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Development of Renal Podocytes Cultured under Medium Perifusion

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BACKGROUND: In the past, podocytes have been described as highly susceptible to dedifferentiation under cell culture. Whether this process resulted from insufficient culture conditions or whether it was a consequence of missing cellular interactions remained unclear. A further reason could be that podocytes within the maturing kidney are irreversibly growth-arrested at a very early point of development because proliferating cells have been detected at the S-shaped body stage but not at the capillary loop stage or in the maturing glomeruli. These were important reasons that hindered the establishment of podocyte cell culture systems.

EXPERIMENTAL DESIGN: The aim of our present study was to culture podocytes under the most organotypic conditions possible to maintain typical cellular characteristics. Cortex explants of neonatal rabbit kidneys consisting of nephrogenic tissue were used as a source for podocytes. No serum additives were given for the whole culture period of 13 days. An organ-specific environment was obtained by keeping the podocytes within the surrounding renal tissue and by ensuring a permanent exchange of medium.

RESULTS: mAb were used to characterize podocytes and the other glomerular cell types. Cultured podocytes and parietal cells of Bowman's capsule were identified by EnPo 1. Ks 19.2.105, a marker for cytokeratin 19, was used to discriminate among these epithelial cells because cytokeratin 19 is expressed by the parietal cells of Bowman's capsule but not by podocytes. The Ab EC1 specifically detected endothelial cells. Glomerular endothelium cultured under medium perifusion expressed these typical Ag and thus could be unequivocally discriminated. Furthermore, by means of the proliferation marker Ki-67, it could be demonstrated that glomerulus-like structures developed under culture by proliferation of visceral and parietal cells of Bowman's capsule.

CONCLUSIONS: A culture model is presented that allows the maintenance of developing podocytes within the organ-specific tissue environment and under permanent medium perifusion.

Additional key words: Glomerulus, Kidney, mAb, Perifusion cell culture, Rabbit.

Cell culture models would be of immense benefit in the investigation of events leading to glomerular injury. Until now, cultures have generally been established using glomeruli that have been obtained from adult renal tissue by sieving or centrifugation techniques or a combination of both (1–6). However, typical cellular characteristics of cells in primary culture showed a distinct tendency to undergo change in a dedifferentiation process (7–10). Thus, the loss of morphologic and immunohistologic features not only of the tubular but also of the glomerular cells during culture made it difficult to identify the different cell types (11, 12). For example, renal podocytes (synonymous with visceral cells of Bowman's capsule) showed a strong reaction with anti-GD3 Ab, whereas cultured glomerular cells did not (13). The expression of smooth muscle α-actin by cultured mesangial cells was strongly dependent on medium supplements (8).

Markers possessing exclusive specificity for visceral or parietal glomerular cells are scarce and are strictly species-specific (14). After first reports were published that described the distinct expression patterns of intermediate filaments (12, 15, 16), these markers were used frequently for the discrimination of the different glomerular cell types. Within the kidney, podocytes are positive for vimentin but do not express cytokeratins, whereas the parietal glomerular epithelium shows just the opposite labeling pattern. Holthöfer and coworkers (13) reported that the cultured cells no longer showed podocyte characteristics with respect to the expression of vimentin. Furthermore, it was demonstrated that the cultures obtained from mature glomer-

uli were primarily composed of parietal cells of Bowman's capsule and mesangial cells (17), whereas no viable podocytes were detectable.

Because of the strong tendency of adult glomerular cells to dedifferentiate under culture, the aim of the present study was to develop a technique that would make it possible to isolate immature podocytes and to bring them in culture while still maintaining typical podocytic protein expression patterns for prolonged culture periods. The outer cortex of the neonatal rabbit kidney was the source for differentiating renal cells (18, 19). Compared with other cell cultures, the outer cortex of neonatal rabbit kidney provided several advantages: the cells were grown on a kidney tissue-specific support, the tissue could be kept under permanent medium perifusion for prolonged culture periods without subculturing, and the perfusion culture leads to excellent tissue maintenance even under serum-free conditions (20).

EXPERIMENTAL DESIGN

Cortex explants were prepared from neonatal kidneys by stripping off the fibrous organ capsule. The explants were mounted in a set of holding rings, inserted in a perfusion culture container, and cultured for 13 days. The culture medium did not include any serum but was supplemented with aldosterone and 1.25-dihydroxyvitamin D3. To investigate the time course of podocyte development, kinetic experiments were carried out. The Ab listed in Table 1 were used to discriminate among the different glomerular cell types.

RESULTS AND DISCUSSION

Detection of Developing and Mature Glomerular Cells by Different mAb

Unexpectedly, no specific markers were available for podocytes of the rabbit kidney. For instance, podocalyxin antisera (21) or the anti GD3-marker (14) showed strong species specificity for the rat Ag and did not react in rabbit kidney, as seen in our own experiments with these Ab. Thus, new mAb directed against rabbit glomerular Ag had to be raised (20, 22). After immunization with kidney homogenate, two Ab (EnPo 1 and EC1) were obtained that could be used as markers for the following culture experiments. EC1 showed exclusive specificity for endothelial cells, and EnPo 1 detected both endothelial cells and glomerular epithelium of the rabbit kidney (20, 22). In the present study, EnPo 1, EC1, and an Ab-labeling cytokeratin 19 (Ks 19.2.105) (16) were used for the discrimination of the different cell types within the glomerulus (Table 1, Fig. 1).

