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Mouse Glomerular Epithelial Cells in Culture with Features of Podocytes in Vivo Express Aminopeptidase A and Angiotensinogen but not Other Components of the Renin-Angiotensin System

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Abstract. The binding of antibodies to podocytic antigens such as the Heymann antigen or aminopeptidase A may lead to the induction of a membranous glomerulonephritis in several species. To study the possible future interactions of antibodies with antigens on these podocytes, epithelial cells from isolated mouse glomeruli were cultured. By indirect immunofluorescence, the cells were positive for cytokeratin, vimentin, desmin, and the ZO-1 protein, a component of the tight junction complex. When rat monoclonal antibodies were used, the cells were also positive for the hydrolases aminopeptidase A and dipeptidyl peptidase IV, and they stained with ASD-33, a monoclonal antibody that recognized an epitope only present on the cell membranes of mouse podocytes. They were negative for the von Willebrand factor and did not stain with a monoclonal antibody (ASD-13) that binds to endothelial cells of glomeruli and peritubular capillaries. By electron microscopy, the cells showed tight junctions but lacked Weibel-Palade bodies (endothelium), desmosomes, and cilia (parietal epithelium). The mRNA expression of several components of the renin-angiotensin system was also examined, and some factors indirectly coupled to the renin-angiotensin system component angiotensin II in this podocytic culture by RT-PCR analysis. mRNA Expression for the angiotensin II degrading hydrolase aminopeptidase A and angiotensinogen was found, but this was not found for any other component of this system, such as renin, angiotensin-converting enzyme, or the angiotensin II receptors AT1a, AT1b, and AT2. Low mRNA expression for dipeptidyl peptidase IV was observed. In addition, expression of the growth factors transforming growth factor-β and interleukin-7, and the extracellular matrix components fibronectin, laminin B2, perlecan, and collagen IVal, was observed. Given these characteristics, a glomerular epithelial cell culture with features of podocytes in vivo that will allow future studies on the interaction of anti-aminopeptidase A monoclonal antibodies and angiotensin II with aminopeptidase A was established. This is of interest in light of the observation that injection of mice with anti-aminopeptidase A antibodies causes an acute albuminuria. (J Am Soc Nephrol 8: 706-719, 1997)

The study on cultured glomerular cells has greatly extended our knowledge of the individual cells in the normal and damaged glomerulus (1–5). Most studies have been carried out on mesangial cells because they are easy to propagate in culture, in contrast to their low proliferation rate observed in vivo under normal or pathological conditions (6–9). On the other hand, endothelial cells are difficult to grow outside their normal glomerular environment, notwithstanding their ability to divide in vivo (10,11). Glomerular epithelial cells (GEC) share the low proliferation tendency in vivo with glomerular endothelial cells, although better results in culturing these latter cells have been reported in recent years, probably because of better-defined culture conditions (2,3,5,7,12,13). Podocytes have primarily been cultured from rat or human glomeruli (2,3,5,7,12–17). GEC from glomeruli of normal mice are hard to obtain, and stable GEC lines have only been isolated from transgenic mice (18), indicating major species differences in establishing glomerular cells in culture. An important drawback that has hampered the study of GEC in culture so far is the ill-defined characterization of the epithelial outgrowths, because they may be of parietal or visceral origin and the available morphological and phenotypic markers are not able to differentiate between them. Most studies simply assume that the mentioned GEC in culture are derived from visceral epithelial cells. This assumption, however, is highly questionable because even the nude glomeruli from which the cultures are derived might still contain fragments of Bowman’s capsule as a source of parietal GEC. In addition, small fragments of proximal tubules that adhere to the glomeruli can be the origin of the proliferating epithelial cells.

Recently we reported on the nephritogenic properties of
Monoclonal antibodies (mAb) directed against the hydrolase amino peptidase A (APA), an enzyme present on the cell membranes of podocytes (19). A single intravenous injection of one of these mAb induced an acute albuminuria in mice, which was mediated by angiotensin II (Ang II) but not by systemic mediators of inflammation known from other experimental models of glomerulonephritis, such as complement, neutrophils, monocytes, platelets, or fibrin (19,20). The aim of this study was to establish a glomerular visceral epithelial cell line from isolated mouse glomeruli to create an in vitro model to examine the interaction of our nephritogenic mAb with their cell membrane-bound targets on mouse podocytes. In this study, we report the culture of epithelial cells from mouse glomeruli that could be maintained for more than 25 passages. By using a mAb that specifically detects an epitope only present on podocytes in vivo in combination with morphological and other well-established phenotypic markers for glomerular epithelial cells, we obtained evidence that these epithelial cells share characteristics with podocytes in vivo. In addition, the presence of mRNA for APA and other components of the renin-angiotensin system (RAS) in these cultured cells, as well as that of some growth factors and extracellular matrix components that are indirectly connected to Ang II, was examined.

