Novel Three-Dimensional Organoid Model for Evaluation of the Interaction of Uropathogenic Escherichia coli with Terminally Differentiated Human Urothelial Cells

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Human bladder 5637 cells cultivated under microgravity conditions formed organoids that displayed characteristics of in vivo tissue-specific differentiation. Uropathogenic Escherichia coli (UPEC) strain CP9 colonized and penetrated the organoids and induced α-hemolysin-mediated exfoliation of urothelial cells. We propose these uro-organoids as models that simulate the interactions between UPEC and terminally differentiated human urothelium.

Uropathogenic Escherichia coli (UPEC) is the most common cause of urinary tract infections (UTIs) in otherwise healthy individuals. A UTI usually begins when UPEC successfully colonizes the most superficial layer of cells that cover the urinary tract. The normal human urothelium (formerly called transitional epithelium) is five to eight cell layers thick and is comprised of basal, intermediate, and superficial cell types. This polarized cytoarchitecture increases its morphological complexity and differentiation from base to surface (11). The terminally differentiated superficial cells, also known as umbrella cells, display two unique characteristics: a subapical trapezoidal network of cyto-keratins and an apical plasma membrane fine with numerous rigid-looking mannosylated glycoproteins called uroplakins (27, 28). To date, four major uroplakins (UPIa, UPIb, UPII, and UPIII) have been identified on the outer leaflet or asymmetric unit membrane (8, 28). This highly specialized membrane provides the permeability barrier function to the urothelium (13). Moreover, type 1 pili, which are expressed by more than 90% of UPEC strains (16, 17), bind to the urothelial uroplakin Iα to facilitate bacterial uptake (17, 29). Therefore, terminally differentiated urothelial cells are critical for many urothelial tasks and for initiation of UTIs.

Most in vitro models used to investigate interactions between UPEC and urothelium have failed to recreate the differentiated tissue components and structure seen in normal human urothelium. One potential approach to creating differentiated uroepithelial cells is through the use of a suspension culture technology designed by National Aeronautics and Space Administration engineers. The key component of the system is a rotating wall vessel (RWV) bioreactor that allows cells to remain in suspension with bubble-free aeration. Such conditions simulate microgravity and are optimal for assembly of individual cells into three-dimensional (3-D) organoids (18, 26). These organoids are characterized by cell polarity, extracellular matrix production, and organ-specific differentiation (7, 18, 26). Furthermore, the RWV technique may potentially be used to culture a wide variety of normal and neoplastic cells whose aggregation within this RWV can be promoted by the addition of specific scaffolds. These scaffolds can be collagen-coated glass beads or acellular biomaterials, such as a derivative of porcine small intestine submucosa (SIS). The resulting macroscopic 3-D structures display many of the cellular attributes of the corresponding in vivo tissue (7, 18, 26) and thus provide a more biologically relevant system for studying certain aspects of microbial pathogenesis than do two-dimensional tissue cultures. In this study, we sought to establish such a 3-D organoid model of human urothelium and to assess the interaction of the resultant tissues with UPEC strains that had previously been evaluated for infectivity and pathological effects in murine bladders challenged intraurethrally with the organisms (20).

