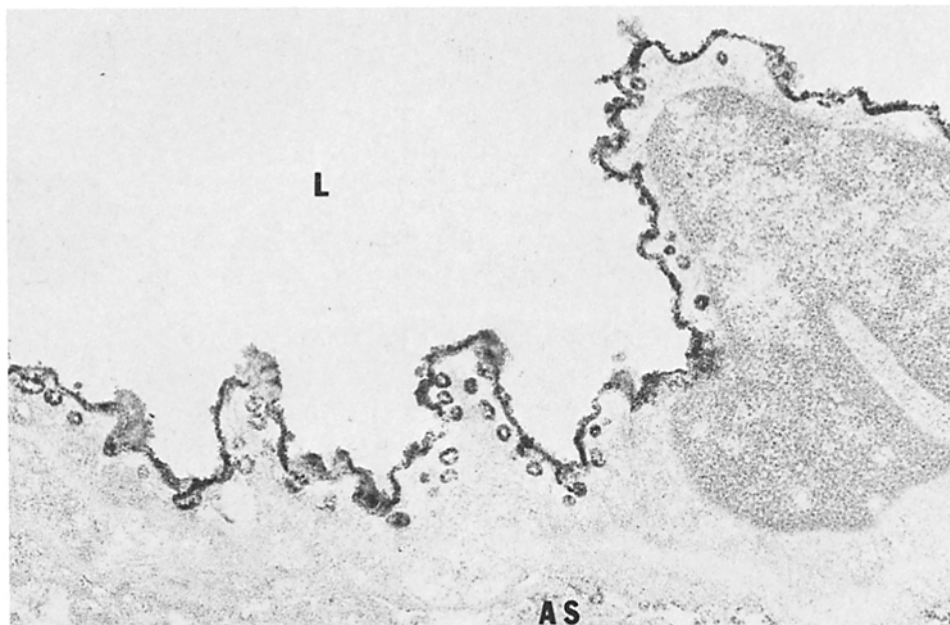

FIGURE 6.   Electron immunocytochemistry of rat lung capillaries shows specific decoration of mAb G4 on the luminal surface of the endothelium. The concave surface of caveolae is also decorated. AS, alveolar space lined by type 1 pneumocytes. L, capillary lumen. 26,800×.
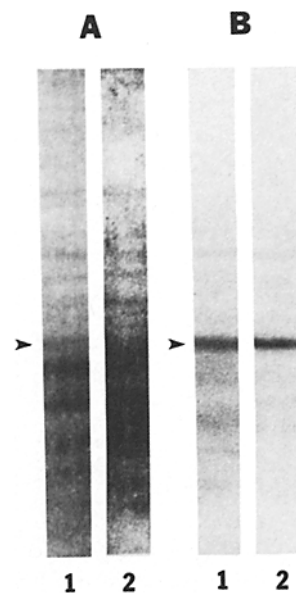
FIGURE 7.   SDS-PAGE of glomerular extracts. Lane 1, extract with Triton X-100; lane 2, extract with SDS-containing sample buffer. (A), gels stained with Coomassie blue. The banding patterns are similar between two gels. (B), transblot onto nitrocellulose paper and immune overlay with mAb G4. Lanes 1 and 2 show single bands of ~62 kD. The antigen (podoendin) band corresponds with a Coomassie blue-stained band (arrowhead) in A.

(lane 2) and lung (lane 3) extracts shows an additional band of 62 kD on both Coomassie blue-stained gels and immune overlay on the nitrocellulose transblot. If the first antibody (G4) is omitted, and only the second antibody is applied in immune overlay, the 62 kD band was not decorated. The light and heavy chains remain decorated, although the decoration is extremely weak.

On polyacrylamide gels, PAS or alcian blue reaction fails to stain the 62 kD band in the glomerular extracts or in immune-complex purified by the protein A column. The absence of polyanions is best shown with spot tests, as in Fig. 9. The SDS buffer extracts from glomeruli (Fig. 9, spot 2) and lungs (spot 4) reveal positive colloidal iron reactions, while protein A column–purified mAb G4 (spot 1) and antigen-antibody complexes purified from kidneys (spot 3) and lungs (spot 5) give negative results. Similar results are obtained by staining with 0.1% alcian blue in 3% acetic acid.

