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Perinuclear Distribution of Plectin Characterizes Visceral Epithelial Cells of Rat Glomeruli

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Plectin is an intermediate-filament-associated protein identified over a wide range of tissue and cell types. The distribution of this protein in glomerular visceral epithelial cells (VECs) during the differentiation and growth of rat kidneys was studied in comparison with that of vimentin. By immunofluorescence microscopy, preferential localization of these two cytomatrix elements was different, although both were observed in the cell body and primary processes of VECs. Strong staining of plectin was always found in the perinuclear region of the VEC body in kidneys of young and adult rat, but vimentin stained distinctly only in the primary processes of young rats yet in both cell bodies and primary processes of the adults. This perinuclear staining was unique to VECs, that is, was absent from other cells. In the neonatal kidney, plectin staining during differentiation of VECs changed from weak and diffuse throughout the cytoplasm in the S-shaped body to prominently perinuclear in the maturing stage. However, after the differentiation of VECs, the staining intensity of plectin did not change further. In contrast, that of vimentin increased conspicuously in parallel with the growth of VECs rather than at differentiation. After a long period of culture and during aminonucleoside nephrosis, situations when VECs lose differentiated phenotypes, most of the cells had no perinuclear plectin. These findings indicate that the perinuclear distribution of plectin may play an important role in the differentiation of VECs. (Am J Pathol 1996, 149:319–327)

Visceral epithelial cells (VECs) of renal glomeruli, also called podocytes, are believed to be an especially differentiated cell type that rarely undergoes cell division in the adult.[1–3] They have long cytoplasmic processes extending from the main cell body toward the glomerular basement membrane (GBM). The long primary processes divide and are affixed to the GBM by numerous secondary processes (foot processes). Their complicated shape has been studied extensively by electron microscopy to understand the mechanisms that maintain the well developed cytoskeleton of VECs.[4,5] Microtubules and intermediate filaments (IFs) are clear, especially within the cell body and the primary processes, as are numerous clustered microfilaments in the foot processes. Among the cytoskeletal proteins, IF proteins are the most characteristic of VECs, which contain vimentin but not cytokeratins.[6–10] Vimentin-containing IFs are typically found in most non-epithelial and non-neuronal cell types, whereas cytokeratins are present in most epithelial types.[11–13] VECs in the rat also express the muscle-type IF protein desmin, and the intensity of their desmin staining increases with the animal's age and in conditions of nephrosis.[14]

Although the function of IFs has not been resolved, IF structure and dynamics are becoming clear, and the number of IF-associated proteins (IFAPs), proteins considered to interact with IFs, is steadily growing.[15,16] One such IFAP, called plectin, is found in numerous cell types.[17] Based mainly on biochemical and immunolocalization studies, plectin is postulated to function as a general cytoplasmic cross-linking element of IFs, eg, joining them to the plasma membrane via spectrim, to the nuclear envelope via nuclear lamins, and to microtubules via

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microtubule-associated proteins.[18,19] Furthermore, by self-association, plectin can form its own network, which may convey stability to cytoplasmic areas devoid of cytoskeletal filaments.

The purpose of the present study was 1) to examine the distribution of plectin in rat VECs in comparison with that of vimentin and 2) to determine whether the expression of plectin changes in relation to differentiation and pathological or culture conditions. Our results show that plectin in VECs has a unique cytoplasmic distribution with a distinct tendency to accumulate in perinuclear regions. This perinuclear distribution occurs during the differentiation of VECs but diminishes under conditions of nephrosis and culture.

Materials and Methods

Animals and Antibodies

WKY rats were purchased from Charles River Japan (Atsugi, Japan) and used in this experiment at the ages of 1 and 11 days, 4 and 8 weeks, and 5 months. Murine monoclonal antibodies to plectin (10F6, 1A2, 6B8, 5C6, and 7A8) and rabbit polyclonal antiserum to plectin (P21) were previously characterized.[20,21] Murine monoclonal anti-vimentin antibody (V9) was obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). Guinea pig polyclonal antiserum to vimentin was kindly provided by Dr. W. W. Franke, German Cancer Research Center (Heidelberg, Germany). Rabbit antisera to desmin and laminin were products of Bio-Science Products (Emmenbrück, Switzerland) and Serotec (Oxford, UK), respectively. For secondary antibodies, fluorescein isothiocyanate (FITC)- or tetramethylrhodamine isothiocyanate-conjugated goat anti-mouse IgG was purchased from Cooper Biomedical (Malvern, PA). Texas Red-conjugated goat anti-guinea-pig IgG, and FITC-conjugated goat anti-rabbit IgG were from Dianova (Hamburg, Germany) and from Medical and Biomedical Laboratories (Nagoya, Japan), respectively. These secondary antibodies were mixed with normal rat serum and allowed to stand overnight for absorption before use.