Glomerulogenesis has been described as including five steps: the vesicle stage, the S-shaped body stage, the capillary loop stage, and the maturing glomerulus stage, which finally leads to the differentiated glomerulus (23). First, podocyte precursor cells (synonymous with precursors of visceral cells of Bowman's capsule) could be detected by morphologic and immunohistologic means within the lower limb of the S-shaped body (Figs. 1*c*, 2*a*). At this developmental step, the podocytes are cuboidal, and, at their apico-lateral cell poles,

Table 1. The Reaction Pattern of EnPo 1, EC1, and Ks 19.2.105 Observed in Neonatal and Adult Rabbit Kidneys

	EnPo 1	EC1	Ks19.2.105
Visceral epithelium of Bowman's capsule (syn:podocytes)	+++	−	−
Parietal epithelium of Bowman's capsule	++	−	++↑
Glomerular endothelium	++	++	−
Mesangial cells	−	−	−
Afferent and efferent arterioles	++	++	−
Small vessels	++	++	−
Large vessels	(+)	++	−
Developing vasculature, outer cortex	++	++	−
Developing visceral and parietal epithelium of Bowman's capsule	+++	−	(−)
Mesenchymal cells surrounding the collecting duct ampulla	++	+/−	−
Endothelial cells in culture	(+)↓	++	−
Parietal cells of Bowman's capsule in culture	++	−	++↑
Visceral cells of Bowman's capsule in culture (podocytes)	+++	−	−

−, no labelling; (−), few positive spots; (+), weak staining; +/−, partially positive; ++, clear staining; +++, very intensive staining; ↑, up-regulation; ↓, down-regulation.

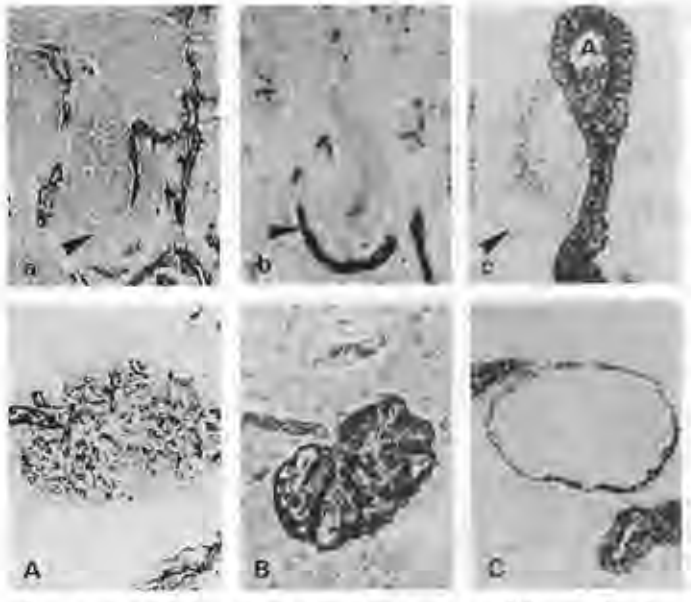

Fig. 1. Discrimination of glomerular cells at different developmental stages by mAb. *a* and *A*, The newly developed mAb EC1 with exclusive specificity for endothelial cells intensively labeled endothelium within the S-shaped bodies (*a*) and mature glomeruli (*A*). (*Arrowhead*: visceral glomerular epithelium, *syn*: podocytes). *b* and *B*: Endothelium and glomerular epithelial cells expressed the EnPo 1 Ag. Developing visceral (*arrowhead*) and parietal epithelium located in the lower limb of the S-shaped body (*b*) is arranged in the form of a hemisphere. Longitudinal sections of those structures labeled by EnPo 1 appear in the form of a U. Mature glomeruli showed intensive EnPo 1 labeling (*B*). *c* and *C*: Cytokeratin 19 was detectable within the developing (*a*) and mature collecting duct epithelium. Cells of the S-shaped body (*c*) only showed a spotty cytokeratin 19 labeling. In contrast, the parietal epithelium of the mature glomerulus (*C*) was strongly positive for this cytoskeletal component. Visceral glomerular epithelium (*arrowhead*) was never observed to express cytokeratin 19. Magnification: ×365.

