Primary Uroepithelial Cultures

A MODEL SYSTEM TO ANALYZE UMBRELLA CELL BARRIER FUNCTION*

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Despite almost 25 years of effort, the development of a highly differentiated and functionally equivalent cell culture model of uroepithelial cells has eluded investigators. We have developed a primary cell culture model of rabbit uroepithelium that consists of an underlying cell layer that interacts with a collagen substratum, an intermediate cell layer, and an upper cell layer of large (25–100 μm) superficial cells. When examined at the ultrastructural level, the superficial cells formed junctional complexes and had an asymmetric unit membrane, a hallmark of terminal differentiation in bladder umbrella cells. These cultured “umbrella” cells expressed uroplakins and a 27-kDa uroepithelial specific antigen that assembled into detergent-resistant asymmetric unit membrane particles. The cultures had low diffusive permeabilities for water (2.8 × 10⁻⁴ cm/s) and urea (3.0 × 10⁻⁷ cm/s) and high transepithelial resistance (>8000 Ω cm²) was achieved when 1 mM CaCl₂ was included in the culture medium. The cell cultures expressed an amiloride-sensitive sodium transport pathway and increases in apical membrane capacitance were observed when the cultures were osmotically stretched. The described primary rabbit cell culture model mimics many of the characteristics of uroepithelium found in vivo and should serve as a useful tool to explore normal uroepithelial function as well as dysfunction as a result of disease.

The epithelium that lines the urinary bladder provides an effective barrier between the urine and the underlying connective tissue. This uroepithelium is comprised of multiple cell layers including a basal cell layer that attaches the uroepithelium to the connective tissue substratum, an intermediate cell layer that is 1–2 thick, and a superficial cell layer composed of large (up to 100 μm in length) highly differentiated “umbrella” cells that line the luminal surface of the bladder (1). The umbrella cells of many mammals have a specialized apical plasma membrane, in which the outer leaflet appears twice as thick as the inner one (1). This asymmetric unit membrane (AUM),¹ a hallmark of the uroepithelium (2, 3), is comprised of at least 3 major integral membrane proteins, the uroplakins, that assemble into a paracrystalline hexagonal array of 16-nm protein particles (4–6). Uroplakins are also present in cytoplasmic vesicles (5, 7), which are discoidal or fusiform in appearance, and are believed to be involved in modulating the surface area of the bladder by recycling membrane to and from the apical surface of the umbrella cells (8–10). The fusion of these vesicles with the apical surface of the umbrella cells, along with the ability of the bladder mucosa to unfold, provides the bladder with a tremendous reserve capacity to accommodate changes in urine volume (1). This ability is a crucial aspect of bladder barrier function.

The barrier imparted by the uroepithelium is the result of several additional specializations. High resistance junctional complexes present in the umbrella cell layer provide an effective barrier to paracellular ion flux (11, 12). In addition, the umbrella cell apical plasma membrane is highly impermeable to water and small solutes (13, 14) and contains an array of glycosaminoglycans (15). The glycosaminoglycans may prevent bacterial adhesion to the epithelium and are thought by some to affect membrane permeability although their exact role remains uncertain (16). Umbrella cells also possess a sodium channel within their apical plasma membrane along with other ion transport systems (12, 17). These channels regulate transcellular ionic flux across the epithelium and may be important in maintaining the large osmotic gradient between the urine and the underlying tissue. As a result of these specializations, the umbrella cell forms one of the tightest and most impermeable barriers in the body. Any disruption of the umbrella cell barrier could result in the infiltration of toxic solutes from the urine and subsequent inflammation and breakdown of underlying tissue. Thus, the umbrella cells play a pivotal role in the normal physiology of the bladder.

Our current understanding of the development and maintenance of the permeability barrier, the assembly of the AUM, and the formation of discoidal vesicles and their stretch-induced fusion with the apical plasma membrane is hampered by the lack of a suitable cell culture model. Thus far, the study of

¹ The abbreviations used are: AUM, asymmetric unit membrane; TER, trans-epithelial resistance; PBS, phosphate-buffered saline; Pipes, 1,4-piperazinediethanesulfonic acid; Ω, ohm.
umbrella cells has been limited to whole tissue preparations of excised bladder. With these systems, the accurate measurement of uroepithelial function is limited by access, the need to sacrifice multiple animals for each experiment, and the presence of underlying connective tissue and muscle layers. In the past 25 years numerous culture systems of uroepithelium have been described (18–56). However, many of these systems lacked several of the terminal differentiation markers that are signposts of polarized umbrella cell development. In addition to morphological shortcomings, no culture system has exhibited functional properties similar to whole tissue preparations such as low permeability to urea and water, the development of high transepithelial resistance to ion flux, and sensitivity to stretch. Hence, a cell culture model system that mimics both the form and function of intact tissue has not yet been developed.