Immunofluorescence Microscopy

The indirect immunofluorescence technique was applied to frozen kidney sections, isolated glomeruli, and cultured glomerular cells as described previously.[14,22,23] In brief, the rat kidneys were snap-frozen at −70°C, sectioned at a thickness of 4 μm in a cryostat, and fixed in acetone at 4°C for 5 minutes. Air-dried sections were processed for double-label immunostaining. Glomeruli isolated from these kidneys, which were perfusion fixed with periodate-lysine-paraformaldehyde for 5 minutes, and cultured cells were processed in the same fixative for 10 minutes, permeabilized with 0.3% Triton X-100 in phosphate-buffered saline (PBS) for 5 minutes, and stained with antibodies. For double-label immunofluorescence microscopy, primary and secondary antibodies were applied simultaneously. PBS, normal rabbit or guinea pig serum, or murine IgG1 monoclonal antibody (against rotavirus), shown not to react with rat glomeruli, were used as negative controls for the primary antibodies. Immunofluorescence of the sections and cultured cells was performed with a Vanox AH-2 microscope (Olympus, Tokyo, Japan). Isolated glomeruli and cultured cells were monitored for immunofluorescence by a laser scanning confocal microscope (MRC-1000 and MRC-1024, Bio-Rad Laboratories, Hemel Hempstead, UK).

Isolation of Glomeruli and Culture Conditions

Glomeruli from rat kidneys were isolated as described previously.[22] In brief, glomerulus-enriched tissue was obtained by serial sieving of renal cortical pieces. The tissue consisted mainly of decapsulated glomeruli but also contained a small number of encapsulated glomeruli and tubular fragments. Decapsulated or encapsulated glomeruli were selected by sucking them into a micropipette under an inverted tissue culture microscope with phase-contrast optics and then cultured on type I or type IV collagen-coated Lab-Tek glass slides (Miles Scientific, Naperville, IL) in RPMI 1640 medium supplemented with 5% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml).

Induction of Aminonucleoside Nephrosis

Eight-week-old rats were injected intraperitoneally with a single dose of puromycin aminonucleoside (Sigma Chemical Co., St. Louis, MO), 10 mg/100 g body weight as a 1% solution in PBS. The animals were then examined as described previously.[14,24]

Results

Immunolocalization of Plectin in Glomeruli of Normal 5-Month-Old Rats

The localization of plectin in glomeruli was examined by double-label immunostaining with anti-plectin and

anti-laminin antibody (Figure 1, a and a′). A number of monoclonal antibodies to plectin recognizing different epitopes[20] as well as a rabbit antiserum to plectin antibody stained the renal tissue similarly. Although all of the cells in glomeruli were stained diffusely in the cytoplasm with anti-plectin antibodies, prominent fluorescence specific to plectin was observed in VECs located on the GBM facing Bowman's space. Endothelial and mesangial cells showed weaker plectin staining. Confocal laser scanning microscopy used in a double-label technique with anti-vimentin antibody clarified the differing distributions of plectin and vimentin in VECs on isolated glomeruli (Figure 1, b and b′). Intense staining of plectin appeared in the cell body, whereas that of vimentin was in both the primary processes and the cell body. Immunofluorescence microscopy at high magnification revealed the accumulation of plectin in the perinuclear region of VECs (Figure 1, c and c′). The nuclei of VECs that had deep indentations were distinctly outlined by plectin staining (Figure 1, d and e). The perinuclear staining was unique to VECs, as parietal epithelial cells (PECs) of Bowman's capsule and other tubular cells showed diffuse staining throughout the cytoplasm with or without intense labeling at or around the plasma membrane. It should be noted that, in a previous immunolocalization study[21] performed with an antiserum to plectin, the occurrence of plectin in VECs of rat kidney went undetected, probably due to technical insufficiencies in tissue preparation.

