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E. Yaoita · H. Kurihara · T. Sakai · K. Ohshiro
T. Yamamoto

Phenotypic modulation of parietal epithelial cells of Bowman's capsule in culture

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Abstract The origin of cobblestone-like polygonal cells (the most numerous in renal glomerular cell culture) remains controversial; they could be either dedifferentiated podocytes or parietal epithelial cells (PECs) of Bowman's capsule. Poor cellular outgrowth from glomeruli devoid of Bowman's capsule (decapsulated glomeruli) hinders podocytes being obtained without contamination of PECs in culture. Since podocytes are easily damaged during the isolation of glomeruli by the conventional sieving method, we devised a gentle isolation method without forced sieving, resulting in substantial numbers of arborized cells growing out from decapsulated glomeruli. The cells were distinctly different from cobblestone-like polygonal cells in their irregular and often arborized shape and extended long cytoplasmic processes that often crossed over adjacent cells. The arborized cells from decapsulated glomeruli showed intense staining for a podocyte-specific marker, podocalyxin, but no staining for markers specific to PECs (pan cadherin), mesangial cells (Thy-1) or endothelial cells (von Willebrand factor, RECA-1), indicating their podocyte origin. Polygonal cells growing out from encapsulated glomeruli were negative for podocalyxin and positive for pan cadherin at the peripheral cell-cell contact. Thus, the cell population from decapsulated glomeruli is distinctly different from that from encapsulated glomeruli, supporting the idea that polygonal cells originate from PECs, although immunocytochemical markers specific to podocytes in vivo such as WT1, synaptopodin, HSP27 and P-31 antigen were expressed significantly in the polygonal cells. Occasionally, large irregular-shaped cells appeared at the periphery of the outgrowths from encapsulated glomeruli. They were similar in shape to the arborized cells from decapsulated glomeruli but were identical in antigenic properties to cobblestone-like polygonal cells and thus may be named "pseudo-arborized cells". We conclude that PECs in culture modulate their phenotype to resemble podocytes.

Keywords Glomerulus · Podocyte · Parietal epithelial cells · Bowman's capsule · Cell culture · Rat

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E. Yaoita (✉) · K. Ohshiro · T. Yamamoto
Department of Structural Pathology, Institute of Nephrology, Faculty of Medicine, Niigata University, 1-757 Asahimachi-dori, Niigata, 951-8510 Japan

H. Kurihara · T. Sakai
Department of Anatomy, Juntendo University School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo, 113-8421 Japan

Introduction

Visceral epithelial cells of renal glomeruli, referred to as podocytes, are believed to be an especially differentiated cell type with an elaborate morphology. They consist of cell bodies, primary processes, and foot processes, are situated on the glomerular basement membrane (GBM) as the terminal element in the ultrafiltration barrier, and contribute significantly to glomerular permeability (Daniels 1993). In adult rats, podocytes rarely undergo cell division even after subtotal nephrectomy (Fries et al. 1989; Pabst and Sterzel 1983; Rasch and Nørgaard 1983). In contrast, parietal epithelial cells (PECs) of Bowman's capsule are squamous in shape and have a central cilium. Occasionally, they show cell replication under normal conditions (Pabst and Sterzel 1983). When podocytes are lost for any reason, they cannot be replaced by newly replicated cells. Detachment of damaged podocytes from the GBM leaves denuded areas of the membrane that come into contact with PECs of Bowman's capsule, triggering glomerular tuft adhesion and destruction (Kihara et al. 1990; Kondo and Akikusa 1982; Kriz et al. 1998; Nagata and Kriz 1992).

The culturing of glomerular cells has provided a specific approach to defining the actions of individual glomerular cell types under physiological and pathological conditions (Floege et al. 1994; Kreisberg and Karnovsky 1983; Lovett and Sterzel 1986; Striker and Striker 1985).

However, certain problems are inherent to this approach. First, glomeruli isolated from the renal cortex are almost never a pure population because of contamination with Bowman's capsule and tubular fragments (Yaoita et al. 1991). Second, cell types in culture show phenotypic modulation, i.e., the loss or gain of certain phenotypic properties. These changes hamper the identification of cells in culture. At least two cell types have been reported as cultured podocytes. The first group are regularly shaped polygonal cells with a cobblestone-like appearance and high rates of replication and constitute the main outgrowths from glomeruli for the first 6 days of culture (Cybulsky et al. 1987; Foidart et al. 1979; Harper et al. 1984; Kreisberg et al. 1978; Mendrick et al. 1991). The second are irregular and arborized and are often multinucleated cells with long cytoplasmic extensions and a very limited proliferative capacity (Bernik 1969; Hancock and Atkins 1983; Holdworth et al. 1978; Morita et al. 1980; Nørgaard 1983, 1987; Yaoita et al. 1995). The latter cells occur as a very small population, constituting no more than 5% of glomerular outgrowths (Yaoita et al. 1995) and are presumably derived from podocytes, since they express podocyte-specific markers, e.g., podocalyxin, synaptopodin (Hancock and Atkins 1983; Yaoita et al. 1995). With respect to the former type, some have argued that the structural and antigenic features of these polygonal cells indicate their origin from PECs (Holthöfer et al. 1991; Nørgaard 1983, 1987; Weinstein et al. 1992; Yaoita et al. 1991). Others have described these polygonal cells as dedifferentiated podocytes, because they lack cell markers expressed by podocytes in vivo (Cybulsky et al. 1987; Foidart et al. 1979; Harper et al. 1984; Kreisberg et al. 1978; Mundel et al. 1997a). Mundel and coworkers (1997a) have showed that polygonal cells express WT-1, which is a podocyte-specific marker in vivo. Since the polygonal cells have been used in most biochemical and functional studies of the kidney, an accurate interpretation of experimental results critically depends on clarifying whether these cells are derived from podocytes or PECs.