Human urothelial cells cultivated under conditions of microgravity. Initially, five different human uroepithelial cell lines were evaluated for their capacity to form organoids. Four of the lines were obtained from the American Type Culture Collection: SV-HUC-1 (CRL-9520), 5637 (HTB-9), J82 (HTB-1), and T24 (HTB-4). The fifth line, α-E7, was the kind gift of Catherine Reznikoff (19). Each cell line was grown in the appropriate medium as recommended by the ATCC or, in the case of the α-E7 cell line, by Reznikoff (19). Cells were maintained in a humidified incubator at 37°C and 5% CO2, cultured until confluent, and then detached from the flask by trypsin treatment. Next, cells from each line were resuspended in the appropriate medium (except that the concentration of fetal bovine serum was reduced to 5%) at ~1 × 106 cells/ml and then introduced into a 50-ml volume of disposable RWV (Synthecon, Inc., Houston, TX) along with 2- to 3-mm² pieces of extracellular matrix (SIS; Cook Biotech Incorporated, Bloomington, IN). The RWV were attached to the rotator base of a rotary cell culture system (Synthecon, Inc., Houston, TX). The initial rotation speed was set at 12 rpm and then increased as needed to ensure that the cell aggregates remained in a state of
free fall as the masses of the organoids increased. The vessels were routinely checked and any visible air bubbles removed. Medium was replaced twice weekly. Organoid pieces were removed from the RWVs after \(10\) days of growth and fixed in \(5\%\) buffered formalin (Fisher Scientific, Pittsburgh, PA). Paraffin-embedded \(5\)-\(\mu\)m-thick organoid sections were cut, and slides were stained with Masson’s trichrome and analyzed by light microscopy. Histological examination revealed that the cell lines T24 and J82 did not form 3-D structures analogous to that of the urothelial organization found in vivo (data not shown). The SV-HUC-1 and α-E7 cell lines appeared to develop organized aggregates; however, the formation of these aggregates was neither reliable nor reproducible. By contrast, the well-studied 5637 cell line (5, 14, 15) developed into tissue-like arrangements that under microscopic analysis appeared as closely packed layers of four to six cells that were heterogeneous in size and shape (Fig. 1A and B). This morphology was reminiscent of normal human urothelial cells in vivo (Fig. 1C). Consequently, only the 5637 cell line was used to form organoids for further characterization and infectivity studies.

**Immunostaining.** Cell type and differentiation marker expression by the 5637 cells grown either as tissue organoids or monolayers was assessed by immunofluorescent staining. Five-micrometer sections of 5637 3-D aggregates were deparaffinized and subjected to antigen retrieval by microwave oven treatment for \(15\) min in \(1\times\) AntigenPlus buffer, pH 10 (Novagen/EMD Biosciences, Inc., San Diego, CA). Nonspecific binding sites were blocked with \(1\%\) bovine serum albumin (Sigma, St. Louis, MO). Sections were then incubated with the appropriate primary and secondary antibodies (Table 1). Human bladder tissue sections (Novagen/EMD Biosciences, Inc., San Diego, CA; Asterand, Detroit, MI) were similarly stained and used as positive controls. Negative controls consisted of organoid sections, human tissue sections, or monolayers treated with \(1\%\) bovine serum albumin instead of the primary antibody. All samples were analyzed with an Olympus BX60 system microscope with a BX-FLA reflected light fluorescence attachment. Images were acquired using a SPOT RT charge-coupled-device digital camera (Diagnostic Instruments, Inc., Miami, FL) and assembled for presentation in Adobe Photoshop. The presence of immunostaining in the organoids was recorded as positive or negative. The location of any positively staining cells within the epithelium was noted.

Overall, the 5637 organoids (Fig. 2A to D) exhibited increased and more-selective expression of several markers that are typically present in differentiated human urothelium (Fig. 2E to H) compared to the same cell line grown in monolayers (Fig. 2I to L). For instance, E-cadherin (25), a cell adhesion molecule present in normal epithelial tissues, appeared to be more highly expressed in the 5637 organoids (Fig. 2A) and normal human urothelium (Fig. 2E) than in its monolayer

**TABLE 1. List of antibodies**

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Antibody host(s) (description)</th>
<th>Source</th>
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</thead>
<tbody>
<tr>
<td><strong>Primary antisera</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E-cadherin</td>
<td>Mouse</td>
<td>Zymed Laboratories, Inc., San Francisco, CA</td>
</tr>
<tr>
<td>Cingulin</td>
<td>Mouse</td>
<td>American Research Products, Inc., Belmont, MA</td>
</tr>
<tr>
<td>Cytokeratin 20</td>
<td>Mouse</td>
<td>Dako Corporation, Carpinteria, CA</td>
</tr>
<tr>
<td>Uroplakin Ia</td>
<td>Goat</td>
<td>Santa Cruz Biotechnology, Santa Cruz, CA</td>
</tr>
<tr>
<td><strong>Secondary antisera</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AlexaFluor 488</td>
<td>Goat (antimouse), donkey (antirabbit), chicken (antigoat)</td>
<td>Molecular Probes, Eugene, OR</td>
</tr>
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</table>

