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Selective culture conditions for different types of primary human bladder cells

Several protocols for the culture of urothelial cells have been presented over the past years. The ability of establishing an in vitro model that mimics in vivo responses by growing epithelial and mesenchymal cells, provides the opportunity to study interactions between the stroma and the urothelium as well as to study normal cellular processes and abnormal disease states in the bladder. In this respect, different techniques have been published for the culture of either animal or human bladder cells. De Boer et al. established an organoid-like model for normal urothelium by growing human and mouse urothelial cells on Cyclopore membranes [5]. Baskin et al. isolated and cultured fetal bovine bladder smooth muscle and epithelial cells [2], whereas Noguchi et al. investigated epithelial-stromal interactions by using rat urothelial and stromal cells [11]. Another in vitro model used to study the contractility of primary smooth muscle cells of the human bladder was published by Kropp et al. [10].

In the last few years, more and more interest has been focused on tissue engineering and bladder reconstruction. The goal is to culture and expand cells from autologous bladder tissue in vitro and to transplant them in vivo to create new organs. So far, in terms of bladder reconstruction, several approaches in animal models have been published. For instance, Oberpenning et al. cultured urothelial and smooth muscle cells from beagle dogs and transferred them into animals to reconstruct a canine neo-bladder organ [12]. Other groups were successful in growing urothelial tissue grafts in New Zealand white rabbits and rats [1, 7, 9]. In this issue, Atala et al. describe a method for bladder substitution by tissue engineering and there is another report of Rohrmann et al. that provides first clinical experience with an artificial bladder.

In this report, we describe techniques for the isolation and in vitro short-term culture of different cell types of the human bladder. In contrast to most cell culture protocols that use enzymatic digestion of tissue material, we mechanically dissect bladder specimens. This provides the advantage of establishing cell cultures from very small tissue pieces without substantial loss of material. Using selective media, we culture epithelial (urothelial) cells, and fibroblasts as well as smooth muscle cells (SMCs) of human bladder tissue specimens. This provides an opportunity of studying different cell types of the bladder separately, and rearranging them into cocultures in order to investigate stromal-epithelial interactions.

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Preparation of human urothelial and mesenchymal cells for primary culture

Fresh tissue specimens were obtained from transurethral resections or from radical cystectomies from patients of various ages (mean age: 64 years, range: 43–80 years). Representative portions of the tissue were submitted for histological classification. Bladder mucosa was removed from the remaining tissue with fine scissors. Subsequently, tissue was minced into small pieces (about 1–2 mm^3) and transferred into 25-cm^2 tissue culture flasks (Sarstedt, Newton, CA). Explants were allowed to

adhere to the plastic surface before they were gently covered with medium. For initial outgrowth of cells the following medium was found to be most effective: MEM (Minimum essential medium), containing 2 mM L-Glutamine, (Hyclone, Logan, Utah) supplemented with 20% fetal calf serum (FCS, PAA Laboratories GmbH, Linz, Austria), penicillin/streptomycin (100 µg/ml, 100 U/mL) (PAA Laboratories GmbH, Linz, Austria), and 2% MEM non essential amino acids (PAA Laboratories GmbH, Linz, Austria). Cultures were grown in a humidified atmosphere of 5% CO_2 in air at 37 °C. Medium was changed every other day.

The procedure works well for nonmalignant and malignant tissue, depending upon the nature of the specimens. Soft tumor tissue samples, however, were not minced but directly seeded into 25-cm^2 culture flasks (Sarstedt, Newton, CA) after suspending thoroughly in initial outgrowth medium.

The time from tissue preparation until initial outgrowth of cells was calculated for 40 samples derived from donors of various ages. Mean time was 7 days with a range of 5–20 days. No cell cultures could be established from 8 out of these 40 specimens. The effectiveness of cell outgrowth was not significantly dependent on the age of the donors. There was no correlation between the age of the donors and the time until initial outgrowth of cells or success of culture initiation as shown in Fig. 1 (correlation coefficient: $r = -0.074$).

Short-term culture of epithelial and mesenchymal cells of the human bladder in cell type-specific media

As soon as cells were grown out from tissue specimens, initial growth medium was exchanged against selective media to ensure proliferation of either epithelial cells, fibroblasts, or smooth muscle cells (SMC), respectively.