Materials and Methods

Animals

Normal BALB/c, and BALB/c, nu/nu mice were originally obtained from the Jackson Laboratory (Bar Harbor, ME), and were kept by continuous brother-sister matings in the breeding facility of the Central Laboratory of Animals of our university. Lou rats used for the production of mAb were obtained from Harlan Olac Ltd. (Blackthorn, Bicester, UK).

Monoclonal Antibodies

Four rat mAb—ASD-4, ASD-13, ASD-33, and ASD-43—that recognize different epitopes on the cell membranes of mouse glomerular cells were generated in our laboratory by fusing spleen cells of Lou rats that were immunized with a solubilized kidney homogenate with SP 2/0 mouse myeloma cells as described before (19). The mAb were produced in larger amounts by intraperitoneal inoculation of the cloned hybridomas in BALB/c, nu/nu mice pretreated with pristane and were subsequently purified by ammonium sulfate precipitation (19). Two mAb, ASD-4 and ASD-43, have previously been characterized and are directed against the hydrolases APA (EC 3.4.11.7) and dipeptidyl peptide IV (DPP IV; EC 3.4.14.5), respectively (19). Both hydrolases are present on the brush borders of proximal tubular epithelial cells and on the podocytes but are not present on glomerular endothelial or mesangial cells (21). ASD-33 was tested on all mouse tissues, such as the cardiovascular system, gastrointestinal tract, urogenital tract, respiratory system, liver, spleen, pancreas, and brain, by immunofluorescence (IF), as described before for APA and DPP IV (21).

Immunoelectron Microscopy

The binding of the two mAb used for the characterization of the glomerular outgrowths ASD-13 and ASD-33 to a normal BALB/c kidney was examined by indirect immunoelectron microscopy (IEM) using immunoperoxidase labeling on 20-μm frozen sections as described (19). In brief, small cortical pieces from a kidney, fixed for 10 min by perfusion with a mixture of periodate, lysine, and paraformaldehyde (PLP), were immersed for an additional 3 h in PLP. Fragments cryoprotected by immersion in 2.3 M sucrose, pH 7.2, for 1 h were frozen in liquid nitrogen. Twenty-micrometer-thick sections were incubated with ASD-13 or ASD-33 (5 μg protein/ml and 2.5 μg protein/ml, respectively) for 18 h at 4°C, followed by several washes with phosphate-buffered saline (PBS) by incubation with a peroxidase-labeled sheep anti-rat Ig Fab fragment (Seralab, Sussex, UK) for 2 h. After being rinsed in PBS, the sections were incubated in diaminobenzidine (DAB) medium for 10 min, followed by incubation in DAB containing 0.03% H2O2 for 7 min. The sections were washed in distilled water, postfixed in cacodylate-buffered 1% OsO4 for 30 min at room temperature, dehydrated, and embedded in Epon 812 (Merck, Darmstadt, Germany). Thin sections were prepared on an ultratome (LKB Instruments, Bromma, Sweden) and examined unstained in the electron microscope (Jeol, Tokyo, Japan).

Western Blotting

A kidney homogenate, solubilized with 1% sodium deoxycholate, was separated by a 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (22) and subsequently electrophoretically transferred to a nitrocellulose membrane (23). After transfer, the nitrocellulose membrane was first blocked in 5% delipidized milk powder/0.1% anti-foam (Sigma, St. Louis, MO) and solubilized in PBS/0.05% Tween-20 for 2.5 h at room temperature. After several washes with PBS/Tween-20, the membrane was incubated with 1 μg/ml primary mAb, again followed by several washes in PBS/Tween-20. Next, the membrane was incubated in 0.1 μg/ml peroxidase-conjugated rabbit (Fab fragments) anti-rat Ig (heavy and light chains) from Seralab. After several washes in PBS/Tween-20, detection was carried out with chemiluminescence Western blotting reagents according to the instructions of the manufacturer (Boehringer, Mannheim, Germany) and exposed to preflashed x-ray films (Kodak XR-Omat, Chalon, France).