We describe a primary culture model system that more closely resembles whole tissue uroepithelium. Characteristics of this system that mimic those of bladder epithelium in vivo include: the presence of an apical umbrella cell layer with terminal differentiation markers (e.g. the uroplakins), an AUM, discoidal vesicles, low permeability to water and urea, development of high transepithelial resistance (TER; > 8000 Ω cm²), the presence of an apical sodium transport pathway, and the ability to alter apical surface area in response to stretch.

**EXPERIMENTAL PROCEDURES**

**Materials—**Unless specified otherwise, all chemicals were of reagent quality or better and were obtained from the Sigma.

**Isolation of Epithelial Cells from Rabbit Bladders—**Animal experiments were performed in accordance with the Animal Use and Care Committee of the University of Pittsburgh. Urinary bladders were obtained from New Zealand White rabbits (3–4 kg). Typically, two rabbits were used per culture. Each rabbit was euthanized with 250 mg of pentobarbital, the bladder was exposed, the ends of the bladder were clamped with hemostats, an incision was made lengthwise along the bladder and the opened bladder was surgically excised and washed in Krebs solution (110 mM NaCl, 5.8 mM KCl, 25 mM NaHCO₃, 1.2 mM KH₂PO₄, 2.0 mM CaCl₂, 1.2 mM MgSO₄, 11.1 mM glucose, pH 7.4). The bladder was trimmed of excess fat and stretched on a rack (see Fig. 1b in Ref. 57), mucosal side down, in the same solution at 37 °C. The smooth muscle layers were carefully removed by dissection using a scalpel and forceps and the stripped mucosa was transferred to a 10-cm square dish containing an 8-cm plastic rack with 10 sharp metal pins along each edge. The tissue was stretched mucosal side up in Krebs solution (110 mM NaCl, 5.8 mM KCl, 25 mM NaHCO₃, 1.2 mM KH₂PO₄, 2.0 mM CaCl₂, 1.2 mM MgSO₄, 11.1 mM glucose, pH 7.4). The bladder was trimmed of excess fat and stretched on a rack (see Fig. 1b in Ref. 57), mucosal side down, in the same solution at 37 °C. The smooth muscle layers were carefully removed by dissection using a scalpel and forceps and the stripped mucosa was transferred to a 10 × 10-cm square dish containing an 8 × 8-cm plastic rack with 10 sharp metal pins along each edge. The tissue was stretched mucosal side up across the metal pins and then incubated overnight at 4 °C in sterile minimal essential medium (Life Technologies, Inc., Grand Island, NY) containing 1% (v/v) penicillin/streptomycin/fungizone (Life Technologies, Inc.), 20 mM HEPES, pH 7.4, and 20 mM HEPES, pH 7.4, to weaken the association between the epithelium and the connective tissue and to facilitate manual separation of the mucosa.

Following treatment, the stripped mucosa was transferred to a sterile tissue culture hood, the minimal essential medium/dispase solution was aspirated, and the epithelial cells were scraped from the underlying connective tissue using two flexible cell scrapers (number 83.1830, Sarstedt, Inc., Newton, NC). The scraped cells were transferred to a tissue culture dish, resuspended in 20 ml of 0.25% (v/v) trypsin-1 mM EDTA (Life Technologies, Inc.), and incubated 15–30 min at 37 °C, resuspending once during the incubation. After trypsinization, the single cell suspension was brought up to 50 ml with minimal essential medium containing 1% (v/v) penicillin/streptomycin/fungizone, 20 mM HEPES, pH 7.4, and 5% (v/v) fetal bovine serum (HyClone Laboratories, Logan, OR) in a sterile conical tube and spun down in an IEC Centra CL2 Centrifuge (International Equipment Co., Needham Heights, MA) at 1,000 rpm for 5 min to pellet the cells and remove the trypsin. The supernatant was aspirated carefully and the cells were resuspended in 50 ml of the same minimal essential medium/penicillin/streptomycin/fungizone/fetal bovine serum solution and washed an additional two times. The third time the antibody was then washed. The cell suspension was resuspended in minimal essential medium (Life Technologies, Inc.) and then resuspended in the appropriate volume of defined keratinocyte medium to yield a final concentration of 700,000–800,000 cells/ml, as determined by cell counting in a hemocytometer chamber (Hausser Scientific, Horsesham, PA). This cell density was crucial to obtaining highly differentiated cultures as plat- ing at a higher (>900,000 cells/ml) or lower density (<500,000 cells/ml) resulted in poorly developed cultures. Two bladders yielded enough cells to plate approximately 50–60 12-mm Transwell filters (Corning Costar, Cambridge, MA).