Conventionally, one obtains glomeruli-rich fractions of renal tissue by pressing small pieces of the cortex through a sequence of sieves. Pure fractions of glomeruli devoid of Bowman's capsule (decapsulated glomeruli) result from further selection by suction of glomeruli into a micropipette under a phase-contrast microscope (Holthöfer et al. 1991; Nørgaard 1987; Weinstein et al. 1992; Yaoita et al. 1991). When these purified decapsulated glomeruli are cultured, cell outgrowths appear in less than 1% of the glomerular preparation (Weinstein et al. 1992; Yaoita et al. 1991). This low rate of outgrowths further complicates the identification of podocytes and PECs in culture. Moreover, as reported previously, podocytes may be damaged or undergo apoptosis during the sieving process (Ishikawa and Kitamura 1998; Nørgaard 1987).

In the present study, we have isolated glomeruli with a minimum of damage to podocytes by avoiding pressure while sieving. In the resulting glomerular cell cultures, we have found that podocytes differ morphologically from the regularly shaped polygonal cells, and that PECs express some markers specific to podocytes in vivo. Some of this work was presented at 32nd Annual Meeting of the American Society of Nephrology in abstract form (Yaoita and Yamamoto 1999).

Materials and methods

Animals and antibodies

WKY rats were purchased from Charles River Japan (Ataugi, Japan) and used when aged 8–12 weeks. The following antibodies were used: (1) antibodies specific to podocytes in vivo: monoclonal anti-podocalyxin antibody (1A; generous gift of Dr. M. G. Farquhar, University of California, San Diego, Calif., USA; Miettinen et al. 1990), monoclonal anti-synaptopodin antibody (G1D4, PROGEN Biotechnik, Heidelberg, Germany; Mundel et al. 1991, 1997b), rabbit polyclonal anti-Wilms' tumor protein WT-1 antibody (C-19, Santa Cruz Biotechnology, Santa Cruz, Calif.), monoclonal anti-heat shock protein 27 (HSP 27) antibody (courtesy of Dr. W. E. Smoyer, University of Michigan, Mich.; Smoyer et al. 1996), monoclonal antibody against a novel intermediate-filament-associated protein in rat podocytes (P-31; Kurihara et al. 1998), monoclonal anti-nephrin antibody (5-1-6; kindly provided by Dr. F. Shimizu, Niigata University, Niigata, Japan; Topham et al. 1999); (2) antibodies specific to mesangial cells: monoclonal anti-Thy-1 antibody (Sera-Lab, Crawley Down, Sussex, UK); (3) antibodies specific to endothelial cells: rabbit anti-human von Willebrand (vWF) factor antibody (kindly provided by Dr. M. Handa, Keio University, Tokyo, Japan); monoclonal antibodies recognizing the cell surface antigen of rat endothelial cells (RECA-1; Serotec, Oxford, UK); (4) antibodies specific to rat monocytes and macrophages: monoclonal antibody recognizing cytoplasmic antigen of rat monocytes and macrophages (ED-1; Serotec); (5) miscellaneous: monoclonal anti-pan cadherin antibody (CH-19; Sigma, Saint Louis, Mo.), rabbit anti-ZO-1 antibody (Zymed Laboratories, South San Francisco, Calif.), rabbit anti-laminin antibody (Serotec). For the secondary antibodies, fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG was purchased from Immuno-Biological Laboratories (Gunma, Japan). FITC-conjugated and tetramethylrhodamine isothiocyanate (TRITC)-conjugated goat anti-mouse IgG were obtained from Cooper Biomedical (Malvern, Pa.). These secondary antibodies were mixed with normal rat serum and allowed to stand overnight for absorption before use.

Isolation of glomeruli and culture conditions

Glomeruli were isolated from rat kidneys by a modified procedure as described previously (Yaoita et al. 1991). The kidney cortex was dissected free and cut into small pieces with a surgical blade in culture medium. The tissue suspension was then poured onto a stainless steel 60-mesh screen (pore size: 250 µm). To avoid podocyte damage, this step was carried out by only rinsing with phosphate-buffered saline (PBS) instead of the usual pressing with a spatula. Glomeruli-enriched tissue was retained on 200-mesh screen (pore size: 75 µm) placed under a 60-mesh screen. The tissue fragments were collected and suspended in RPMI 1640 medium (Nissui Pharmaceutical, Tokyo, Japan), supplemented with 5% fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 µg/ml). The suspension was placed under an inverted tissue culture microscope with phase-contrast optics. Decapsulated glomeruli, encapsulated glomeruli without visible adhering pieces of tubules or vascular vessels, or tubular fragments were selected from the tissue suspension by sucking into a micropipette and explanted into eight-well glass chamber slides (Lab-Tek, Miles Scientific, Naperville, Ill.) or onto glass coverslips, both having been

coated with type I collagen (Cellmatrix TypeI-P, Niita Gelatin, Osaka, Japan).

Scanning electron microscopy

The cell outgrowths on collagen-coated glass coverslips were fixed with 2.3% glutaraldehyde for 1 h at room temperature. The coverslips with cells were postfixed in 1% osmium tetroxide for 30 min and dehydrated through an ethanol series. Dehydrated cells were dried in a critical-point drier (HCP-1; Hitachi Koki, Katuta, Japan) by using isoamyl acetate and CO_2. Dried specimens were mounted on aluminum studs and coated with gold in an ion-coater (8IB-3; Eiko Engineering, Mito, Japan). The surfaces of these specimens were observed by using a scanning electron microscope (X-560; Hitachi, Tokyo, Japan).

Immunofluorescence microscopy

The indirect immunofluorescence technique was applied to frozen kidney sections and outgrowths from glomeruli or tubular fragments as described previously (Yaoita et al. 1995). In brief, the rat kidneys were snap-frozen at –70°C, sectioned at a thickness of 3 µm in a cryostat, fixed in 2% paraformaldehyde in PBS for 5 min, and processed for double-label immunostaining. Outgrowths from explants cultured on eight-well glass chamber slides were fixed in methanol for 5 min, or fixed in 2% paraformaldehyde in PBS for 5 min, permeabilized with 0.3% Triton X-100 in PBS for 2 min, and stained with antibodies. For double-label immunofluorescence microscopy, rabbit antisera and murine monoclonal antibodies were mixed and applied as primary antibodies simultaneously. After being washed with PBS, the sections were stained with FITC-conjugated anti-rabbit IgG, rewashed with PBS, and subsequently reacted with TRITC-conjugated anti-mouse IgG. PBS, normal rabbit 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 were observed with a Olympus microscope (BX50) equipped with epi-illumination optics and appropriate filters.

