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ISOLATION AND *IN VITRO* CULTIVATION OF HUMAN UROTHELIAL CELLS FROM URINE OF PATIENTS

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ABSTRACT

In clinical practice the number of urothelial cells collected by biopsy are limited and the procedure requires general anaesthesia. Therefore, in order to acquire enough urothelial cells for *in vitro* engineering of the urothelium, in this research we aim to isolate urothelial cells from human urine by an alternative, effective, low-cost and safe technique rather than using the indicated method. Sixty human urine samples had been collected from patients and volunteers, cells then were precipitated by centrifugation and cultured. Following the isolation process, these cells were characterized by the immunocytochemical method using some specific antibodies. There are 2 types of cells were successfully isolated from with different shape and morphology, one of them grew randomly while the others formed smooth-edge contours and cobblestone-like cell morphology. These cells were characterized by immunostaining with a specific marker, both of these cells were positive for urothelial marker cytokeratin 7. All these results were taken into consideration, the isolated cells were urothelial cells observed in the urine-derived cell population. These results will be used for in vitro studies in toxicological and clinical research, and it will be the premised research to determine the cell mechanical properties and then develop a promising method for early diagnosis of bladder cancer.

Keywords: Bladder cancer, cell culture, mechanical properties, urothelial cells.

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INTRODUCTION

Bladder cancer is the most commonly urothelial carcinoma and the 4th common noncutaneous malignancy in the United States (Resnick & Chang, 2013). However, its cost per patient is known as the highest of all the cancer types, and it is approximately 200,000 U.S. dollars per patient from diagnosis to death (Lokeshwar et al., 2005). According to GLOBOCAN statistics, it has been estimated that in 2018 approximately 1,502 new cases of bladder cancer will be diagnosed with 883 deaths in Vietnam. Bladder cancer is common in the age of 40–70 (78%) with male per female ratio of 6:1. When being treated promptly in the superficial stage of the bladder mucosa, the 5-year survival is 51–79%, however, for the stage of bladder cancer ingrained in the muscle layer, the 5-year survival rate is only 25–47%.

The progress of tumour invasion in the bladder wall and also a key component of the stage is time dependent (Wallace et al., 2002), so early detection of bladder cancer is very important. In order to diagnose bladder cancer, it mainly relies on urine cytology and cystoscopy. The mainstay of the bladder diagnosis is cystoscopy, however; this method is an invasive procedure. While urine cytology has high specificity, low cost, but it has low sensitivity especially in low-grade, low-stage tumours. Many urine-mased markers have been developed and are being used in clinical practice and shown definitely a better sensitivity in the detection of bladder cancer (Lotan et al., 2003).

Urine is a liquid medium that travels across the entire urinary tract from the renal pelvis to the urethra. In addition, the kidney contains an extensive network of tubules whose total surface is bigger than the skin, and there are approximately 2000 to 7000 cells from this tubular system and downstream parts of the urinary tract (ureter, bladder and urethra) detach and are excreted in urine daily (Rahmoune et al., 2005). Therefore, the urine contains a broad sample of exfoliated urinary epithelial cells which are not damaged and also fully functional (Inoue et al., 2003). The urine cells are further used for *in vitro* studies and lend themselves easily for cytological examination. There are five types of urine specimens including voided urine, catheterized urine, bladder washings and brushings, upper urinary tract brushings and washings and ileal loop urine. While the cheapest and widely used specimen is the voided urine, it is used as a common initial tool in the assessment of the entire urinary tract.

