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Reconstructive Urology

In Vitro Investigations of Tissue-Engineered Multilayered Urothelium Established from Bladder Washings☆

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Abstract

Objective: Human urothelial cells (HUCs) are commonly isolated from native urothelium requiring open or endoscopic surgery. The aim of this study was to raise primary monolayer cultures of HUCs from bladder washings, to generate multilayered urothelial sheets in vitro, to characterise the sheets immunologically, and to prove their viability.
Methods: Irrigation fluids were taken from 29 adult patients. Isolated cells were cultured in serum-free keratinocyte medium. Confluent monolayer cultures were stratified, and evolved cell sheets were harvested after 10–16 d. Pancytokeratins and cytokeratin20 (CK20) in the stratified cultures and the detached sheets were immunologically detected. To exclude the presence of mesenchymal cells, antibodies against fibroblast surface antigen and smooth muscle alpha-actin were used. In addition, expression of p63 and uroplakin III was investigated. The viability of the detached cell sheets was proven by establishing explant cultures of small sheet sections.
Results: Confluent primary HUC cultures were established in 55.2% of the collected bladder washings between days 15–20. Multilayered urothelium developed in 62.5% of the monolayers. Histology revealed stratified cell layers similar to native urothelium. Both stratified cultures and detached sheets stained 100% positive for pancytokeratins and partially for CK20, indicating differentiation into superficial cells. No positive staining was observed with the mesenchymal markers used. p63 was expressed partially. Uroplakin III expression was not observed. Cell sheet viability was confirmed by rapid cell outgrowth in explant cultures.
Conclusions: Isolation of HUCs from bladder washings is a minimally invasive approach to establish primary urothelial cultures for creating autologous multilayered urothelial sheets.
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1. Introduction

Reconstructive surgery is a growing field in urology, and several methods of autologous tissue transfer are used. For reconstruction of bladder or ureter, mainly gastrointestinal or bowel segments are used, but urethral defects are usually reconstructed with free or vascularised flaps, mainly vascularised genital skin or buccal mucosa grafts. The disadvantage of these approaches is the development of functional problems such as metabolic complications, mucus production, and urolithiasis; the limited amount of transferable tissue, scar formation, and shrinking; and malignant potential [1–3]. Tissue engineering could expand the possibilities of reconstructive urological surgery because of physiological advantages and an almost unlimited quantity of producible tissue.

The application of human multilayered autologous urothelium generated in vitro might be a new option for sustained urothelial regeneration. Because autologous urothelial cells are not immunogenic, tissue-engineered urothelial constructs do not carry the risk of immunological reactions followed by inflammation and fibrosis. Urine separation is achieved by covering wounds completely with tissue-engineered autologous urothelium. Their insertion without complication-bearing matrices might be a treatment option for sustained urethral reconstruction, especially in patients for whom other free grafts are not available.

HUCs are commonly isolated from tissue biopsies of the lower urinary tract, requiring open surgery, or from cold biopsies taken during cystoscopy [4–7]. Isolation of HUCs from bladder washings offers a less-invasive method for the establishment of primary urothelial cell cultures. Fossum et al [8] described the cultivation of HUCs from bladder washings using a cell culture system based on mitomycin-treated 3T3 mouse fibroblasts as feeder cells.

Southgate et al [9] reported the establishment of multilayered human urothelium in vitro. Under serum-free conditions, monolayered urothelial cell cultures were stratified by increasing exogenous calcium. Analysis of the morphology and function of urothelial constructs identified a morphological structure similar to that of native urothelium, as well as the same barrier characteristics against the principal urine components, although terminal differentiation was incomplete [9–11]. Up-to-date surgical techniques for urethral reconstruction in humans do not yet include the use of autologous urothelial constructs generated in vitro.