Results

Early outgrowths from decapsulated glomeruli

When glomeruli were isolated from renal tissues by gentle flushing without pressing against sieves, the rate of cell outgrowth from decapsulated glomeruli in culture was substantial compared with the negligible outgrowth from decapsulated glomeruli isolated by conventional sieving methods (Weinstein et al. 1992; Yaoita et al. 1991). In the present study, 22.8%–29.0% of decapsulated glomeruli (mean: 25%) grew projections after 5 days of culture in five individual experiments.

Glomerular outgrowths were first observed by phase-contrast microscopy on day 2 of culture. Initially, these cells extended long thin cytoplasmic processes to the bottom of the culture flask (Fig. 1a–c), after which cell bodies emerged. In contrast, initial outgrowths from encapsulated glomeruli did not show such long thin processes, exhibiting instead wider lamellipodia (Fig. 1h). Most cells from decapsulated glomeruli retained long cytoplasmic processes that often lay over adjacent cells (Fig. 1d, e). Many of the cells then dispersed on the culture dishes (Fig. 1f). These cells differed morphologically from the regularly shaped polygonal cells observed in cultures of encapsulated glomeruli (Fig. 1i). They assumed a variety of shapes including a long narrow form, a small rounded form with long arborized processes, and a large form with filamentous strands radiating to the periphery in the cytoplasm. These cells tended to become elongated to as much as 100–200 µm, most becoming larger than polygonal cells from encapsulated glomeruli and tubular fragments after the same period of culture (Fig. 1i, j; Yaoita et al. 1991). Even when crowded, they remained large without a cobblestone-like appearance (Fig. 1g). Most of them, especially the large cells, were multinucleated and resembled previously described porcine podocytes in culture (Nørgaard 1983).

As described in our previous reports, scanning electron-microscopic study revealed that the cultured decapsulated glomeruli were covered with cells extending long cytoplasmic processes without cilia (Yaoita et al. 1995; data not shown). In the cultures of encapsulated glomeruli, cilia were seen on cells covering the glomeruli and growing out onto the culture flask, which was common to PECs in vivo (Yaoita et al. 1991; data not shown).

Because glomeruli contain mesangial cells, endothelial cells, and macrophages in addition to podocytes, the outgrowths were examined by immunofluorescence microscopy after staining with antibodies against Thy-1 (mesangial cell marker; Ishizaki et al. 1980; Yaoita et al. 1985), ED-1 (macrophage marker; Dijkstra et al. 1985), and vWF or RECA-1 (endothelial cell markers; Duijvestijin et al. 1992). None of the outgrowing cells stained with these antibodies, with the exception of a few ED-1-positive small rounded cells as described previously (Yaoita et al. 1995; data not shown).

Pan cadherin staining as a PEC marker

Cadherins are a group of cell adhesion proteins that mediate homophilic Ca^{++}-dependent cell-cell adhesion, and their expression is strikingly tissue-specific (Takeichi 1995). We found that anti-pan cadherin antibody reacted with PECs but not with podocytes in kidney sections, indicating that pan cadherin staining is a marker for PECs and that it distinguishes them from podocytes in vivo (Fig. 2a, a'). Pan cadherin staining was observed at cell-cell contact sites of polygonal cells from encapsulated glomeruli, but not at those sites of outgrowths from decapsulated glomeruli (Fig. 2b, c). In contrast, the cell-cell contact sites of both cell types were distinctly stained by anti-ZO-1 antibody (Fig. 2b', c'), whose reactivity has been detected in the both cell types in vivo (Schnabel et al. 1990).

Specificity of podocyte markers in culture

To evaluate the specificity of several podocyte markers, we compared their immunostaining between cell outgrowths from decapsulated and encapsulated glomeruli

