Glomerular Endothelial Cells Form Diaphragms during Development and Pathologic Conditions

Koichiro Ichimura,* Radu V. Stan,† Hidetake Kurihara,* and Tatsuo Sakai*

*Department of Anatomy, Juntendo University School of Medicine, Tokyo, Japan; and †Angiogenesis Research Center, Department of Pathology, Department of Microbiology and Immunology, Dartmouth Medical School, Lebanon, New Hampshire

ABSTRACT

Unlike most fenestrated capillary endothelial cells, adult glomerular endothelial cells (GEnC) are generally thought to lack diaphragms at their fenestrae, but this remains controversial. In this study, morphologic and immunocytochemical analyses demonstrated that, except for a small fraction, GEnC of adult rats lacked diaphragmed fenestrae, which contain the transmembrane glycoprotein PV-1. In contrast, the GEnC in embryonic rats exhibited diaphragmed fenestrae and expressed PV-1 protein. The luminal surface of the fenestral diaphragm possesses a high density of anionic sites, thereby compensating for the functional immaturity of the embryonic glomerular filtration barrier. In addition, GEnC with diaphragmed fenestrae and PV-1 expression were significantly increased in adult rats with Thy-1.1 nephritis, presumably reflecting a process of restorative remodeling of the glomerular capillary tuft after injury; therefore, the reappearance of PV-1 expression and diaphragmed fenestrae may serve as a marker of glomerular capillary remodeling.


Capillary endothelial cells are morphologically and biochemically specialized to maintain the specific functions of individual organs, and a number of studies have investigated the relationship between their ultrastructural features and their permeability.1,2 In the capillary, where transendothelial exchange vigorously takes place, numerous transcellular pores or fenestrae exist to form “sieve plates” at this highly attenuated portion of endothelial cells.3 Such fenestrated endothelial cells are found in the endocrine and exocrine glands, intestinal villi, choroid plexus, liver sinusoid, kidney (both glomerular and peritubular capillaries), and so forth.4,5

Fenestrated capillaries with diaphragmed fenestrae show a remarkably high permeability to water and other small hydrophilic solutes4; however, their permeability to plasma proteins does not exceed that of the nonfenestrated capillaries or continuous capillaries.7 The impermeability of the fenestrated capillary to plasma proteins is attributed to the existence of fenestral diaphragms and endothelial basement membrane.6 Each fenestral diaphragm morphologically consists of a central mesh (or knob) and several fibrils radiating from mesh to fenestral rim.6,9 Among the several regions of endothelial cell surface, the luminal surface of fenestral diaphragm possesses the highest density of anionic sites, which is derived mainly from heparan sulfate proteoglycans.10–13 These anionic sites presumably contribute to the impermeability of the fenestrated capillary to anionic plasma proteins.

Caveolae and transendothelial channels are also involved in transcapillary exchanges1,14 and are fur-
nished with stomatal diaphragms at their orifices in fenestrated capillaries. Stomatal diaphragms are morphologically similar to fenestral ones, but the former do not possess anionic sites. Caveolae exist in various cell types, but stomatal diaphragms are found only in the caveolae of specific endothelial cell types. These facts suggest that stomatal diaphragms of caveolae may play a specific role in specific endothelial cell types, although their exact functions remain unknown.

Fenestral and stomatal diaphragms have a common structural component, PV-1, a type II transmembrane glycoprotein that forms a homodimer in vivo. In cultured endothelial cells lacking both PV-1 and diaphragms, treatment with phorbol myristate ester induces expression of PV-1 and de novo formation of the fenestral and stomatal diaphragms. Moreover, knockdown of PV-1 expression using an small interference RNA approach prevents the formation of stomatal diaphragms and the formation of fenestrae and transendothelial channels in the cultured endothelial cells treated with phorbol myristate ester. These findings strongly suggest that PV-1 is an essential molecule to the formation of both stomatal and fenestral diaphragms.

In this article, we demonstrate that (1) glomerular endothelial cells (GEnC) in rat adult kidney, apart from a small fraction, do not furnish diaphragms with their fenestrae and caveolae; (2) most GEnC in the immature glomeruli of rat embryos have diaphragmed fenestrae and caveolae; and (3) the number of GEnC with diaphragmed fenestrae and caveolae is increased in the glomeruli of Thy-1.1 nephritis rats. Finally, we discuss the physiologic significance of the diaphragmed fenestrae in GEnC.