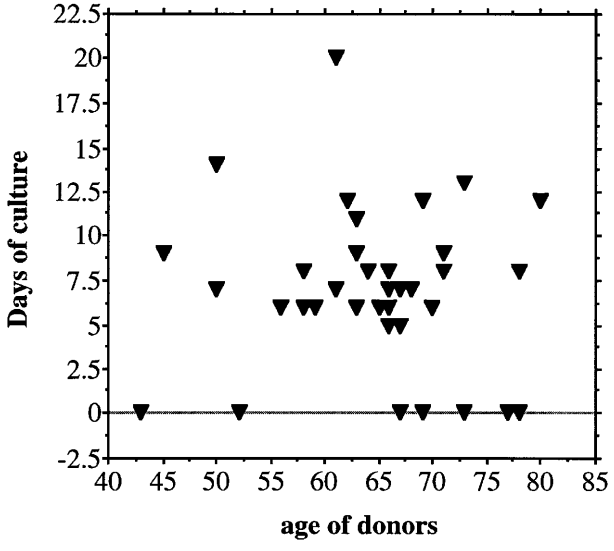


Fig. 1 Scattergram showing the correlation between the age of donors and days until initial outgrowth of cells from tissue specimens. Correlation coefficient was calculated with Stat View 4.1 program and resulted in a value of $r = -0.074$

Table 1 Selective growth medium for the culture of primary human bladder epithelial cells

MCDB-153 (Sigma, St Louis, MO) (Molecular and Cellular Development Biology)	Basal medium
Fetal calf serum (PAA Laboratories, Linz, Austria)	1%
Insulin, Transferrin, Sodium selenite (ITS, Bectin Dickinson)	0.1%
Hydrocortisone (Sigma, St Louis, MO)	200 nM
Triiodothyronine (Sigma, St Louis, MO)	10 nM
MEM-Non essential amino acids (100×, without L-Glutamine) (PAA Laboratories, Linz, Austria)	1%
Epidermal growth factor (Gibco BRL, Life Technologies Ltd)	5 ng/ml
Penicillin (PAA Laboratories, Linz, Austria)	100 U/ml
Streptomycin (PAA Laboratories, Linz, Austria)	100 µg/ml

Epithelial cells were gradually transferred to MCDB-153, supplemented with various components that are summarized in Table 1. The MCDB formulation is the most commonly used medium for the culture of epithelial cells. This medium has a low calcium concentration and therefore should prevent differentiation but stimulate proliferation of epithelial cells at the same time [4, 15]. Moreover, we chose this medium as high percentages of fetal calf serum (FCS) are necessary to the overgrowth of epithelial cells with fibroblasts [16]. Epithelial cells grown in modified MCDB as described in Table 1, presented a characteristic polyglonal morphology (Fig. 2A).

To obtain bladder fibroblasts, cultures were maintained in MEM supplemented with 10% FCS and antibiotics. Morphological analysis by phase contrast microscopy revealed typically elongated fusiform cells (Fig. 2B).

Cells with SMC characteristics were obtained from bladder detrusor tissue pieces maintained in EMEM (Eagle's MEM modification) supplemented with 10% FCS, 1 × nonessential amino acids solution (PAA Laboratories, Linz, Austria), and antibiotics. Cells grew in the characteristic hill and valley pattern and showed a typical SMC morphology under phase contrast microscopy (Fig. 2C). When grown on dishes coated with a semi-fluid matrix (Cell Tak, Collaborative Biomedical Products, Bedford, MA), cells were contractile and contractions were stimulated by acetylcholin in a dose dependent manner [6].

All cell cultures were free from mycoplasma contamination. Sensitive detection of mycoplasmal contaminants was achieved with a Mycoplasma PCR ELISA (Boehringer Mannheim, Mannheim, Germany) that combines polymerase chain reaction (PCR) with an ELISA.

Subculture of primary bladder cell cultures

As soon as cells reached subconfluency in 25-cm^2 culture flasks, they were washed with phosphate buffered saline

(PBS, PAA Laboratories GmbH, Linz, Austria) and detached with trypsin/EDTA (0.05%, 0.02%; PAA Laboratories GmbH, Linz, Austria). Enzyme activity was blocked with medium containing 10% FCS. Cells were centrifuged gently at 1000 rpm for 5 min, washed another time with PBS, and seeded in selective growth medium. During the first passage, fibroblasts were split in a ratio of 1:2, epithelial and smooth muscle cells were split in a ratio of 1:1. For further passages, split ratios of 1:3 and 1:2 were used for mesenchymal and epithelial cells, respectively.

Primary epithelial cells were subcultured up to four times. Only 5 out of 26 (19%) epithelial cell cultures could not be passaged. In contrast, mesenchymal cultures of human bladder tissue seemed to be more suitable for long-term cultures and could be passaged more than 10 times without loss of viability.