**Plating and Cell Culture—**Cells were plated on either 12-mm Transwell or 12-mm Snapwells (Corning-Costar) coated with collagen. The coating solution was prepared by mixing 10 mg of type IV collagen (Sigma, type VI), 200 μl of glacial acetic acid, and 100 ml of H₂O₂ and incubating overnight at 4 °C without stirring. The collagen solution was sterile filtered and stored at 4 °C. Prior to use, the collagen solution was diluted 1:9 in 10 mM Na₂CO₃-HCl, pH 9.0, and 500 μl of the resultant solution was added to each filter and incubated for 60 min at room temperature to allow the collagen to bind to the filters. Prior to plating, the collagen solution was aspirated and 0.5 ml of cell suspension was added to the apical chamber and 2 ml of keratinocyte medium was added to the basal chamber. For Snapwells, 0.5 ml of the cell suspension was added to the apical chamber and 4 ml of keratinocyte medium was added to the basal chamber. The third day after plating, the apical medium was aspirated and replaced with 1 ml of keratinocyte medium for the Transwells and 0.5 ml of medium for the Snapwells. The basolateral medium was exchanged for 1.5 or 4 ml of defined keratinocyte medium per Transwell or Snapwell, respectively. The cells were fed in this manner every 2–3 days. Cells whose TER reached levels of approximately 200 Ω cm² or higher (between 3 and 6 days after plating) were switched to keratinocyte medium containing 1 mM calcium chloride in order to achieve high TER (8000 Ω cm² or greater). Adding 1 mM calcium chloride before day 3 resulted in poorly developed cultures, while adding calcium chloride after day 6 resulted in cultures with suboptimal TER (<8000 Ω cm²). Successful cultures were attained approximately 85% of the time.

**Antibodies and Other Labeled Reagents—**Reagents used included: mouse anti-human AE1 supernatant (diluted 1:20) recognized a 27-kDa AUM-associated antigen that is a marker of terminally differentiated umbrella cells (7); purified mouse anti-keratin antibody AE1 antibody (Chemicon, Temecula, CA) recognizes multiple acidic cytokeratins (58) and was diluted 1:200; rabbit anti-Z0-1 antibody R40.76 supernatant (Dr. D. A. Goodenough, Harvard University, Cambridge, MA) recognizes a tight junction-associated protein and was used at 1:5 dilution; mouse anti-tubulin antibody DM1a ascites (Sigma) recognizes α-tu- bulin and was diluted 1:500; rabbit anti-AUM serum, which recognized uroplakins I and III (5), was used at a 1:500 dilution; fluorescein isothiocyanate-phalloidin (Molecular Probes, Eugene, OR) was reconstituted in methanol as described by the manufacturer and diluted 1:50 prior to use; propidium iodide was made up as a 5 mg/ml stock in PBS and diluted 1:1000 just prior to use; affinity-purified and minimal secondary antibodies (Jackson Immunoresearch Laboratories, West Grove, PA) were diluted 1:200 prior to use; chicken anti-AUM serum, which recognizes uroplakins I and III (5), was diluted 1:10,000 for use in Western blots; mouse anti-uroplakin III hybridoma is a newly developed cell line that produces an IgG, that specifically recognizes the cytoplasmic domain of uroplakin III. Its characterization will be described at a future date. Secondary antibodies (Jackson Immunoresearch Laboratories, West Grove, PA) were diluted 1:500. For immunofluorescence for uroplakin III, anti-uroplakin III was used at 1:50; fluorescence isothiocyanate-phalloidin (Molecular Probes, Eugene, OR) was reconstituted in methanol as described by the manufacturer and diluted 1:100. For Western blotting, affinity-purified and horseradish peroxidase-conjugated goat secondary antibodies (Jackson Immunoresearch Laboratories) were diluted 1:25,000 prior to use in Western blotting.

**Immunofluorescence Analysis—**Indirect immunofluorescence was performed as described (59). Cells grown on 12-mm Transwell filters were washed with Pipes buffer (80 mM Pipes/KOH, pH 6.8, 5 mM EDTA, 2.0 mM MgCl₂) and fixed in pH 6.5 fixative (4% paraformaldehyde dissolved in Pipes buffer readjusted to pH 6–6.5 with HCl) for 5 min with shaking at room temperature. The 6.5 fixative was aspirated, replaced with pH 11 fixative (4% paraformaldehyde dissolved in 100 mM sodium borate, pH 11), and the cells were incubated with shaking for an additional 10 min at room temperature. The cells were washed with PBS, and the paraformaldehyde was quenched and the cells were per- meabilized with PBS containing 20 mM glycine, pH 8.0, 75 mM ammonium chloride, 0.1% (v/v) Triton X-100 for 10 min at room temperature with shaking. The cells were washed with PBS and then incubated in block solution (PBS containing 0.7% (v/v) fish skin gelatin and 0.01% (v/v) saponin) including 5% (v/v) goat serum and 100 μg/ml boiled RNase A for 10 min at 37 °C. Cells were then incubated at 37 °C for 30 min with 1% (v/v) affinity-purified and minimal secondary antibodies (Jackson Immunoresearch Laboratories) were diluted 1:500. The cells were washed with block solution 3 times for 5 min with shaking and then incubated for 30 min with the appropriate secondary antibody diluted in block solution. The cells were washed 3 times for 5 min with block solution and then washed 2 times in PBS and post-fixed (4% paraformaldehyde in 100 mM sodium cacodylate, pH 7.4) for 10 min at room temperature with shaking. The cells were washed in PBS and mounted...
on slides with p-phenylelenediamine mounting medium as described (59).