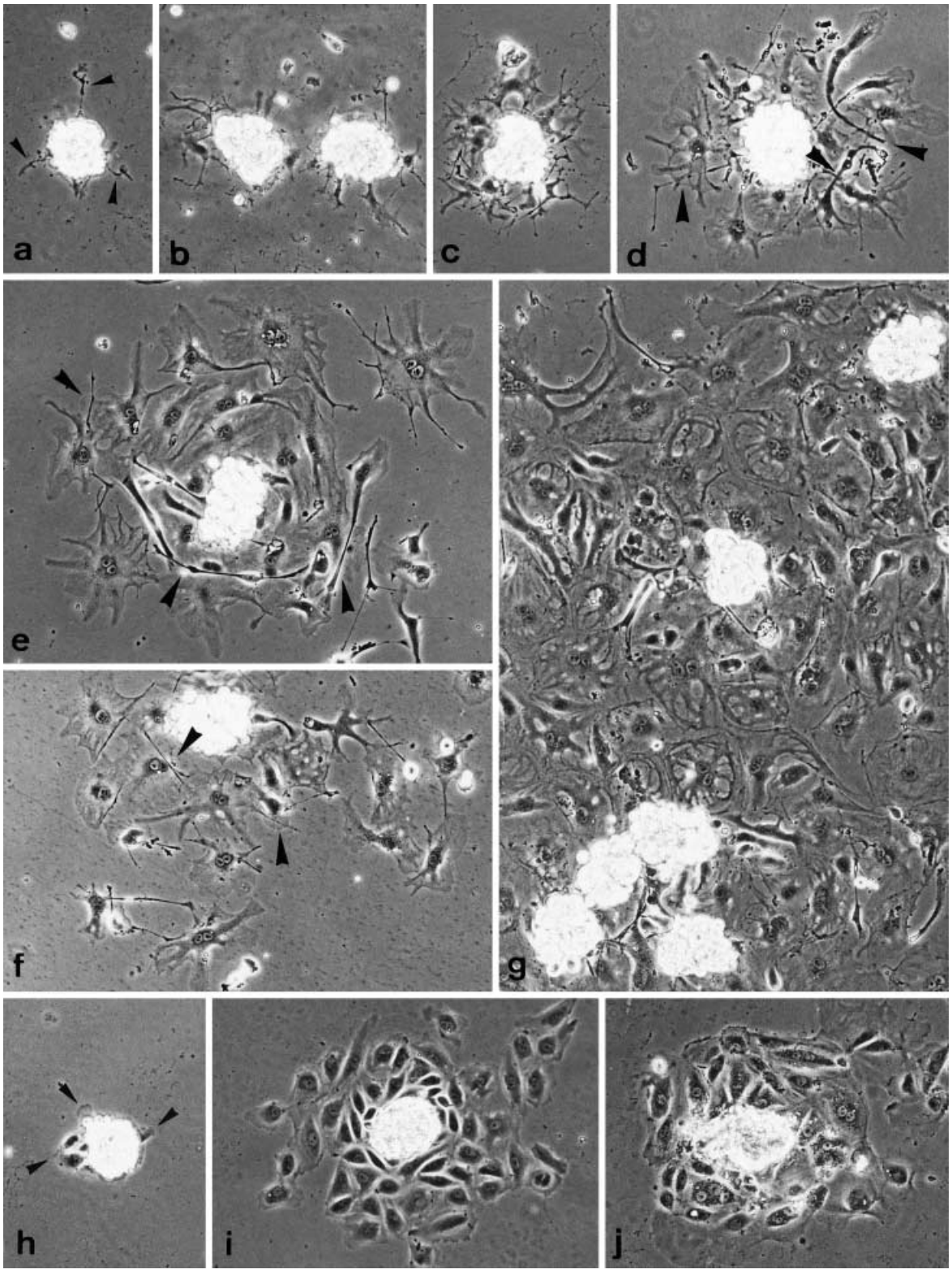

Fig. 1 Phase-contrast microscopy of outgrowths from decapsulated glomeruli after 2–7 days of culture (**a–g**), from encapsulated glomeruli after 2 days (**h**) and 5 days (**i**) of culture, and from tubular fragments after 5 days of culture(**j**). At the beginning of outgrowth from decapsulated glomeruli, cells extend long thin cytoplasmic processes from the glomeruli to the bottom of the culture flask (*arrowheads* in **a**). Cytoplasmic processes often lie over neighboring cells (*arrowheads* in **d–f**). Cells of decapsulated glomeruli (**a–g**) have completely different shapes from cells of encapsulated glomeruli and tubular fragments(**h, i, j**). Initial outgrowths from encapsulated glomeruli show lamellipodia (*arrowheads* in **h**) but not long thin processes. ×110