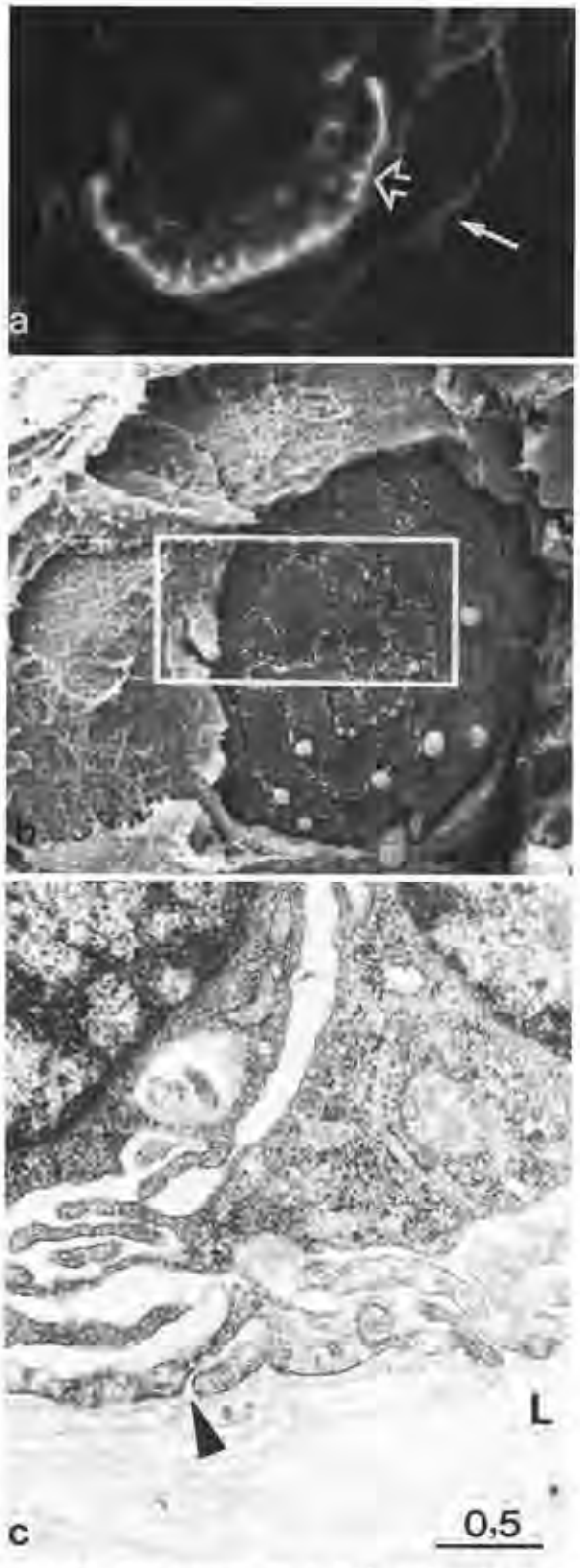

FIG. 2. Glomerular development. *a*: At the S-shaped body stage of glomerulogenesis, the EnPo 1 Ag is first expressed at the apico-lateral cell poles of the developing podocytes (*open arrow*). This could be demonstrated on the light and electron microscopic level. (*Arrow*: capillary). *b*: A top view of the lower pole of a S-shaped body is shown in this scanning electron micrograph. Glomerulogenesis includes several steps. First, glomerular epithelial cells can be distinguished in the lower limb of the S-shaped body. These cells are the precursors of the visceral and parietal epithelium of Bowman's capsule. At this point, the cells of the visceral epithelium (*syn*: podocytes) appear to be cuboidal. Foot processes are absent, but small villous structures can be observed at the apico-lateral cell sites (*white square*). *c*: Renal cortex explants perifusion cultured for 13 days were analyzed by electron microscopy. Cell clusters were composed of cuboidal cells, which have produced an extremely thick layer of extracellular matrix material (*L*). Adjacent to this layer, numerous plasma membrane interdigitations have developed. Only few interdigitations were observed between neighboring cells. Tips of interdigitations appeared to be slightly sunken in the extracellular matrix (*arrowhead*). *Scale bar*: 0.5 μm. Magnification: *a*, ×776; *b*, ×1600; *c*, ×19,400.