In the present study, a feeder cell-free culture system including serum-free culture conditions was used. Stratification was induced in confluent monolayered urothelial cell cultures by increasing exogenous calcium. With the focus on clinical application, the aim of the study was to raise primary monolayer cultures of HUCs from bladder washings, generate multilayered urothelial sheets in vitro, characterise the sheets immunologically, and prove their viability.

2. Materials and methods

2.1. *Bladder washing procedure*

Institutional ethics committee approval (No. 100/2006V) was obtained for the collection of irrigation fluids from adult patients who needed cystoscopy. Patients suffering from tumours of the lower urinary tract and with persistent urinary tract infection were not included in this study. The urinary bladders of 29 adult patients, ages 34–88 yr, were washed with either isotonic saline solution (Fresenius, Bad Homburg, Germany; 8 patients) or an electrolyte-free irrigation fluid (Freka-Drainjet Purisole, Fresenius; 21 patients), depending on the primary reason for the endourological procedure. The irrigation fluid was poured into the bladder cystoscopically, aspirated manually with a syringe, and collected in sterile containers. Volumes from 250 to 600 ml (mean volume, 373) were obtained. Irrigation fluids were immediately transported to the laboratory.

2.2. *Isolation and culture of urothelial cells*

Bladder irrigation fluids were transferred into 5–12 sterile 50-ml tubes and centrifuged for 5 min at 250*g*. The supernatant was discarded, and the remaining cells were resuspended and pooled in complete keratinocyte serum-free medium (KSFMc) (Invitrogen, Carlsbad, CA, USA) containing 50 μg/ml bovine pituitary extract, 5 ng/ml human recombinant epidermal growth factor, and 30 ng/ml cholera toxin (List Biological Laboratories, Campbell, CA, USA) to enhance cell attachment. Following another centrifugation (5 min, 250*g*), cells were resuspended in 1.5 ml KSFMc, and seeded into a 3.5-cm diameter petri dish coated with collagen A (Biochrom, Berlin, Germany) at 50 $\mu g/cm^2$ before use. Primary urothelial cell cultures were maintained at 37 °C in a humidified atmosphere with 5% CO_2 in air. The culture medium was replaced the following day and thereafter three times a week.

At a confluence state of more than 50%, the primary urothelial cells were passaged as described previously [6]. Cells of the subsequent passages were seeded into 6.0-cm diameter petri dishes for immunohistology of stratified cell sheets and onto 8-well Permanox chamber slides (Nunc, Roskilde, Denmark) for immunocytochemical investigations of stratified urothelial cultures. All culture dishes except the Permanox slides were previously coated with collagen A. For all of the following in vitro experiments involving the monolayers (histology, immunostaining, and explant cultures), cells in passage two and passage three were used.

Table 1 – The primary antibodies

Specificity	Clone	Dilution	Source
Pancytokeratin	AE1/AE3	1:200	Chemicon, Temecula, CA, USA
Cytokeratin 20	K_s 20.8	1:300	Dako, Glostrup, Denmark
p63	4A4	1:300	Lab Vision, Fremont, CA, USA
Uroplakin III	AU1	1:4	Progen, Heidelberg, Germany
Fibroblast surface antigen	TE-7	1:200	Chemicon, Temecula, CA, USA
Smooth muscle α-actin	1A4	1:2000	Sigma-Aldrich, St Louis, MO, USA

2.3. *Stratification of urothelial cell cultures in vitro*

Well-established monolayer cultures were microscopically observed for growth behaviour. At achievement of a confluence state of 100%, defined as a gap-free cell monolayer, stratification was induced. Therefore, the cell culture medium was enriched with calcium chloride to a final concentration of 1.509 mmol/l. The stratification medium was changed three times a week. Ten to 14 d after induction of stratification, urothelial sheets were detached by incubation with 2.5% dispase II (Invitrogen, Carlsbad, CA, USA) at 37 °C for 7 min. To demonstrate the viability of the detached cell sheets, explant cultures were established. Small parts of the cell sheets were adhered to the bottom of a culture flask. The tissue pieces were carefully covered with KSFMc and cultivated at 37 °C and 5% CO_2 in air.