Glomerular Cell Culture

Primary cultures of epithelial cells were established from glomeruli isolated from the kidneys of six BALB/c mice. Glomeruli were isolated with a magnet from homogenized kidneys that were loaded with magnetic iron oxide (Fe3O4) by perfusion, as described (24). Isolated glomeruli were plated in six-well culture plates (Costar, Cambridge, MA) coated with a collagen gel (Vitrogen 100; Celtrix, Santa Clara, CA) in a K-1 medium composed of 45% (vol/vol) Dulbecco’s modified Eagle medium (Gibco, New York, NY), 45% (vol/vol) Ham-F10 (Gibco) containing a hormone mix (5 μg/ml insulin, 25 ng/ml proglandelin E1, 5 × 10^-10 M triiodothyronine, 1 × 10^-8 M Na2SeO3, 5 μg/ml transferrin, 18 ng/ml hydrocortisone, (all from Sigma), 100 U/ml penicillin (Gibco), 100 μg/ml streptomycin (Gibco), and 10% Nu-serum (Collaborative Biomedical products, Bedford, MA), as described (25). The glomerular explants were cultured at 37°C in a humidified atmosphere of 5% CO2 and 95% air. After 3 days, the medium was replaced with K-1 medium with 5% Nu-serum, and nonbound glomeruli were gently washed away. After 10 days, a few outgrowths around glomeruli with “cobblestone-like” appearance were present. These outgrowths were excised and digested with 0.2% (550 U/mg) collagenase type Ia (Sigma) for 30 min at 37°C, followed by three washes to minimize the collagenase concentration. Glomeruli that were still present after the last washing step were removed with a magnet. During the next 6 wk, small outgrowths with cobblestone appearances were excised and collectively passed using 0.2% collagenase type Ia, followed by two washes with me-
dium. After these passages, the epithelial cells that grew in small fields and were morphologically homogeneous were excised as described above and transferred to uncoated wells, which were unable to maintain stable cultures on vitrogen-coated plates. The small explants on the uncoated wells, which grew very slowly in the next 3 months, were transferred by trypsinization with 0.5% trypsin (Difco, Detroit, MI) containing 2.5 mM ethylenediaminetetraacetate (EDTA; Merck, Darmstadt, Germany) for 5 min at 37°C. Only after the tenth passage (about 4 months after the first outgrowth) did the growth rate of the epithelial-like cells increase in such a way that the cells could be cultured in culture flasks, reaching confluency in approximately 5 to 7 days. All experiments described in this study were performed between passages 15 and 25.

Immunohistochemistry

Because of the low proliferation rate of the initial outgrowths, the cultured epithelial cells could only be examined by IF after the tenth passage. The immunoreactivities were tested on confluent cell cultures grown on uncoated plastic Slideflasks (Nunc, Roskilde, Denmark). The cell-surface immunoreactivity of the four ASD mAb was tested on vital cells at 4°C by adding the mAb (100 μg/ml) directly to the medium for 30 min. After the slides were gently rinsed with PBS, the cells were fixed in 90% acetone for 10 min at 4°C. The immunoreactivity of cytoplasmic proteins was tested on prefixed cells. Therefore, the cells were gently washed in warm PBS (37°C) and subsequently fixed in 90% acetone for 10 min at 4°C. After the fixation step, the slides were washed with PBS several times and incubated with commercially available antibodies for 30 min at room temperature. The antibodies used were rabbit anti-human cytokeratin, rabbit anti-human vimentin, rabbit anti-human desmin (Organon Technika, Boxtel, The Netherlands), all known to cross-react with rabbit anti-human vimentin, rabbit anti-human desmin (Organon Technika), or FITC conjugated swine anti-rabbit Ig (Dako A/S, Glostrup, Denmark) for 30 min at room temperature. Negative controls were performed by omitting the primary antibodies. All antibodies were also tested by indirect IF on 2-μm frozen sections of a kidney of a normal BALB/c mouse as described (19).

Electron Microscopy

For transmission electron microscopy (TEM), cells were cultured to confluency in K-1 medium with 5% Nu-serum on 6.5 mm, 0.4 μm pore-size, transwell filters (Costar). After the filters were rinsed with culture medium, the cells were fixed with 2% glutaraldehyde for 2 h, washed in PBS, postfixed in cacodylate-buffered 1% OsO4 for 30 min, and finally embedded in Epon 812. Ultrathin sections, cut on an ultratome (LKB Instruments), were contrasted with aqueous uranyl acetate and lead citrate.

For scanning electron microscopy (SEM), GEC were cultured to confluency in K-1 medium with 5% Nu-serum on melinex (Agar Scientific Ltd., Essex, UK) as a support for the growing cells. After being washed in culture medium, the cells were fixed in 2% glutaraldehyde for 2 h, washed in PBS, postfixed in cacodylate-buffered 1% OsO4 for 30 min, and rinsed with PBS. After the cells were dehydrated through ethanol to acetone, the cells were critical-point dried with CO2, mounted on stubs, and finally sputter-coated with gold. All specimens were examined in the electron microscope equipped with scanning abilities (Jeol).

Puromycin Aminonucleoside Cytotoxicity

Cells in culture were incubated with 40 μg/ml puromycin aminonucleoside (PAN; Sigma) for 72 h and subsequently examined under an inverted light microscope (Leitz, Letzlar, Germany).

Table 1. Sequences of the primers used for RT-PCR analysis on cultured cells and normal mouse kidney