Fig. 2A–C Microphotographs of primary epithelial cells **A**, fibroblasts **B**, and SMCs **C** of human bladder tissue grown in selective culture media

Fig. 3A–C Immunohistochemical detection of cytokeratins 8, 18, and 19 in bladder epithelial cells: Immunoreactivity was determined using the Vectastain ABC immunoperoxidase system and diaminobenzidine as substrate (Vector Laboratories, Burlingame, CA). Briefly, subconfluent monolayers of either epithelial or mesenchymal cultures were grown in plastic chamber slides (Merck, Vienna, Austria). Epithelial cells were fixed with 3.7% formaldehyde for 10 minutes at room temperature and subsequently permeabilized with 1% triton-X-100 for 1 h. Chambers were removed and unspecific binding sites were blocked with nonfat dry milk for 1 h. Slides were incubated with primary monoclonal antibody staining with cytokeratin 8, 18, and 19 (Monosan) for 2 h at room temperature. Biotinylated secondary anti-mouse IgG was added for a 1-h incubation period. Extensive washing with PBS was performed between each step. Primary epithelial cells grown in MCDB-153 **A** and the urothelial tumor cell line HT1197 **B** stained positive for cytokeratins. Primary mesenchymal cells **C** were cytokeratin negative

Immunohistochemical detection of cell-type specific proteins

Besides morphological characterization of cell cultures by phase contrast microscopy, expression of cell type-specific marker proteins was assessed by immunohistochemistry. For characterization of urothelial cells, expression of cytokeratin 8, 18, and 19 was determined by using the Vectastain ABC immunoperoxidase system (Vector Laboratories, Burlingame, CA) and a primary mouse monoclonal antibody (Monosan). Results are presented in Fig. 3.

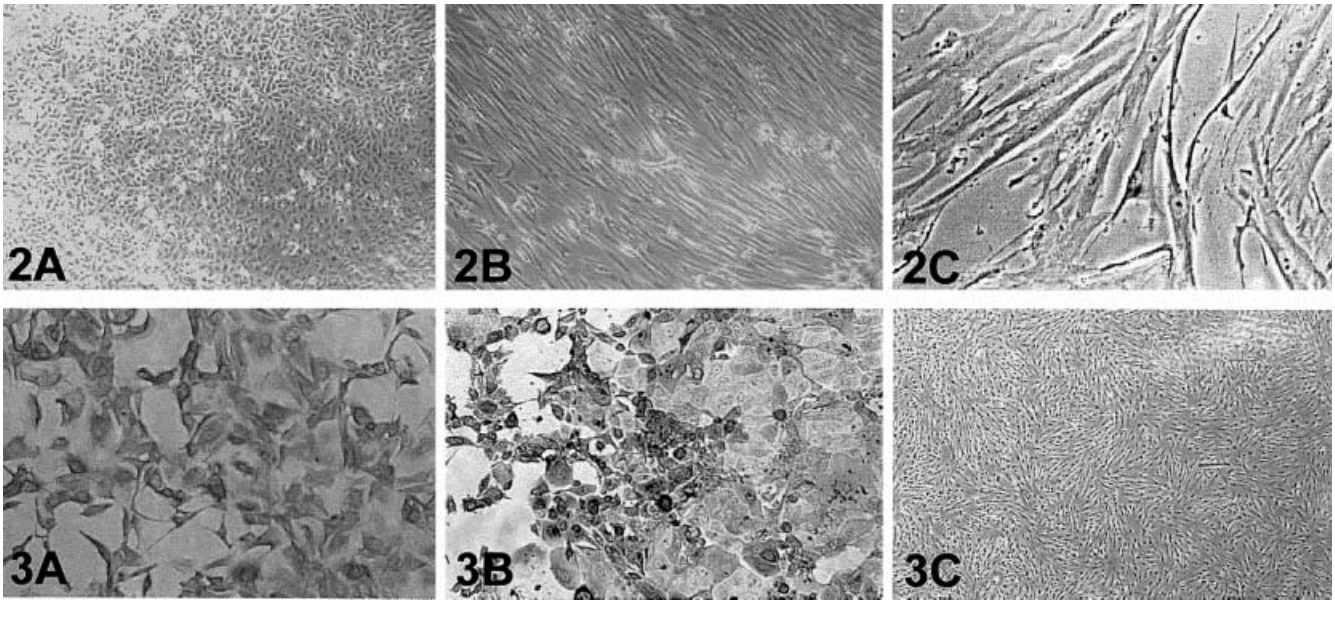
Cytokeratin expression was strong and uniform in primary human epithelial cells (Fig. 3A). HT-1197, a human urothelial tumor cell line that was used as a positive control, showed similar cytokeratin expression (Fig. 3B). In contrast, primary mesenchymal cultures from human bladder tissue grown in MEM with 10% FCS were negative for cytokeratin staining (Fig. 3C).

In the bladder, smooth muscle cell cultures expression of SMC-specific marker proteins were investigated. The cultures were positive for α-actin, SMC-myosin and desmin. No immunohistochemical staining was seen with the antibody DIA100 (Dianova, Hamburg, Germany) recognizing a fibroblast-specific surface marker [6].