For routine histology, harvested cell sheets were washed twice in phosphate-buffered saline (PBS), placed between two foam plastic sheets, fixed in 4% neutral buffered formalin for 24 h, and embedded in paraffin. Then 5-μm sections were deparaffinised in xylene, rehydrated in ethanol, and stained with hematoxylin-eosin.

2.4. *Immunoassaying*

2.4.1. *Immunohistology*

Samples of native tissue and harvested cell sheets were fixed in 4% neutral buffered formalin, dehydrated through a graded series of ethanol to xylene, and embedded in paraffin. Sections 5 μm thick were deparaffinised in xylene and rehydrated.

2.4.2. *Immunocytochemistry*

Monolayer and multilayer chamber slide cultures were gently rinsed with PBS and subsequently fixed in 3.7% neutral buffered paraformaldehyde for 10 min to label cell-surface antigens. For labeling of intracellular antigens, a permeabilisation step with PBS containing 0.1% saponin was performed after fixation with paraformaldehyde.

2.4.3. *Antibodies*

The mouse monoclonal anti-pancytokeratin antibody AE1/AE3 was used to identify all cells of epithelial origin. To exclude the presence of mesenchymal cells, we applied a mouse monoclonal anti–smooth muscle α-actin antibody (1A4) and mouse monoclonal anti-fibroblast surface antigen–specific antibody (TE-7). For p63 expression, the mouse monoclonal anti-p63 antibody 4A4 was used. CK20 expression was detected with K_s 20.8, a mouse monoclonal anti-CK20 antibody; uroplakin III was detected with AU1, a mouse monoclonal anti–uroplakin III antibody. Dilutions and suppliers for specific antibodies are listed in Table 1.

2.4.4. *Immunohistochemical and immunocytochemical staining*

For immunohistology with AE1/AE3, TE-7, 4A4, and K_s 20.8, microwave epitope retrieval was performed in citrate buffer pH 6.0 (3 times, 15 min each). Subsequently, the samples were incubated with appropriate diluted primary antibody (Table 1) for 40 min at room temperature in a humidified chamber. Binding of the primary antibodies was assessed with the DakoCytomation LSAB2 system detection kit (Dako, Glostrup, Denmark). As a negative control, the primary antibody was substituted with PBS. A nuclear counterstain with hematoxylin was used.

3. Results

3.1. *Cell culture*

Primary urothelial monolayer cultures were established in 16 (55.2%) of the 29 bladder washing samples investigated. In 13 (44.8%) of the bladder washing samples, no adherent cells could be detected microscopically up to 10 d after initial seeding. The washings in isotonic saline solution resulted in establishment of a successful primary culture in 5 of 8 (62.5%) samples; washings in Purisole resulted in establishment in 11 of 21 (52.4%) samples. In this study, the success rates decreased in samples from patients older than 70 yr ($n = 4$); however, even samples from this age group resulted in successful primary urothelial cell cultures in 25% of the bladder washings performed. Rate in patients younger than 50 was 57% ($n = 7$); in the age group 50–70 yr, rate was 61% ($n = 18$). No statistical significance between the volume of bladder washing samples and the success rate of primary cultures was identified.

The average time required from the initial seeding of the cells up to the detachment of multilayered urothelial sheets was 36 d (range, 30–49; median, 33). Primary urothelial monolayer cultures reached a confluence state of more than 50% between days 6 and 19 after initial seeding, followed by up to four culture passages. In 62.5% of