In glomerular culture, outgrowth of large polygonal cells is observed after 1 wk. By 2 wk, a corona of 5 to 20 cells is seen surrounding a glomerulus (Fig. 10). The cell surface of podocytes on a glomerulus, as well as a rim of multilayered cells immediately surrounding the glomerulus is intensely and diffusely decorated by mAb G4 2 wk after commencement of glomerular culture. The monolayer cell outgrowth surrounding the glomerulus, however, showed a rapid decrease in surface decoration and became completely devoid of cell surface decoration. The absence of cell surface decoration is associated with the appearance of scattered decorated cytoplasmic granules (Fig. 10).

In newborn rats, a nephrogenic zone is observed in the subcapsular renal cortex. The earliest identifiable glomerular anlage is on one end of the S-shaped
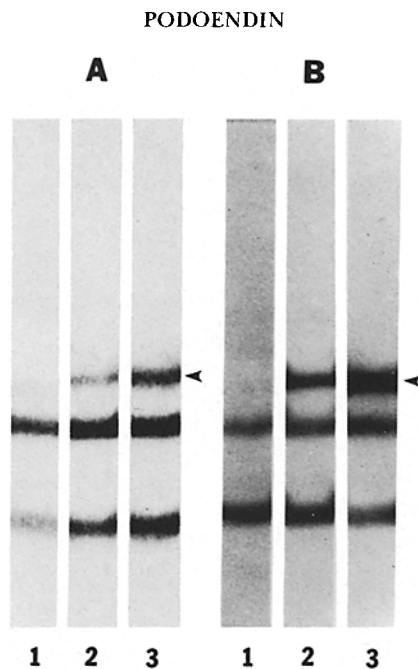
A   **B**

1 2 3   1 2 3

FIGURE 8. SDS-PAGE of protein A column–purified mAb G4 (lane 1) and antigen-antibody complex isolated from kidneys (lane 2) and lungs (lane 3). (*A*), gels stained with Coomassie blue. (*B*), transblot and immune overlay with mAb G4 of a duplicate gel. mAb G4 is composed of light and heavy chains of ~23 and ~45 kD, respectively. The antigen-antibody complexes isolated from kidneys and lungs show an additional 62 kD band (arrowheads) on both Coomassie blue–stained gels, and transblot followed by immune overlay.
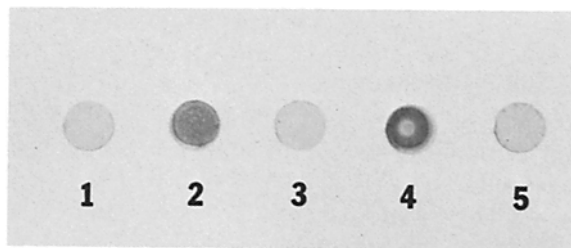

1   2   3   4   5

FIGURE 9. The spot test for polyanions with colloidal iron stain. (*1*), mAb G4. (*2*), Extract from glomeruli. (*3*), Purified antigen-antibody complex from glomeruli. (*4*), Extract from lungs. (*5*), Purified antigen-antibody complex from lungs. Both extracts (*2* and *4*) give intense positive results, while antibody alone (spot *1*) or antigen-antibody complex (spots *3* and *5*) are completely negative.

metanephrogenic vesicle in the immediate subcapsular area (Fig. 11*a*). The anlage is composed of a crescent-shaped slit containing a thin outer epithelium and tall inner columnar epithelium. The former give rise to the parietal epithelium of Bowman's capsule; the latter, glomerular podocytes (28). The capillary endothelial surface in the nephrogenic zone is diffusely decorated by mAb G4 with equal intensity as elsewhere in kidneys (Fig. 11). Among the metanephrogenic vesicles adjacent to the renal capsule, the earliest decoration of glomerular anlage is seen when the urinary side of the visceral epithelium changes from a flat to a convex surface. The decoration is limited to the urinary surface of both
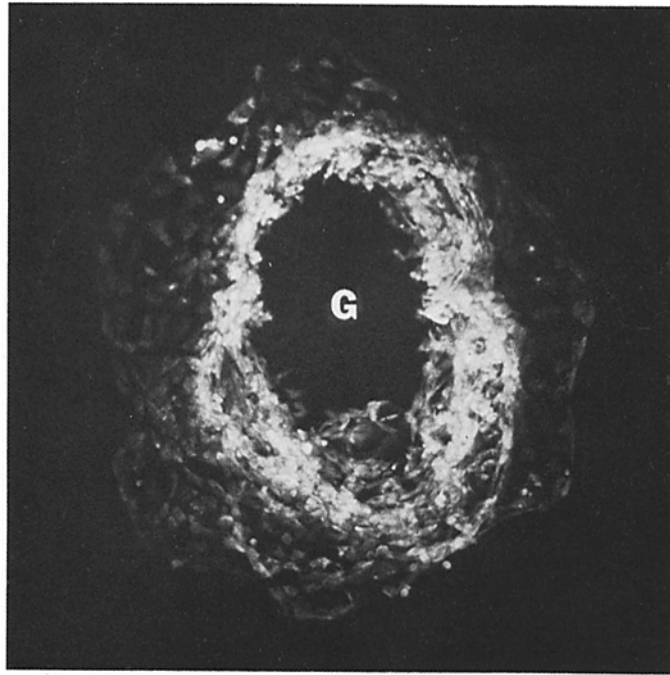
FIGURE 10. Indirect immunofluorescence study of glomerular culture. A glomerulus (G), which fell off from the slide, is surrounded by a corona of large polygonal cells after 2 wk in culture. Cells immediately adjacent to glomeruli appear multilayered and show a diffuse, intense decoration by mAb G4. The decoration decreases and completely disappears from monolayer cells. The latter contain decorated cytoplasmic granules. 160×.