**Scanning Laser Confocal Analysis of Fluorescently Labeled Cells**—The samples were analyzed using an argon-krypton laser coupled to a Molecular Dynamics (Mountain View, CA) Multiprobe 2001 confocal, attached to a Diaphot microscope (Nikon, Melville, NY) with a Plan Apo 60×1.2 NA oil immersion objective. Images were taken with the appropriate filter combinations. Collection parameters were as follows: laser output set at 50 milliwatts, PMTs set to 750 mV, laser attenuation at 3%, 50 μm slt. The images (512 × 512 pixels, 0.8 μm pixel size) were acquired using ImageSpace software (Molecular Dynamics). The images were converted to tag-information-file-format and the exact levels of the images adjusted in the Photoshop program (Adobe Co., Mountain View, CA) on a Power PC Macintosh G3 computer (Apple, Cupertino, CA). Every attempt was made to collect and process images in an identical manner. The contrast-corrected images were imported into Freehand 8.0 (Macromedia, San Francisco, CA) and printed from a Kodak 8650 PS Color Printer (Kodak, Rochester, NY).

**Transmission Electron Microscopy**—Freshly isolated tissue or 7-day-old cell cultures were fixed in 100 mM cacodylate buffer containing 1.5% (v/v) glutaraldehyde, 2% (v/v) paraformaldehyde, 1 mM CaCl₂, 0.5 mM MgCl₂ for 1–2 h at room temperature. The samples were then osmicated 1–2 h with 1.5% (v/v)/OsO₄, in 100 mM cacodylate, washed several times with distilled water, and then block stained overnight in 0.5% (v/v) aqueous uranyl acetate. Cells were dehydrated in a graded series of ethanol, the epoxy resin was embedded (Ladd Research Industries Inc., Burlington, VT), and sectioned with a diamond knife (Di-atomex US, Fort Washington, PA). Sections, pale gold in color, were mounted on butvar-coated nickel grids, contrasted with uranyl acetate and lead citrate, and viewed at 80 kV in a JEOL (Japan) 100 CX electron microscope.

**AUM Purification and EM Negative Staining**—AUM was isolated using a modification of the protocol described by Wu et al. (4). Freshly isolated rabbit bladder was cut open and stretched, epithelial side up, on a rack as described above. The epithelium was scraped (as described above) and the cells were transferred to two 1.5-ml Eppendorf tubes that were then brought up to 1.5 ml with ice-cold PBS. Alternatively, 7-day-old uroepithelial cells cultured on 75-mm Transwells (plated using 20 ml of cell suspension per Transwell) were scraped into ice-cold PBS and collected into two 1.5-ml Eppendorf tubes. Both the tissue-derived and cultured epithelium were collected by centrifugation at 1000 x g for 5 min at 4 °C in a GP-6R centrifuge (Beckman, Palo Alto, CA). The supernatant was aspirated and the cells were washed three additional times with ice-cold PBS. The supernatant was aspirated after the final wash, and each cell pellet was homogenized in 1 ml of homogenization buffer (10 mM HEPES containing 1.5 M NaCl, 0.5 M NaCl, 25 mM Tris-HCl, pH 8.0, and 0.05% (v/v) Triton X-100) using a Teflon-glass homogenizer (Potter-Elvehjem, Teflon, Dounce). The homogenate (12 mm Snapwell filters were switched to 1 mM CaCl₂. When high TER (>8000 Ω cm²) was achieved, the filters were placed in Ussing chambers modified to accept Snapwell filters (Vertical Diffusion Chambers, Navi-tech, Sparks, NV). The basal medium was aspirated and the apical medium was left in the apical well of the Snapwell during transfer to prevent sudden and dramatic falls in TER due to the pressure/stretch sensitivity of the cultures. The Snapwell insert was placed in the physiological (basolateral) hemichamber and the apical (apical) hemichamber was clamped into place, and 5 ml of keratinocyte medium was added to the mucosal hemichamber. The complete chamber was tilted upright and 5 ml of keratinocyte medium was then added to the mucosal hemichamber. The spontaneous potential difference (Pd) and short circuit current (Isc) across the cell layers were measured by four agar-bridge electrodes connected to a VCC MC6 voltage clamp (Physiologic Instruments, San Diego, CA). Humidified 5% CO₂, 95% O₂ gas was bubbled into the chambers. The TER of the filters was calculated from the Pd and Isc using Ohms law and was measured throughout the experiment to confirm that the cultures maintained high TER.