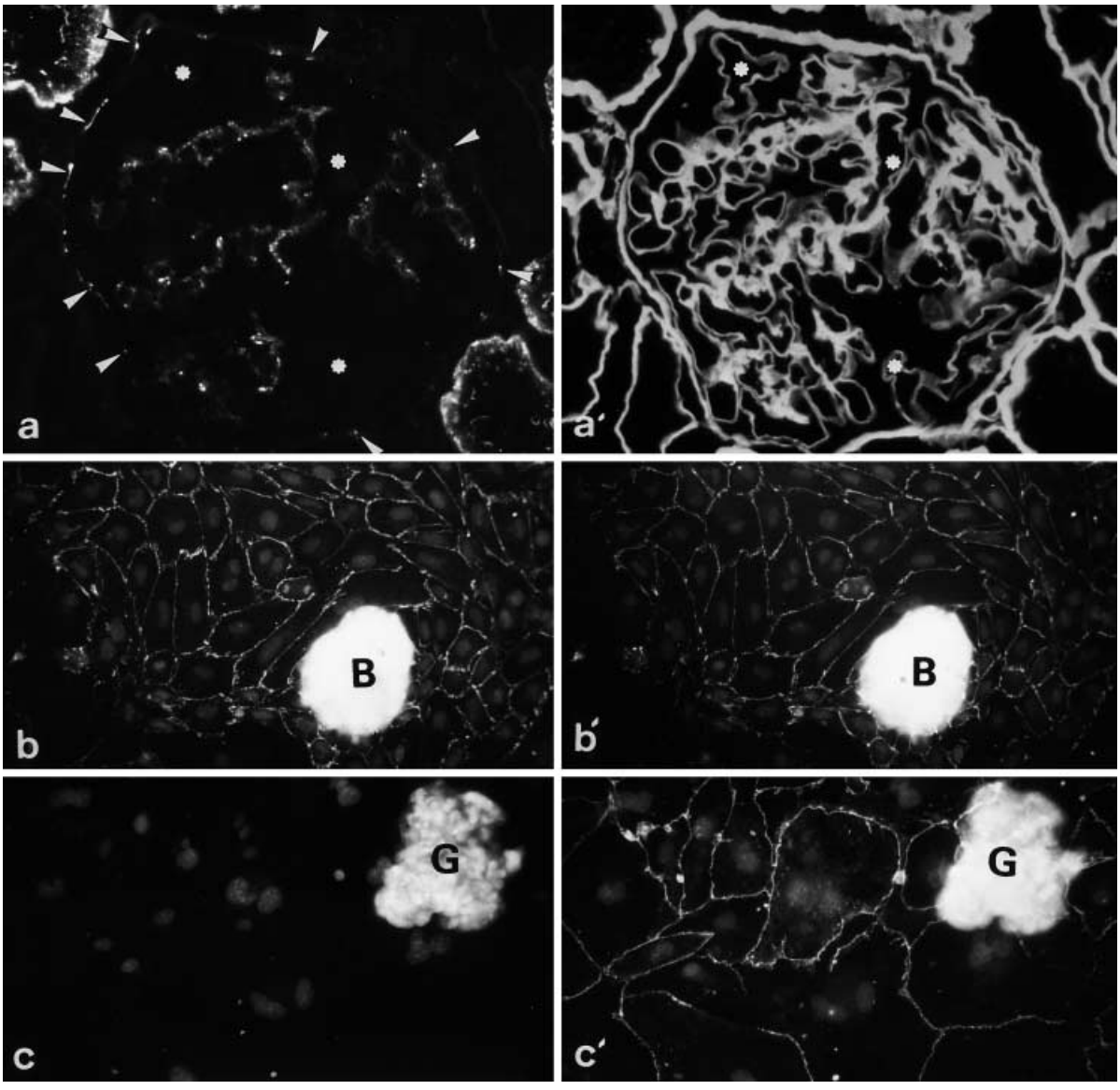

Fig. 2 Immunofluorescent localization of pan cadherin staining in a kidney section (**a, a'**), in outgrowths from encapsulated glomeruli (**b, b'**) and from decapsulated glomeruli (**c, c'**). **a, a'** Double staining with antibodies against pan cadherin (**a**) and laminin (**a'**). Pan cadherin localizes along Bowman's capsule (*arrowheads*) and in the mesangium, but not along the glomerular capillary wall (*asterisks*). **b, b', c, c'** Double staining with antibodies against pan cadherin (**b, c**) and ZO-1 (**b', c'**). Pan cadherin is detected at cell-cell contact sites of outgrowths from encapsulated glomeruli (**b**), but not at those from decapsulated glomeruli (**c**). Both the cell-cell contact sites stain with anti-ZO-1 antibody (**b', c'**). *G* Decapsulated glomeruli. *B* Encapsulated glomeruli. *a, a'* ×500, **b, b', c, c'** ×175

and tubular fragments. Podocalyxin, WT-1, synaptopodin, HSP 27, P-31 antigen, and nephrin were examined in this study because of their demonstrated ability to localize on podocytes but not on PECs or tubular epithelial cells (TECs) in kidney sections (Kerjschki et al. 1984; Kurihara et al. 1998; Mundel et al. 1991, 1997b; Mundlos et al. 1993; Orikasa et al. 1988; Smoyer et al. 1996).

Double-labeled immunostaining for podocalyxin and WT-1 discriminated the three types of outgrowths from the explants (Fig. 3a–c, a'–c'). Cell outgrowths from decapsulated glomeruli were stained intensely by both antibodies (Fig. 3a, a'). Polygonal cells from encapsulated glomeruli were negative for podocalyxin but positive for WT-1, although the latter staining was weaker than that of outgrowths from decapsulated glomeruli (Fig. 3b, b'). Outgrowths from tubular fragments did not stain for either antigen (Fig. 3c, c'). Synaptopodin staining was intense in all cells from decapsulated glomeruli and faint but noticeable in some polygonal cells from encapsulated glomeruli (Fig. 3d, e). None of cells from tubular fragments was labeled by the antibody (Fig. 3f). Surprisingly, antibodies against HSP 27 and P-31 antigen reacted significantly with all cells from the explants (decapsulated glomeruli, encapsulated glomeruli, and tubular fragments), although the staining patterns in podocytes were different from those in other cell types (Fig. 3g–l). No significant staining for nephrin was detected in any of the cells (data not shown).

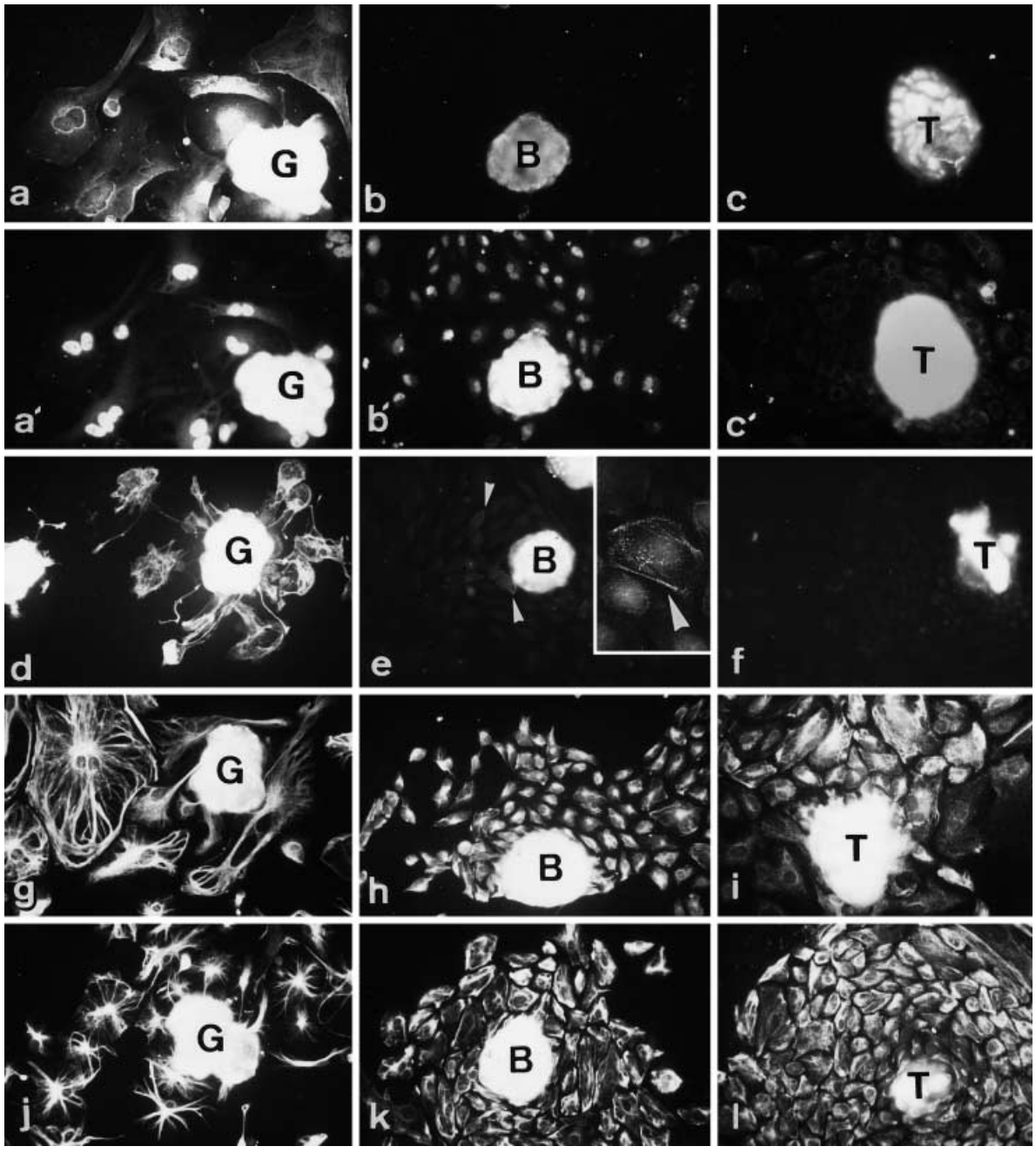

Fig. 3 Podocytic markers in outgrowths from decapsulated glomeruli (**a, a', d, g, j**), encapsulated glomeruli (**b, b', e, h, k**) and tubular fragments (**c, c', f, i, l**) after 5 days of culture. The localizations of podocalyxin (**a–c**), WT-1 (**a'–c'**), synaptopodin (**d–f**), HSP 27 (**g–i**), and P-31 (**j–l**) are shown by immunofluorescence microscopy (see especially **a–c** and **a'–c'**: double-labeled immunostaining; *arrowheads* in **e** faint but significant staining for synaptopodin). *G* decapsulated glomeruli, *B* encapsulated glomeruli, *T* tubular fragments. ×120, *Inset* in **e** ×240