Change of Plectin Staining during the Differentiation and Growth of VECs

Neonatal kidneys were examined to determine whether the perinuclear staining of plectin related to the differentiation of VECs. During the S-shaped stage at the beginning of glomerulogenesis, the presumptive VECs showed very weak diffuse staining of plectin in the cytoplasm (Figure 2, a and a′). In the capillary loop stage, when capillaries formed in glomeruli, plectin staining in VECs became more distinct, and perinuclear accumulation of plectin was recognizable in some cells (Figure 2, b and b′). Young, 11-day-old rats with glomerulogenesis almost complete had some glomeruli in the subcapsular region of the renal cortex yet still remained in the maturing stage. In these glomeruli, intense perinuclear fluorescence specific to plectin was observed in every VEC (Figure 2, c and c′) and was not different in young and adult rats (Figure 2, c–d′). Comparatively, changes of vimentin expression in VECs were much more conspicuous after the maturation of glomeruli compared with that in the developing ones. VECs in the S-shaped body showed weak but significant staining for vimentin (Figure 3, a and a′). Then, during the maturation of glomeruli, the intensity of vimentin staining increased mainly in the primary processes of VECs (Figure 3, b–c′). However, staining in the cell body remained weak even in differentiated VECs of the young rats (Figure 3, c and c′). Adult rat VECs showed extremely enhanced vimentin staining, especially in the cell body, compared with that in the young rat (Figure 3, d and d′), a striking contrast with the unchanged plectin staining of young and adult rats.

Change of Plectin Staining in Glomerular Cell Culture

In addition to cytoplasmic staining of plectin, most large irregular cells just outgrowing from glomeruli exhibited embossed nuclear staining (Figure 4, a and c) and sharp outlines of the nucleus (Figure 4e) by conventional and confocal immunofluorescence microscopy, respectively. In contrast to these large irregular cells, polygonal cells of cobblestone-like appearance had no such distinct staining on the nucleus or, if any, had much weaker staining (Figure 4, b, d, and f). Cytoplasmic staining was prominent in those cells. The unique plectin staining on the nucleus became obscured for longer periods of culture. After 10 days of culture, we no longer found this staining pattern (Figure 4, g–i).

Change of Plectin Staining in Aminonucleoside Nephrosis

Rats given a single intraperitoneal injection of puromycin aminonucleoside began to develop massive proteinuria within 4 days. Urinary protein excretion attained a maximum of 700 mg/day within 2 weeks. In animals manifesting a proteinuric state, the perinuclear dominance of plectin was obscured in many VECs due to weaker staining in the perinuclear region or enhanced staining throughout the cytoplasm (Figure 5, a–b′).

Discussion

The unexpected results of this study to locate the sites of plectin binding compared with those of vimentin in rat glomerular VECs showed plectin attached predominantly to the perinuclear region. These sites were distinct from those where vimentin