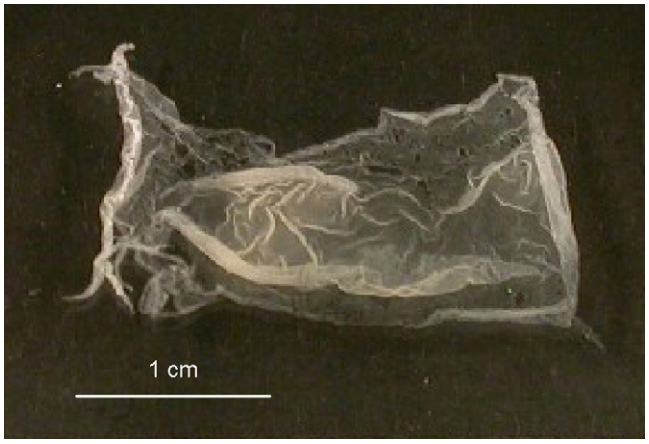

Fig. 1 – Enzymatically detached urothelial cell sheet established from isolated urothelial cells of a bladder irrigation fluid at day 15 after induction of stratification.

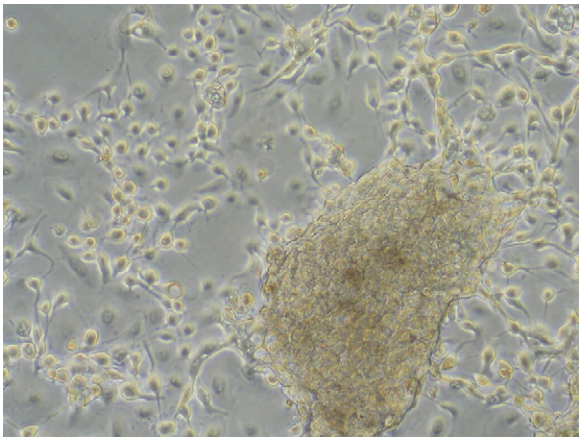

Fig. 3 – Explant culture established from sheet fragments showing cell outgrowth at day 9 after enzymatic detachment of the multilayered urothelial construct (original magnification: ×10).

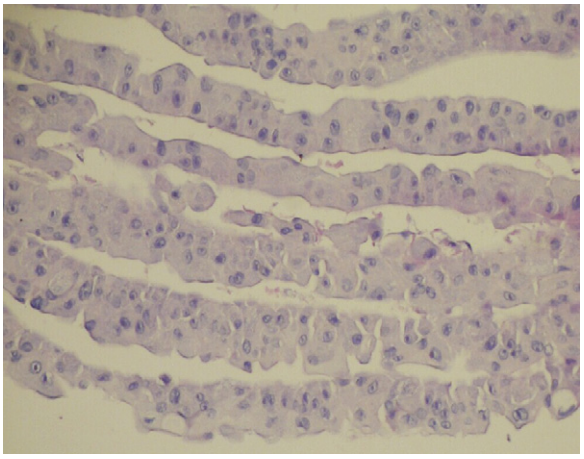

Fig. 2 – Histology of an in vitro–grown and enzymatically detached multilayered urothelial construct showing stratified cell layers similar to those of native urothelium (5-μm-thick paraffin section, hematoxylin-eosin staining; original magnification: ×10).

the established primary urothelial cell cultures, stratification was induced at a confluence state of 100%. After enzymatic detachment with dispase II at days 10–14 after induction of stratification, the sheets shrank to about one third of the original size of the petri dishes or culture flasks used (Fig. 1).

The detached sheets were quite fragile and showed the same tensile strength described for urothelial cell sheets established from tissue biopsies of the lower urinary tract [10–12]. In addition, routine histology of detached sheets revealed stratified cell layers similar to those of native urothelium (Fig. 2). To demonstrate that urothelial constructs consist of viable cells after enzymatic detachment, explant cultures were established from small parts of the detached sheets. Cell outgrowth was observed 4 d after explanting the sheet fragments, demonstrating a maintained proliferation potential of the stratified urothelial constructs when replaced in KSFMc with low-calcium concentration (Fig. 3).