Urothelial cells are one of the specialized cells types that line the inner surface of urinary passages including the surface of the renal pelvis, the ureters, the urinary bladder and proximal parts of the urethra (Pavelka et al. 2010). The exfoliated cells from urine were first cultured and these samples were obtained from newborn infants (Sutherland & Bain, 1972). These cells were described with epithelial-like shape and formed colonies after cultured (Linder, 1976; Herz, 1980; Herz et al., 1979, 1985). The colonies of these cells were observed in two distinct types and designated as type - 1 or type - 1 cell colonies (Felix & Littlefield, 1979, 1980). Type - 1 cell colonies were discriminated by irregular contour with a random arrangement of cells, whereases type - 2 cell colonies presented smooth-edged contour and small cells (Felix & Littlefield, 1979). In addition, the cultivable urinary cells were obtained from the patients with diabetes mellitus free of complications and healthy adults but found only a few cultivable cells in most urine specimens (Detrisac et al., 1983). In another study, the urinary cells were cultured from various patient urine specimens including healthy adults, the newborn infant with meconium ileus, children with cystic fibrosis and elderly patients with cystic fibrosis (Sens et al., 1984). The urinary cells from healthy adults and elderly patients with cystic fibrosis were failed cultured, there is only a few cultures were initiated from the urine of the children with cystic fibrosis. Recently, cultures of urinary sediments from three groups of healthy adults, elderly multimorbid patients and urological patients were isolated and cultured (Angelika et al., 2000). The successfully cultured colonies of these 3 groups revealed means of 3 or 4 colonies.

Therefore, the aim of this study is to establish and optimize a processing for isolation and culture urothelial cells from the voided urine specimens from glaucoma patients. Then further investigate successful cultured urothelial cells with a biomechanical change in order to predict bladder cancer.

MATERIALS AND METHODS

Materials

Phosphate buffered saline (PBS) was purchased from Sigma Life-Aldrich (St. Louis, MO, USA). Tripton X-100 was obtained from AppliChem, Germany. Bovine serum albumin (BSA) was purchased from Sigma, USA. Paraformaldehyde solution was obtained from USB, United States. Nucleic acid stain 4', 6-diamidino-2-phenylindole DAPI are from Sigma, USA. Cell harvesting solution TrypLE express, fetal bovine serum (FBS), Penicillin-Streptomycin and Bovine Pituitary Extract (BPE) were obtained from Sigma, USA. Medium Keratinocyte-SFM was purchased from Gibco Invitrogen, Scotland. Fetal bovine serum (FBS) purchased from Biochrom AG, Germany. Vimentin and Cytokeratin 7 was purchased from Abcam, Germany.

Methods

Patient recruitment

Sixty patients and volunteers were selected from the clinical practice of Angelo Tanna of the Northwestern Ophthalmology Department. They were divided into three groups: (1) a glaucoma patient group - patients have an open angle, an intraocular pressure greater than 25 mmHg on two successive occasions, and visual field and optic nerve damage; (2) a glaucoma suspect group - patients have an open angle, an intraocular pressure between 24 and 30 mmHg on two successive occasions, and no indications of visual field or optic nerve damage (ocular hypertensives); and (3) a control group - volunteers are spouses of members of glaucoma and glaucoma suspect group, have a pressure less than 18 mmHg and normal optic discs and visual field. All patients and volunteers do not have urothelial carcinoma or know problems in the urinary tract.

Cell isolation and culture

Voided urine samples from patients and volunteers were collected, kept on ice (4 ^{o}C) and transferred to the Center for Advanced Molecular Imaging (CAMI) for processing within 3 hours. First, voided urine was transferred into sterile 50 ml tubes and centrifuged at 1500 rpm for 5 minutes at room temperature. The supernatant was aspirated, leaving 2 ml in the tube. The pallets were resuspended gently and individually in the remaining 2 ml of urine and then transfer into a single 50 ml tube. Second, phosphate-buffered saline (PBS) supplemented with 100 U.ml^{-1} of penicillin, 100 µg.ml^{-1} of streptomycin (washing buffer) was added into the tube to fill up 50 ml. The sample was centrifuged again at 1500 rpm for 5 minutes at room temperature. The supernatant was also discarded, leaving 1 ml in the tube. Third, the pellet was resuspended and transferred into a sterile 15 ml tube for washing the second time. The supernatant was removed, leaving only 0.5 ml plus the pellet. Finally, primary medium (Keratinocyte-SFM supplemented with 5 ng.ml^{-1} of human recombinant Epidermal Growth Factor 1-53 (EGF 1-53) and 50 µg.ml^{-1} of Bovine Pituitary Extract (BPE), 10% (vol/vol) Fetal bovine serum (FBS), 100 U.ml^{-1} of penicillin and 100 µg.ml^{-1} of streptomycin) was added to resuspend the cell pellet, and then transfer the volume into a single well of a 12-well plate (Corning Inc., Corning, NY, USA) containing a sterile 18 mm diameter round cover glass. The culture was incubated at 37 ^{o}C and 5% CO_2 and added 1 ml of primary medium every 2 days until cell colonies appeared (normally in 7 days).