\(a\) Antibodies dilutions were done as per the manufacturer’s recommendations.
counterpart (Fig. 2I). Similarly, immunodetection of the tight junction-associated protein cingulin (2) in the 5637 3-D aggregates (Fig. 2B) and in normal urothelium (Fig. 2F) showed increased staining around the cell periphery compared to cells in monolayers (Fig. 2J). Moreover, the immunostaining pattern in the 5637 organoids for cytokeratin 20 (27), a protein component of the intermediate filament cytoskeleton, was more intense in superficial layer cells of the organoid (Fig. 2C) and normal urothelium (Fig. 2G) than the reduced and more irregular expression of the same marker in the 5637 monolayers (Fig. 2K). Furthermore, immunohistochemical staining with the antibody to uroplakin Ia (8, 28), a major integral membrane protein often considered a biochemical marker of urothelial differentiation, confirmed that this uroplakin was specifically and abundantly expressed in the normal human urothelium (Fig. 2H) and by the superficial layer of cells in the 3-D aggregates (Fig. 2D) compared to the irregular expression of this marker by the same cell line grown as a monolayer (Fig. 2L). Immunochemical evaluation of the negative controls did not reveal a staining pattern (data not shown).

**FIG. 2.** Expression of cellular markers in normal human urothelium, 5637 monolayers, and organoids. The 5637 urothelial cells were cultured as organoids (A-D) or monolayers (I-L). Expression of E-cadherin (A, E, and I), cingulin (B, F, and J), cytokeratin 20 (C, G, and K) or uroplakin Ia (D, H, and L) was analyzed by immunofluorescence microscopy. Note that cytokeratin 20 (C and G) and uroplakin Ia (D and H) are located apically in the superficial cells of the 5637 organoids and normal human urothelium. Magnification, ×40.

**UPEC infection of 5637 cell monolayers and organoids.** The response of 5637 monolayers and organoids to infection with strain CP9, a human blood isolate of *Escherichia coli* (04/H5/K54) that expresses cytotoxic necrotizing factor type 1 (CNF1) (22), and its cnf1-negative isogenic mutant (20) was tested. This selection of strains was based on previous observations that...
CNF1 can induce apoptosis of 5637 cells (15) and that mutation of $cnf_1$ has an attenuating effect on UPEC virulence in a murine model of ascending UTI (20). *E. coli* CP9 also produces other virulence factors, such as Prs pilus (class III PapG adhesin), type 1 pili (21, 22), and $\alpha$-hemolysin, which is often coexpressed with CNF1 (1, 6). To discriminate between effects attributable to CNF1 and hemolysin on 5637 cells, we mutated $hlyA$ in CP9 as described below. All bacterial strains were grown in static Luria-Bertani (LB) broth (Fisher Scientific, Pittsburgh, PA) at 37°C for 24 h prior to use in experiments.

CP9 carries two hemolysin genes (10); one $hlyA$ copy is located within an operon immediately upstream of $cnf_1$ (unpublished observation), as is seen in other uropathogenic *E. coli* strains, such as J96 (04/H5/K*H11002*) (1, 10, 12, 24). In this study, we introduced a nonpolar mutation in $hlyA$ upstream of $cnf_1$ by the Lambda Red recombination technique (3) with modifications as noted below. Oligonucleotides KG03F and KG04R (Table 2) were used to amplify by PCR the chloramphenicol acetyltransferase gene (*cat*) sequence in pACYC184 (New England Biolabs, Ipswich, MA). Oligonucleotides KG01F