**RESULTS**

**GEnC of Normal Mature Glomeruli in Adult Rats**

Most GEnC exhibited nondiaphragmed fenestrae and caveolae in mature glomeruli of normal 15-wk-old rats, as reported previously (Figure 1, A through D), but a small part of GEnC exhibited diaphragms at almost all of their fenestrae and caveolae in normal mature glomeruli (adult rat) (Figure 1, E through G). We found these GEnC with diaphragmed fenestrae in 2.0 ± 0.2% (n = 3) of total glomerular capillary cross-sections with transmission electron microscopy (TEM). In the GEnC with diaphragmed fenestrae and caveolae, transendothelial channels with two diaphragms (closed arrows in E) exist in the capillary cross-sections denoted by the arrow. D and G show crossing sections of non-diaphragmed and diaphragmed fenestrae, respectively. All of the micrographs except for A are of the same magnification. CL, capillary lumen; P, podocyte. Bar = 20 μm in A; 200 nm in B through G.

Double-immunofluorescence staining for PV-1 and intercellular adhesion molecule-2 (ICAM-2), which is a useful positional marker for endothelial cells including GEnC, showed that most GEnC do not exhibit the immunoreactivity for PV-1 in normal 15-wk-old rats (Figure 2, A through D), but a small part of GEnC exhibited PV-1 immunoreactivity in 1.6 ± 0.2% (n = 3) of total glomerular capillary cross-sections.
which were visualized by the anti–ICAM-2 antibody, as observed in TEM samples (Figure 2, C and C’). Immunoreactivity for PV-1 was also found in endothelial cells of peritubular capillary, vasa recta, and intrarenal vein.

**GEnC of Immature Glomeruli in Rat Embryos**

We next observed the GEnC of immature glomeruli in day 18 rat embryos, where all stages of glomerulogenesis except for the completely mature one were found. In the S-shaped body stage, a simple capillary reticulum was situated in the vascular cleft and was close to the columnar epithelial layer of immature podocytes via minute basement membrane (Figure 3A). GEnC from this stage were continuous and had diaphragmed caveolae on both their luminal and abluminal surfaces (Figure 3, B and C). In rare cases, diaphragmed fenestrae and transendothelial channels with two stomatal diaphragms were seen (Figure 3D).

In the capillary loop stage, each glomerular capillary loop protruded toward Bowman’s space, and podocytes developed foot processes and filtration slits. Diaphragmed fenestrae and transendothelial channels were occasionally found, and most caveolae were furnished with stomatal diaphragms.

In the maturing glomerulus stage, capillary reticulum became more complicated, and primitive mesangium became arborized (Figure 3E). GEnC had increased numbers of fenestrae and transendothelial channels. In some GEnC, all of the fenestrae and caveolae were furnished with diaphragms (Figure 3, F and G), whereas in others, diaphragmed and non-diaphragmed fenestrae and caveolae were observed to coexist within the same individual cells (Figure 3H). We also found GEnC whose fenestrae and caveolae were virtually devoid of diaphragms (Figure 3I).

Immunoreactivity for PV-1 was detected in immature glomeruli from the S-shaped body stage to the maturing glomerulus stage and was co-localized with that for ICAM-2 (Figure 4). We further confirmed that PV-1 was predominantly localized at the stomatal and fenestral diaphragms of GEnC in immature glomeruli by the use of immunogold electron microscopy (Figure 5, A through D). Immunogold labelings for PV-1 were also observed at the fenestral diaphragms of pored domes, which form a fenestrated cytoplasmic sheet protruding into capillary lumen (Figure 5E).

**GEnC in Thy-1.1 Nephritis Rats**

Reconstructive remodeling of injured glomerular capillary tuft can be observed in Thy-1.1 nephritic glomeruli as shown in our previous study, then we examined the GEnC in the recovery phase of this nephritis (5 to 30 d after the antibody injection). Enlargement of mesangium including proliferated mesangial cells and increased matrices was observed from day 5 to day 14 in Thy-1.1 nephritis rats (Figure 6A). TEM quantification showed that the percentage of the glomerular capillary cross-sections containing the GEnC with diaphragmed fenestrae was three to four times larger in Thy-1.1 nephritic glomeruli than in control 6-wk-old ones (Figure 6H). In the case of GEnC with diaphragmed fenestrae, almost all of their fenestrae and caveolae were furnished with diaphragms in some cells, whereas in others, diaphragmed and nondiaphragmed fenestrae and caveolae were observed to coexist within the same individual cells. Transendothelial channels were also found in both types of GEnC. Furthermore, some GEnC with diaphragmed fenestrae seemed to be morphologically intact and adhered to glomerular basement membrane (GBM; Figure 6, B through D), but others were detached from GBM (Figure 6E). Diaphragmed fenestrae (50 to 60 nm in diameter) were more uniform in shape and size than nondiaphragmed ones (60 to 160 nm in largest diameter), as shown in normal mature glomeruli (Figure 6, F and G).