<table>
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<tr>
<th>Component</th>
<th>Sense</th>
<th>Antisense</th>
</tr>
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<tr>
<td>Aminopeptidase A</td>
<td>5'-TTC ACA TCC AGT GTT CGT CA-3'</td>
<td>5'-TGG AGA GAG CCT CGA TCC ACC AGT GCC CTG CA-3'</td>
</tr>
<tr>
<td>Dipeptidyl peptidase IV</td>
<td>5'-AAT GAG TAC CAC AGG CTG GG-3'</td>
<td>5'-CTG CAG CAT TCT ATC CAA AGC-3'</td>
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<tr>
<td>Angiotensinogen</td>
<td>5'-AGA AGA CCC TGC ATG ATC AAC-3'</td>
<td>5'-TTT TCT CCT ACG GAA AAA CT-3'</td>
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<tr>
<td>Renin</td>
<td>5'-CTG TGG GTG GAA TCA CTG TG-3'</td>
<td>5'-CCA GTG TCC ACC ACT ACC G-3'</td>
</tr>
<tr>
<td>ACE</td>
<td>5'-TAA CTC GAG TGC CGA GGT G-3'</td>
<td>5'-CCA GCA GGT GGC AGT CTT-3'</td>
</tr>
<tr>
<td>AT1α receptor</td>
<td>5'-GCA TCA TCT TTG TGG G-3'</td>
<td>5'-ATC AGC ACA TCC AGG AAT G-3'</td>
</tr>
<tr>
<td>AT1β receptor</td>
<td>5'-GCA TCA TCT TTG TGG G-3'</td>
<td>5'-ATG AGC ACA TAC AGG AAA C-3'</td>
</tr>
<tr>
<td>AT2 receptor</td>
<td>5'-ATG CTC GAT GGT CTG CTG G-3'</td>
<td>5'-AAC ACA GCT GTT GGA TCC-3'</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>5'-TGA GTG GCT GCT TTT TGA CG-3'</td>
<td>5'-ATG GTA GCC CTT GGG CTC-3'</td>
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<tr>
<td>PDGF</td>
<td>5'-CTT TTC TCT GAG ATG CGT TGG-3'</td>
<td>5'-CTC CCG TTA TGG TTA AAG GT-3'</td>
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<tr>
<td>IL-7</td>
<td>5'-ATT GCC CGA ATA ATG AAC CA-3'</td>
<td>5'-GGC CCA AAC ATG ATG AGC-3'</td>
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<tr>
<td>Perlecan</td>
<td>5'-ACG GTG ACT TTG ATG CGT ACG-3'</td>
<td>5'-CAT GCC CCT ACA GCT GTA TG-3'</td>
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<tr>
<td>Fibronectin</td>
<td>5'-GCC TTT ACA GGT CCA AGT TCC-3'</td>
<td>5'-GGT GCT TCA TGG GGA TCG-3'</td>
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<tr>
<td>Collagen IV α1</td>
<td>5'-CTC TGG GGA CAA CAT CGG-3'</td>
<td>5'-TCT TCT CAT GCA CAC TTG GC-3'</td>
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<tr>
<td>Laminin 2B</td>
<td>5'-TGA AAA GTG CCT GCC TTT CT-3'</td>
<td>5'-GCA TCT TCT ACA GGT GAA GCC-3'</td>
</tr>
</tbody>
</table>

*a* ACE, angiotensin-converting enzyme; AT, angiotensin; TGF-β1, transforming growth factor-β1; PDGF, platelet-derived growth factor; IL-7, interleukin-7.
**Fluorimetric Enzyme Assay**

Cells were cultured in a 24-well tissue culture plate (Costar). For the determination of the APA, DPP IV, and aminopeptidase N (APN; EC 3.4.11.1) enzyme activities, culture medium was replaced by 0.5 ml of the following substrates (10^{-5} M), l-glutamic acid-α-7-amido-4-methylcoumarin (Glu-AMC; APA), and l-alanine-7-amido-4-methylcoumarin (Ala-AMC; APN) in 0.1 M Tris, 1.25 mM CaCl_2, pH 7.0, and glycyl-l-proline-7-amido-4-methylcoumarin-HBr (Gly-Pro-AMC; DPP IV) in 0.1 M Tris pH 8.0 (all from Bachem, Bubendorf, Switzerland). After incubating for 1 h at 37°C, 0.5 ml 0.1 M Na_2CO_3 was added to the wells to stop the reaction, after which the supernatants of each well were centrifuged at 13,000 g for 5 min to remove cellular debris. The enzyme activities were determined by measuring the fluorescence of 7-amino-4-methylcoumarin cleaved from the substrates (27). The fluorescence was measured at 375 nm (excitation) and 440 nm (emission) using a luminescence spectrometer LS-5 (Shimadzu Benelux, 's-Hertogenbosch, The Netherlands). For an inhibition study, 100 μg of ASD-4 was added to the culture medium for

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**Figure 1.** Indirect immunofluorescence and immunoelectron microscopy of a normal mouse kidney section incubated with ASD-33 (A, B) and ASD-13 (C, D). ASD-33 stains the periphery of the capillary loops by immunofluorescence (A; magnification, ×1000). All other renal structures are negative. Immunoelectron microscopy demonstrates a diffuse staining of the cell membranes of the podocytes (B; magnification, ×5100). ASD-13 stains the inner side of the capillary loops and the peritubular capillaries (arrows) by immunofluorescence (C; magnification, ×900). Immunoelectron microscopy shows a diffuse staining restricted to the cell membranes of the endothelial cells (arrow, D; magnification, ×8000). P, parietal epithelium; V, visceral epithelium.
30 min, followed by measurement of APA enzyme activity as described above.