### Table 2. Oligonucleotide primers

<table>
<thead>
<tr>
<th>Primer designation</th>
<th>Primer sequence</th>
<th>Target</th>
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<tbody>
<tr>
<td>KG01F 5'-CAGATTTCAATTTTCCATTAACAG-3'</td>
<td>40 bp upstream of $hlyA$ and first 20 bp of cat</td>
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</tr>
<tr>
<td>KG02R 5'-CAGGCCCTAGAACATGTGCATT-3'</td>
<td>5' last 33 bp of $cat$ and 20 bp downstream of $hlyA$</td>
<td></td>
</tr>
<tr>
<td>KG03F 5'-ATGGAGAAAAATCCTGAGG-3'</td>
<td>$cat$</td>
<td></td>
</tr>
<tr>
<td>KG04R 5'-TTACGCCCGGCTCCTGAC-3'</td>
<td>Downstream NcoI site, $hlyA$</td>
<td></td>
</tr>
<tr>
<td>KG05F 5'-TACACCTGAGTGCCTGAC-3'</td>
<td>Downstream NcoI site, $hlyA$</td>
<td></td>
</tr>
<tr>
<td>KG06R 5'-TTATATGCTATGCTACAGAT-3'</td>
<td>Upstream of $hlyA$</td>
<td></td>
</tr>
<tr>
<td>KG07F 5'-CCGAGTATGCTTGTGACG-3'</td>
<td>Downstream of $hlyA$</td>
<td></td>
</tr>
<tr>
<td>KG08R 5'-GGAATTAACCTGAGGTCATC-3'</td>
<td>$hlyA$</td>
<td></td>
</tr>
<tr>
<td>KG09F 5'-ATATGTATGCTGAC-3'</td>
<td>$cnf_1$</td>
<td></td>
</tr>
<tr>
<td>KG10F 5'-GATACCACTCATTGACCTCA-3'</td>
<td>$cnf_1$</td>
<td></td>
</tr>
<tr>
<td>a F, forward; R, reverse.</td>
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</table>

FIG. 3. Construction and characterization of an isogenic hemolysin mutant, CP9Δ$hlyA::cat$. (A) The Lambda Red recombination system was used to replace a copy of the $hlyA$ gene with a chloramphenicol resistance gene within the $hly$ operon. (B) Southern blot analysis showed the deletion of the copy of the $hlyA$ gene that is located upstream from $cnf_1$. (C) Western blot analysis demonstrated that the $hlyA$ mutant produced CNF1 at wild-type levels.
and KG02R were designed from the published hly operon sequence of UPEC strain J96 (GenBank M10133) and were used to add sequences that flank hlyA to the cat gene. The resulting 720-bp PCR product, which was generated to create a precise in-frame deletion of hlyA and insertion of cat in place of that gene (Fig. 3A), was gel purified using the Qiaex II gel extraction kit (QIAGEN, Valencia, CA) and transformed into CP9(pKD46) by electroporation at 2.5 V, 200 µF, and 25 µF. Hly mutants were selected on LB agar with 30 µg/ml chloramphenicol, screened by PCR for acquisition of the cat gene (KG03F-KG04R), and screened for reduced hemolysis on blood agar. The insertion of cat in the hlyA locus was verified by amplification of PCR products with primers that extended from within cat downstream (KG03F-KG08R) or upstream (KG07F-KG04R) into the hlyA-flanking regions. Primers specific for the regions flanking hlyA (KG07F-KG08R) were used to confirm the replacement of the 3.1-kb hlyA gene with the 660-bp cat gene.

Southern blot analysis (ECL direct nucleic acid labeling and detection system; Amersham Biosciences, Piscataway, N.J.) of NcoI-digested chromosomal DNA from the hlyA mutant, CP9, and CP9cnf1 was done (Fig. 3B). A probe specific for the hlyA fragment downstream of an internal NcoI site was amplified by PCR (KG05F-KG06R). The two native hlyA genes were found on NcoI fragments of approximately 7.9 kb and 8.8 kb in the wild-type chromosomal digest. An expected band shift up was

FIG. 4. Interaction of UPEC CP9, CP9cnf1, and CP9ΔhlyA::cat with 5637 monolayers. The 5637 urothelial cells were grown as confluent monolayers (A and B), infected with either E. coli CP9 (C and D), CP9cnf1 (E and F), or CP9ΔhlyA::cat (G and H), and incubated at 37°C in 5% CO2 for 90 min or 120 min. Cells were fixed and Leukostat stained before microscopic analysis (magnification, ×41).
seen in the hlyA-cnf1-associated band (from ~8.8 kb to ~12 kb) in the cnf1 mutant (Fig. 3B, lane 2) due to the loss of an NcoI site within the deleted portion of the cnf1 gene. The cnf1-linked hlyA gene was absent in the CP9ΔhlyA::cat mutant (Fig. 3B, lane 3). The location of the hlyA mutation within the hlyA operon adjacent to cnf1 was confirmed by long-range PCR with PFU TURBO (Stratagene, La Jolla, CA) using the following primer sets: KG03F-KG10R and KG09F-KG10R (data not shown).