3.2. *Immunostaining*

All monolayered urothelial cell cultures examined stained 100% positive for the anti-pancytokeratin antibody AE1/AE3 (Fig. 4a), whereas no positive staining was observed for the anti–smooth muscle α-actin antibody (1A4) or for the anti-fibroblast antibody TE-7 (data not shown). Expression of CK20 was not detected (Fig. 4c). In contrast, stratified urothelial cell cultures revealed partially positive CK20 expression at days 10–14 after induction of stratification (Fig. 4f). The presence of uroplakin III was not detected in either the monolayered or stratified urothelial cell cultures (data not shown). After reaching 100% confluence, urothelial monolayer cultures showed homogeneously distributed expression of p63 (Fig. 4b). When stratification was induced in 100% confluent urothelial cell cultures, p63 was expressed only partially 14 d after induction of stratification (Fig. 4e). All cell layers of the urothelial sheets investigated stained 100% positive for the anti-pancytokeratin antibody AE1/AE3, demonstrating the epithelial phenotype of the urothelial construct (Fig. 4g). Cells in the superficial umbrella cell layer stained positive for CK20 (Fig. 4i), although in a more nonhomogeneous distribution compared with native urothelium. Investigation of

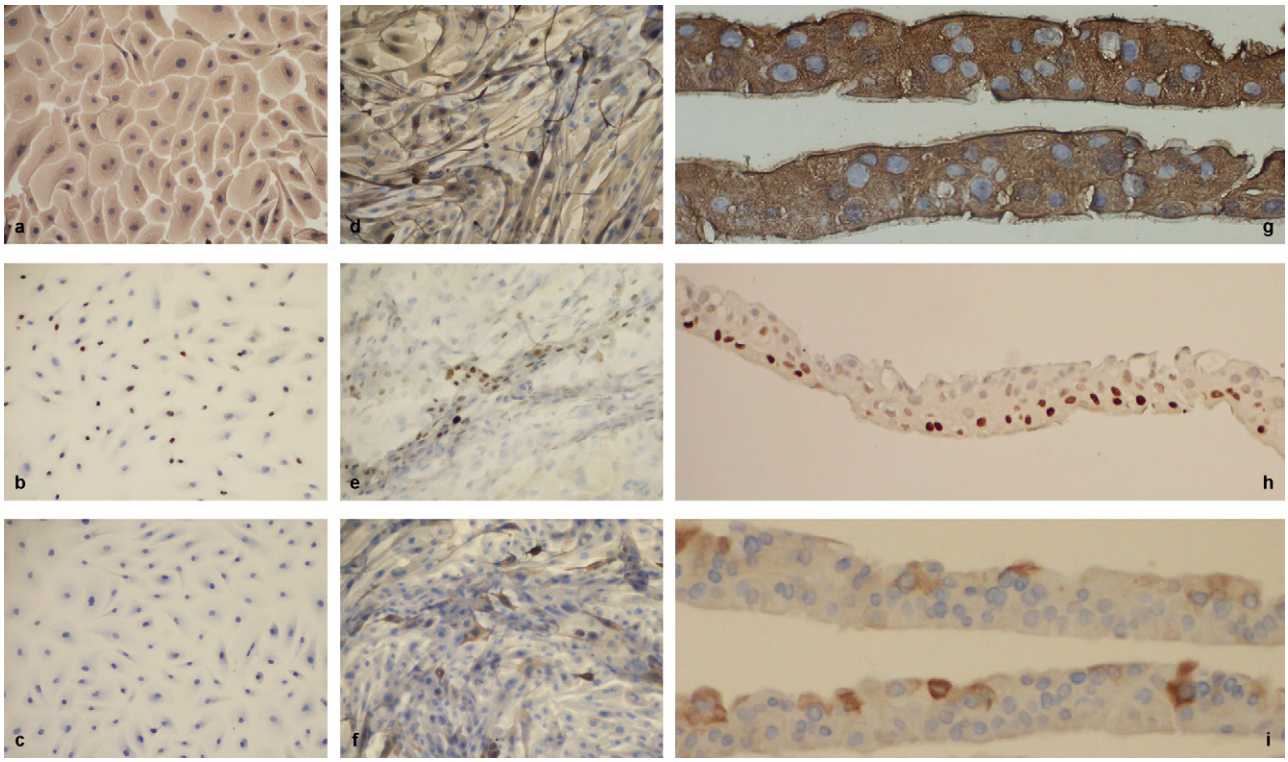

Fig. 4 – Immunocytochemistry of urothelial cell cultures before stratification (a–c) and at day 14 after induction of stratification (d–f), and immunohistological investigation of detached multilayered cell sheets (g–i). Staining with anti-pancytokeratin antibody (top), anti-p63 antibody (centre), and anti-CK20 antibody (bottom; original magnification: ×10 for a–f, h, and ×40 for g, i). Binding of the primary antibodies was assessed with the LSAB2 system detection kit (Dako). 3,3′-Diaminobenzidine was used as substrate. Brown colour indicates positive staining with the respective antibody. Hematoxylin was applied as nuclear counterstain.

p63 revealed partial distribution of p63-positive cells with highest intensity in the basal cell layer (Fig. 4 h).

4. Discussion

Although surgical techniques for urological reconstruction have advanced considerably in recent decades, the quest for the ideal substitute for sustained urothelial regeneration continues. Depending on the genesis, location, and severity of the organ defect associated with the surgical technique applied, different tissue-engineering techniques, using cultured urothelium, might expand the reconstructive toolbox.

