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ORIGINAL ARTICLE

A new and reliable culture system for superficial low-grade urothelial carcinoma of the bladder

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Abstract Several bladder cancer culture systems have been developed in recent years. However, reports about successful primary cultures of superficial urothelial carcinomas (UC) are sparse. Based on the specific growth requirements of UC described previously, we developed a new and reliable culture system for superficial low-grade UC. Between November 2002 and April 2006, 64 primary cultures of bladder cancer specimens were performed. After incubating the specimens overnight in 0.1% ethylenediaminetetraacetic acid solution, tumour cells could easily be separated from the submucosal tissue. Subsequently, cells were seeded in a low-calcium culture medium supplemented with 1% serum, growth factors, non-essential amino acids and glycine. The malignant origin of the cultured cells was demonstrated by spectral karyotyping. Overall culture success rate leading to a homogenous tumour cell population without fibroblast contamination was 63%. Culture success could be remarkably enhanced by the addition of glycine to the culture medium. Interestingly, 86.4% of pTa tumours were cultured successfully compared to only 50% of the pT1 and 38% of advanced stage tumours, respectively. G1 and G2 tumours grew significantly better than G3 tumours (86, 73 and 41%, respectively). Up to three passages of low-grade UC primary cultures were possible. We describe a new and reliable culture system, which is highly successful for primary culture and passage of low-grade UC of the bladder. Therefore, this culture system can widely be used for functional experiments on early stage bladder cancer.

Keywords Bladder cancer · Low-grade urothelial cancer · Primary culture

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Introduction

Superficial low-grade urothelial carcinomas (UC) of the bladder correspond to 70% of all UC and are characterised by multiple recurrence in up to 50–70% of the cases. Therefore, a reliable tumour model system would be extremely useful for testing chemotherapeutics preventing cancer recurrence. Besides animal models, analysis of UC is almost exclusively employed on immortal cell lines [1–3]. Unfortunately, cell lines established from papillary UC like RT4 or RT112, which represent an often-used model for superficial UC to date [4, 5], exhibit quite similar genetic alterations as advanced bladder cancer cell lines [6]. Cell lines derived from advanced UC carry typical molecular and chromosomal alterations like loss of chromosomes 3p, 8p, 9 and 13q; gain of 5p, 6p, 8q and 20q; and mutations of p53 and other tumour suppressors [7–10]. To solve the limitations of cancer cell lines, several primary culture systems of UC have been studied using tumour tissue pieces [11–15] or urinary exfoliated cells [16, 17]. However, the

majority of these studies used tumour explants from advanced bladder cancer specimens and there were only few reports about the short-term culture of low-grade superficial UC.

Recently, we observed substantial differences in the mechanisms that drive proliferation of normal urothelial cells and established UC cell lines [18]. Our findings support the assumption that the growth requirements of superficial low-grade UC resemble those of normal urothelial cells, rather than those of advanced cancers. Based on our findings, we modified the culture system described for normal urothelial cells by Southgate et al. [19] allowing us to culture superficial low-grade UC under almost serum-free conditions.

Materials and methods

Media and chemicals

Dulbecco Minimum Essential Medium (DMEM) and Keratinocyte serum free medium (KSFM) were purchased from Gibco Life Technologies (Gaithersburg, MD, USA). Serum-free EpiLife-medium and coating matrix was from Cascade Biologics (Portland, OR, USA). Bovine pituitary extracts, epidermal growth factor, insulin, transferrine, selenium (ITS) as well as non-essential amino acids were products from Gibco. All other chemicals, if not specified differently, were products of Sigma-Aldrich (St. Louis, MO, USA).

Patients and tissue specimens

Between November 2002 and April 2006, 64 primary cultures of UC specimens were performed from patients who underwent transurethral resection. After establishing an optimised culture procedure, a series of 46 UC primary cultures was analysed prospectively regarding culture success and passage rate. Median age of these patients was 72 years (range 52–95 years). Thirty-five patients were males and 11 were females. The study was approved by the ethics committee of the Heinrich-Heine University. Twenty-two UC were staged as pTa, 8 as pT1 and 16 tumours as at least pT2 according to the UICC 1997 classification. G1, G2 and G3 grading was found in 7, 22 and 17 patients, respectively.

Preparation of tumour pieces and primary culture

The specimens were stored in room temperature in Hank's Balanced Solution (HBSS). Under stereomicroscopic control, the specimens were freed from thermally damaged parts and incubated overnight at 4°C in 0.1% ethylenediaminetetraacetic acid (EDTA) solution containing 10 mM HEPES and 20 kIU/ml aprotinin. The following day, tumour cell layers were stripped from the submucosal tissue using Pasteur pipettes (Fig. 1a–d). After washing in the medium and pelleting for 5 min at 1,000 × *g*, the tumour cells were resuspended in fresh medium and plated in 75 cm^2 culture flasks (T-75) precoated with a culture matrix. At this stage, most of the cells were present as clusters of 10–100 cells.

Cell culture and passage

Approximately 100,000 cells were seeded in a T-75 flask. Cells were routinely grown in keratinocyte media with a series of supplements (Table 1). Routinely, 20 ml medium was used per T-75 flask. Cultures were grown in a humidified atmosphere of 5% CO_2 in air at 37°C. When the primary cultures reached subconfluence (3–7 days after seeding), the cells were detached using accutase I (PAA Laboratories, Pasching, Austria). Cells were pelleted by centrifugation and washed twice with medium before reseeding. Culture success and passage success were defined if more than 30% cell confluence was reached.