visceral and parietal epithelia (Fig. 11 a). The basal as well as lateral surface is not decorated. As the apical bulge of visceral epithelial cells becomes more prominent and assumes a hub nail shape, the decoration on the urinary surface becomes more intense and extends to the lateral surface of visceral epithelial cells (Fig. 11 b). The basal aspect of podocytes is not decorated until developing glomeruli assume a spherical form accompanied by lobulation of capillary tufts (Fig. 11 c). The basal decoration is very intense and follows the pattern of the epithelial basal lamina. In 5-d-old rats, the findings are similar, except undecorated glomerular anlage are not found. This set of observations is in complete agreement with the results of immunofluorescence studies on fresh frozen sections.

30 min after left ventricular injection, the direct immunofluorescence revealed that the mAb G4 was present in the glomerular capillary wall and endothelial surface of extraglomerular endothelium (Fig. 12). The fluorescence intensity of Fig. 12 b is comparable with the in vitro indirect immunofluorescence shown in Fig. 1 a. The decoration is not accompanied by fixation of complement C3. Electron immunocytochemistry of isolated glomeruli reveals diffuse decoration of glomerular podocytes cell surface and endothelial cell surface (Fig. 13). The glomerular basal lamina is not decorated.

There are no discernible alterations of kidneys by light microscopy 30 min after intracardiac injections. The ultrastructural observations are equally unre-
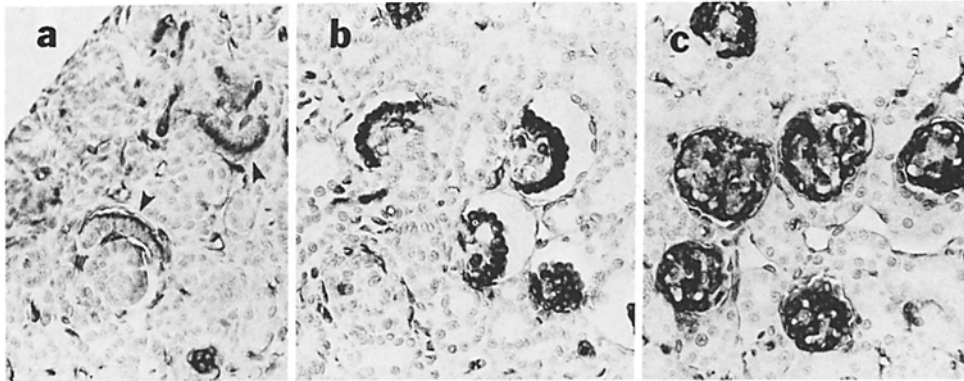
FIGURE 11.   Glomerulogenesis and emergence of podoendin. Newborn rat kidneys decorated with mAb G4, which is revealed by an avidin-biotin HRP technique. (a) A nephrogenic zone is seen in the subcapsular renal cortex of the newborn. The capillary endothelium is diffusely decorated irrespective of their locations. Podoendin first appears on the urinary surface of the visceral epithelial cells of glomerular anlagen (arrowheads) when they change from a flat to a convex surface. The parietal epithelial cell surface decoration appears simultaneously with that of the visceral epithelial cells. (b) In the midzone of the renal cortex, where developing glomeruli assume a spherical shape, the intensity of decoration for podoendin on visceral epithelial cell surface is increased. (c) In deep cortex, mature glomeruli with lobulation of capillary tufts show additional intense decoration of the basal surface of visceral epithelial cells facing glomerular capillary wall. All sections counterstained light green. 220×.
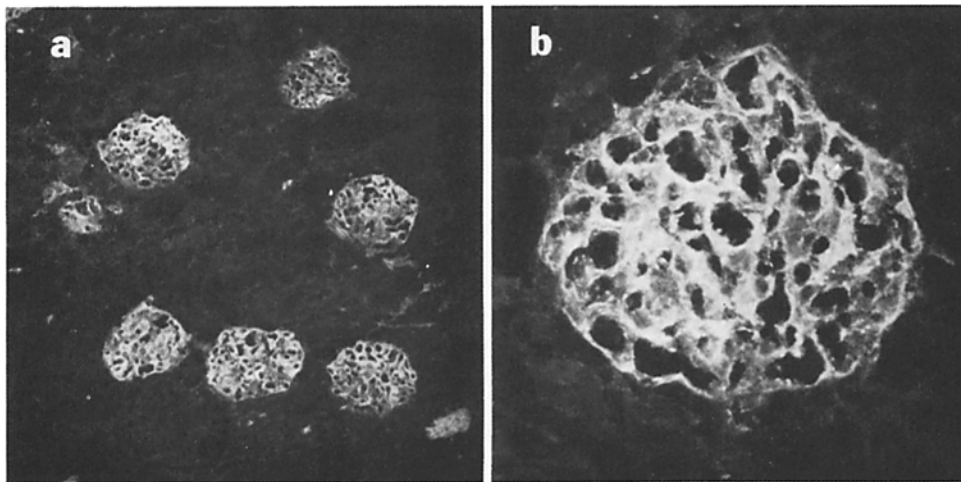

