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Isolation and Primary Culture of Urothelial Cells from Normal Human Bladder*

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Summary. Trypsinization (WT) was employed to disaggregate urothelial cells from normal human urinary bladder mucosa for the preparation of primary cultures. Urinary bladders of two male adults both 25 years old were obtained autopsy 1–2 h after death. The mucosa was incubated in HBSS containing 0.25% trypsin at 37 °C. Mean cell yield, viability, and attachment were 14.6×10^7, 76%, and 42.5% after WT. In histologic sections of treated mucosa, most of the urothelium was removed by WT. Following plating and attachment, cells obtained by WT formed a monolayer of flattened epithelial-like cells. When stained with polyclonal antikeratin antibody using the indirect immunoperoxidase technique, all of the cells were immunoreactive indicating an epithelial origin. In conclusion, based on morphology and immunocytochemistry, WT removed virtually all of the urothelial cells from the mucosa and no contamination by mesenchymal cells resulted from this procedure. Thus, WT is an appropriate technique for the isolation of urothelial cells and initiation of primary cultures of normal human urothelial cells for subsequent study.

Key words: Human bladder, Urothelial cells, Cell isolation, Cell culture.

Introduction

Previous studies of mammalian urothelial cells in vitro have focused on the rat [3, 7, 21] and bovine bladder [16]. Investigators who have worked with human urothelium have primarily used explant culture techniques to obtain primary outgrowth cultures [6, 8, 23]. Most of the other investigative efforts have been directed towards establishment and characterization of cell lines derived from transitional cell carcinomas of human bladder. Techniques used to isolate cells from neoplastic urothelial tissues explant culture [2, 9, 17], mechanical separation [20], enzymatic isolation [10] or a combination of mechanical and enzymatic isolation [24].

Although there is one report of successful isolation of normal human urothelium from ureters [22] and many reports of successful isolation and growth of neoplastic bladder tissue [2, 9, 10, 17, 24], techniques for reproducibly isolating human urothelial cells from normal human urinary bladder and establishing these in primary culture have not been reported.

In the present study, warm (WT) and cold trypsinization (CT) were compared as a means of obtaining disaggregated urothelial cells from normal human urinary bladder mucosa. The parameters compared were morphology of treated tissue pieces, cell yield, attachment, viability by dye exclusion, and morphology and origin of isolated cells by phase contrast microscopy and immunocytochemistry.

Materials and Methods

Tissue

Human urinary bladders were obtained from one white (Case 1) and one black male (Case 2) adults, both 25 years old, at immediate autopsy [26] 1–2 h after death. Bladders were transported to the tissue culture laboratory in cold L-15 medium [12]. Mucosa was separated from muscle and connective tissue by blunt dissection using scissors and forceps. The mucosa was then placed in L-15 containing 600 U/ml Penicillin/600 μg/ml Streptomycin (Biofluids Inc., Rockville, MD) and 1.25 μg/ml Amphotericin B (GIBCO, Grand Island, NY) at 37 °C for 2 h, to reduce the possibility of subsequent bacterial or fungal contamination of cultures. The mucosa from each bladder was then divided to compare WT and CT, and trimmed into small pieces of about 0.5 cm^2. In Case 1, approximately one-half of the total bladder mucosa was available for study, and in Case 2, the entire bladder mucosa was used.

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Warm Trypsinization

The technique was performed as outlined by Fresney [4]. Pieces of tissue were placed in a trypsinization flask (Bellco, Vineland, NJ), 20 ml of 0.25% trypsin in Hanks Balanced Salt Solution (HBSS) (Mg^{++} and Ca^{++} free) (GIBCO) were added in a 1:5 (v/v) ratio, and the flask incubated at 37 °C. The contents of the flask were continuously stirred with a magnetic stirrer. After 30 min, tissue pieces were allowed to settle 3–5 min and the incubation medium was aspirated and discarded, as it contained mostly red blood cells. Fresh trypsin solution was added and the flask incubated for an additional 30 min. After settling of tissue pieces, disaggregated urothelial cells were present in the supernatant. Trypsin in the aspirate was then inactivated by adding an equal volume of M-199 (GIBCO) containing 50% fetal bovine serum (FBS) (GIBCO). Residual tissue was trypsinized further for an additional 30 min, the solution aspirated, and the enzyme inactivated as above. The trypsinates containing disaggregated cells were then pooled, centrifuged at 1,100 rpm for 5 min, and the cell pellet was resuspended in 10 ml of M-199 with 10% FBS to determine cell counts and viability as described below.