There are several possible applications of cultured multilayered urothelium. Direct application of multilayered urothelial sheets could replace flaps in open urethral surgery and might be used in endoscopic urethroplasty [13]. The use of fibrin glue instead of suturing, as described by Barbagli et al [14], probably helps to handle the fragile urothelial construct. Romagnoli et al [15] proposed a one-step treatment of proximal hypospadias by an autologous graft of cultured squamous urethral epithelium. Endoscopic examination in three patients 12–18 mo postoperatively revealed a normal-appearing, well-stratified squamous epithelium. Fraser et al [16] reported composite cystoplasty using porcine urothelial cell sheets transferred to a polyglactin carrier, and sutured to de-epithelialised colon and uterus segments. In all animals, there was relining of smooth muscle tissue with autologous cultured urothelial sheets. In a dog model, cultured urothelial sheets were autografted onto de-mucosalised gastric flaps without fixation in eight animals, followed by the rapid formation of a native-like epithelium in five of the dogs [12]. Urothelial reconstruction using an acellular matrix derived from bladder submucosa and covered by bladder cells has been reported in the rabbit model [17]. Our intent is to use the cell sheets without carrier material, but in combination with a stabilisation factor (experiments are still in progress).

The advantages of cell harvesting by the bladder-washing procedure used in the current study are its low morbidity and the possibility to repeat it several times. The procedure may be continued with an

endourological investigation as recommended before a reconstructive approach [18], or can be done by a urethral catheter or via an indwelling suprapubic catheter. No special additional endourological equipment was used for harvesting the irrigation fluid. Irrigation of the bladder is less traumatic than biopsy, the procedure itself can be performed without general anaesthesia, and thus a second attempt in cases of cell culture failure is tolerable [8,19–21]. We identified no complications in our patients from the washing procedure, such as irritation, persistent haematuria, or urinary tract infections. These advantages help outweigh the relatively smaller amount of cells obtained from a bladder washing compared with the amount obtained from a tissue biopsy.