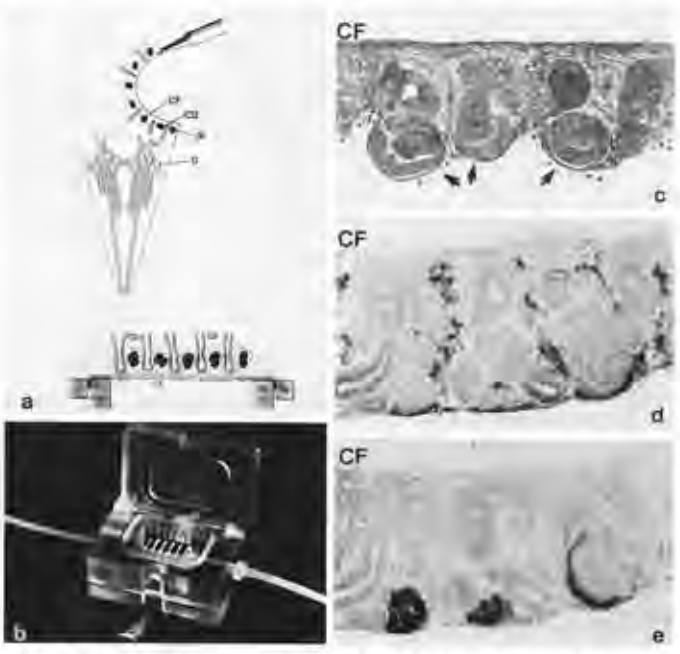

FIG. 3. Preparation and culture of cortex explants from neonatal rabbit kidneys. *a*: Cortex explants from neonatal rabbit kidneys can easily be prepared by stripping off the fibrous organ capsule. Freshly prepared explants were mounted in a set of holding rings (*R1, R2*). *CF*, capsula fibrosa; *CD*, collecting duct; *iN*, developing nephron; *G*, glomerulus. *b*: Mounted explants were inserted in a perfusion culture container and perfused with a constant flow rate of 1 ml/hour. Serum-free Iscove's modified Dulbecco's medium supplemented with aldosterone and 1.25-dihydroxyvitamin D3 served as the culture medium. *c*: Freshly prepared kidney cortex explants consist of the capsula fibrosa (*CF*), collecting duct ampullae, mesenchymal cells, nephrons in different developmental stages (*arrows*), and endothelial cells. (Semithin section, methylene blue staining). *d*: Endothelial cells were detectable in cortex explants by means of the mAb EC1. (Cryosection of an explant cultured for 1 day). *e*: In a consecutive section, it could be demonstrated that intensively EnPo 1-labeled cells did not colocalize with EC1-positive endothelium. *In vivo*, the EnPo 1 Ag is expressed by endothelial cells and glomerular epithelium. However, under culture, the Ag is down-regulated by endothelial cells, and only cultured glomerular epithelium was detected by EnPo 1. Magnification: *c-e*, ×185.

numerous villous structures are visible (Fig. 2*b*). As revealed by electron microscopic immunocytochemistry, the EnPo 1 Ag was detectable on the villous structures and on all cell sites facing the urinary space (Fig. 2*c*, *d*). During the differentiation process, the podocyte precursor cells, which are located in the lower limb of the S-shaped body, undergo a profound change in their polarization. Like all epithelial cells, immature podocyte-epithelium express tight junctions at the api-

cal cell poles (24). The differentiation of the podocytes is accompanied by a shift of the junctional complex from the apical to the basal cell pole. With respect to EnPo 1 Ag expression, the following observation was made: Immature podocytes are mainly positive for EnPo 1 at their apical cell membrane, whereas maturing cells showed Ab labeling at the apical and lateral cell sites (Figs. 2*c*, *d*). This characteristic labeling pattern could be observed in cultured S-shaped bodies also (Figs. 3, 4).

The lower limb of the S-shaped body forms a hemisphere (Fig. 2*b*) (25). As can be observed in S-shaped bodies and in precapillary loop-stage glomeruli, the walls of the hemisphere consist of the precursors of visceral and parietal glomerular epithelium. Longitudinal sections of the hemispheric structure will appear in most sections in form of a U (Figs. 1*c*, 2*a*, 3*e*, 4).

The EnPo 1 Ag is expressed by parietal and visceral cells of Bowman's capsule as well as by endothelial cells. To discriminate between the epithelial and endothelial cell populations, the newly developed Ab EC 1 with exclusive specificity for endothelium (20) was used in this study. EC 1 labeling was never observed on developing or mature cells of the Bowman's capsule (Fig. 1*a*, *b*), but EC 1 reacted with large and small vessels as well as with glomerular capillaries (Table 1, Fig. 1*a*, *b*).

To further discriminate between the visceral and parietal glomerular cells, the mAb Ks 19.2.105 was used. Neither endothelial nor mesangial cells express cytokeratin 19 (Fig. 1*e*, *f*) (12, 13, 16, 17). Within rabbit kidney, the neonatal and mature collecting duct epithelium is intensively stained by the anti-cytokeratin 19 Ab (Fig. 1*e*, *f*) (26). However, except for a few positive spots, the developing cells of the visceral and parietal epithelium of Bowman's capsule abundant in S-shaped bodies were not labeled by Ks 19.2.105 (Table 1, Fig. 1*e*). In contrast, within mature glomeruli, the parietal cells are strongly positive for this Ab (Fig. 1*f*). The first parietal cells to express cytokeratin 19 were detected at the capillary loop stage of glomerulus development. Mesangial cells were not labeled by any of the Ab described above, so we could clearly distinguish podocytes within tissue sections (Table 1).