**Enzyme Histochemistry**

To correlate the in vitro enzyme activities with the in vivo situation, we examined the activities of APA, DPP IV, and APN in 5-μm thick frozen kidney sections from a normal BALB/c mouse by using enzyme histochemistry, as described by Lojda and Gossrau (28). Acetone-fixed sections were incubated for 10 min at 37°C with specific substrates (Bachem): 1.6 mM N-Glutamyl-4-methoxy-3-naphtylamide in 0.1 M Tris, pH 7.0, containing 2.5 mM CaCl₂ (APA), 1.4 mM Glycyl-Prolyl-4-methoxy-3-naphtylamide in PBS, pH 7.5 (DPP IV), and 2 mM N-αlanyl-4-methoxy-β-naphthylamide in PBS, pH 7.0 (APN). In addition 1.1 mM Fast Blue B salt (Serva) was added to the various substrates. The sections were rinsed in distilled water and fixed in 4% buffered formalin for 15 min at room temperature. After being rinsed in distilled water, the sections were counterstained with a hematoxylin solution for 1 min and rinsed in tap water for 5 min. The sections were embedded in aquamount (BDH, Poole, UK), and examined in a conventional light microscope (Leitz, Letzlar, Germany).

**RNA Isolation and RT-PCR**

For RNA isolation, confluent GEC culture in a 75 cm² flask were trypsinized with 0.5% trypsin and 2.5 mM EDTA for 5 min at 37°C and subsequently pelleted by centrifugation for 5 min at 750 g. Total cellular RNA was subsequently isolated using the RNAzol B method (29), and the isolated RNA was controlled by agarose gel electrophoresis for integrity, and measured and checked for purity by optical density in the Genequant® DNA/RNA calculator (Pharmacia, Uppsala, Sweden).

For the RT-PCR, primers were selected on basis of the murine cDNA sequences available from the EMBL sequence databank (release 42) using the PRIMER software program (version 0.5, MIT, Cambridge, MA), or on the basis of published primer sets (ATIa, AT1b) (30), and synthesized using the 30SB DNA synthesizer (Applied Biosystems, Foster City, CA). The sequences of the sense and antisense oligonucleotides used in this study are shown in Table 1. For the RT-PCR, 1 μg of isolated total cellular GEC RNA was reverse-transcribed for 1 h at 37°C, using the antisense primers described in Table 1, in the following RT-mixture (20 μl): 75 mM KCl, 50 mM Tris-HCl pH 8.3, 6 mM MgCl₂, 10 mM β-mercaptoethanol, 0.2 mM dNTP each, 1.25 μM antisense primer, and 4.5 U avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI). After completion of the RT reaction 80 μl of a PCR reaction mixture was added, resulting in the final concentrations of 65 mM KCl, 20 mM Tris-HCl pH 8.3, 1.3 to 4.8 mM MgCl₂ (optimized per primer set), 2 mM β-mercaptoethanol, 0.1% NP-40, 0.3 U Thermoperfect polymerase (Integro, Zaandam, The Netherlands), 500 nM antisense primer, 500 nM sense primer, and 0.2 mM dNTP. The final samples of 100 μl were overlaid with two drops of mineral oil, and, after an initial denaturation step at 94°C for 5 min, the mixture was amplified for 35 cycles at 94°C, 55°C, and 72°C each for 1 min in a Mastercycler 5330 plus thermal cycler (Eppendorf, Hamburg, Germany). Twenty microliters of the PCR products were analyzed on a 2% agarose gel (Boehringer) and stained with ethidium bromide. pGEM DNA Markers (Promega) were used as a length marker.

**Results**

**Characterization of the ASD-13 and ASD-33 Antigens**

The localization of the epitopes to which ASD-13 and ASD-33 are directed were examined by indirect IF and indirect IEM (Figure 1). ASD-33 (IgM subclass) has a highly restricted localization, i.e., solely on the cell membranes of the podocytes (Figure 1, A and B). All other renal structures and other organs were negative, except for a faint staining of some cells in the cerebral cortex. ASD-13 (IgG1 subclass) was directed against an antigen located on the cell membranes of the endothelial cells of glomeruli and peritubular capillaries but not of arteries and veins, as seen in Figure 1, panels C and D. Podocytes and mesangial cells were negative. The molecular weight of the putative antigens were determined by Western blotting. Western blotting with ASD-13 on solubilized renal proteins showed a single band of approximately 55 kd (Figure 2, lane 1). A Western blot with ASD-33 on solubilized renal proteins showed a band of approximately 150 kd (Figure 2, lane 2). A Western blot incubated with a control rat antibody was completely negative (Figure 2, lane 3).

**Glomerular Cell Culture**

The glomerular suspension used for the primary outgrowths showed only a minor contamination of tubular and vascular fragments of less than 3%, with about 20% of the glomeruli still encapsulated. Around a small number of glomeruli, seeded in vitrogen-coated six-well culture plates, fields of polygonal epithelial-like cells with a cobblestone pattern could be observed.