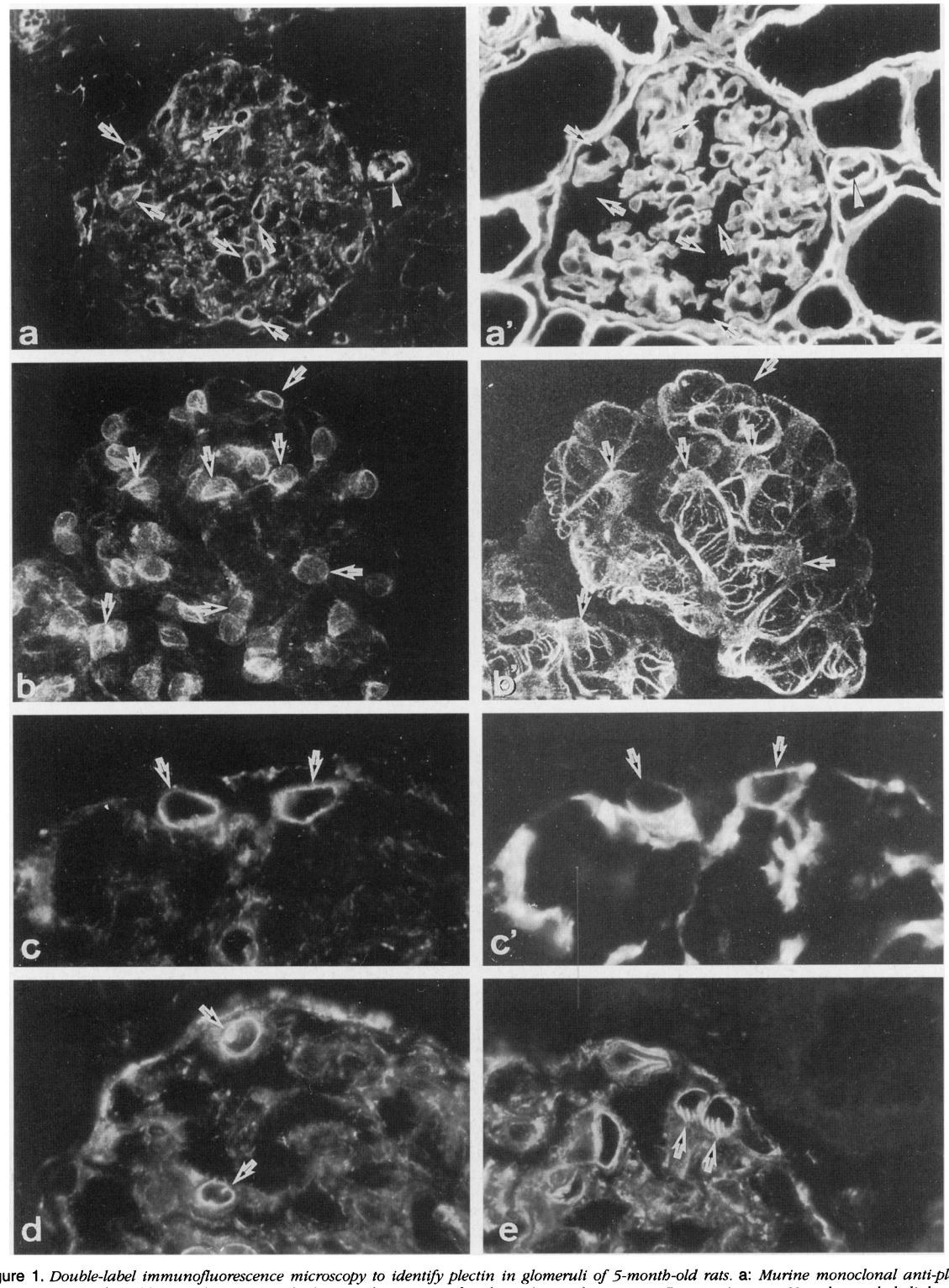

Figure 1. *Double-label immunofluorescence microscopy to identify plectin in glomeruli of 5-month-old rats.* **a**: *Murine monoclonal anti-plectin antibody (7A8).* **a'**: *Rabbit anti-laminin antibody. Circular staining for plectin* (**arrows**) *is seen in Bowman's space. Note that endothelial cells of arterioles* (**arrowheads**) *express plectin intensely.* **b**: *Rabbit anti-plectin antibody.* **b'**: *Murine monoclonal anti-vimentin antibody. Project images combining a three-dimensional Z-series in laser scanning microscopy of isolated glomeruli show that plectin is located mainly in the cell body of VECs, whereas vimentin is detected in the primary processes rather than the cell body.* **Arrows** *indicate the same VECs.* **c**: *Murine monoclonal anti-plectin antibody (7A8).* **c'**: *Guinea pig anti-vimentin antibody. Plectin in the cell body is concentrated around the nucleus, whereas vimentin is distributed throughout the cytoplasm of the cell body.* **d** *and* **e**: *Murine monoclonal antibodies (10F6, 1A2). Indented sites* (**arrows**) *of the nucleus of VECs are distinctly outlined. Magnification, ×440* (**a** *and* **a'**)*; ×460* (**b** *and* **b'**)*; ×1100* (**c** *and* **c'**)*; ×830* (**d** *and* **e**).