FIGURE 12.   Rat glomeruli 30 min after left ventricular injection of mAb G4. Direct immunofluorescence study with FITC-conjugated rabbit anti–mouse IgG. (a) Diffuse decoration of glomeruli and extraglomerular vascular endothelium. 40×. (b) Higher magnification shows intense decoration of glomerular capillary walls. 162×.

vealing. The interdigitating pedicels of podocytes are discrete, without evidence of retraction or effacement. The slit diaphragm, basal lamina, and endothelial cells are all normal (Fig. 14). There are no deposits. 3 d after intracardiac injection of mAb G4, the results of immunofluorescence and ultrastructural studies remained unchanged, except for the presence of hyaline droplets in renal
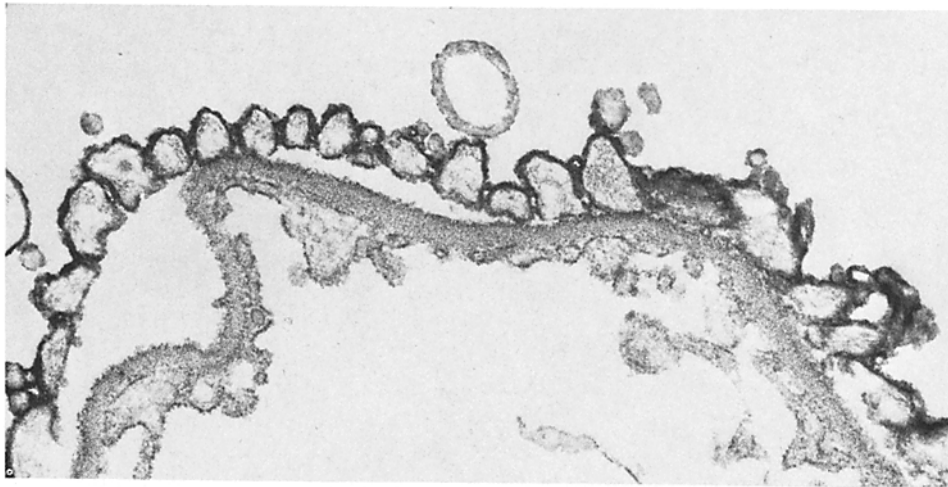
FIGURE 13.   Electron immunocytochemistry of fresh, isolated glomeruli from rats 30 min after left ventricular injection of mAb G4. There is a diffuse and intense decoration of the podocyte surface. 26,800×.
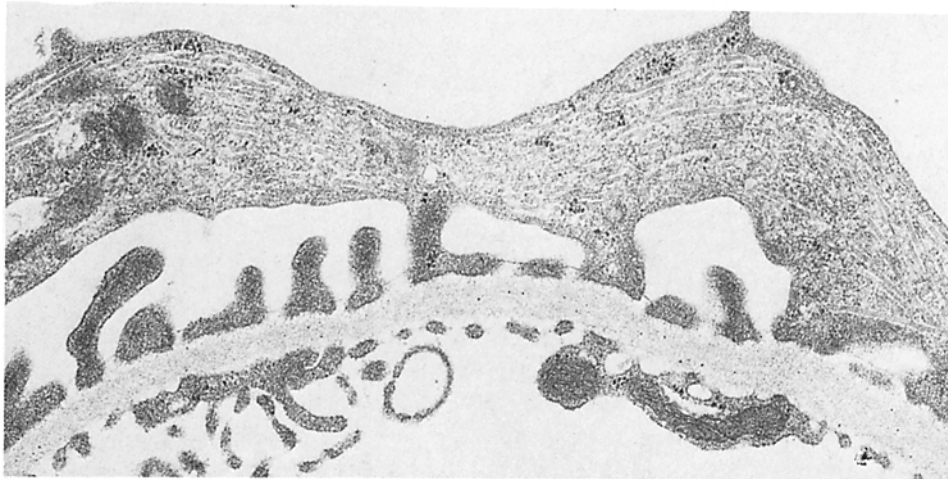