![Figure 2](image-url)
served after 5 days (Figure 3A). The concentration of Nu-serum added to the K1 culture medium was lowered to 5% because we found that this had an inhibiting effect on the outgrowth of fibroblasts or mesangial-like cells, which very quickly overgrew the slowly growing polygonal cells upon longer culturing with the addition of 10% Nu-serum (Figure 3B). All of the passages performed with these selected primary cultures were indeed not hampered by the rapid growth of these elongated cells. The initial passage on vitrogen-coated wells yielded small outgrowths of poorly proliferating cells. After approximately 8 wk, the cells were transferred to uncoated plastic wells and still showed a low proliferation rate. Not until the tenth passage, more than 4 months after the start of the cultures, did the cells enter a more proliferative phase and grow in confluent monolayers (Figure 3C), which could be transferred every week at a 1:4 split ratio. The polygonal flat cells varied in size; this was especially observed in nonconfluent cultures. Dispersed in the monolayers, foci of small, multilayered cells were seen, from which cells were growing to a larger size. Particularly at the edge of the fields in nonconfluent monolayers, very large cells were observed, sometimes containing two or three nuclei.

Cultured cells were also very sensitive for the toxic effect of PAN. The cells rounded up and detached from the culture dishes after incubation of PAN for 3 days (Figure 3D). PAN concentrations higher than 40 μg/ml were lethal to the cells. This growth pattern was also examined by TEM, for which cultured cells were grown on transwell filters. Areas with flat polygonal cells, interdigitating with each other to form monolayers, alternated with areas in which these flat cells formed multilayers (Figure 4, A and B). The cells were connected with each other by junctional complexes of the tight junction type, mostly situated at the luminal side of the cells (Figure 4C). At the luminal surface, small villi were present (Figures 4, B and C). No desmosomes, Weibel-Palade bodies, or cilia could be observed. By SEM, the polygonal form of the flat, interdigitating
Figure 4. Transmission electron microscopy of glomerular epithelial cells (GEC) in culture, forming either monolayers (A; magnification, ×10,300) or multilayers (B; magnification, ×9900). The cells are connected by tight junction-like complexes (arrow), and lack Weibel-Palade bodies, desmosomes, or cilia (C; magnification, ×26,500). Scanning electron microscopy of cultured GEC shows flat, polygonal, interdigitating cells growing in monolayers (D; magnification, ×3200), with small villi at their luminal side (inset; magnification, ×65,000).
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cells, with their small villi, could be nicely confirmed (Figure 4D, inset).

**Immunohistological Characterization**

Because of their initial low proliferation rate, the cultured cells could not be characterized until the tenth passage. The findings on the cultured cells were compared with the results on frozen sections of a normal BALB/c mouse kidney (Table 2). GEC in culture stained for the intermediate filaments cytokeratin (Figure 5A) and vimentin (Figure 5B) and, to a lesser extent, for desmin (Figure 5C). Cytokeratin filaments ran through the entire cytoplasm, whereas vimentin fibers were primarily located around the nucleus. In vivo, podocytes are strongly positive for vimentin, whereas parietal epithelial cells stained for cytokeratin, and tubular epithelial cells were focally positive for cytokeratin, but always negative for vimentin (Table 2). Desmin was predominantly present in mesangial cells and only occasionally in podocytes (data not shown). GEC in culture were positive for two membrane-bound hydrolases, APA, and DPP IV, using rat mAb developed in our laboratory (Figure 5, D and E). Both hydrolases were present on cultured cells in a fine punctate pattern. In vivo, APA and DPP IV are present on parietal, visceral, and proximal tubular epithelial cells (19). With ASD-33, a highly specific marker for mouse podocytes that detects an as-yet uncharacterized epitope on their surface, GEC in culture were also stained in a very fine granular way (Figure 5F). GEC in culture were also positive for the ZO-1 protein, an integral component of the tight junctions located at the cytoplasmic side (Figure 5G). ZO-1 could also be observed in vivo in parietal and tubular epithelial cells and in a fine granular way along the glomerular capillary wall, presumably in the slit pores of podocytes, as reported in the rat (26,31). The cultured GEC were negative for the von Willebrand factor and for ASD-13, excluding an endothelial origin (Figure 5 I). Control incubations with the anti-rat or anti-rabbit antibodies used as second layer antisera were completely negative (Figure 5H).

**Enzymatic Activity**

We have measured the enzyme activity of the three hydrolases APA, DPP IV, and APN (Figure 6). The enzyme activity values of APA, DPP IV and APN were 125.5, 267.1, and 491.1 relative fluorescence U/well, respectively. The enzyme activity of APA could be almost completely blocked with ASD-4 (7% residual activity), which is known to be able to block the APA enzyme activity in vivo and in vitro (19), although it had no effect on the other two enzymes (Figure 6). ASD-43, which cannot inhibit the DPP IV enzyme activity in vitro on isolated renal brush borders of mice (19), likewise had no inhibiting effect on the DPP IV activity (data not shown).