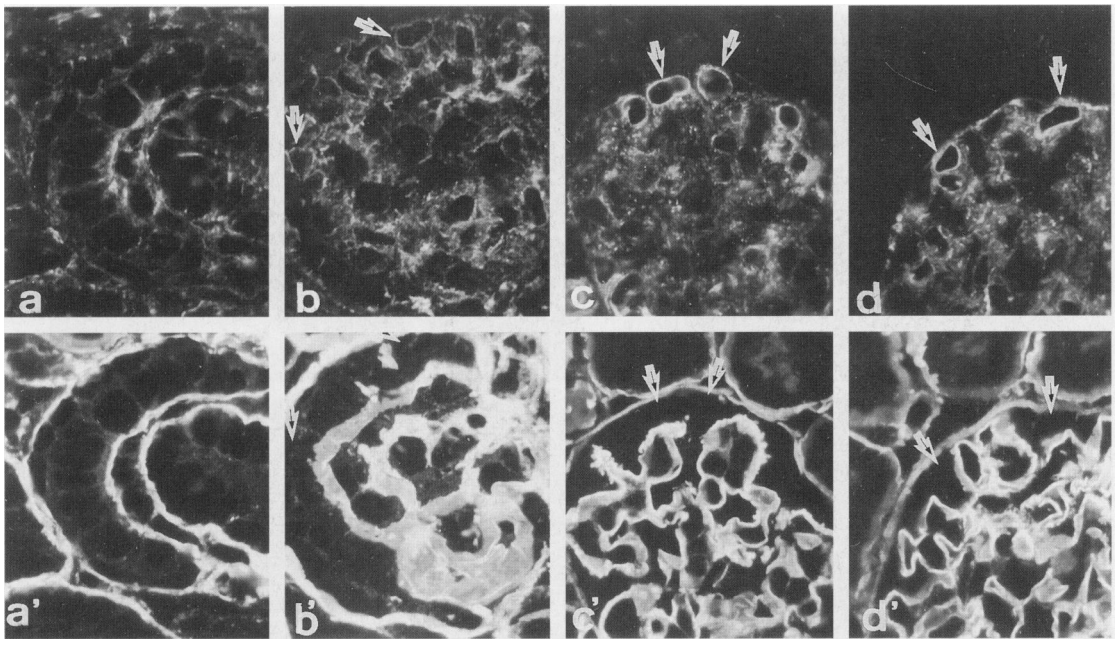

Figure 2. *Double-label immunofluorescence microscopy of kidney sections of 1-day-old* (a *to* b'), *11-day-old* (c *and* c'), *and 8-week-old* (d *and* d') *rats using monoclonal anti-plectin antibody* (*7A8*) (a *to* d) *and rabbit anti-laminin antibody* (a' *to* d'). *Weak and diffuse staining of plectin is seen in the cytoplasms of VECs in the S-shaped body* (a *and* a'). *In the capillary loop stage, plectin staining becomes distinct, and some VECs show perinuclear staining* (arrows) (b *and* b'). *In the maturing stage, perinuclear distribution of plectin is complete* (c *and* c'). *VECs in the adult show the same plectin staining as those in the maturing stage* (d *and* d'). *Magnification,* ×*580.*