FIGURE 14.   Rat glomeruli 30 min after left ventricular injection of mAb G4. Absence of ultrastructural alterations. 30,600×.

tubules (Fig. 15). The tubular epithelial cells contained increased number of pinocytotic vesicles visible in electron microscopy.

Urine analysis of the experimental animals revealed that the first-day urine contained trace protein. On the second day, one rat had 1+ proteinuria (30 mg/dl protein) and trace blood in the urine; two others, trace proteinuria but no blood. On the third day, all three rats had 2+ proteinuria (100 mg/dl protein). The rat with trace blood in the urine on the second day also had trace blood in the urine on the third day. Ketone and glucose tests were all negative. The control group consistently showed negative results with all tests, except for the first-day urine, one of which showed a trace proteinuria.
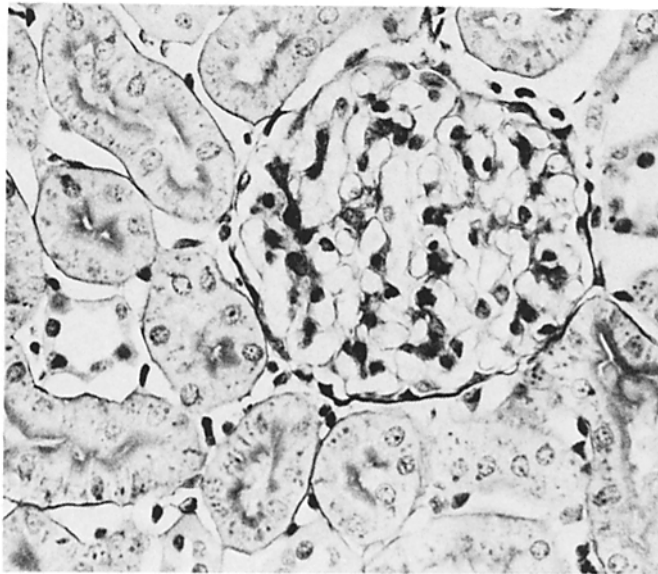
FIGURE 15.   Rat kidneys 3 d after left ventricular injection of mAb G4. Glomeruli and vessels appear normal. Large hyaline droplets are present in the renal tubules. PAS reaction with hematoxylin counterstain. 425×.

Discussion

The hybridoma G4 is one of hybridomas developed in our laboratory during the course of investigating molecular and immunochemical heterogeneity of glomerular basal lamina (27, 29). The glomerular basal lamina preparation used in immunization of mice presumably contains the membrane debris of podocytes adherent to the lamina rara externa and endothelial cell membrane, which remains in the glomerular capillary lumens. The cell surface antigen identified by the mAb G4 had been known as PE (podocyte/endothelium) antigen in our laboratory until our successful isolation and identification of the antigen. It was subsequently renamed "podoendin".

Podoendin is a membrane protein; detergents such as Triton X-100 or SDS are required for extraction. The epitopes for mAb G4 binding are accessible from extracellular space in both fresh and glutaraldehyde-fixed cells. Thus the domain of the molecule extends beyond the outer leaflet of the lipid bilayer and constitutes part of the cell surface fuzzy coat. Ethanol fixation causes precipitation and reaggregation of membrane proteins due to extraction of membrane lipids. Lipid extraction and membrane protein reaggregation presumably make additional epitopes accessible to the antibody.