Large irregular-shaped cells in the culture of encapsulated glomeruli

After 5 or 6 days of culture, large-sized cells appeared at peripheries of the colonies of polygonal cells with cobblestone-like appearance (Fig. 4a, b). They were variously sized and irregular in shape. Some of them were multinucleated and occasionally extended cytoplasmic processes. Their morphology was similar to that of large arborized cells from decapsulated glomeruli. Repeated phase-contrast microscopic monitoring, however, suggested that polygonal cells were converted into the large cells as reported by Mundel et al. (1997a). In addition, intermediate cells were observed at the peripheries of the colonies.

Their phenotypic features were examined by immunofluorescence microscopy after staining with antibodies against podocalyxin, synaptopodin, and pan cadherin. They were negative for podocalyxin (Fig. 5a, a') and sometimes showed faint but noticeable staining for synaptopodin (Fig. 5b, b'), which was identical to the

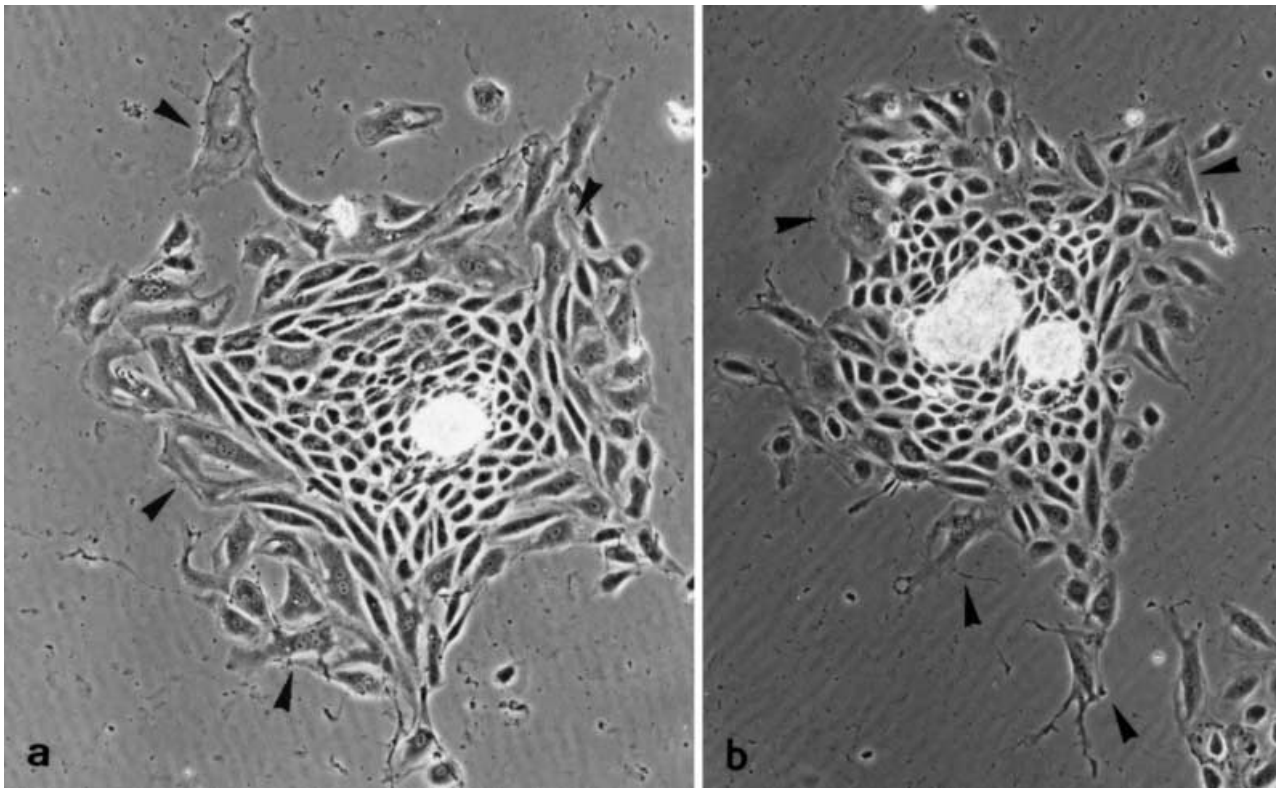

Fig. 4a, b Phase-contrast microscopy of outgrowths from encapsulated glomeruli after 6 days of culture. Large irregularly shaped cells (*arrowheads*) are observed at the margins of polygonal cell colonies. Some of them are multinucleated, and some extend cytoplasmic processes. ×110

staining of cobblestone-like polygonal cells. Pan cadherin staining was detected at the cell-cell contact sites of the cells (Fig. 5c, c'). Cell-cell contact sites between the large irregular-shaped cells and cobblestone-like cells were also stained with anti-pan cadherin antibody (arrowheads in Fig. 5c, c'). To examine whether pan cadherin antibody reacted at cell-cell contact sites between large arborized cells from decapsulated glomeruli and cobblestone-like polygonal cells, decapsulated glomeruli and encapsulated ones were mixed and co-cultured. Immunostaining study revealed that the border between both types of cell contained ZO-1 but not pan cadherin (Fig. 5d, d', d").