Double-immunofluorescence staining for PV-1 and ICAM-2 showed that the percentage of the glomerular capillary cross-sections with PV-1 immunoreactivity was three to six times larger in Thy-1.1 nephritic glomeruli than in control 6-wk-old ones (Figure 7). We confirmed that PV-1 was also specifically localized at the stomatal and fenestral diaphragms.

**Figure 2.** Protein expression of PV-1 in GEnC of mature glomeruli. In normal glomeruli of 15-wk-old rat, most GEnC, which are visualized with the anti–ICAM-2 antibody, show no immunoreactivity for PV-1 (red; A, A’, B, and B’). Only a small number of GEnC exhibit immunoreactivity for PV-1 (arrows in C and C’). Endothelial cells of peritubular capillaries (arrowheads in A, A’, C and C’) and intrarenal vein (asterisk in B and B’) show immunoreactivity for PV-1; however, those of intrarenal artery do not (arrowhead in B and B’). Bar = 50 μm.
study, we demonstrate that GEnC, apart from a small fraction, do not possess diaphragms at their fenestrae and caveolae in adult kidney by precise TEM observation, in addition to the immunocytochemical demonstration of absence of PV-1, the only known component of endothelial diaphragm. These results support the previous morphologic studies.22,23

Rostgaard and Qvortrup28 clearly demonstrated filamentous sieve plugs, which presumably consist of proteoglycans and contribute to impermeability of glomerular capillary wall to plasma proteins at nondiaphragmed fenestrae of GEnC. For visualization of the sieve plug, they used a new fixative containing oxygen-carrying blood substitute and a contrast enhancement method with K$_3$[Fe(CN)$_6$], tannic acid, and uranyl acetate. In this study, we were able to improve visibility of endothelial dia-
phragms containing PV-1 by the use of tannic acid and uranyl acetate as contrast enhancers; however, our simple method was not capable of visualizing the sieve plug at nondiaphragmed fenestrae of GEnC.

We furthermore showed that the GEnC with diaphragmed fenestrae and caveolae frequently appeared in embryonic and Thy-1.1 nephritic kidneys, as summarized in Figure 8. The GEnC form the S-shaped body stage exhibited dia-
phragmed caveolae but rarely diaphragmed fenestrae and transendothelial channels, indicating that the most part of the immunofluorescence signals for PV-1 in these GEnC represent the stomatal dia-
phragms of caveolae. We frequently found diaphragmed fenestrae at the maturing glomerulus stage, and some previ-
ous researchers also reported that dia-
phragmed fenestrae were found in the GEnC of immature glomeruli.23,29,30 Our detailed observations further revealed that the diaphragmed and nondiaphragmed fenestrae coexist in some individual cells at the maturing glomerulus stage, in which fenestrae are vigorously formed. During glomerular development at least, nondiaphragmed fenestrae may hence be formed by the disappearance of diaphragms from existing diaphragmed fenestrae.

In matured glomeruli, permselectivity of capillary wall to plasma proteins is controlled by endothelial glycocalyx, GBM, and podocyte slit diaphragms.27,31–34 In fenestrated capillary,
Fenestral diaphragms are believed to contribute to permselectivity of capillary wall to plasma proteins, because fenestral diaphragms possess abundant anionic sites on their luminal surface and these anionic sites presumably repel plasma proteins.\(^1,11,12\) Fenestral diaphragms in immature glomeruli also carry abundant anionic charges, as seen in other endothelial cell types.\(^23\) It is thus conjectured that fenestral diaphragms may compensate for the functional immaturity of developing glomerular filtration barrier in embryonic kidney; however, for verification of this hypothesis, glomerular permselectivity in PV-1-null mutant mouse embryos will need to be evaluated in future research.

Fenestral diaphragms are probably involved in determining the size of fenestrae in a certain small range, because the diameter of diaphragmed fenestrae was smaller than that of nondiaphragmed ones in immature glomeruli, as shown here. The disappearance of diaphragms from fenestrae and the concomitant enlargement of fenestral diameter are linked to the increase of glomerular filtration coefficient (\(K_f\)). These changes thus contribute to efficient production of a considerable amount of primary urine in mature glomeruli. Similar changes are also seen in the developing liver. Liver sinusoidal endothelial cells (LSEnC) typically have large nondiaphragmed fenestrae in normal adult liver, whereas, in embryonic liver, LSEnC exhibit smaller diaphragmed fenestrae.\(^35\) Such larger nondiaphragmed fenestrae in LSEnC of adult liver enhance the exposure of hepatocytes to circulating blood and permit more efficient clearance of chylomicron remnants.