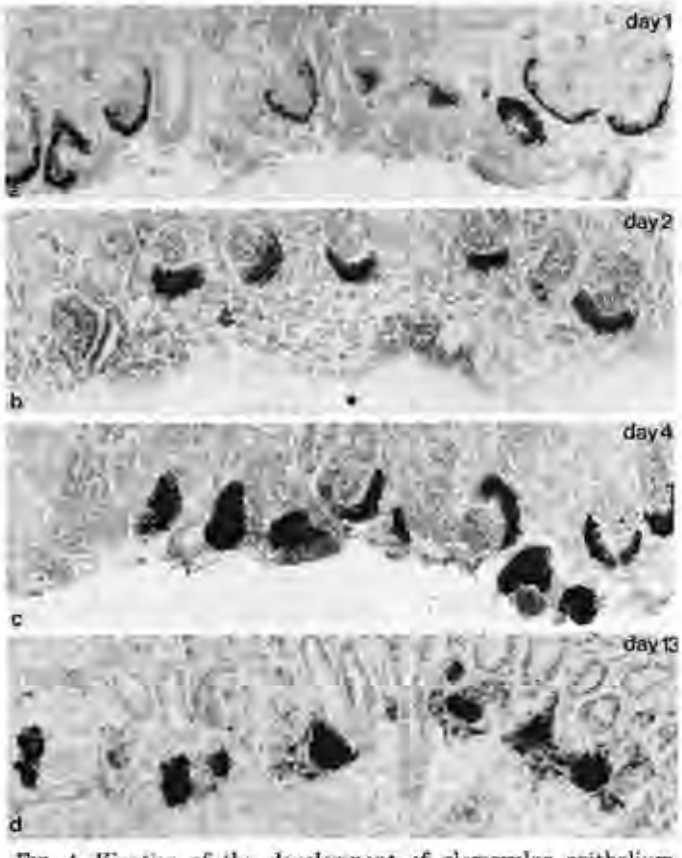

FIG. 4. Kinetics of the development of glomerular epithelium under perifusion culture. Renal cortex explants of neonatal rabbits have been cultured under serum-free conditions for various length of time. The mAb EnPo 1 was used to detect glomerular epithelium in cryosections of cortex explants. *a*: Day 1: EnPo 1-labeled cells mostly appeared as U-shaped cell aggregates. These aggregates resemble the cellular arrangement found in the lower limb of the S-shaped body. *b*: Day 2: The U-shaped cell aggregates, which were positive for EnPo 1, have broadened. *c*: Day 4: Besides U-shaped aggregates, an increasing number of round EnPo 1-labeled clusters were found. *d*: Day 13: No U-shaped aggregates labeled by EnPo 1 were found in explants cultured for 13 days. Magnification: ×212.

PODOCYTE DEVELOPMENT UNDER PERIFUSION CULTURE

As a source for immature podocytes, the embryonic tissue zone of the outer cortex of neonatal rabbit kidney was prepared by stripping off the fibrous organ capsule (Fig. 3*a*). This thin tissue layer consisted of mesenchymal cells, developing nephrons, embryonic collecting duct epithelium, and developing endothelial cells (Fig. 3*c*). The tissue was cultured in a medium without serum additives and under permanent perifusion of the medium for varying periods of time up to 13 days. In samples taken after 1 day of culture, small streaks of endothelial cells were detectable after immunoincubation with EC1 (Fig. 3*d*). A comparison of the EC1 and EnPo 1 Ag expression in consecutive explant sections revealed that the EnPo 1 labeling of endothelial cells was found to have decreased. EC 1-positive cell streaks were only very weakly labeled by EnPo1. Obviously, a rapid down-regulation of the EnPo 1 Ag in endothelial cells occurred under culture (Fig. 3*e*). In contrast, intensive EnPo 1 labeling was abundant on round cell clusters and U-shaped structures resembling developing glomerular epithelial cells (Fig. 4). No co-localization of intensive EC1 and EnPo 1 labeling was detected in consecutive tissue sections, indicating that no endothelial cells were labeled by EnPo 1. In contrast, cell groups that morphologically resembled developing glomerular epithelium continuously expressed the EnPo 1 Ag in large amounts.

To investigate the time course of the development of the parietal and visceral glomerular epithelium, kinetic experiments were carried out (Fig. 4). After 1 day of culture, morphologic criteria showed that nearly all of the epithelial cells of the glomerulus displayed the characteristic spatial arrangement that is found in the lower limb of the S-shaped body (Fig. 4*a*). Longitudinal sections of tissue explants, which were incubated with EnPo 1 Ab, revealed a great number of labeled,

U-shaped structures resembling the lower segment of the S-shaped body. Only few labeled cell clusters were found. The mAb Ks 19.2.105 was used to distinguish between parietal and visceral glomerular cells. All cells detected by EnPo 1 were negative for cytokeratin 19 on culture Day 1, indicating that no mature parietal cells of Bowman's capsule were present at this point in time. On the second day, the U-shaped structures had broadened slightly (Fig. 4*b*) but remained negative for cytokeratin 19 staining.

Under ongoing culture between Days 2 and 13, more and more U-shaped cell aggregates tended to build clusters of EnPo 1 Ag-bearing cells. From Days 2 to 4, nearly all of the U-shaped aggregates turned completely into EnPo 1-immunopositive clusters (Fig. 4*c*). However, none of these cell groups were positive for cytokeratin 19. No further changes in the shape of the glomerulus-like clusters were observed from Days 7 to 13 (Fig. 4*d*). Cytokeratin 19-expressing cells, which co-localized with EnPo 1-labeled structures, were found on Day 13 (Fig. 5). These cells appeared in the form of spottily labeled cell rings lining the periphery of EnPo 1-positive clusters like mature parietal cells of Bowman's capsule (Fig. 1*f*).

Electron Microscopic Analysis of Cultured Cortex Explants

Cortex explants cultured for 13 days under permanent medium exchange were examined. Besides well developed tubular structures, we frequently found clusters of cuboidal cells with numerous membrane interdigitations. A further typical feature of these clusters were extremely broad bands of extracellular matrix material deposited on one side of the cell layer (Fig. 2*c*). Adjacent to this thick layer of extracellular matrix material, the cells have formed the numerous interdigitations resembling immature podocyte footprocesses. The tips of these interdigitations, which were in contact with the extracellular matrix, seemed to be slightly sunken in the matrix material (Fig. 2*d*). However, footprocesses with slit membranes known from mature podocytes were not observed. At the lateral sites of neighboring cells, only a few interdigitations had developed. With respect to morphology, the cells resembled immature podocytes of the S-shaped body stage (Fig. 2*b*).