Characterization of urothelial cells

In order to characterize the isolated urothelial cells, these cells were cultured on coverslips and stained with specific antibodies such as cytokeratin 7, vimentin (Abcam, Germany) by immunocytochemical techniques. In this research, cytokeratin 7 antibody was used as a urothelial cell marker,

vimentin antibody was additionally used as selectable markers for human mesenchymal stem cells. At the initial step of the immunocytochemical analysis, urothelial cells were cultured in the normal glass base dishes (Thermo, USA) were rinsed with PBS and then fixed in 4% paraformaldehyde solution (USB, United States) for 30 minutes at room temperature. Following the fixation process, the cells were permeabilized with 0.3% TritonX-100 (AppliChem, Germany) solution for 5 minutes at room temperature and then washed with PBS. In order to prevent nonspecific antibody binding, urothelial cells were incubated with primary antibodies overnight in a humidified chamber at 4 ºC. On the next day, the cells were incubated with fluorescein-conjugated secondary antibodies and nucleic acid stain 4', 6-diamidino-2-phenylindole (DAPI) (Sigma, USA) for 1 hour in dark at room temperature. Then, specimens were observed by fluorescence microscopy (IX81, Olympus).

RESULTS AND DISCUSSION

Voided urine collection and cell culture

In 60 patients and volunteers, we had 27 males and 33 females with ages between 41 and 83 years old (mean 69.9 ± 9.9 years old). They were arranged into 3 groups with 25 glaucoma patients, 3 glaucoma suspect patients and 32 volunteers in the control group. The number of processed samples was 56 due to errors in collecting 4 samples. Cell colonies were first observed approximately 7 days after seeding (Fig. 1) and 23 samples were succeeded in obtaining cells (41.1%). However, 8 out 23 samples were contaminated and discarded before going to AFM measurements. Cells were cultured until ready for measuring cell stiffness. In 15 successful samples, we measured 5 samples of glaucoma patients, 1 sample from the glaucoma suspect group and 9 samples in the control group.

Table 1. Voided urine processed samples statistic

Donors	Voided urine samples (n)	Samples with cell proliferation (n)	Samples with AFM measurement (n)	Mean age of donors (years)[a]
Glaucoma patients				
Total (25)	23	8	5	67.2 ± 7.7
Male (10)	10	3	3	62.7 ± 6.0
Female (15)	13	5	2	74.0 ± 2.8
Glaucoma suspect patients				
Total (3)	3	1	1	70
Male (1)	1	0	0	
Female (2)	2	1	1	70
Control group				
Total (32)	30	14	9	73.0 ± 6.3
Male (16)	16	7	2	77.0 ± 5.7
Female (16)	14	7	7	71.9 ± 6.4

Note: [a] The mean age of donors whose voided urine samples could be successfully cultured and measured cell stiffness.

Within one week of cell seeding, only small amounts of cells in voided urine sample attached to the cover glass surface, proliferated, formed small colonies and grew quickly. Normally, two distinct types of colonies were observed. Cells in type 1 colonies arranged randomly while cells in type 2 colonies had the regular appearance, grew closely attached to neighbour cells forming smooth-edged contours and cobblestone-like cell morphologies (Figure 1).