Discussion

The notable finding in this study is the improved rate of cell outgrowth from decapsulated glomeruli isolated by our gentle procedure. Few or no outgrowths have been reported from decapsulated glomeruli isolated by the conventional sieving method, which involves forceful pressure being brought to bear on renal cortical fragments (Weinstein et al. 1992; Yaoita et al. 1991). Nørgaard (1987) has stated that podocytes are the cells most susceptible to damage during the isolation procedure and that many podocytes are stripped away from decapsulated glomeruli, whereas podocytes in encapsulated glomeruli are well preserved. Ishikawa and Kitamura (1998) have reported that about 80% of podocytes undergo apoptosis immediately after the isolation of glomeruli. In the present study, we have separated glomeruli from renal cortical pieces merely by cutting the cortices into small pieces with a surgical blade. The enhanced cellular outgrowth that results corroborates the idea that podocytes sustain severe damage during the sieving process.

The substantial outgrowths from decapsulated glomeruli have enabled us to compare cells growing from encapsulated glomeruli with those from decapsulated glomeruli. Cells from decapsulated glomeruli extend long cytoplasmic processes and tend to become large and arborized. Polygonal cells rarely grow out from decapsulated glomeruli. On the other hand, outgrowths from encapsulated glomeruli manifest a polygonal cobblestone-like appearance. In addition to the morphological difference, immunostaining for pan cadherin and podocalyxin discriminates between the two types of outgrowth. These findings demonstrate that the cell population from encapsulated glomeruli is completely different from that from decapsulated glomeruli. Based on the morphological resemblance to their counterparts in vivo and on the results of immunostaining, it is reasonable to assume that cells growing from decapsulated and encapsulated glomeruli are derived from podocytes and PECs, respectively. We have observed the cell outgrowths from decapsulated glomeruli over a 4-week period. Although the cells increase in size with time, they do not exhibit a cobble-

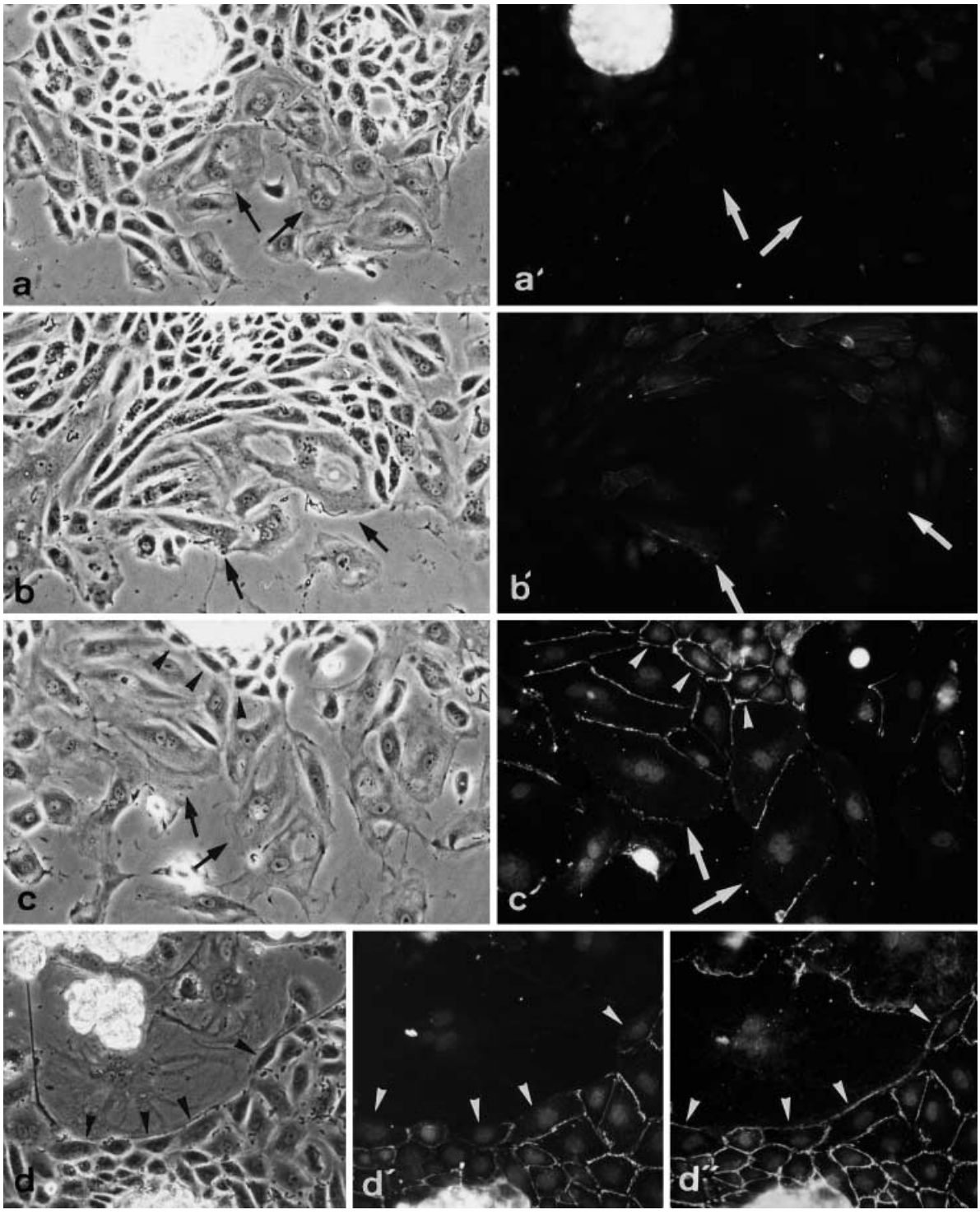

Fig. 5 Phase-contrast and immunofluorescence microscopy of large irregular-shaped cells in the culture of encapsulated glomeruli (**a, a', b, b', c, c'**) and large arborized cells in the co-culture of encapsulated and decapsulated glomeruli (**d, d', d"**). **a, a'** Antibody raised against podocalyxin does not stain the large cells. **b, b'** Some of the large cells show faint staining for synaptopodin. **c, c'** Cell-cell contact sites between all cells are stained with anti-pan cadherin antibody. **d, d', d"** In the co-culture of encapsulated and decapsulated glomeruli, double staining with antibodies against pan cadherin (**d'**) and ZO-1 (**d"**) clearly show that the border between the polygonal cells and large arborized cells is negative for pan cadherin (*arrows* the same cells in **a** and **a', b** and **b'**, and **c** and **c'**, respectively; *arrowheads* in **c, c', d, d', d"** border between polygonal cells and large-sized cells). **a–d** ×130, **a'-d', d"** ×175

stone-like appearance. Thus, we have found no relationship between podocytes and polygonal cells, in spite of previous reports (Cybulsky et al. 1987; Foidart et al. 1979; Harper et al. 1984; Kreisberg et al. 1978; Mendrick et al. 1991).