Cold Trypsinization

The technique was performed using the procedure of Hodges et al. [7]. Pieces of tissue were put into a sterile 4015 cup (Becton Dickson, Oxnard, CA) containing 20 ml of 3% trypsin (GIBCO) and 1% pancreatin (GIBCO) in HBSS (Mg^{++} and Ca^{++} free) (GIBCO). This cup was placed at 4 °C for 2 h and then at 37 °C for 30 min. Afterwards an equal volume of M-199 (GIBCO) with 50% FBS (GIBCO) was added to inactivate the enzymes. Cells were separated from the tissue by gentle pipetting. The solution containing disaggregated cells was centrifuged at 1,100 rpm for 5 min, and the cell pellet was resuspended in 10 ml of M-199 with 10% FBS to determine cell counts and viability as described below.

Cell Counts and Viability

The number of isolated cells was determined with a hemocytometer. Viability was estimated by mixing 0.8 ml of each cell suspension (WT or CT) with 0.2 ml of 0.4% filtered trypan blue (GIBCO) followed by determination of the percentage of viable cells which excluded the dye.

Attachment

The cell suspensions from both isolation methods were then centrifuged at 1,100 rpm for 5 min. The supernatant was discarded and the cell pellet resuspended in M-199 with 10% FBS, giving a final concentration of 10^6 cells/ml. Three ml of this suspension was pipetted into a 35 mm culture dish (Costar, Cambridge, MA). The percentage of cells attached to the surface of the dish was determined at intervals up to 48 h using an inverted, phase contrast microscope (Leitz-Wetzlar, Germany).

For immunocytochemistry (see below), cells were diluted to a concentration of 3×10^5 cells/ml and 1 ml of cell suspension was carefully layered onto a sterile 22 mm^2 glass cover slip in a 35 mm culture dish. The cells were allowed to settle for 5 min and an additional 2 ml of medium was added to the cultures. The cells were allowed to attach and spread for 24 h and were then fixed in absolute ethanol at room temperature.

Morphology

Following enzyme digestion, tissue remnants were fixed in 4F1G [13] for light microscopy. Paraffin sections were stained with hematoxylin and eosin (H&E). The morphology of urothelial cells during attachment and at intervals thereafter, was studied and photographed by phase contrast microscopy.

Immunocytochemistry

To identify the origin of isolated cells, coverslips with attached cells were removed at intervals during culture and fixed in absolute alcohol. Following fixation, cells were stained with polyclonal antikeratin antibodies using the indirect immunoperoxidase technique. Initially, absolute ethanol-fixed coverslips with attached cells were treated with absolute methanol containing 1.5% H_2O_2 for 15 min to abolish endogenous peroxidase, followed by washing in tap water, then in phosphate buffered saline (PBS) (pH 7.3). Cells were then treated with guinea pig polyclonal anti-bovine hoof prekeratin antibody (Miles-Yeda, Israel), diluted 1:30 in PBS, for 1 h at room temperature, then washed twice in Tris buffer (0.05 M, pH 7.6), 5 min each. Cells were then treated with peroxidase-conjugated rabbit anti-guinea pig IgG (Accurate Chemicals, Westbury, NY), diluted 1:80 in PBS, for 30 min at room temperature. Treated cells were washed in Tris buffer and incubated for 10 min in freshly prepared and filtered 0.05% 3,3'-diaminobenzidinetetrahydrochloride (DAB-HCl) (Sigma Chemical Co., St. Louis, MO) in 0.5 M Tris buffer (pH 7.6), containing 0.03% H_2O_2 at room temperature, then rinsed and counterstained with hematoxylin for 30 s. Immunostained cells were examined and photographed by routine light microscopy using an Olympus Photomax (Olympus Optical Co., Ltd., Tokyo, Japan).