Urothelial primary cell culture techniques vary in terms of the applied culture conditions, comprising medium supplements such as bovine serum, bovine pituitary extract (BPE), and the use of stromal feeder cells. Cytostatic drug–treated or lethally irradiated 3T3 mouse fibroblasts are used as feeder cells [8,12,22–24]. Animal serum has traditionally been added to support cell proliferation in vitro. Serum-free media contain defined levels of nutrients and are supplemented with mitogens. BPE, containing a variety of growth factors and hormones, results in approximately 70 times the mitogenic activity of animal serum and is therefore commonly used to support cell proliferation [25]. In the present study, a feeder cell–free cell culture system including serum-free cell culture conditions was used. These conditions are important regarding good medical practice (GMP) requirements. To conform to a GMP laboratory setting, investigators have to substitute the animal components used [6]. Fossum et al [26] reported no primary culture success of HUCs isolated from bladder washings cultured in KSFM supplemented with 25 μg/ml BPE and 1 ng/ml endothelial growth factor (EGF). In our study KSFM was supplemented with 50 μg/ml BPE, 5 ng/ml EGF, and cholera toxin (30 ng/ml). The success in primary culture must be due to the supplements' concentration.

Increased exogenous calcium induces stratification in monolayered urothelial cultures (eg, by desmosome formation), as described by Southgate et al [9]. The cell sheets obtained showed good viability after enzymatic detachment, resulting in a rapid cell outgrowth from established sheet explant cultures. The sheets consisted of three to four cell layers, similar to neo-urothelium established from human biopsies taken from the lower urinary tract [9,11]. In addition, the sheets exhibited mechanical properties similar to those described for urothelial sheets created in vitro and established from tissue biopsies of the lower urinary tract [10].

Urothelial cytokeratin expression alters with regard to the stratification and differentiation status of the tissue. CK20 is restricted to the superficial umbrella cells and very occasionally to intermediate cells [27]. The apical surface of mammalian urothelium is covered with membrane plaques containing uroplakins as the major differentiation products in terms of maintaining the urinary permeability barrier [28]. Uroplakin III is present in all urothelial plaques. Thus, the expression of CK20 and the presence of uroplakin III give evidence of the differentiation status and allusively of the function of urothelial tissue.

The present investigation of multilayered urothelial constructs established from bladder washings revealed expression of CK20 in the superficial cell layers, whereas uroplakin III was not detected. The results agree with the expression pattern of CK20 and uroplakin III of urothelial constructs created from bladder or ureter biopsies as recently reported by our group [29]. In stratified urothelial cell cultures and in detached urothelial sheets, CK20 was expressed only partially, not continuously as in native tissue.

Terminal differentiation is crucial for the functionality of the urothelium, including the prevention of bacterial infection. Urothelial transplants, applied in a surgical model before reaching terminal differentiation in vitro did not reach a terminally differentiated status in vivo [16]. These authors therefore concluded that urothelium generated in vitro and having superior differentiated and functional properties should be used for transplantation to limit the exposure of non-barrier tissues to urine.

p63 regulates genes with various roles in cellular function, including proliferation, cell-cycle control, and induction of apoptosis in many stratified epithelial structures. In native urothelium, p63 expression is found in the basal and intermediate cell layers. Commonly, p63 is not expressed in the most superficial cells of the urothelium, the well-differentiated umbrella cells [30,31]. With a focus on urothelial development, Urist et al [32] demonstrated that p63-deficient mice develop a nontransitional cuboidal epithelium lacking the apical layer of umbrella cells. They thus concluded that p63 is not essential for the formation of a bladder epithelium, but is essential for the specific terminal differentiation of the superficial urothelial cell layers. Tissue-engineered urothelial constructs exhibiting p63 expression in the same pattern as in native urothelium indicate a good regenerative potential and differentiation capacity [29].

5. Conclusions

This study demonstrated that HUCs isolated from bladder washings can be established in a feeder cell–free culture system and subsequently that multilayered urothelial sheets can be generated. The cell sheets showed partial differentiation, but terminal differentiation was not achieved. Additional studies must be done to improve the yield of primary cultures, to induce terminal differentiation in vitro, and to surgically apply tissue-engineered urothelial sheets in an animal model. Because of an intended clinical application, current work has to focus on substitution of bovine pituitary extract and cholera toxin used in this study, which still do not conform to GMP requirements.