At the start of the experiment, 25 μl of [H]water (1 μCi/ml) and 25 μl of [3H]urea (1 μCi/ml) were added to the mucosal and serosal sides of the chambers at 15-min intervals and placed into 22-ml scintillation vials. Duplicate samples were taken for each side of the chamber to account for inaccuracy due to pipetting errors. At each time point, the amount of medium was replaced with the same volume of keratinocyte medium and the TER was measured prior to each 15-min sample. 0.1% Triton X-100 (v/v) was added to the mucosal side at the time point to the contribution of the unstirred layers to the diffusive permeability. The number of counts in the apical and basolateral samples were determined by adding 10 ml of ScintiSafe scintillation fluid (VWR, West Chester, PA) to each vial and counting for 5 min per sample in a Wallac 1409 scintillation counter (Wallac OY, Turku, Finland).

The measured diffusive water and urea permeabilities (Pd) were calculated by assuming that the membrane was uniform and polymeric as described previously (13): P = A(ΔC)/D where ΔC is the concentration gradient and D is the diffusional permeability in the absence of Triton X-100 (P) or in the presence of Triton X-100 (P): P = P + ΔP/isotopic as described in Ref. 13. In all flux measurements the flux rate was linear (R > 0.98) and corrections were made for sample dilution.

**Capacitance Measurements**—Capacitance was measured as a means of estimating surface area (where 1 microfarad = 1 cm² of actual membrane area) as described previously (10, 12). Because the apical membrane of the umbrella cell is the major site of resistance, changes in capacitance reflect primarily changes in the apical cell surface area (10, 12). Briefly, a square current pulse of 1 μAmp, generated by a MacLab 8 A/D converter (AD Instruments, Victoria, Australia) interacted with a 400 MHz PowerPac Macrointosh computer (Apple) and a VCC MC6 current/voltage clamp. Physiologic (basolateral) hemichambers were switched across the cell layers (grown on Snapwells and mounted in vertical chambers) for 250 ms following a 50-ms delay. The voltage response of each filter was digitized and then recorded every 100 μs using the Scope program (AD Instruments). An average of 8 sweeps was recorded for each current pulse. The time constant, τ, was determined by calculating the length of time required to reach 63% of the steady state voltage.
using a curve fitting routine (10, 60). The capacitance was determined using the formula: 
\[ C = \frac{t}{R}, \]
where \( C \) is the capacitance and \( R \) is the resistance. Resistance was determined by dividing the amplitude of the steady-state voltage response by the amplitude of the square current pulse.

**RESULTS**

**Differentiated Uroepithelial Cells Can Be Cultured on Transwell Filters**—In the past 25 years, in vitro culture of many polarized epithelial cells has become feasible. These advances are a result of increased understanding of cell-extracellular matrix interactions (50, 61), realization that permeable filter supports allow for epithelial polarization to occur (62), and the development of specialized growth media (e.g., defined keratinocyte medium) that contain appropriate concentrations of growth factors and other additives that control differentiation and stratification (e.g., \( \text{Ca}^{2+} \)) (63). With these advances in mind, isolated uroepithelial cells were plated on type IV collagen-coated Transwell filters and then cultured in a defined serum-free keratinocyte medium. Following 1 day in culture, virtually 100% of the cells stained positively with an antibody that recognizes multiple cytokeratins (AE1 hybridoma supernatant, see Ref. 58) confirming their epithelial origin (data not shown).

To ascertain the differentiation of the uroepithelial cultures over time, we examined cultures by indirect immunofluorescence and confocal microscopy. Using AE31 (a mouse monoclonal antibody that recognizes a uroepithelial-specific 27 Kd antigen) as a marker of terminally differentiated umbrella cells (7), ZO-1 as a marker of tight junctions, and propidium iodide as a stain for nuclei, we could assess the growth and differentiation of the uroepithelium in culture over time. After 1 day of plating, the cultures were mostly one cell layer thick and were composed primarily of basal cells (these were defined as the 80% of cells that directly adhered to the filter) with an occasional island of AE31 positive cells sitting atop the basal cell layer (Fig. 1, A–D). AE31-positive umbrella cells spanned the majority of the epithelial depth at this early stage of development. ZO-1 was expressed on the periphery of umbrella cells and at the periphery of adjacent basal cells that had not yet been covered by umbrella cells (see Fig. 1D). Although ZO-1 is normally considered a marker of tight junctions, in non-polarized cells ZO-1 is known to interact with adherens junctions (64). It is possible, therefore, that in these early cultures ZO-1 is also interacting with components of the adherens junction. By day 3, the cultures had differentiated into at least two distinct cell layers: a basal cell layer that by this time had completely covered the filter and an apical cell layer that was AE31 positive (data not shown). ZO-1 was found at the periphery of the AE31-positive umbrella cells and had largely disappeared from the basal cell layer. Although umbrella cells covered most areas of the underlying basal cell layer, there were some regions where basal cells remained exposed on their apical surface (data not shown). By day 7, some regions of the cultures had fully matured into 3 distinct layers of uroepithelium including an intermediate cell layer that has formed above the basal layer (Fig. 1, E–H). The normal ultrastructure of rabbit umbrella cells (3) was not recognized by this antibody in either the tissue or the cultures (data not shown).