Results

The data for cell yield, viability and attachment of urothelial cells following WT and CT of the mucosa of urinary

Fig. 1. Cells freshly isolated from normal human bladder mucosa by enzymatic disaggregation. Spherical to irregular-shaped cells are seen. Some cells are in the process of flattening. Phase contrast, x64 ▶

Fig. 2. WT cells 6 days after isolation and plating. Cells form a monolayer of epithelial-like cells. Phase contrast, x64

Fig. 3. CT cells 9 days after isolation and plating. Most of the cells are spindle shaped and resemble fibroblasts. A single, multinucleated giant cell is present (*arrow*). Phase contrast, x40

Fig. 4. Example of cells isolated from normal human bladder mucosa by WT and stained with polyclonal, antikeratin antibodies using the indirect immunoperoxidase technique. All cells are immunostained for cytokeratin; both granular and filamentous patterns are displayed. Hematoxylin counterstain, x280

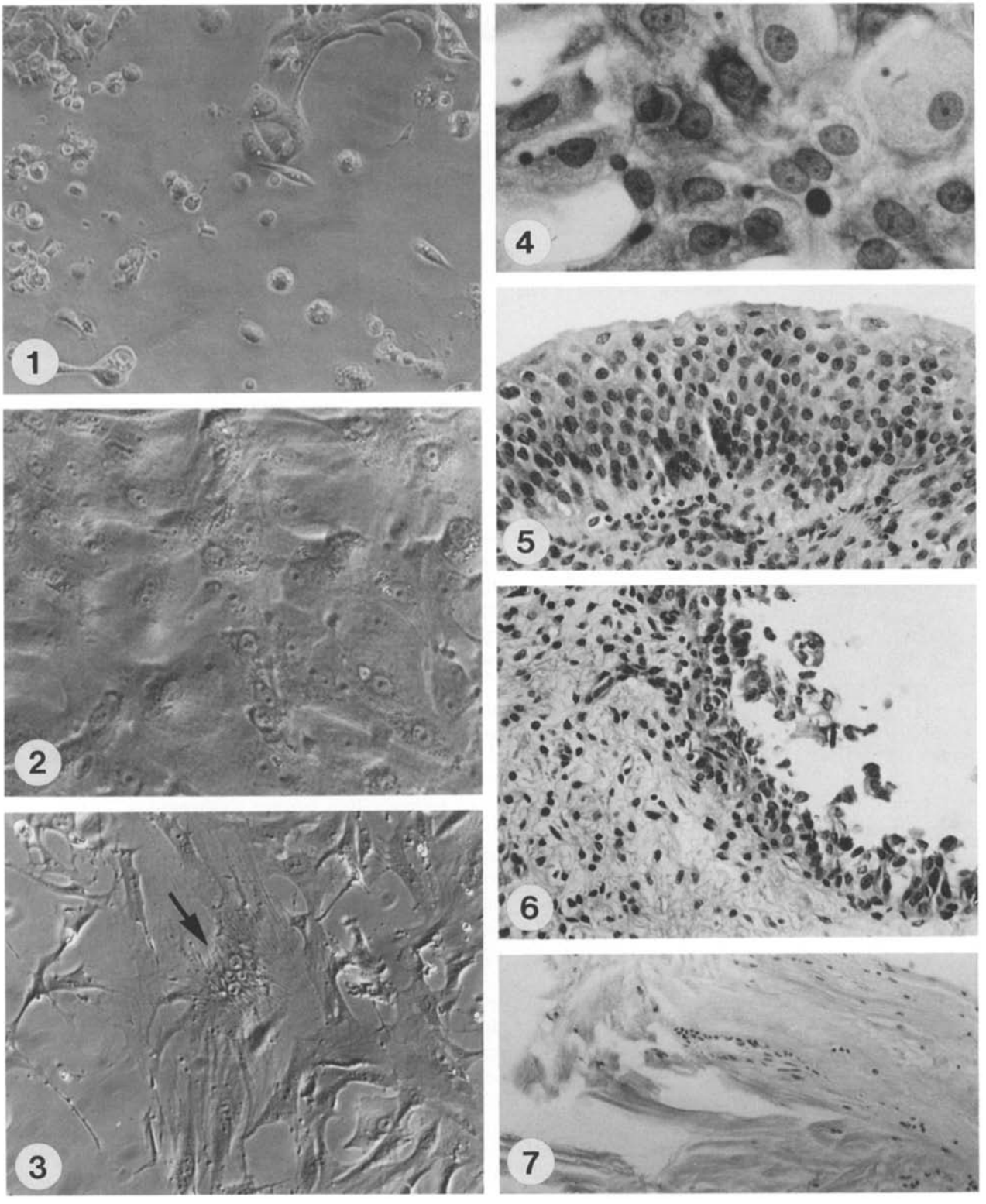

Fig. 5. Normal human urinary bladder mucosa before enzymatic disaggregation. Urothelium is intact and shows the normal pattern of cell differentiation. Hematoxylin and eosin, x280