The immunoreactivity of podoendin is stable in the noncrosslinking fixatives such as 100% ethanol or Carnoy's fixative. Even after Carnoy's fixation and paraffin embedding, podoendin preserves sufficient immunoreactivity to be decorated in the avidin-biotin–conjugated immunoperoxidase method viewed with light microscopy. The pattern of decoration is identical with the immunofluorescence method used on fresh frozen sections fixed with 100% ethanol. Therefore, this became a favored method for immunohistochemical demonstra-

tion of podoendin in our laboratory. This approach, however, abolishes the decoration of sinusoidal lining cells in the liver.

Podoendin is relatively unstable when treated with crosslinking fixatives such as glutaraldehyde or paraformaldehyde, but crosslinking fixatives are required for electron immunocytochemistry. In a preliminary study, fixation in 3% glutaraldehyde in PBS for 1 h at 20°C followed by frozen sectioning completely abolished the immunoreactivity by an indirect (two steps) immunoperoxidase method. Because of the relative susceptibility to crosslinking fixatives, two methods are employed to resolve the structural components in the glomerular capillary wall that is decorated by mAb G4. The first methods is to incubate freshly isolated glomeruli with antibodies, in a manner similar to the method of Mendrick et al. (30) at 4°C. Maintenance of low temperature is of critical importance in preventing potential shifting of cell surface molecules caused by ligand binding. The results clearly show diffuse decoration of the podocyte surface except for the basal aspect of pedicels, which is only weakly decorated. Capillary endothelium is poorly preserved with this method. The most notable finding is the apparent lack of decoration of the glomerular basal lamina. This is colloborated by the set of experiments in which mAb injected into left ventricle of the heart traverse the glomerular basal lamina to decorate podocytes (Fig. 12 and 13) without decorating the basal lamina. Therefore, the absence of basal lamina decoration is real and not due to inaccessibility of basal lamina to the antibody.

Another method we used is to briefly fix the glomerular capillary wall with aldehyde fixatives under well-controlled conditions, as done by Kerjaschki and Farquhar (9). This approach is highly satisfactory in terms of preservation of ultrastructure and immunoreactivity of podoendin. In light microscopy, the indirect immunoperoxidase method, as done for electron immunocytochemistry, shows an identical decoration pattern with immunofluorescence on fresh frozen sections and avidin-biotin–conjugated immunoperoxidase method on Carnoy-fixed paraffin sections. In electron microscopy, the podocyte surface is diffusely decorated, except for the basal surface of pedicels, where the decoration is weak. In addition, there is successful decoration of the endothelial surface. Because of the potential technical pitfalls of immunoperoxidase procedures (31), various experimental conditions such as brief reaction time, or low enzyme or substrate concentration were performed to assess the possibility that the distribution of podoendin may be more restricted to certain parts of the podocyte surface. The results are similar. to those of the standard reaction condition reported above.

We also surveyed the pattern of mAb G4 decoration of brain, heart, skin, skeletal muscle, intestines, and liver with an indirect immunofluorescence method. The results have conclusively demonstrated the specificity of mAb G4 to the surface fuzzy coat of glomerular epthelial cells, the urinary surface of parietal epithelial cells of the Bowman's capsule, and luminal surface of the endothelium. The antibody is also species-specific, and does not crossreact with human or mouse kidneys or other tissues. The tissue reactivity of mAb G4 is very similar to PHM 5 mAb against human glomeruli, reported by Hancock and Atkins (32). They described antibody decoration of podocyte surface and renal endothelial cells, but not glomerular capillary tuft endothelium. Since electron

immunocytochemistry was not performed, comparison with the decoration pattern of our antibody is difficult. Podocalyxin is the major sialoprotein of rat podocyte fuzzy coat, and has been well characterized (11). Distribution of podocalyxin is very similar to podoendin, except that the former is absent from parietal epithelial cells of Bowman's capsules. Therefore, podendin may represent one of a group of podocyte fuzzy coat molecules that are also shared by the endothelium. Because of the unique specificity to the endothelium in the extrarenal tissues, podoendin and podocalyxin can be used as specific cell markers for the endothelium. The endothelial specificity of mAb G4 far exceeds mAb against the endothelial cell surface antigen E92 described by Kaplan et al. (33).