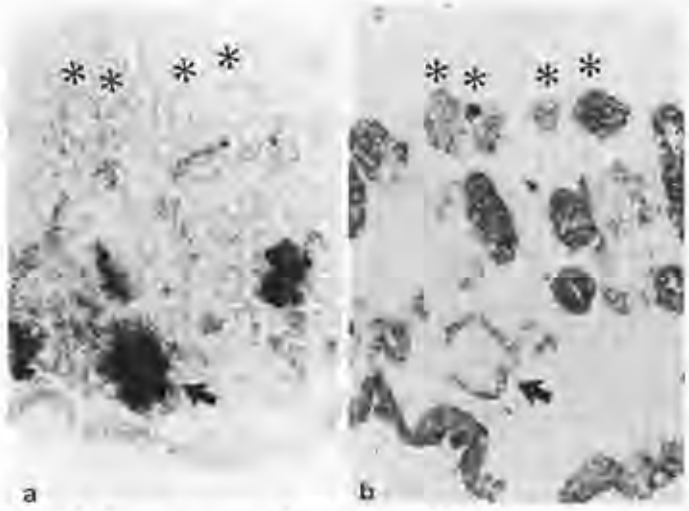

FIG. 5. The expression of cytokeratin 19 by cells abundant in EnPo 1-labeled clusters (*a, arrow*) was observed after 13 days of perifusion culture. In serial cryosections of cultured explants, it could be demonstrated that cells located at the periphery of EnPo 1-positive clusters expressed cytokeratin 19 (*b, arrow*). This arrangement of cytokeratin 19-labeled cells closely resembled the situation found in neonatal kidneys (for comparison, see Fig. 1*f*). (Asterisks, position of cross sections of collecting duct.) Magnification: ×248.

Detection of Proliferating Cells in Neonatal Rabbit Kidneys and Cultured Cortex Explants

To further investigate whether EnPo 1-labeled cell clusters were formed under culture by mere cell dislocation or whether these clusters included proliferating cells, the proliferation marker Ki-67 was used. The mAb Ki-67 has been characterized as a marker that labels cells in all phases of the cell cycle except in the G_0-phase (27). We used Ki-67 to detect proliferating cells within the developing zone of the renal cortex and within tissue explants.

Proliferating cells were detectable in all stages of nephrogenesis. Mesenchymal cells, cells of the renal vesicle and the S-shaped body, as well as cells of the developing collecting duct epithelium were labeled by Ki-67. Podocyte precursor cells abundant within the lower limb of the S-shaped body were strongly positive for Ki-67 labeling (Fig. 6*b*). In the more highly developed glomeruli of the neonatal kidney, *i.e.*, glomeruli at the capillary loop stage, labeled podocytes were never detected.

Within cortex explants, numerous different Ki-67-positive cells were detectable during the whole culture period. Intensive Ki-67 labeling of EnPo 1-positive cells was observed on culture Days 2 to 4 (Fig. 6*b*). The number of Ki-67-positive, U-shaped aggregates and clusters decreased under ongoing culture. By Day 13, the Ki-67 Ag was no longer detectable in any of the EnPo 1-labeled cell clusters.

Discussion

It is a general problem in cell culture techniques that typical cellular features of highly differentiated cells are lost under *in vitro* conditions (28, 29). Specific cell characteristics, such as morphologic and functional parameters, are radically influenced first by tissue preparation and then by the culture procedures (7). Like many other cell types, the renal podocytes (synonymous with visceral cells of Bowman's capsule), which are characterized by very specific morphologic and functional features within the glomerulus, showed a strong tendency to dedifferentiate during culture (11–13). Recently, it was shown that the principle morphologic characteristics of human renal podocytes can be maintained in an organotypic culture system (30).

In the present study, three mAb were used to identify podocytes and to monitor the dedifferentiation process in nephrogenic tissue that was kept under perifusion culture. Because of the species specificity of

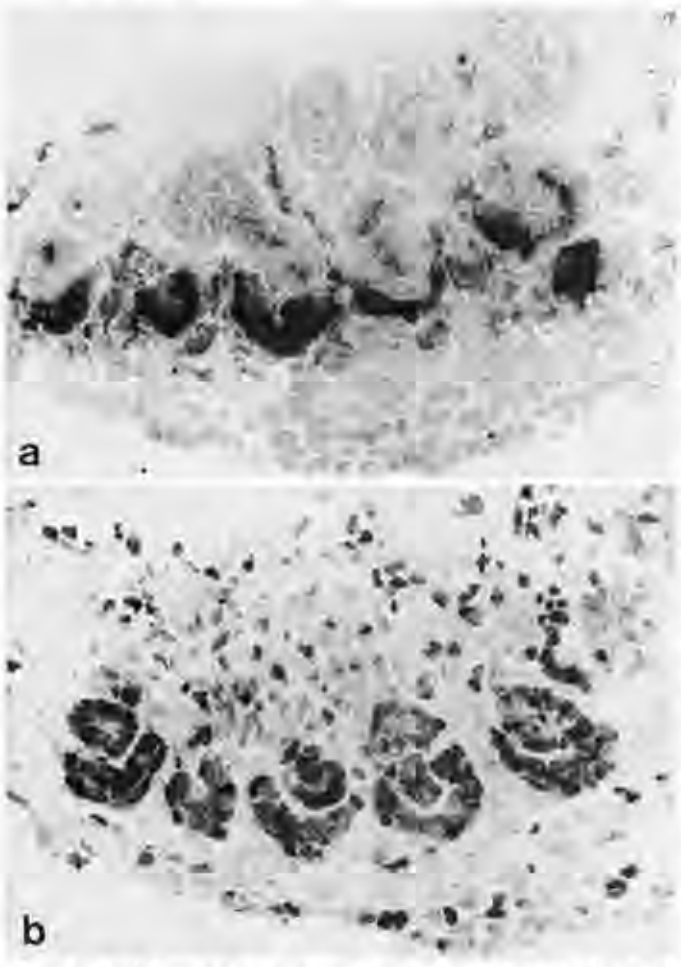

FIG. 6. Proliferating cells located in EnPo 1-labeled, U-shaped aggregates or round clusters (*a*) were detected by the proliferation marker Ki-67 (*b*). The greatest amount of proliferation in perfusion-cultured glomerular epithelium was observed on Days 2 to 4. Consecutive cryosections are shown. Magnification: ×350.