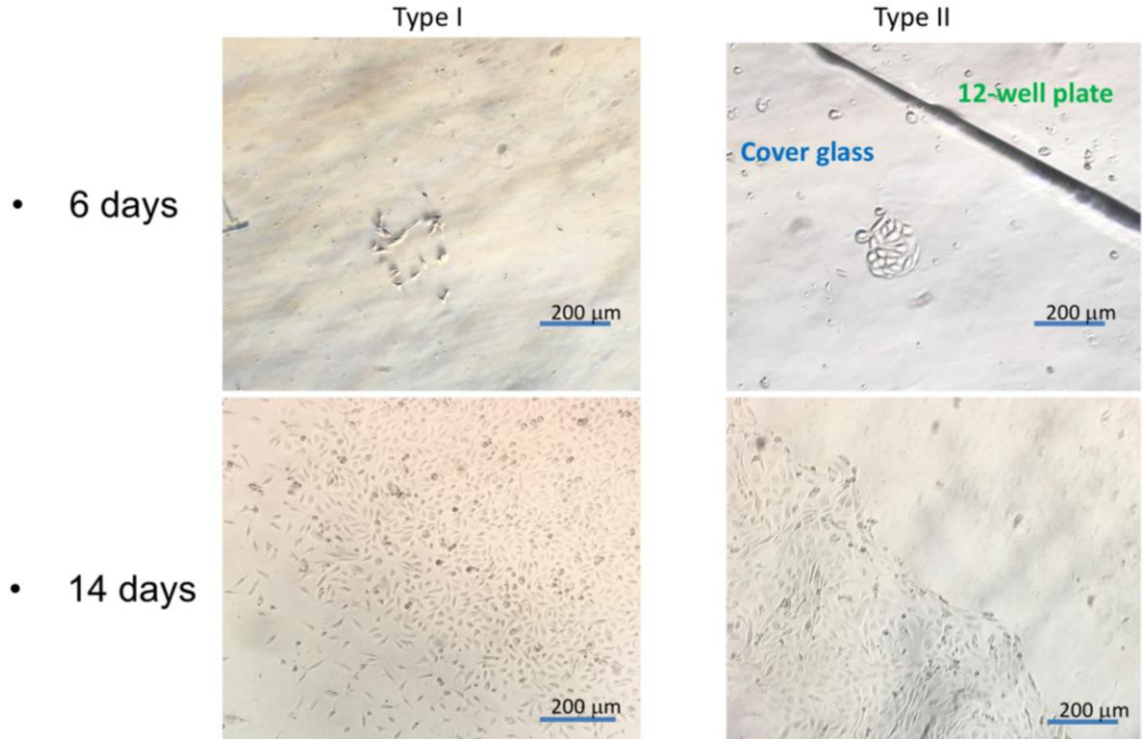

Figure 1. Examples of cell colonies cultured from voided urine, type 1 and type 2 after 6 days and 14 days

The overall success rate of cell culture in this experiment was 25%, lower than previous reports from other groups (Dorrenhaus et al., 2000, Zhou et al., 2011). However, please note that glaucoma patients and their spouses are usually elderly people (the average age of the glaucoma group was 67.2 and the control group was 73). This factor would affect the quality of cells in void urine samples. Further, the volume of voided urine samples was another factor that reduced the success rate. Only 16 out of 60 samples had a volume of 200 ml or more. Due to the extremely low cell density in voided urine, especially in male samples, a large volume of urine should be collected.

Urothelial cell characterization

In order to characterize the collected cells from voided urine, these isolated cells were stained with fluorescence labelled specific antibodies using immunocytochemical techniques. In this experiment, cytokeratin 7 were used as the marker for urothelial cells and vimentin antibody were known as the negative selectable marker for human fibroblast cells. Human urothelial cells, human renal proximal tubular epithelial cells and human dermal fibroblast cells were used as control. As shown in Figure 2, both urine cells in type 1 and type 2 were positive for an epithelial marker - cytokeratin 7, while we observed weak staining for the fibroblastic - like maker vimentin. These results supported that both cell types have an epithelial origin and suitable for a further step in our study.

We successfully isolated urothelial cells from freshly urine specimens obtained from glaucoma patients and glaucoma suspect people. In this study, age and health status are important factors in the rates of success in isolation and cultivation of urothelial cells. For example, cells that were obtained from the glaucoma donors were at a higher rate than the glaucoma suspect donor group. Contrary to other studies, higher isolated urothelial

cells were observed in the patients with urolithiasis, hematuria (80%) and kidney stones (75%) (Dorrenhaus et al., 2000). The isolated urothelial cell cultures were pooled reaching high density and split for further characterization by immunofluorescence microscopy with specific antibodies. Therefore, the results improved the cells were stained with epithelial maker - cytokeratin indicating their epithelial origin.