The reemergence of the GEnC with diaphragms in Thy-1.1 nephritic glomeruli presumably reflects a process of reconstruction of the glomerular capillary tuft in injured glomerulus. Glomerular capillary loops destroyed in Thy-1.1 nephritis undergo restorative reconstruction in an intussusceptive angiogenesis manner, as shown in our previous reports.\(^24,25\) Reconstructing capillaries in Thy-1.1 nephritic glomeruli are likely to recapitulate the following processes as seen in glomerulonephritis: The diaphragmed fenestrae are first formed, then the expression of PV-1 is downregulated, and finally the diaphragms disappear from the fenestrae. Reemergence of diaphragmed fenestrae has also been reported in the LSEnC of cirrhotic liver, in which the organization of the hepatocyte and sinusoidal network undergo remodeling.\(^36\)

Vascular endothelial growth factor (VEGF), which is secreted from podocytes in mature glomeruli, plays a crucial role in the formation and maintenance of fenestrae in GEnC, as well as in various endothelial cell types.\(^37-40\) VEGF has also been reported to upregulate the expression of PV-1 mRNA and protein in human endothelial cell\(^11,42\); however, most GEnC in mature glomeruli do not express PV-1, as confirmed in this study. This inconsistency presumably indicates that the intracellular signaling pathways for the formation of fenestrae and the upregulation of PV-1 are not completely identical. Upregulation of PV-1 requires the activation of phosphatidylinositol 3-kinase and p38 mitogen-activated protein kinase.\(^41,42\) VEGF–phosphatidylinositol 3-kinase–p38 mitogen-activated protein kinase pathway is a candidate for the mechanisms involved in the upregulation of PV-1 in the GEnC of immature and Thy-1.1 nephritic glomeruli. In most GEnC without diaphragms, however, this pathway may be inactivated in adult mature glomeruli by some yet unknown mechanisms. Conversely, the formation of fenestrae requires the thinning of the cell periphery by de-
polymerization of actin filaments, but the pathway linking VEGF and the depolymerization of actin filaments in GEnC remains to be elucidated.

In conclusion, the GEnC in mature glomeruli, except for a small fraction, exhibit nondiaphragmed fenestrae and caveolae. During glomerulogenesis, diaphragmed fenestrae and caveolae, which exist in most GEnC of immature kidney, are replaced by nondiaphragmed ones. Furthermore, the remer-

Figure 5. Localization of PV-1 in GEnC of immature and Thy-1.1 nephritic glomeruli. Localization of PV-1 was examined in GEnC by the use of immunogold electron microscopy. (A through E) E18 rat embryos. Immunogold labelings for PV-1 are predominantly localized at stomatal diaphragms of caveolae (arrows in A and B) and fenestral diaphragms (arrowheads in C and D). Immunogold labelings for PV-1 are also localized at the fenestral diaphragms of pored dome, which form a fenestrated cytoplasmic sheet that protrudes into capillary lumen (arrowheads in E). (A and B) S-shaped body stage. (C through E) Maturing glomerulus stage. (F through H) Thy-1.1 nephritis rats (day 8). Immunogold labelings for PV-1 are predominantly localized at fenestral diaphragms (arrowheads in F and G) and stomatal diaphragms of transendothelial channel (arrows in H). All of the micrographs are of the same magnification. Bar = 200 nm.

Figure 6. Ultrastructure of GEnC in Thy-1.1 nephritic glomeruli. (A) Mesangium (M) is enlarged by proliferated mesangial cells and increased matrices at day 8. Diaphragmed fenestrae (arrowheads in B through E), diaphragmed caveolae (open arrows in B and C), and transendothelial channels with two diaphragms (closed arrows in B) are frequently found in GEnC. GEnC with diaphragms seem to adhere to GBM normally in some sites (B through D) but are separated from GBM in other sites (E). Grazing sections of nondiaphragmed (F) and diaphragmed (G) fenestrae are shown. All of the micrographs except for A are of the same magnification. (H) The percentages of glomerular capillary cross-sections containing the GEnC with diaphragms are significantly large in all of the Thy-1.1 nephritis groups except for day 30, in comparison with control (control 3.1 ± 0.2%; day 5 10.5 ± 0.9%; day 8 12.7 ± 1.0%; day 14 12.6 ± 1.6%; day 30 7.5 ± 1.1%; n = 3 in each group). Bar = 50 μm in A; 200 nm in B through G. *P < 0.05 versus control.
gence of diaphragmed fenestrae and caveolae in injured (Thy-1.1 nephritic) glomeruli presumably reflects the postinjury remodeling of the glomerular capillary tuft.