Ab directed against podocytic Ag (14, 31), a newly developed Ab (EnPo 1) was used. EnPo 1 detected an Ag (MW 110, pI 6.5) (22) that is abundant on glomerular epithelial cells as well as on the endothelium of the rabbit kidney. Under perifusion culture conditions, the EnPo 1 Ag was constistently and intensively expressed by the glomerular epithelium (Fig. 4). In contrast, it was down-regulated by cultured endothelial cells (Fig. 3). It is concluded that with respect to EnPo 1 Ag expression, endothelial cells behave differently than the glomerular epithelium. However, the organ-specific environment and the permanent medium exchange positively influenced EnPo 1 expression by glomerular epithelial cells.

To discriminate unequivocally between endothelial cells and the glomerular epithelium, the mAb EC1 was used, which is specific for endothelial cells (20). The EC1 Ag is continuously expressed by endothelial cells *in vivo* (32) and under culture, but it must be pointed out that the permanent exchange of medium is a prerequisite for the excellent maintenance of endothelial tissue in renal cortex explants (20).

For the discrimination of parietal and visceral glomerular cells, an Ab directed against cytokeratin 19 was applied (16). An expression pattern of cytokeratin 19 that corresponded to the *in vivo* situation was observed in cultured explants. Also, the time course of cytokeratin expression *in vitro* is in agreement with observations made *in vivo*, where first expression of cytokeratin 19 was reported for later developmental stages of glomerulogenesis (16). No reaction of mesangial cells with one of the described Ab was detected (12, 13, 17, 20, 32). Thus, a combination of three defined markers made a clear-cut discrimination of the different glomerular cell types possible (Table 1).

Electron microscopical analysis of renal explants revealed that indeed immature podocytes could be generated under perfusion culture. The cells developed an extremely thick layer of extracellular matrix material and numerous plasma membrane interdigitations. However, there were no mature footprocesses. As shown by immunohistology, the clusters did not include endothelial cells (Fig. 1). Even with the electron microscope, cells expressing morphologic characteristics of endothelium were not detected within the clusters. The lack of endothelial cells could be the explanation for the immature status of the cultured podocytes as well as for the extremely thick layer of extracellular matrix material produced by these cells. It has been reported by several authors that the glomerular basement membrane is synthetized and architecturally controlled by both endothelial cells and podocytes (33, 34). Otherwise, we could speculate that the differentiation process of the podocytes is not yet completed at culture Day 13. First, parietal cells of Bowman's capsule expressing typical features of the mature glomerular cells were detectable on the last day of culture. Cytokeratin-positive parietal cells of Bowman's capsule were detectable at the capillary loop stage in the developing kidney for the first time.

This work has shown for the first time that podocytes are able to proliferate and that typical cell features are expressed under perfusion culture conditions. The culture medium used did not include any serum additives but was supplemented with aldosterone and vitamin D3. As reported earlier, this medium also supported the development of the renal microvasculature (20). It must be further analyzed which role aldosterone and vitamin D3 may play in the development of the different tissue constituents. However, it seems likely that cortex explants of immature kidneys might be used as a source of proliferating podocytes.

Primary cultures of glomerular cells can consist of at least five different cell types if contaminations with tubular epithelium or other tissue components are not taken into account. Intensive characterization of glomerular cultures obtained from adult tissue by different authors revealed that those cultures were mainly composed of descendants of parietal epithelial cells and mesangial cells (13, 17, 35). No proliferating visceral cells were ever found no matter which culture protocol was applied. It has been concluded that podocytes were irreversibly growth arrested at a very early point of

differentiation (13). This assumption has been supported by results obtained recently (36). In these experiments, the proliferating cell nuclear Ag was last detectable in developing podocytes at the capillary loop stage. Our results, obtained using the proliferation marker Ki-67, pointed in the same direction. Although podocyte precursors in the lower limb of the S-shaped body were positive for Ki-67, no labeled cells were detected in glomeruli of the capillary loop stage. It is well known that proliferating cell nuclear Ag (PCNA) expression continues slightly beyond the proliferative cell cycle phase (37). Thus, we have to conclude that podocyte proliferation ceased between precapillary loop and capillary loop stages of glomerulus development within the kidney. This developmental period cannot be prolonged with organotypic culture conditions.

MATERIALS AND METHODS

ORGAN MATERIAL

Neonatal rabbits (Days 1 to 3) were anesthetized with diethylether and were killed by cervical dislocation. For immunohistologic incubations, the kidneys were removed and immediately frozen in liquid nitrogen. For the preparation of renal cortex explants, the kidneys were immersed in sterile PBS (pH 7.2) before further treatment.