To confirm that the primary uroepithelial cultures expressed uroplakins and were able to assemble these proteins into AUMs, we isolated detergent-insoluble AUM from mature 7-day cultures, blotted them, and probed with a polyclonal anti-AUM serum. Major proteins with approximate molecular masses of 30 and 47 kDa were identified (Fig. 4A, right lane), consistent with the molecular weights of uroplakins I and III, respectively. Proteins with identical molecular weights were identified in purified AUM isolated from intact tissue (Fig. 4A, left lane). Uroplakin II, which has a molecular mass of 15 kDa, was not recognized by this antibody in either the tissue or the cultures (data not shown). The identity of the 47-kDa molecular mass species as uroplakin III was confirmed by probing Western blots of purified AUM with a monoclonal antibody that recognizes an epitope in the cytoplasmic domain of this protein. A protein with a molecular mass of 47 kDa was recognized in AUM purified from both intact tissue (Fig. 4B, left lane) as well as in primary cell cultures (Fig. 4B, right lane). When detergent-insoluble AUMs purified from primary uroepithelial cultures were negative stained and examined in the electron microscope they were found to form paracrystalline arrays of proteins identical to those observed in native tissue (Fig. 4C).

Additional immunofluorescence experiments demonstrated that all of the superficial umbrella cells of the primary cultures reacted with the anti-AUM antibody (Fig. 4D).

**Cultured Uroepithelium Achieves High TER and Functions as a Tight Barrier to Ion and Solute Flux**—Uroepithelium in vivo has one of the highest TERs in the body with values ranging from 8000 \( \Omega \) cm\(^2\) to 75,000 \( \Omega \) cm\(^2\) (11, 12). These high values reflect the primary function of the bladder, which is to maintain a tight barrier to ion flux. To assess the barrier function of our cultures, we monitored TER over time. As described under “Experimental Procedures,” these primary cul-
munities are grown in defined keratinocyte medium. This medium is formulated to have a low calcium concentration (<0.1 mM) that prevents keratinocyte stratification and differentiation. When grown in unsupplemented keratinocyte medium, uroepithelial cells failed to achieve a high TER. We therefore supplemented the keratinocyte medium with 1 mM calcium chloride to promote cell stratification and formation of high resistance tight junctions.

**Fig. 1.** Distribution of AE31, ZO-1, and nuclei in primary uroepithelial cultures 1-day and 7-days post-plating. Cells were fixed with paraformaldehyde and AE31 (green staining), ZO-1 (thin red lines) and nuclei (stained red with propidium iodide) were simultaneously detected by indirect immunofluorescence following 1 day (A–D) or 7 days (E–H) in culture. Individual optical sections, obtained with a scanning laser confocal microscope are shown. The nuclei of the basal cell layers are shown in panels D and H. Panels C and G are optical sections taken approximately 3 μm above panels D and H. Panels B and F are approximately 4 μm above panels C and G, and panels A and E are approximately 2 μm above panels B and F. Bar = 10 μm.

The timing of the addition of Ca^{2+} was crucial to the ability of the cultures to develop a high TER. Adding supplemental calcium too early (before a TER of ≥200 Ω cm²) or too late (after day 6) prevented the cultures from developing a high TER. For example, cultures grown in medium supplemented with 1 mM calcium from day 1 never achieved high TER and rapidly senesced (data not shown). Although the time point at which supplemental calcium chloride was added to the medium var-
panels A–D

primary uroepithelial cultures. Cells were fixed with paraformaldehyde and cytokeratins (stained with AE1 ascites; panels A–D), actin (stained with fluorescein isothiocyanate-phalloidin; panels E–H) and microtubules (stained with DM1a ascites; panels I–L) were detected by indirect immunofluorescence. Individual optical sections, obtained with a scanning confocal microscope, are shown from the apical pole of the umbrella cell layer (A, E, and I), 2–3 μm below this level near the nuclei of the umbrella cell layer (B, F, and J), at the level of the nuclei of the basal cell layer (C, G, and K), and near the base of the basal cell layer (D, H, and L). Bar = 20 μm.

Fig. 2. Distribution of cytokeratins, actin, and microtubules in primary uroepithelial cultures. Cells were fixed with paraformaldehyde and cytokeratins (stained with AE1 ascites; panels A–D), actin (stained with fluorescein isothiocyanate-phalloidin; panels E–H) and microtubules (stained with DM1a ascites; panels I–L) were detected by indirect immunofluorescence. Individual optical sections, obtained with a scanning confocal microscope, are shown from the apical pole of the umbrella cell layer (A, E, and I), 2–3 μm below this level near the nuclei of the umbrella cell layer (B, F, and J), at the level of the nuclei of the basal cell layer (C, G, and K), and near the base of the basal cell layer (D, H, and L). Bar = 20 μm.

ied, we found it best to add it after the umbrella cells had formed a confluent layer atop the basal cells and the TER had reached levels of at least 200 Ω cm² (typically after day 3). Fig. 5 shows that the addition of 1 mM calcium on day 5 rapidly increased TER to greater than 5 times the levels of untreated cultures. Approximately 85% of our cultures achieved a TER of 8000 Ω cm² or greater and in some cases reached levels as high as 20,000 Ω cm² (data not shown). Addition of calcium had no obvious morphological effect on the differentiation markers expressed by these cells when they were examined by light microscopy (data not shown).