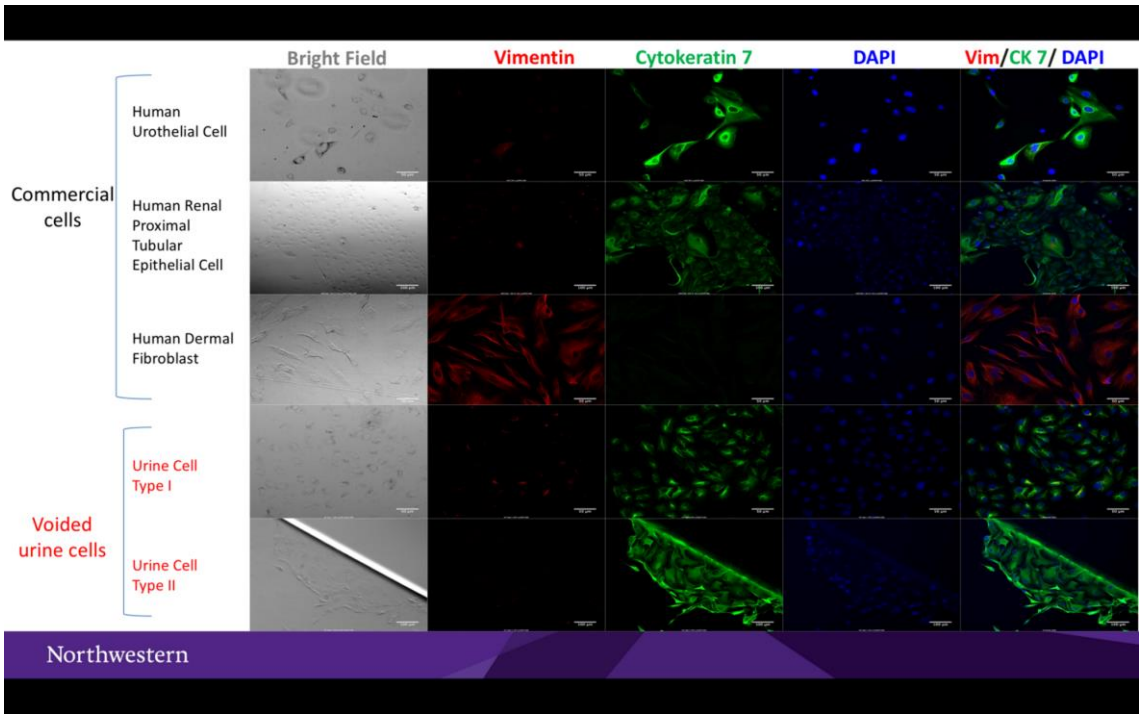

Figure 2. Immunofluorescence staining for commercial cells and isolated urine cells. Vimentin (red fluorescence) is a marker for fibroblast cells, Cytokeratin 7 (green fluorescence) is an epithelial marker, DAPI (blue fluorescence) for staining cell nucleus, each scale bar indicated 100 μm

It comes to bladder cancer diagnosis, urine cytology is now the most accessible method to examine the urothelial cells which are the potential to be urothelial cancer cells. In recent years, many researchers have improved the mechanical differences between normal and cancer cells are possible to be used as a diagnostic or prognostic marker of cancer progression. The individual cancer cell has been characterized that they were more deformable than normal cells because of the disruption and/or reorganization of actin filaments. Thus, the mechanical features of cancer cells were changed and softer than normal cells (Alibert et al., 2017). Base on this characterization of cancer cells, the determination of the mechanical features of cancer cells could be used for early cancer detection. Some papers demonstrated that human bladder cancerous cells are softer than non-malignant bladder cells and it happens at an early stage of the malignancy process (Liu et al., 2013; Ramos et al., 2014; Abidine et al., 2015). The voided is able to obtain some bladder cancer cells shed from the lining of the bladder. Particularly, the high-grade cancer cells are detected in urine which are reported to be over 80% (Gregoire et al., 1997). In addition, there are no research to determine the mechanical properties of the urothelial isolated from urine. Therefore, the successful isolated urothelial cells are going

to be examined for their mechanical stiffness and it could possibly detect early stage of bladder cancer.

In conclusion, we successfully isolate and culture the urothelial cancer cells from urine specimens obtaining from the glaucoma patients, then these cells will be further examined their mechanical properties to detect bladder cancer.

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