By enzyme histochemistry on frozen mouse kidney sections, APA and DPP IV activity was shown on glomerular and tubular epithelial cells (Figure 7, A and B), identical to the immunostaining with ASD-4 and ASD-43, respectively. In vivo APA activity, however, was restricted to the brush borders of the proximal tubular epithelial cells and could not be demonstrated on the glomerular visceral GEC (Figure 7C).

**RT-PCR on Cultured GEC and Normal Mouse Kidney**

GEC in culture express the mRNA of two of the components of the RAS, i.e., the Ang II-degrading hydrolase APA and angiotensinogen, as examined by RT-PCR (Figure 8A). This technique, however, could not detect mRNA for any other RAS component, such as renin, angiotensin-converting enzyme, or the Ang II receptors AT1a, AT1b, and AT2 (Figure 8A). RT-PCR on total kidney RNA was positive for all RAS components except the AT1b receptor, indicating the validity of the RAS primers used (Figure 8A). We also found expression for

<p>| Table 2. Indirect immunofluorescence on podocytes in culture and on normal mouse kidney sections¹ |
|------------------------------------------|----------|--------|--------|--------|</p>
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<th><strong>Antigen</strong></th>
<th><strong>GEC in Culture</strong></th>
<th><strong>In Situ</strong></th>
<th><strong>VGE</strong></th>
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¹GEC, glomerular epithelial cells; VGE, visceral glomerular epithelial cells; PGEC, parietal glomerular epithelial cells; MES, mesangial cells; ENDO, glomerular endothelial cells; ASD-13, rat mAb against a cell surface-bound epitope on glomerular endothelial cells; APA, aminopeptidase A; DPP IV, dipeptidyl peptidase IV; ASD-33, rat mAb against a cell surface-bound epitope on podocytes; ZO-1, component of tight junction complex.
Figure 5. Immunophenotyping of the cultured GEC showed a strong, filamentous staining for cytokeratin (A; magnification, ×350) and vimentin (B; magnification, ×350). A less intense and granular staining was seen for desmin (C; magnification, ×350). Staining of the cultured GEC with the mAb against aminopeptidase A (D; magnification, ×350) and dipeptidyl peptidase IV (E; magnification, ×350) showed a membranous staining in a punctate pattern, suggesting crosslinking effects of the mAb. All cells were positive, although the intensity varied from cell to cell. Staining of the cultured GEC with the podocyte-specific marker ASD-33 also showed a membranous, punctate staining (F; magnification, ×350). Staining with an antibody against the ZO-1 protein showed a very strong, circumferential staining at the contact sites of the GEC (G; magnification, ×350). A control incubation of the cultured GEC with the fluorescein isothiocyanate-labeled anti-rat antibody was completely negative (H; magnification, ×350). ASD-13, an endothelial specific mAb, did not stain the cells (I; magnification, ×350).

DPP IV (Figure 8A) and for some components indirectly influenced by Ang II, such as the extracellular matrix components fibronectin, perlecan, laminin 2B, and collagen IVα1, and the growth factors transforming growth factor-β and interleukin-7 (Figure 8B). No expression was seen for platelet-derived growth factor (Figure 8B). The length of the RT-PCR products for all components found matched the predicted lengths (see legend to Figure 8). All controls, cycled without reverse transcriptase, were negative.

Discussion
Glomerular visceral epithelial cells, more properly called podocytes, have many diverse functions in normal and pathological conditions that are related to their position on the outer
Figure 6. Enzymatic activities of aminopeptidase A (APA), dipeptidyl peptidase IV (DPP IV), and aminopeptidase N (APN) were measured on cultured podocytes incubated either with or without anti-APA mAb. The enzymatic activities are given as percentages of the activities on cultured podocytes without anti-APA mAb (100%). The original enzyme activities were 125, 267, and 491 relative fluorescence U/well for APA, DPP IV, and APN, respectively.

The notion of the nonproliferative state of the podocyte was recently questioned in passive Heymann nephritis (47). In this model, which is characterized by a primary immunologic injury of the podocytes in rats, some proliferative response was observed in the damaged podocytes (47). Parietal epithelial cells, on the other hand, have been shown to be the most proliferative cells of the glomeruli under normal and pathological conditions (46).