In addition to maintaining a high resistance to ion flux, uroepithelium must maintain steep urine/blood osmotic gradients by restricting the flow of water and other small nonelectrolytes that readily cross most biological membranes. To measure the barrier to solute diffusion imparted by the uroepithelium, cells plated on Snapwells were mounted in modified Ussing chambers designed to accept Snapwells. The diffusive permeability of water and urea were determined using isotopic flux measurements and corrected for the unstirred layer as described previously (13). Table I compares the permeability values of various barrier epithelia found throughout the body. Although the permeability of water in the cultures was greater than that shown for isolated tissue (13), permeability values for the primary cultures were still much lower than those of other barrier epithelia including the collecting ducts of the kidney (65–67). The urea permeability was actually lower than tissue and bore similarity to values reported for purified bladder apical membrane endosomes and gastric vesicles (14, 68). These results indicate that our cultures maintained a low permeability to solute flux.

Cultured Uroepithelium Contains a Sodium Transport Pathway—Umbrella cells express the epithelial Na⁺ channel in their apical membranes (11, 12). These channels function in transepithelial Na⁺ reabsorption from the urine, and are selectively inhibited by the diuretic amiloride at a concentration of 10 μM. To determine whether primary uroepithelial cultures expressed this sodium transport pathway, we mounted filter-grown uroepithelial cells in modified Ussing chambers and the effects of 10 μM amiloride on the spontaneous potential difference (Fig. 6A; a measure of the asymmetric distribution of charge across the epithelium), short-circuit current (Fig. 6B, a measure of active ion transport) and TER (Fig. 6C) were assessed. As shown in Fig. 6, A and B, both the potential difference and the short-circuit current were markedly decreased (approximately 90%) by the addition of amiloride to the mucosal (apical) hemichamber. Addition of amiloride to the serosal (basal) hemichamber had no effect (data not shown). As expected, addition of amiloride significantly increased the TER by blocking the major pathway for active transepithelial ion transport (Fig. 6C).

Cultured Uroepithelium Exhibits a Stretch Response—An important feature of uroepithelium is its ability to accommodate changes in bladder volume (1). One mechanism believed to be important in modulating bladder volume is the insertion of
were grown in keratinocyte medium (●) and were measured at the indicated number of days post-plating. Mean ± S.E. is shown (n = 12).

**DISCUSSION**

In the past 25 years numerous attempts have been made to develop a cell culture model of uroepithelium (18–56). Despite continual effort, few systems have been described that resemble uroepithelium in vivo, and in many cases the culture models share almost no similarity to native tissue. Table II gives examples of previously described uroepithelial culture models and summarizes the features of these culture systems that mimic uroepithelium in situ. These features include a stratified epithelium with a superficial layer of AE31/uroplakin-positive umbrella cells that form an asymmetric unit membrane. The umbrella cells should have discoidal vesicles as well as junctional complexes. Functionally, the uroepithelium should exhibit a high transepithelial resistance, a low permeability to small solutes, an apical sodium transport pathway, and a stretch response. We have not included culture systems of tumor cell lines as in general these lines do not exhibit any of the hallmarks of uroepithelial differentiation (29, 36, 38, 40, 46, 53). Moreover, we have excluded systems in which the epithelial cells shed into normal urine are cultured (47, 49, 69), as some of these cells are unlikely to be of uroepithelial origin (70).

One of the earliest attempts at uroepithelial culture was that of Elliot et al. (25). They examined cell outgrowths from explants of uroepithelium. These cultures were epithelial in nature but had none of the features expected of uroepithelium. Chlapowski and Haynes (20) were the first group to culture cells that exhibited many morphological similarities to native tissue including an AUM, tight-junction formation, and cytoplasmic vesicles. In their system, explants were cultured on collagen-coated nylon discs in a system that predated the Transwell. However, the cells had leaky junctions and a high permeability to water. A few years later, Reznikoff et al. (39) developed a system to culture human uroepithelium. The epithelium was stratified and the apical cell layers contained junctional complexes, however, there were no data on the barrier function of these systems. Kirk et al. (51) developed a serum-free medium in which to culture human uroepithelial cells. This medium, almost identical to that used to culture keratinocytes, was used by several other researchers. Howlett et al. (27) and Fujiyama et al. (71) developed a system much like that of Chlapowski and Haynes (20), except they plated cells on collagen gels containing embedded fibroblasts. Morphologically, the cultures were stratified, and the upper cell layer had an apical AUM as well as junctional complexes. While these co-culture systems were extremely promising, no assessment has been made on the barrier function of these systems.