CONCISE METHODS

Antibodies

Chicken polyclonal anti-rat PV-1 antibody was raised against the last 12 amino acid residues of the extracellular C terminus of the rat PV-1. Production of mouse monoclonal anti-rat ICAM-2 antibody (clone D12) was carried out as described previously. TRITC-conjugated donkey anti-chick IgY F(ab')2 fragment and FITC-conjugated donkey anti-mouse IgG F(ab')2 fragment were from Jackson ImmunoResearch Laboratories (West Grove, PA). Colloidal gold–conjugated goat anti-chick IgY was from British BioCell (Cardiff, UK).

Animals

In all of the experiments, we used Wistar rats obtained from Charles River Japan (Kanagawa, Japan). For induction of Thy-1.1 nephritis, we intravenously injected mouse monoclonal anti-rat Thy-1.1 antibody (clone E30, 100 µg/kg) into 6-wk-old male Wistar rats, as reported previously. All of the procedures performed on laboratory animals were approved by the institutional animal care committee of Juntendo University School of Medicine, and all of the animal experiments were carried out in compliance with the guidelines for animal experimentation of Juntendo University School of Medicine.

Transmission Electron Microscopy

Rat kidneys were perfused with 2.5% glutaraldehyde fixative buffered with 0.1 M phosphate buffer (PB) under anesthesia with pentobarbital. Fixed samples were processed by modified cold dehydration method, as reported previously. In brief, samples were successively immersed in 0.4% OsO4 in 0.1 M PB for 1 h, 2% low molecular weight tannic acid in 0.05 M maleate buffer for 4 h, and 1% uranyl acetate in 0.05 M maleate buffer for 3 h. Samples were then dehydrated with a graded series of acetone at 0 to −30°C before embedding in Epon 812. Ultrathin sections were stained with uranyl acetate and lead citrate and observed with a JEM1230 transmission electron microscope (JEOL, Tokyo, Japan).

Immunofluorescence and Immunogold Labeling

Rat kidneys were perfused with 4% paraformaldehyde fixative buffered with 0.1 M PB under anesthesia with pentobarbital. Fixed samples were immersed successively in PBS containing 10, 15, and 20% sucrose; frozen in OCT compound (Sakura Finetechnical, Tokyo, Japan); and then cut into 5-µm-thick cryosections. After mounting on glass slides, the sections were incubated for 2 h at room temperature or for 12 h at 4°C with the primary antibodies diluted 1:100 with 1% BSA in PBS. Subsequently, the sections were incubated for 1 h at room temperature with fluorescence dye– or colloidal-gold–conjugated secondary antibodies diluted 1:100 to 1:200 with 1% BSA in PBS. Fluorescence specimens were observed with a LSM510 confocal laser scanning microscope (Carl Zeiss, Oberkochen, Germany). Immunogold-labeled specimens were further dehydrated and embedded in Epon 812. Ultrathin gold sections were observed with a JEM1230. Peritubular capillary endothelial cells were available for good positive control sites. As the negative control experiment, the primary antibodies were omitted from the incubation solution.

Statistical Analysis

We quantified the percentage of the glomerular capillary cross-sections containing the GEnC with diaphragms in the TEM samples. Moreover, we quantified the percentage of the glomerular capillary cross-sections containing PV-1–expressing GEnC in the double-immunolabeling samples for PV-1 and ICAM-2. The anti–ICAM-2 antibody was used to visualize all of the glomerular capillary cross-sections, which were recognized as circular or oval linear signals. In both quantifications, we selected the glomerular cross-sections that were expected to pass through the center of the glomerular capillary tuft.
glomerulus and examined 15 to 20 of these sections per animal. Three rats were examined in each group. Values are presented as means ± SEM. Differences were tested using ANOVA followed by the Bonferroni test as post hoc test. \( P < 0.05 \) was considered significant.

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DISCLOSURES

None.

REFERENCES

21. Stan RV, Tkachenko E, Niesman IR: PV1 is a key structural component
for the formation of the stomatal and fenestral diaphragms. Mol Biol Cell 15: 3615–3630, 2004