The CP9ΔhlyA::cat mutant grew at wild-type levels and exhibited a reduced hemolytic phenotype on blood agar plates compared to CP9 (data not shown). The hlyA mutant produced CNF1 at wild-type levels, as shown by Western blot analysis of equal concentrations of sonicated cellular protein (Fig. 3C). The protein concentration was determined according to the methods of the BCA protein assay kit from Pierce (Rockford, IL). Western blot analysis was done as previously described with goat polyclonal anti-CN1F serum as the probe (15).

Infection of human urothelial 5637 cells in monolayers (shown uninfected in Fig. 4A and B) by UPEC was done essentially as described previously (14). Organoids were infected similarly except that the tissue aggregates were placed into 35-mm Corning cell culture dishes with 3 ml of adhesion medium that contained 70 μl of bacteria diluted in LB broth (A600nm ~0.5). All 5637 cell cultures were incubated at 37°C in 5% CO2. The infected monolayers were incubated for 90 and
120 min, respectively, and then washed three times with Dulbecco's phosphate-buffered saline (Cambrex BioSciences, Walkersville, MD) fixed, stained with Leukostat (Fisher Scientific, Pittsburgh, PA), and examined by light microscopy. The dishes that contained the organoids were incubated for 1, 2, 3, or 6 h, respectively, at 37°C in 5% CO₂ and then washed and fixed for either light or electron microscopy analysis. All infection experiments were done in triplicate, and the results were reproducible.

Infection of 5637 monolayers with either CP9 or CP9\textit{cnf}_1 caused severe damage to the cells after 90 min (Fig. 4C and E), with less than 50% of the monolayer remaining at 2 h postinfection (Fig. 4D and F). We speculated that hemolysin might be responsible for this immediate damage to the urothelial cells based on similar hemolysin-mediated toxic events reported for a variety of other cell types (4, 9, 21). We tested this idea by infecting 5637 monolayers with our newly created isogenic \textit{hly} mutant of CP9 (described above). After 120 min of infection with CP9\Delta\textit{hly}:\textit{cat} (multiplicity of infection, $\sim$10), little or no apparent damage to the 5637 monolayers was observed (Fig. 4H). Taken together, these experiments suggest that hemolysin was likely responsible for the toxicity caused by both the CP9 and CP9\textit{cnf}_1 strains, a supposition supported by the absence of toxicity when 5637 cells were challenged with CP9\Delta\textit{hly}:\textit{cat}. The relative contributions of CNF1 and Hly to uropathogenesis in the CP9 strain remain to be defined.

Light-microscopic examination of formalin-fixed sections of uninfected (Fig. 5A, zero time; Fig. 5B, 6 h of incubation in medium alone) and infected organoids stained with Masson's trichrome showed the angular plasma membrane with asymmetric unit membrane regions, fusiform vacuoles, and dilated vacuoles, which are indicators of well-developed and differentiated urothelium (6, 8) (scale bar, 200 nm). (B) Infected 5637 organoids showed \textit{E. coli} CP9 (b) in close association with the superficial urothelial cells. Some loss of cell structural integrity was evident (asterisks) (scale bar, 1 μm).

FIG. 6. Transmission electron micrographs of 5637 organoids. (A) Uninfected organoids exhibited an angular plasma membrane with asymmetric unit membrane regions (arrowheads), fusiform vacuoles (fv), and dilated vacuoles (dv), which are indicators of well-developed and differentiated urothelium (6, 8) (scale bar, 200 nm). (B) Infected 5637 organoids showed \textit{E. coli} CP9 (b) in close association with the superficial urothelial cells. Some loss of cell structural integrity was evident (asterisks) (scale bar, 1 μm).
constructs that expressed cellular markers and structural organization normally seen in differentiated human urothelium. This unique stage of development makes the uro-organoids well suited for in vitro studies of infection with UPEC strains and provides a more physiologically relevant alternative to the two-dimensional conventional monolayer structure. Moreover, the model system we have recreated in vitro recapitulates for the first time (to our knowledge) a number of observations from in vivo studies of the interactions between well-differentiated uroepithelial cells and uropathogenic E. coli.

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