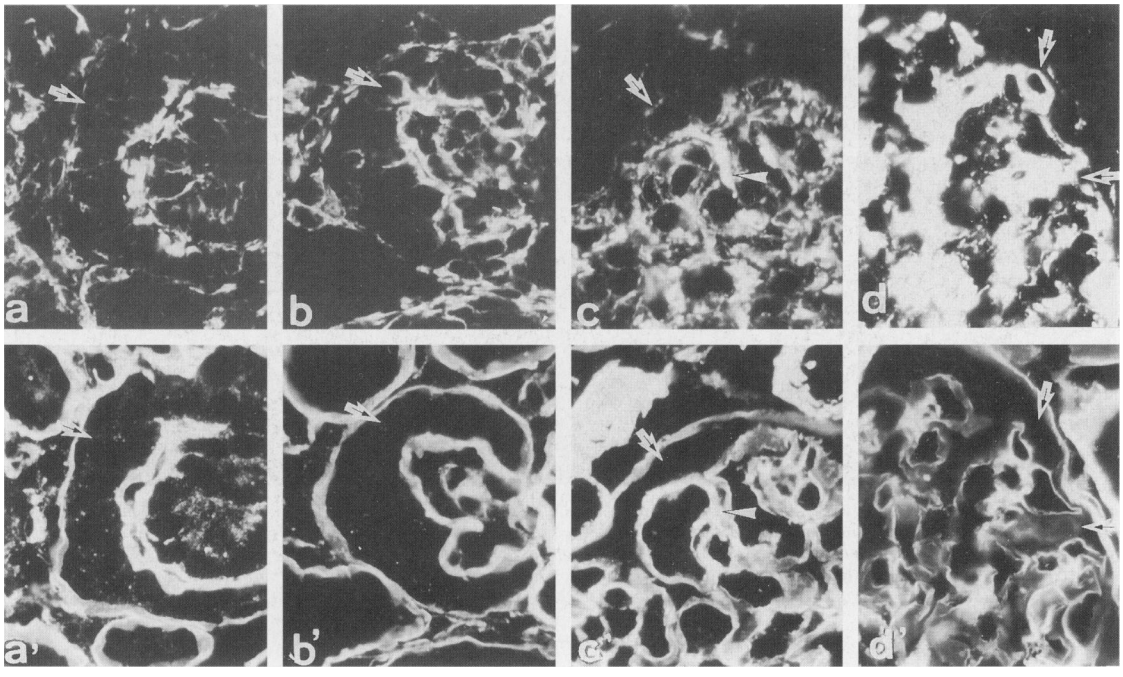

Figure 3. *Double-label immunofluorescence microscopy of kidney sections of 1-day-old* (a *to* b'), *11-day-old* (c *and* c'), *and 8-week-old* (d *and* d') *rats using murine monoclonal anti-vimentin antibody* (a *to* d) *and rabbit anti-laminin antibody* (a' *to* d'). *In the S-shaped body, VECs stain very weakly with anti-vimentin antibody in contrast to invading vessels expressing vimentin intensely* (a *and* a'). *In the capillary loop stage, distinct staining is observed near the GBM in some VECs* (b *and* b'). *In the young rat, vimentin staining is intense in the primary processes* (arrowheads) *but weak in the cell body* (c *and* c'). *In the adult, VECs become much richer in vimentin than those in the young* (d *and* d'). *Photos were taken at the same exposure time. Magnification,* ×*580.*