Fig. 6. Normal human bladder mucosa after CT. Only partial disaggregation of urothelial cell is evident. Hematoxylin and eosin, x280

Fig. 7. Normal human bladder mucosa after WT. No urothelial cells are seen. Hematoxylin and eosin, x140

Table 1. Quantitative comparison of yield, viability and attachment of urothelial cells isolated from normal human urinary bladder mucosa by warm and cold trypsinization

Treatment	Cell yield ($\times 10^7$)	Viability (%)	Cells attached at 48 h (% of viable cells)	Cells attached at 48 h (% of total cells)
Warm trypsinization[a]				
Case 1[c]	6.00	77.0	52.0	40.0
Case 2	14.59	75.0	60.0	45.0
Mean values	–	76.0	56.0	42.5
Cold trypsinization[b]				
Case 1	3.13	85.0	59.0	50.0
Case 2	7.40	82.0	55.0	45.0
Mean values	–	83.5	57.0	47.5

[a] 1.5 h at 37 °C, stirred continuously; isolated cells were harvested every 30 min
[b] 2 h at 4 °C, then 30 min at 37 °C without agitation
[c] Twice as much tissue was sampled from Case 2 compared to Case 1, but approximately equal amounts of tissue were used for WT and CT in each case

bladders of the two cases are compared in Table 1. Differences in data for cell yield between Case 1 and Case 2 was due to the differences in the amount of bladder mucosa available, although the same amount of tissue was utilized for WT and CT for each case. Twice as many cells were isolated by WT in both experiments. However, data on cell viability and attachment are nearly identical for both cases. Compared to WT, CT resulted in a slightly higher mean viability (83.5% vs 76.0%). Although cells isolated by CT attached initially more quickly (20% at 4 h) than those isolated by WT (0% at 4 h), WT and CT showed similar percentages of attached cells after 48 h. The cells isolated by both methods were initially spherical in shape (Fig. 1), but flattened and spread out following attachment. Most of the WT cells formed a monolayer of epithelial-like cells (Fig. 2). In contrast, most of the CT cells were spindle shaped, resembling fibroblasts (Fig. 3). In addition, some WT and CT cells were multinucleated (see Fig. 3). Despite differences in cell morphology, nearly all of the attached cells obtained by WT or CT stained positive for cytokeratin by the indirect immunoperoxidase technique (Fig. 4).

By LM, the urothelium of both cases appeared normal before enzyme treatment (Fig. 5). After enzyme treatment, the urothelium of CT mucosa was only partially disaggregated (Fig. 6), while urothelial cells were absent after WT (Fig. 7). These observations are consistent with differences in cell yields obtained by the two techniques (see Table 1).

Discussion

There are two basic methods of obtaining cells from intact tissues for in vitro studies: primary outgrowth from organ explants or isolation of cells by enzymatic disaggregation or mechanical separation. In organ explant culture, small organs or small pieces of large organs are placed in culture dishes containing medium and maintained under controlled conditions [4, 5]. Cells grow out from the explant and form a monolayer on the adjacent surface of the culture dish, but the cells obtained by this method may be a mixed population of mesenchymal and epithelial cells. In cell culture, cells are first isolated from intact tissue and grown under similar conditions in a culture dish or flask.

One method to isolate cells from intact tissue is by mechanical dispersion in which the tissue is first chopped and then passed through fine screens [4]. However, with this method, a large number of cells may be lost due to physical trauma. A second method of isolating cells from intact tissues utilizes enzyme disaggregation. In this technique, tissue is treated with proteolytic enzymes such as trypsin, collagenase or pancreatin, singly or in combination, to disrupt intercellular junctional complexes and attachment to the basement membrane, as in the case of epithelial cells. Disaggregated cells are then collected by centrifugation and resuspended in culture medium.