Podoendin is readily extractable with buffers containing Triton X-100 or SDS. SDS-PAGE, transblot to nitrocellulose paper, and immune overlay of glomerular extracts (Fig. 7) confirm that podoendin is a single molecular species of 62 kD. The podoendin band is readily stainable with Coomassie blue on polyacrylamide gels. It is orthochromatic (light purple) when stained with carbocyanine ("stain all", Kodak organic chemical No. 2718) (34, 35), in contrast to the solitary metachromatic (blue) band of 140 kD, tentatively identified as podocalyxin in extracts obtained by SDS-containing buffers (11). Susceptibility of immunoreactivity of podoendin to trypsin digestion and aldehyde fixatives suggests that the molecule is proteinaceous.

With the aid of a protein A column, podoendin is copurified and isolated as an immune complex with mAb G4 (Fig. 8). The purified podoendin, which can be identified by Coomassie blue stain and its immunoreactivity confirmed by transblot and immune overlay, is also a single band of 62 kD. By increasing the sample volume, the podoendin band in each gel can be increased to 5-10 μg to facilitate histochemical characterization with cationic dyes. Failure of alcian blue to stain the band indicates podoendin contains insignificant numbers of anionic groups. This is further supported by a negative spot test with colloidal iron (Fig. 9) and alcian blue. Since these are the most commonly used cationic dyes in histochemical demonstration of podocyte polyanions (2-5, 36, 37), it can be conclusively stated that podoendin is not responsible for cationic dye decoration of the glomerular capillary wall in histochemistry. PAS (24) and alcian blue following periodate oxidation (21) also failed to reveal carbohydrate.

Therefore, podoendin is chemically distinct from the major sialoprotein of podocyte fuzzy coat, podocalyxin. The latter is estimated to contain 20% hexose and 4.5% sialic acid (11). Its molecular mass of 140 kD is twice as much as podoendin. Podoendin is also more stable during extraction procedures from tissues. There are no appreciable differences between SDS and Triton X-100 extracts, and protease inhibitors are not required during tissue homogenization and purification by immune absorption. Podocalyxin is unstable in buffers containing Triton X-100 and has a tendency to degrade into 125 kD and 110 kD components (11). The polyclonal antibodies raised against native podocalyxin do not react with molecules of 62 kD in immune overlay (11). Immune precipitation also fails to precipitate molecules of 62 kD (11). Podocalyxin is also absent from parietal epithelial cells of the Bowman's capsule (11). For these reasons, we believe podoendin is a new molecular species distinct from podocalyxin.

Although both endothelium and podocytes arise from mesodermal derivatives

during embryogenesis (28), they are diverse in structural differentiation and cellular physiology. To ascertain potential heterogeneity among molecules sharing an identical epitope, we decided to isolate podoendin from pure endothelial origin. Lungs were chosen because of their large endothelial surface, endothelium is the only source of podoendin (Fig. 6). Podoendin extracted from kidneys or glomeruli has three potential cellular origins: podocytes, endothelium of glomerular and extraglomerular vessels, and parietal cells of the Bowman's capsule. The results of PAGE, immune blot, and histochemical staining on gels indicate that podoendin isolated from diverse cell types is rather homogeneous.

To assess the possibility that podoendin on the endothelial and podocyte surface may derive its origin from circulating podoendin, SDS-PAGE, transblot, and immune overlay of the rat serum was performed. Within the limitation of the sensitivity of the method, which is in the picogram range, no podoendin was detected in the rat serum. More refined approaches may be required to give a definitive answer to the question.

During development of glomeruli, the appearance of podoendin on the endothelial surface is universal, and not dependent on the development of glomeruli. Its appearance on podocytes and parietal epithelial cells of the glomerular anlage seems to coincide with differentiation of glomerular epithelial cells and appearance of the anionic coat on the convex surface of podocytes (38), thus closely related to the state of differentiation of podocytes. In tissue culture, podocytes are known to proliferate and form a monolayer of polygonal cells, with retraction of pedicels in a dedifferentiated state that is accompanied by the loss of anionic fuzzy coat (39). This is well correlated with the disappearance of podoendin from cell surface in our glomerular culture studies. The appearance of cytoplasmic granules containing podoendin is either due to endocytosis of preexisting cell surface podoendin, or to failure of newly synthesized podoendin to translocate to the cell surface. These observations are consistent with the notion that podoendin on podocytes is a cell differentiation–dependent cell surface protein and not a cell lineage marker. Endothelial podoendin and the state of endothelial differentiation is currently under study in our laboratory.