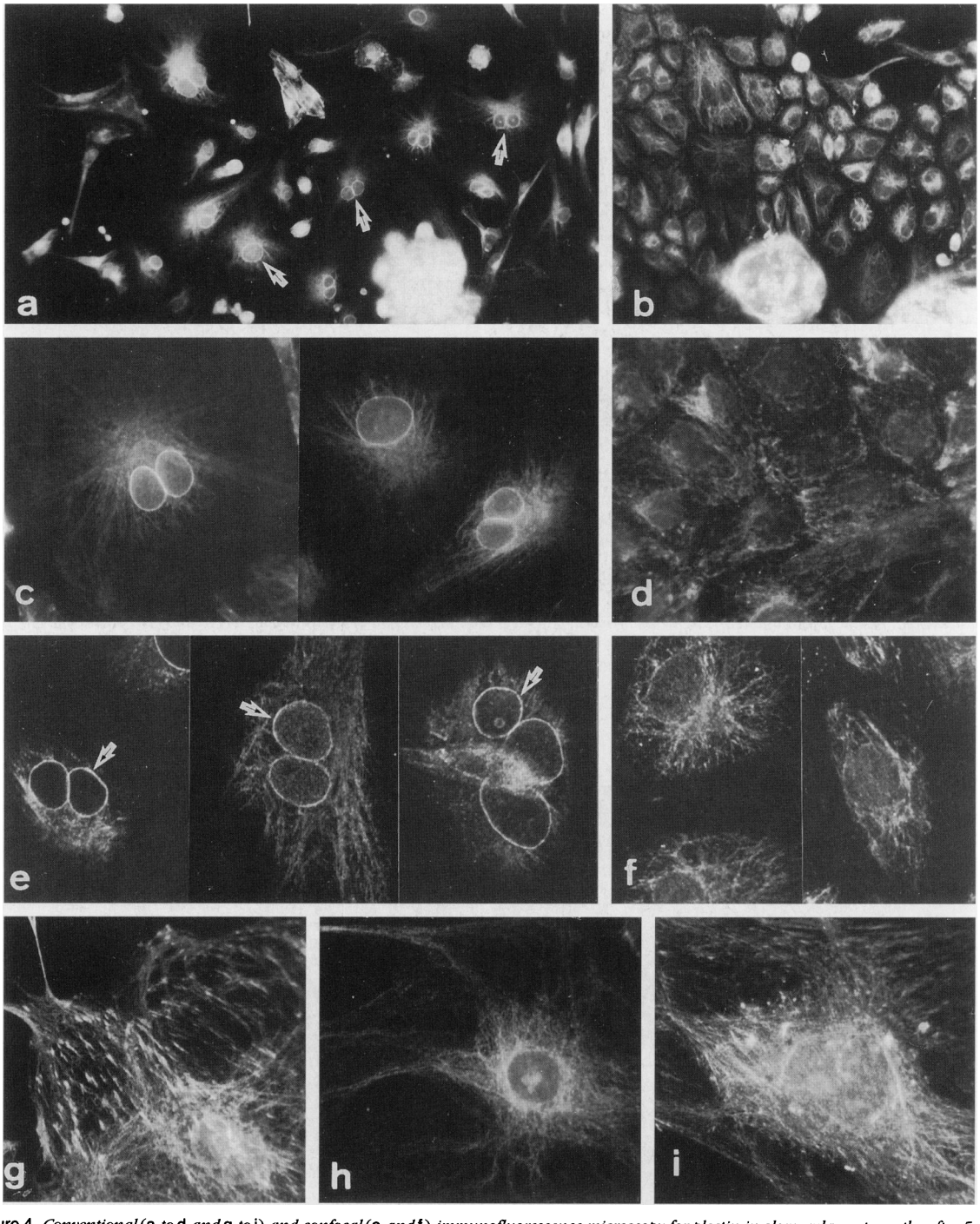

Figure 4. *Conventional* (**a** *to* **d** *and* **g** *to* **i**) *and confocal* (**e** *and* **f**) *immunofluorescence microscopy for plectin in glomerular outgrowths after 5 days* (**a** *to* **f**) *and 10 days* (**g** *to* **i**) *of culture. Two types of cell, large irregular cells* (**a**, **c**, *and* **e**) *and regular polygonal ones* (**b**, **d**, *and* **f**), *are observed in early glomerular outgrowths. Plectin staining emphasizes nuclear shape of the former cells* (**a** *and* **c**). *Confocal microscopy shows sharp outlines of the nuclei* (**e**). *No such distinct staining is observed in the latter cells, whereas cytoplasmic staining is prominent* (**b**, **d**, *and* **f**). *After 10 days of culture, the distinct staining on the nucleus is not observed in any of the cells* (**g** *to* **i**). *Magnification,* ×*170* (**a** *and* **b**); ×*460* (**c**, **d**, *and* **g** *to* **i**); ×*580* (**e** *and* **f**).

was distributed in the VECs. Plectin has been shown to bind to various IF subunit proteins including vimentin,[25] which VECs express prominently. Moreover, immunofluorescence microscopy previously revealed the widespread occurrence of plectin in a variety of cell types.[17,21,26] Depending on that type, the plectin was found primarily either throughout the cytoplasm, at the cell periphery, or in both locations.