In kidney sections, several podocyte-specific markers have been described that do not appear in PECs or TECs in vivo. However, our results have revealed that PECs or TECs do express some of the markers in vitro. Of the markers studied here, only the anti-podocalyxin antibody interacted specifically with cultured podocytes. Intense staining for synaptopodin was clear only in the irregular arborized cells regarded as podocytes, although some polygonal cells from encapsulated glomeruli were weakly but significantly positive for synaptopodin. It is unlikely that these positive cells are podocytes migrating from encapsulated glomeruli, because they are uniformly polygon-shaped, and their staining for synaptopodin is much less intense than that of cells growing out from decapsulated glomeruli. The Wilms' tumor protein, WT-1, was expressed by all polygonal cells from encapsulated glomeruli but none from tubular fragments. Additionally, HSP 27 and P-31 antigens were detected in all cells from encapsulated glomeruli and tubular fragments. These findings indicate that not all markers specific for podocytes in vivo are specific to them in culture, and that the specificity of markers in culture must be evaluated by comparison with PECs and TECs under the same culture condition.

The podocytic markers, WT-1 and synaptopodin, were detected in cultured PECs but not in TECs. Desmin, the muscle-type intermediate filament protein, can also be regarded as this type of podocytic marker (Yaoita et al. 1990), and staining with anti-desmin antibody ranges from negative to significantly positive for cultured PECs, similar to that for podocytes in vivo (Yaoita et al. 1991). Cultured TECs do not show desmin staining (Yaoita et al. 1991). The phenotypic modulation specific to PECs implies that the background of gene expression in PECs has something in common with that in podocytes, despite their completely different morphologies, an idea that may be associated with their close relationship during ontogeny. In addition, PECs have been shown to develop into podocytes under conditions that cause glomerular cyst formation (Ojeda and Garcia-Porreto 1982). Although the phenotypic modulation of cultured PECs may be regarded as transdifferentiation to podocytes, such a change must be carefully delineated from the possible alteration of PECs into a phenotype that is an adaptation to culture conditions, as has previously been suggested for mesangial cells (Floege et al. 1994).

In the culture of encapsulated glomeruli, large irregular-shaped cells appeared at the margins of colonies of cellular outgrowths, and their morphology was similar to that of large arborized cells from decapsulated glomeruli under the phase-contrast microscope. Their staining for podocalyxin, synaptopodin, and pan cadherin, however, was identical to that of cobblestone-like polygonal cells. From repeated phase-contrast microscopic observation, it is likely that cobblestone-like cells are converted to the large cells, as described by Mundel et al. (1997a). Thus, they may be named "pseudo-arborized cells". These findings demonstrate that two types of large-sized cell are present in glomerular cell culture, when starting with isolated glomeruli that are not selected under a phase-contrast microscope. Podocalyxin and pan cadherin are critical markers for distinguishing the two types of cell. From this point of view, it is interesting to examine whether large-sized cells, which have been reported as podocytes in previous papers, are positive for podocalyxin or pan cadherin (Bernik 1969; Holdworth et al. 1978; Morita et al. 1980; Mundel et al. 1997a; Nørgaard 1987).

In addition to HSP 27 and P-31 antigen, vimentin, the mesenchymal intermediate filament protein, is also phenotypically expressed exclusively by podocytes in vivo but is inducible in cultured PECs and TECs (Bachmann et al. 1983; Yaoita et al. 1991). The majority of epithelial cells express only one type of intermediate filament in vivo, that of cytokeratins. However, many epithelial cell lines and primary cultures of epithelial cells co-express vimentin in addition to cytokeratins (Franke et al. 1979). In experiments with epithelial cell lines, vimentin expression is influenced by cell shape, cell spreading, and cell-cell contact (Ben-Ze'ev 1984, 1985, 1987). Sparse monolayer cultures synthesize high levels of vimentin, whereas dense monolayer and suspension cultures make only low levels, i.e., vimentin synthesis is highest in cells that are spread on the substrate and lowest in cultures with minimal cell spread (Ben-Ze'ev 1984, 1987). In addition, levels of vimentin synthesis are extremely low in dense suspension cultures where cell-cell contacts are extensive compared with cultures having sparse cellular suspensions and dense monolayers (Ben-Ze'ev 1985, 1987). The extent of cell spreading on the substratum and cell-cell contacts is likely to change when PECs and TECs are released from the constraints imposed by three-dimensional tissue organization when they grow out from explants onto cell-free culture flasks. In line with this suggestion, we have observed that PECs and TECs at the periphery of outgrowths are exceptionally large. Their expression of HSP 27 and P-31 antigen may be regulated similarly to that of vimentin, because HSP 27 and P-31 have been reported to be associated with vimentin (Kurihara et al. 1998; Perng et al. 1999).

In vivo, each podocyte extends over a wide area of GBM, i.e., one podocyte covers an estimated 1300 μm^2 or three to four times as much area as one PEC covers (Hishiki et al. 1999). The intercellular space between foot processes of podocytes is known as a glomerular slit, from which a huge amount of glomerular filtrate springs. Foot processes are attached to one another by slit diaphragms, bridgelike structures (30–45 nm wide) that are the widest intercellular junctions in the epithelium (Rodewald and Karnovsky 1974). Thus, the width of attachment to the basement membrane and the width of intercellular space may influence expression of some podocyte-specific phenotypes.

In summary, the present study shows that podocytes can retain their in vivo phenotypes when growing out from decapsulated glomeruli in culture, that polygonal cells are derived from PECs or TECs but not podocytes, and that PECs in particular modulate their phenotype to resemble podocytes in culture.

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