Cell proliferation

In vitro proliferation of primary epithelial cell cultures and fibroblasts of the human bladder was determined by counting cell number at several time points, using a hemocytometer. Results were summarized in Fig. 4.

The epithelial cell culture shown in Fig. 4 was passaged 18 days after tissue preparation, when a cell number of 3.2×10^4 cells/cm^2 was evaluated. One week



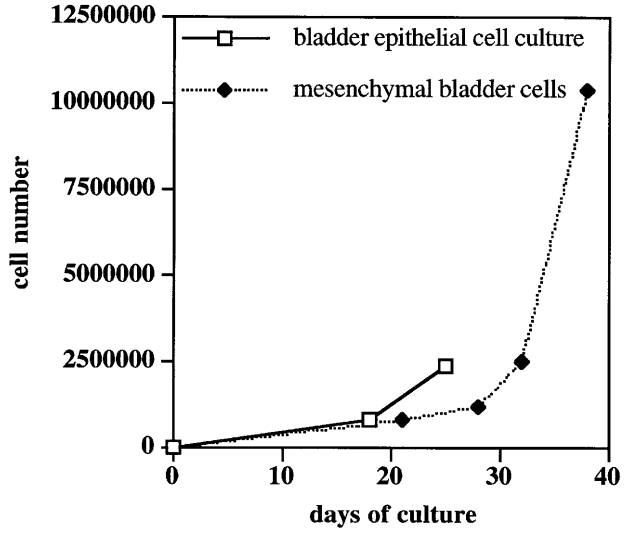


Fig. 4 Cell proliferation was determined in a primary bladder epithelial cell culture as well as in bladder fibroblasts by counting cell numbers at various time points, using a hemocytometer

later, cell numbers increased threefold. This epithelial cell culture could not be subcultured a second time. The fibroblast cell culture was trypsinized at day 21 after tissue preparation and was passaged 3 times for this experiment. Cell numbers increased 1.5 times within the first passage, twice within the second passage, and 4 times within third passage.

Discussion

In this report we describe an efficient method for the isolation and in vitro maintenance of epithelial and mesenchymal cells of the human bladder. The technique we preferred for the isolation of primary bladder cells was mechanical dissection of the tissue specimens. In contrast to various extensive digestion protocols, this method has the important advantage that cell cultures can be established from very small tissue pieces.

A major goal of our study was to establish pure populations of either epithelial cells, fibroblasts or smooth muscle cells (SMCs) of the human bladder with little or no cross contamination. This could be achieved by using different culture conditions. Because of their more rapid growth, fibroblasts tend to overgrow epithelial cultures quite rapidly. To eliminate this problem, we used a selective medium for the culture of epithelial cells with low calcium and serum concentrations in order to prevent overgrowth with fibroblasts. MCDB-153 has been pioneered in this respect for the culture of keratinocytes in the last few years. This medium should stimulate proliferation and inhibit differentiation of epithelial cells [4, 15, 16]. The addition of selective supplements further optimizes epithelial cell culture conditions, especially during subculture [13].

We currently have a success rate of about 80% in culturing epithelial cells from normal human bladder specimens. Life span of epithelial cells is one to four passages from initiation of the culture to senescence. The epithelial nature of the cells was verified by cytokeratin expression. Phase contrast microscopy as well as immunohistochemical staining revealed little or no cross contamination with fibroblasts.

In contrast to epithelial cells, mesenchymal human bladder cells could be maintained in culture for a longer period of time over several passages, with rapid growth rates approximately three passages after culture establishment. SMCs of the bladder were grown and maintained in culture easily. These cells expressed the specific smooth muscle cell markers and were stimulated to contract by the muscarinic receptor agonist acetylcholin, but did not respond to phenylephrine, an α1-adrenergic receptor agonist [6]. These contractile properties are different from SMC cultures derived from prostate tissue, which contract when stimulated with phenylephrine, thus demonstrating the maintenance of specific tissue characteristics of the cultures [5].

The use of selective media provides the opportunity to separately culture different cell types and to investigate their specific physiological and cellular features. This is important in terms of tissue engineering and studying the specific requirements that each cell type needs for the establishment and maintenance of tissue grafts in vivo. Moreover, after expansion, cells can be rearranged in cocultures in order to investigate stromal-epithelial interactions. To perform this, cells can be grown in a culture system separated by a microporous membrane as described by Bayne et al. [3] or as a static culture in a gel matrix with liquid overlay, also called "liquid overlay culture technique" [14]. Another interesting possibility is the growth of cells in rotating bioreactors where cells form three-dimensional spheroidal structures [8]. In the future, we will use different culture techniques with various combinations of cell types in cocultures in order to find optimal conditions for the growth and maintenance of cells and tissue grafts in vivo.

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