It was not until 1990 that markers of uroepithelial differentiation became available. Surya et al. (43) plated a single cell suspension of bovine uroepithelium and used a then recently developed antibody (AE31) to demonstrate that the cells synthesized a component of the AUM. The first attempt to grow...
cells on commercially available permeable filter supports (cyclospore filters) was by De Boer et al. (23). However, they presented no evidence on the expression of differentiation markers or the achievement of barrier function (23). Finally, Perrone et al. (37) have recently described the establishment of an immortalized human bladder cell line from a patient with interstitial cystitis. This multilayer culture model was the only system that formed reasonably tight monolayers (TER of 500–1000 $\Omega$ cm$^2$) (11, 12), as well as stretch responsiveness (8, 10). To achieve these ends, we took advantage of several advances in epithelial cell culture. First, we used permeable filter supports (Transwell or Snapwell filters). It has been known since the landmark work of Cereijido et al. (62) that culture of epithelial cells on permeable supports is necessary for this cell type to polarize and establish specialized apical and basolateral membrane domains. Consistent with this requirement, when the isolated uroepithelial cells used in this study were cultured on collagen-coated plastic dishes, the cells failed to stratify and did not express the AE31 antigen or uroplakins. This is in contrast to the results of Surya et al. (75) whose cultures were grown on dishes yet stratified and expressed AE31 (75). The difference may reflect cell origin or the different culture media used in these studies. An additional component of this culture system was the use of type IV collagen to coat the filters before plating. Type IV collagen is an important component of the basal lamina that separates the epithelia from the underlying connective tissue. In the absence of the collagen coating, the uroepithelial cells did not adhere to the filter. The use of basement membrane collagen was important as type I collagen failed to promote cell binding. Furthermore, we used a serum-free, low calcium growth medium (keratinocyte medium) because it has been shown previously to prevent differentiation and stratification of other cell types including keratinocytes (63, 76). The concentration of cells in the plating suspension is also vital to proper growth and development of the cultures. Too high or too low a concentration resulted in lower basal cell adherence to the filter and poor differentiation of the superficial umbrella cell layer. This may reflect the need for autocrine growth factors that are limiting at low cell number but become growth inhibitory at higher cell number.

The ability of cultures to develop high TER was critically dependent on the addition of supplemental calcium only after the cultures had stratified and attained a TER of $\leq200 \Omega$ cm$^2$. The final TER obtained, typically $>8000 \Omega$ cm$^2$ and as high as 20,000 $\Omega$ cm$^2$, is within the reported range reported for intact uroepithelium (8,000–75,000 $\Omega$ cm$^2$) (11, 12). These extremely high TERs were consistent with these cultured cells forming a tight barrier to ion flux. Moreover, the cultured cells formed an effective barrier to solute flux, as determined by their low diffusive permeabilities to water and urea. The water permeability of the cultured cells was higher than that observed in intact tissue, but the urea permeability was lower (13). Even so, the values for water permeability were still significantly lower than those observed in other barrier epithelia (see Table I). The ion transport properties of cultured epithelium were almost identical to those reported for intact tissue. The uroepithelial cells had a potential difference of approximately negative 40–50 mV (measured relative to the serosal side), and a small short circuit current of 4–6 $\mu$Amps/cm$^2$. The short circuit

### Table I

| Sample          | Primary cultures (this work) | Rabbit tissue (Ref. 13) | Bladder AME$^a$ (Ref. 14) | Gastric vesicles (Ref. 68) | CCD$^b$ (Refs. 65 and 66) | IMCD$^b$ (Refs. 65 and 66) |
|-----------------|-------------------------------|-------------------------|---------------------------|----------------------------|--------------------------|---------------------------|
| $P_{W}$, cm/s   |                               |                         |                           |                            |                          |                           |
| Water ($\times10^6$) | 2.8                           | 0.41                    | 2.3                       | 2.8                        | 20                       | 70                        |
| Urea ($\times10^6$)   | 0.3                           | 4.4                     | 0.8                       | 0.6                        | 4                        | 3.4–69                    |

$^a$ Bladder AME, mammalian bladder apical membrane endosomes.

$^b$ CCD, cortical collecting duct.

$^c$ IMCD, inner medullary cortical collecting duct.

![FIG. 6](image_url) Effect of amiloride on spontaneous voltage (A), short-circuit current (B), and TER (C). See text for explanation. All values were measured relative to the serosa. Shown is mean ± S.E. (n = 6).

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current was significantly blocked (>90%) by addition of amiloride to the apical compartment, confirming the presence of an epithelial sodium channel activity in the apical membrane. This is consistent with previous electrophysiological analyses as well as the recent localization of the epithelial sodium channel in rat urothelial cells (12, 17).

Finally, a well known feature of the bladder mucosa is its sensitivity to mechanical stimulation (8, 10, 17, 60). This is manifested in at least two ways. First, when isolated bladder mucosa is mounted in an Ussing chamber quick removal and manifesting not only uroepithelial development and function, but also the processes by which epithelial cells achieve and maintain their specialized plasma membrane domains, and how these functions are disrupted in disease.

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