To date, no studies have been performed comparing techniques of isolating epithelial cells from normal human tissue. However, Slocum et al. [25] compared both mechanical and enzymatic techniques of cell isolation from human neoplastic tissue. In their study, which involved more than 200 samples of human melanoma, sarcoma and lung tumors, it was shown that enzymatic methods were definitely better than mechanical techniques in terms of cell viability. Although enzymes can separate cells, they may also damage the plasma membrane and induce injury. The advantage of this technique is that a large number of cells can be isolated in a relatively short period of time. By this method, cell yield may depend on the degree of adhesiveness of the cells to be isolated and their ability to withstand the rigors of enzyme treatment.

Freshney [4] compared warm and cold trypsinization for disaggregation of chick embryo cells and obtained somewhat different results than those presented here. In

contrast to our results, he was able to isolate more cells by CT but they showed a lower viability (60–75%) by the dye exclusion method compared to WT (86%). Compared to adult tissue, embryonic tissue contains much less connective tissue, cells are less adhesive, and consequently easier to disaggregate. Moreover, there may be differences between embryonic and adult tissues in sensitivity to enzyme treatment, and in attachment and growth in vitro [4].

Since growth is facilitated if cells are seeded at fairly high concentrations [16], the cells in the present study were seeded at a concentration of 10^6/ml (or 3×10^6/35 mm dish); these cells formed monolayers of epithelial cells. Cells isolated by WT in the present study did not attach until after 12 h, whereas cells isolated by CT were able to attach within a few hours after isolation. This observation may reflect damage to the plasma membrane of urothelial cells by WT.

The luminal surface of human bladder urothelium is particularly impermeable to even small molecules because of the thickened, asymmetric unit membrane (AUM) plaques comprising the plasma membrane, and tight junctions between adjacent cells [1, 14, 18, 27]. In cold trypsinization, tissue and enzymes are placed at 4 °C to allow enzymes to diffuse into the intercellular spaces, with minimal proteolytic activity at that temperature. Because we did not obtain good cell yields with CT it would be reasonable to assume that the AUM and/or tight junctions (zonula occuludens) [1, 17, 25] limited the enzyme diffusion between urothelial cells. In WT enzymes were able to isolate more epithelial cells than in CT, presumably due to the longer incubation time at 37 °C, coupled with the mechanical agitation.

After initial attachment and spreading of cells, a difference in morphology was apparent in cells isolated by WT and CT. This morphological difference in cells isolated by the two methods persists throughout the life of the cultures. This is probably due to the fact that WT allows the establishment of cultures composed of all urothelial cells, while CT cultures are composed mainly of superficial cells (see Figs. 6 and 7).

After about one week in culture, a large number of cells isolated by both WT and CT, became multinucleated and increased many fold in size as compared to other cells in culture. Nevertheless, nearly all cells proved to be of epithelial origin; they were immunoreactive to polyclonal antikeratin antibodies. Cytokeratin has been shown to be a marker of epithelial cells and has been demonstrated in normal and neoplastic human bladder urothelium [11, 15, 19]. In fact, the multinucleated giant cells were more reactive to antikeratin antibodies as compared to other immunostained cells, presumably because they contained greater relative amounts of keratin. The formation of multinucleated giant cells may be analogous to the formation of large multinucleated superficial cells during normal differentiation of basal and intermediate urothelial cells [18].

In the present study, the treatment of normal adult urinary bladder mucosa by WT or CT resulted in the isolation of a nearly pure population of urothelial cells. Almost all of isolated cells stained for polyclonal antikeratin antibodies by the indirect immunoperoxidase technique. Although WT and CT isolated cells showed similar viability and attachment, CT did not remove all of the urothelial cells from the mucosa.

Based on the results of this study, WT should be the technique of choice for disaggregation of urothelial cells from normal adult urinary bladder mucosa since complete removal of these cells was achieved and nearly all of the isolated cells were of epithelial origin. Following isolation as described above, a variety of in vitro studies are now possible using normal human bladder urothelial cells in primary culture. Of particular interest in our laboratory is the investigation of the cytoskeleton and its role in normal and neoplastic human bladder urothelium.

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