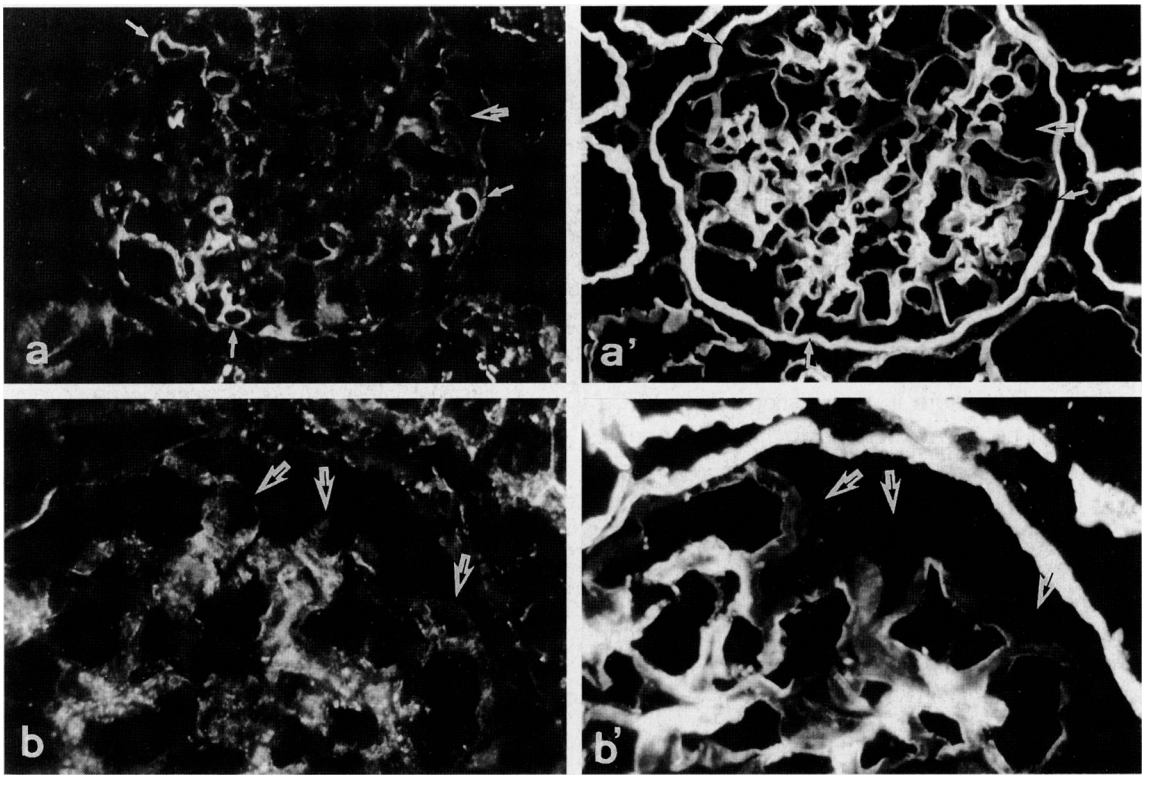

Figure 5. *Double-label immunofluorescence of kidney section at 2 weeks after a single injection of puromycin aminonucleoside. Using murine monoclonal anti-plectin (7A8)* (a *and* b) *and rabbit anti-laminin* (a′ *and* b′) *antibody, some VECs show intense staining of plectin in both the cell body and the primary processes* (small arrows), *and others show weak cytoplasmic staining* (arrows). *Perinuclear pattern of plectin staining as seen in Figure 1,* a *and* c, *is not observed in most of the VECs. Magnification, ×420* (a *and* a′); *×810* (b *and* b′).

However, among these various patterns, the perinuclear staining of plectin is unique to VECs. When we used double labeling with phalloidin or anti-β-tubulin antibody to examine whether other cytoskeletal proteins co-distributed with plectin in the perinuclear region, no other perinuclear staining was evident (data not shown). Whether this perinuclear accumulation of plectin we saw resulted from its association with other structures, such as lamin B,[19] or constituted self-association[17] was not resolved.

During their differentiation and subsequent growth, VECs exhibited various changes of plectin and vimentin staining and intensity. The characteristic differentiation of VECs parallels the development of glomeruli, which is arbitrarily divided into four stages: vesicle, S-shaped body, capillary loop, and maturing stage.[27] The formation of foot processes typical of VECs begins in the capillary loop stage, and VECs largely achieve their normal configuration in foot processes and filtration slits during the maturing stage. Glomerulogenesis is complete on approximately the 10th day of age in the rat.[28] The number of VECs does not increase further after the 6th week of age, maintaining approximately 160 cells per whole glomerulus throughout life.[29,30] Yet after maturation, the glomerular volume continues to increase significantly at least until week 60. The main mechanism by which VECs adapt to this glomerular growth is by increasing their cell volume, in contrast to endothelial and mesangial cells, which increase in number.[31] Our experiments reflected these sequential changes as immunofluorescence specific to plectin and vimentin in VECs changed from very weak in the immature S-shaped body to intense in mature glomeruli. The behavior, however, was completely different for the two molecules. A conspicuous change of plectin staining was observed during differentiation of VECs, but no similar change of vimentin expression occurred until the period of glomerular growth. Plectin staining became distinct in the capillary loop stage, and the shift from a cytoplasmic to a perinuclear distribution was complete in the maturing stage. After that, no additional changes were detected. On the other hand, vimentin staining was much enhanced during glomerular growth rather than glomerular development. Based on these findings, we conclude that the perinuclear staining of plectin can serve as a differentiation marker for VECs, whereas the

changes in expression of vimentin can be considered responses to circumstances such as those of desmin in a nephrotic condition.[14] IFs withstand much greater stretching forces than microtubules or actin filaments.[32] Therefore, abundant vimentin filaments in mature VECs may maintain their integrity by preventing excessive deformity as mechanical tension mounts due to glomerular growth and increasing glomerular filtration rate.[29,33]

In this study, some cells growing out from cultured glomeruli displayed distinct plectin staining on the nucleus. Yet a controversy surrounds the matter of glomerular cell culture in that distinguishing VECs from PECs is not always certain.[22,34–36] We reported earlier[22,23] that regular, polygonal cells were very similar to PECs in morphology and phenotype and that large cells of irregular shape expressed VEC-specific antigens such as podocalyxin and pp44. Thus, the former cells presumably originated from PECs and the latter from VECs. The perinuclear staining of plectin described here was observed in the large irregularly shaped cells, which sometimes extended long cytoplasmic processes and were multinucleated; in contrast, regular polygonal cells did not show such perinuclear staining. These findings sustain the above idea and indicate that the staining pattern of plectin can be considered a specific marker of VECs in culture.

The perinuclear staining shown here tended to disappear after a prolonged period of culture and in VECs from rats with puromycin-aminonucleoside-induced nephrosis. Under both of these conditions, VECs have been reported to lose many differentiated phenotypes.[23,37–41] Most of their foot processes, filtration slits, and some VEC-specific antigens are lost, and junctional complexes are located between VEC processes. These changes, including the loss of plectin's perinuclear localization, seem closely linked to the differentiation of VECs, giving rise to their complicated morphology. Because their unique structure suits them so well for biological filtration processes, the function of plectin in the morphogenesis of VECs deserves full clarification.

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