mAb as a class of antibodies are not highly pathogenic upon reaction with cell or tissue constituents, although scattered reports on cancer cell killing by mAb are known (40, 41). They fail to crosslink antigen molecules unless the latter, in a highly unlikely event, possess two identical epitopes on the same molecule. Thus, mAb do not usually cause cell surface molecule modulation, which requires crosslinking of molecules on the cell surface. Failure of complement fixation, in most instances, also protects tissues from complement-mediated inflammatory reaction and tissue injury (42). Nevertheless, it was decided that useful information may be reaped by intravascular injection of mAb G4. Selection of left ventricular injection is to avoid potential massive trapping of antibody by the immense endothelial surface of lungs during the first passage of the injected antibody. A brief observation period of 3 d also enabled us to observe the direct effect of mAb on kidneys without potential complication of the autologous phase of glomerular injury, when injected rats develop polyclonal antibodies against mouse IgG.

30 min after intracardiac injection of mAb G4, intense binding of antibody to

glomeruli is observed (Fig. 12). The intensity of fluorescence is comparable to in vitro indirect immunofluorescence (Fig. 1 a). This strongly indicates that the injected antibody has traversed through the glomerular filter and bound to the podocyte fuzzy coat, because the fluorescence intensity imparted by binding to endothelial cells alone is normally extremely weak. Binding of antibody to podocyte fuzzy coat is further supported by direct electron immunochemical observation (Fig. 13). It is possible that some of the mouse IgG binding seen in Fig. 13 took place during isolation of glomeruli; its contribution is probably minor, based on the intensity of fluorescence discussed above. The estimated molecular mass of mAb G4 is 136 kD. Its isoelectric point has yet to be determined. Mendrick et al. (30) described an mAb (K9/4) against rat podocyte fuzzy coat that did not cross the glomerular basal lamina barrier upon intravenous injection. Antibody K9/4 does not react with endothelial cells. The relative ease with which our antibody crosses the glomerular filtration barrier remains unexplained.

There are no apparent structural changes to account for pathogenesis of proteinuria. Lack of complement fixation may, in part, account for the absence of inflammatory reaction and complement-mediated tissue injury. We can only speculate that binding of antibody may alter the permselectivity of glomerular filtration barrier, by changing its charge characteristic or by some other unknown mechanisms. Despite proteinuria (100 mg/ml protein on the third day), effacement of podocyte pedicels is not observed. Effacement of podocyte pedicels has been widely accepted as a protective response of glomeruli to prevent protein leakage across glomerular capillaries. Properly functioning contractile protein, actin, is required for retraction and effacement of podocyte pedicels (43). It is also known that binding of polycations to the pdocyte surface causes rapid (within 10 min) effacement of podocyte pedicels (8). It is possible that binding mAb to podoendin may cause an unusual modulation of podocyte behaviors, including absence of pedicel effacement in response to proteinuria.

Summary

A new cell surface protein, podoendin, has been identified in Sprague-Dawley rats, and isolated using monoclonal antibody (mAb) G4. The distribution of podoendin is restricted to the surface of glomerular podocytes, urinary surface of the parietal epithelium of Bowman's capsule, and the luminal surface of endothelial cells. The antibody does not crossreact with podocytes or endothelia of human or mice. In newborn rats, the appearance of podoendin on glomerular epithelium is attendant on podocyte differentiation during glomerulogenesis of metanephrogenic vesicles. It disappears when podocytes retract and efface foot processes in tissue culture. Thus, podoendin appears to be a cell differentiation–dependent surface protein of podocytes.

Podoendin is a protein of 62 kD mobility on 5% polyacrylamide gel electrophoresis. It stains intensely with Coomassie blue, but gives negative reactions to carbohydrate (periodic acid/Schiff reaction) and polyanions (alcian blue, colloidal iron, and carbocyanine). It is distinct from the major sialoglycoprotein of podocyte fuzzy coat, podocalyxin (11). Podoendin isolated and purified from endo-

thelium of lungs appears to be identical with that from podocytes and endothelium of kidneys.

Injection of mAb G4 into left ventricle of rats resulted in intense decoration of the endothelium and podocyte surface within 30 min. The decoration persisted throughout the 3-d period of observation. This was not accompanied by complement (C3) fixation. Preliminary results showed that the rats developed moderate proteinuria (100 mg/ml protein in urine), which was associated with the presence of hyaline droplets in renal tubules, on the third day. The proteinuria was not accompanied by effacement of podocyte pedicels. There were no morphologic alterations indicating glomerular or vascular injury in the kidneys.

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