PERFUSION CULTURE

Renal cortex explants were prepared and mounted as described in detail previously (10, 20). After several washes in sterile buffer and culture medium, the fibrous organ capsule was stripped off of the kidney by a pair of sterile forceps. The explants were mounted in a set of holding rings (Minucells and Minutissue, Bad Abbach, Germany). These mounted explants were placed in a perfusion culture container (Fig. 3b) and were kept under permanent medium exchange for 13 days. The constant flow rate of 1 ml/hour was produced with a conventional peristaltic pump (INP 8, Ismatec, Wertheim, Germany). Culture containers were placed on a warming plate (Medax, Kiel, Germany) at 37° C. The culture medium did not include any serum. It was composed of Iscove's modified Dulbecco's medium, 25 mM HEPES, 100 IU/ml penicillin, 100 μg/ml streptomycin (Gibco-BRL Life Technologies, Eggenstein, Germany), 1×10^{-7} M aldosterone (Aldocorten, Ciba-Geigy, Basel, Switzerland), and 1×10^{-9} M 1.25-dihydroxyvitamin D3 (Biomol, Hamburg, Germany). Supply medium and used medium were stored at 4° C. For immunohistologic analysis, explants were placed on a drop of TissueTek (Plano, Marburg, FRG) and were frozen in liquid nitrogen before cryosectioning.

To investigate the time course of podocyte development *in vitro*, kinetic experiments were carried out. To prove reproducibility, three different 13-day kinetic experiments were analyzed, including three explant samples per day.

IMMUNOHISTOLOGIC ANALYSIS

For indirect immunoperoxidase and immunofluorescence labeling, cryosections of 8 μm were cut with a cryomicrotome (Micron, Heidelberg, Germany). The incubation protocol used was a modification of the method described by Kujat *et al.* (38). Fixation of sections was carried out following a two-step protocol. First, the sections were immersed for 30 seconds in a solution of 4.2% paraformaldehyde (Merck, Darmstadt, Germany), 16% picric acid (Fluka, Buchs, Switzerland), 0.002% cobalt chloride, and 0.1% glutaraldehyde (Serva, Heidelberg, Germany) in PBS, pH 7.2. This procedure was followed by a 15-minute incubation in a solution that included all of the reagents listed above except glutaraldehyde. After a washing step (0.1 M Tris-[hydroxymethyl]-aminomethan, Sigma Chemical Company, St. Louis, MO; 0.8% NaCl, 0.002% Triton × 100, pH 7.4, Pierce, Rockford, ME), the samples were incubated in a blocking solution consisting of 0.1 M Tris buffer, pH 7.4, 25% FCS, 1% NaCl, and 1% Triton × 100 for 45 minutes. Primary Ab (for dilutions see below) were applied overnight. Biotin-SP-conjugated donkey anti-mouse Ig (Dianova, Hamburg, Germany) was diluted 1:600 in blocking solution before application. After this, the sections were washed and incubated for 30 minutes in phenylhydrazine solution (Sigma), including 0.0006% H_2O_2 (Merck) to block endogenous peroxidases. The biotin-detection complex was applied according to the manufacturer's instructions (Vectastain, Vector, Burlingame, CA). The enzyme reaction was then started by adding the substrate solution (0.5 mg/ml diaminobenzidine, 0.1 M Tris, pH 7.4, 0.002% cobalt chloride, 0.04% nickel chloride, and 0.012% H_2O_2). A final rinse in wash buffer stopped the reaction. After that, the sections were dehydrated in a graded ethanol series and a 10-minute incubation in xylol (Merck). DePeX (Serva) served as embedding medium. Sections were analyzed with an Axiovert 35 microscope (Zeiss, Oberkochen, Germany). The documentation of the results was carried out using Agfa PAN 25 film material.

PRIMARY AB

The method developed by Köhler and Milstein (39) was used to produce the mAb. The Ab EnPo 1 and EC1 were raised by immunization with whole kidney homogenate. The characterization of the Ab was described in detail previously (20, 22, 30). The labeling pattern of EnPo 1 and EC1 on rabbit tissue is listed in Table 1. For Ag detection, undiluted culture supernatants were used.

The mAb Ks 19.2.105 was raised and characterized, concerning its reactivity with human fetal and adult kidney tissue, by Moll *et al.* (16). The working dilution of Ks 19.2.105 was 1:400. The proliferation marker Ki-67 (27) was applied undiluted as culture supernatant.

CONTROLS

Controls were provided by sections where the primary Ab was omitted and by sections that were incubated with irrelevant primary Ab. None of these control sections showed any labeling of glomerular structures.

SAMPLE PREPARATION FOR ELECTRON MICROSCOPY

Fresh prepared kidneys and explants cultured for 13 days were fixed in culture medium, including 3% glutaraldehyde (Merck), and were postfixed in 1% osmium tetroxide diluted in PBS. After dehydration in a graded ethanol series, the samples were passed through propylene oxide and were embedded in Epon (Serva). Ultrathin sections were stained with uranyl acetate and lead citrate. The specimens were examined with a Zeiss electron microscope (EM 902). For scanning electron microscopy, the fixed samples were dehydrated in a graded series of ethanol, critical point dried with CO_2 and sputter-coated with gold. Examination was carried out using the DSM 940 A microscope (Zeiss).

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