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Editorial Comment on: In Vitro Investigations of Tissue-Engineered Multilayered Urothelium Established from Bladder Washings

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The genitourinary tract can be involved in congenital abnormalities or damaged by several pathologic conditions such as trauma, cancers, inflammation, infections, or iatrogenic injuries. The repair of injured organs requires not only many options of surgical technique but especially alternative tissue sources, when autologous tissue is lacking or not available. Pioneer works carried out few years ago have challenged the poor attitude to in vitro regeneration of human urothelial tissue [1,2]. However, since the laboratory techniques of cell culture and differentiation have been refined, reconstructive urology has largely embraced the field of tissue engineering. Starting from achieving an adequate amount of cells for tissue substitution, subsequent efforts concentrated on finding a carrier material that could make the renewing tissue suitable for surgical handling and repair. The present study reports for the first time to the urologic community a way of building multilayered urothelial sheets using urothelial cells collected from bladder washings [3]. This approach is simple and reproducible and collects a sufficient quota of cells for in vitro expansion. A questionable point is why a quite high rate (44.8%) of bladder washings did not result in successful cultures. Assuming that the laboratory protocol was consistent for all urine samples processed, the different rate of cell growth may depend on heterogeneous cell quality and viability among patients. As evidenced by the authors, patients older than 70 yr produced 2-fold fewer successful cultures, but they could have had an obstructive condition due to prostate enlargement resulting in alteration of cells exfoliated from the bladder. Thus, it is uncertain if age is a significant limitation for this method even considering that it is easily repricable in the same patient. Other open questions concern the technique for scaffold assembling once the urothelial sheet has been established (ie, acellular matrices, fibrin glue), the potential clinical applications for surgical reconstruction of the urinary tract, and, finally, the cost. However, this study is of considerable interest and large numbers of urologists should be aware of this new option for the future.

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Editorial Comment on: In Vitro Investigations of Tissue-Engineered Multilayered Urothelium Established from Bladder Washings

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In this article, Nagele et al [1] used a quite simple technique to obtain cells for tissue engineering, described by Fossum et al [2]. With bladder washings, either during an endoscopic procedure or by catheter, a smaller amount of cells could be obtained as with biopsy, which is the method used in most studies to obtain material for tissue

engineering. Bladder washing seems to be less traumatic when compared to other techniques; however, in this study sufficient cells to establish a monolayer culture could be gained in only 16 of 29 patients. In 10 of these 29 patients, multilayer urothelium developed after an average of 36 d (range: 30–49 d). Even though this technique was successful in only one third of the initial patients, the procedure can be repeated. The authors demonstrated by immunohistology that viable urothelial cells could develop.

Tissue-engineered urothelium has been in focus for some time [3–5]. For bladder reconstruction, urothelial and muscular cells as well as a matrix are necessary [6,7]. Complete reconstruction of the bladder is not without problems (blood supply, fibrosis, regeneration of the native tissue, etc), some of which are still waiting for a good solution. For urethral reconstruction, a multilayer urothelium or buccal mucosa graft seems to be sufficient. For handling the tissue, a matrix appears to be necessary. At the moment, there is no ideal matrix on which the cells can grow; the surgeon can transplant and fix the graft where it is needed and about 2 wk later the matrix should be gone without causing fibrosis, and leaving the urothelium behind. The idea of fixing the multilayer urothelium with fibrin glue is fascinating; however, I doubt that this will work in reality.

Indications for complex urethral reconstruction with the need for tissue-engineered material are rare. These patients have undergone multiple hypospadias repairs or failed urethral reconstruction and are without sufficient local tissue. Such patients would benefit tremendously from the use of reliable graft material, available in large amounts. The techniques published to date are still very experimental, expensive, and miles away from being of clinical use. However, in the ''near'' future, more reliable and practical techniques may become available. Until tissue-engineered material for urethral reconstruction becomes a reality, we have to trust in the use of free grafts such as buccal mucosa.

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