We succeeded in culturing epithelial cells obtained from isolated mouse glomeruli. After an initial growth on nitrocellulose supports, we could only pass and propagate these cells on uncoated plastic. During the first 4 months, the proliferative rate was extremely low, but this changed after the tenth passage. Morphologically, the polygonal cells were flat, grew in mono- as well as multilayers, and differed in size. The foci of small, frequently multilayered cells showed outgrowths of larger cells, particularly at the edge of the outgrowth of nonconfluent cultures. This contrasts with the findings of Nørgaard, who found small and large cells corresponding with parietal and visceral epithelial cells, respectively (3). Recently, it was suggested that the different sizes of the outgrowing cells from rat glomeruli correspond to the differentiation state of the cultured cells, the small cells being differentiated podocytic cells and the larger cells being differentiated podocytic cells (48). Immunophenotypically, the cells contained vimentin, cytokeratin, and—to a lesser degree—desmin. They were also positive for ZO-1, an integral component of tight junctions (26). In addition to these cytoplasmic markers, they contained the surface-associated enzymes APA and DPP IV. All positive markers cannot differentiate between visceral and parietal epithelial cells or proximal tubular epithelial cells. However, the cells in culture could be stained with ASD-33, which detects an epitope that is highly specific for podocytes. This epitope, which has not yet been characterized, could only be localized on podocytes and on a few cells in the cerebral cortex. Its localization resembles that of a recently described renal glomerular epithe- lial cell membrane-bound protein tyrosine phosphatase GLEPP1 (49) and that of a podocyte-specific ganglioside (50). This suggests that our cultured glomerular epithelial cells most likely are of podocytic origin because they are stained by ASD-33, a marker not present in parietal or proximal tubular epithelial cells in vivo. Definition of the in vivo origin of cultured cells is, however, hampered by the fact that the unphysiological conditions of cell culture induce variable dedifferentiation of the cells, with the gain or loss of some phenotypic or functional features. This has been described for vimentin, cytokeratin, desmin, and APN, markers frequently used for the characterization of glomerular cells in culture (51–56). In the normal in vivo situation, vimentin can only be detected in podocytes, whereas cytokeratin is present in parietal and tubular epithelial cells (54,55). However, in various pathological conditions, coexpression of these intermediate filaments can be seen, explaining the frequent coexpression mentioned in many reports on GEC in culture. The same holds true for desmin, which is normally present in mesangial cells but which in various pathological conditions is also expressed in podocytes but not in other epithelial cells (53,54).
together, our glomerular epithelial cells in culture share a number of characteristics with podocytes in vivo and thus seem attractive for further in vitro studies of visceral epithelial cell pathophysiology.

In addition to the morphologic and immunologic characterization of the cultured GEC, we also analyzed the mRNA expression of components of the RAS and some components that are indirectly coupled to the function of one of the RAS components, i.e., Ang II. Apart from APA and angiotensinogen, the presence of mRNA for other components of the RAS could not be demonstrated in our cultured podocytes. Apart from the angiotensinogen expression, this finding is an additional support for the podocytic origin of the GEC in culture, because the presence of the RAS components at the protein and mRNA level have been reported for the glomerular parietal epithelial cells and proximal tubular epithelial cells but not for podocytes (57–64). Although expression for the AT1a and AT2 Ang II receptor isoforms could be demonstrated in the kidney, we could not detect such an expression in cultured podocytes (59–61). AT1b receptor expression was absent in both the kidney and the podocytes as reported (60). We found also mRNA expression for the growth factors transforming growth factor-β and interleukin-7, the synthesis and regulation of which can be influenced by components of the RAS (65–67). No mRNA expression was seen for platelet-derived growth factor, which corresponds to the findings of Abboud (68). However, Floege et al. (47) demonstrated production of platelet-derived growth factor-B chain in visceral glomerular epithelial cells under certain pathological conditions. Furthermore, we discovered a high mRNA expression for the extracellular matrix components fibronectin, perlecan, laminin 2B, and collagen IVα1, proteins that can be produced by podocytes via the interaction of the above-mentioned growth factors (47,68–71).

In this report, we describe an epithelial cell line from isolated glomeruli of normal BALB/c mice, with immunological, morphological, and phenotypic characteristics that suggest a podocytic origin. The immunologic characterization was particularly based on the staining of the cultured cells with a rat mAb that recognizes a membrane-bound epitope only present on podocytes. As shown with the inhibition studies with ASD-4, which could inhibit the enzyme activity of APA on our
Figure 8. Detection of mRNA for podocytic molecules, renin-angiotensin system components, and angiotensin II (Ang II)-related components by RT-PCR analysis on RNA isolated from both normal mouse kidney and cultured podocytes. For each component, the amplification product on normal mouse kidney is shown in the first lane, followed by the amplification product on the cultured podocytes in the second lane. The amplification products were separated on an ethidium bromide-stained 2% agarose gel. pGEM DNA Markers are indicated on the left. In panel A, amplification products of the following are shown: aminopeptidase A (APA; 561 bp), dipeptidyl peptidase IV (DPP IV; 474 bp), angiotensinogen (Aogen; 509 bp), renin (363 bp), angiotensin-converting enzyme (ACE; 348 bp), and the Ang II receptors AT1a (690 bp), AT1b (690 bp), and AT2 (328 bp). In panel B, the amplification products for the following components are shown: the extracellular matrix components perlecan (HSPG; 414 bp), fibronectin (FN; 449 bp), laminin 2B (LAM; 463 bp), and collagen IVα1 (COL-IV; 394 bp), and the growth factors platelet-derived growth factor (PDGF; 336 bp), transforming growth factor-β1 (TGF-β1; 465 bp), and interleukin-7 (IL-7; 288 bp).

cultured cells, these cells can be used as an in vitro model for future studies on the interaction of antibodies with surface-bound antigens, components of the RAS, growth factors, and extracellular matrix proteins of podocytes.

Acknowledgments
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