Cell culture of human bladder fibroblasts

Human bladder fibroblasts were prepared using selective culture conditions [20]. Bladder fibroblasts were maintained in DMEM supplemented with 30% FBS. Conditioned medium from the human bladder fibroblast cultures was added to the culture medium (Table 1).

Viability assay (MTT test)

Cell viability was determined by the Cell Titer-Glo Luminescent Cell Viability Assay (Promega, Mannheim, Germany) according to the manufacturer's instructions. Results were based on three different experiments.

Detection of cytokeratin

Immunohistochemical detection of cytokeratin 7 and 13 using FITC-conjugated antibodies (Sigma-Aldrich) was performed to demonstrate the urothelial origin of the tumour cells, as described earlier [18].

Cytogenetic analysis

Chromosome banding analysis of first passage cultures of three tumours (pTaG2, pT1G2 and pT4aG3) were performed essentially as reported [21]. Multicolour karyotyping was done using a spectral karyotyping system (Applied Spectral Imaging, Mannheim, Germany) as described [22].

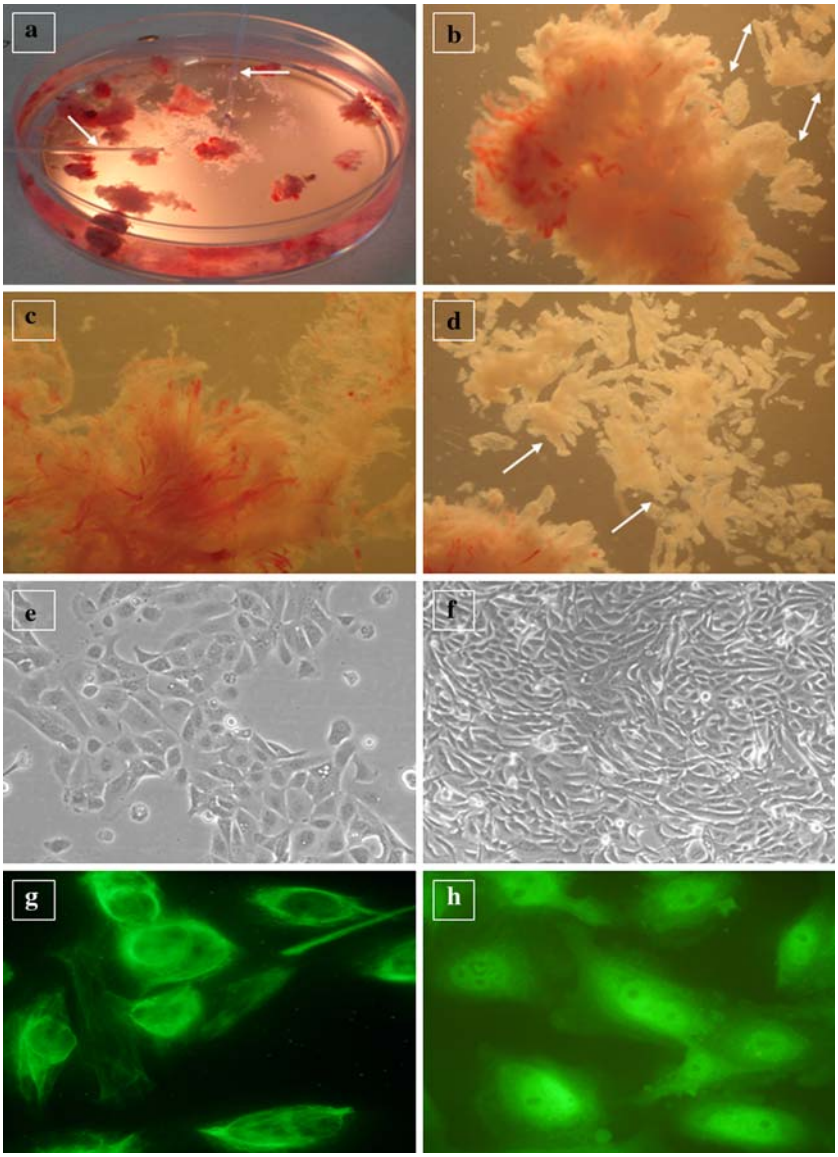

Fig. 1 Preparation technique of the tumour specimens for primary culture. Using two Pasteur pipettes (**a**, *small arrows*, ×3) the tumour cell layers of a superficial UC (**b**, *small arrows*, ×14) were gently stripped from the submucosal tissue under stereomicroscopic control; **c** shows the tumour specimen of **b** after almost all tumour cell layers have been removed. The obtained tumour tissue pieces (**d**, *small arrows*, ×14) were then washed with medium, pelleted and resuspended in culture flasks. **e–f** After processing the tumour cell layers, ~100,000 cells per 75 ml culture flask were seeded. 12 h after seeding, the attached tumour cells started proliferation (**e**, ×250, pTa G2 UC). About 24 h after seeding, a monolayer of tumour cells was established. Confluence was reached after 3–7 days (**f**, day 6, ×100, pTa G1 UC). **g–h** Immunohistochemical detection of cytokeratin 7 (**g**) and 13 (**h**) was performed to demonstrate the urothelial origin of the tumour cells (×400, pTaG2 UC passage 1)

Table 1 Supplemented medium used for primary cultures of superficial low-grade UC

Epilife	Base medium
Bovine pituitary extracts (BPE)	25 µg/ml
Epidermal growth factor (EGF)	0.5 ng/ml
Glycine	3 mM
MEM-non essential amino acids	1% (v/v)
Insulin, transferrine, selenium (ITS)	1% (v/v)
Supernatant human bladder fibroblasts culture	10% (v/v)
Foetal calf serum (FCS)	1% (v/v)
Penicillin	100 U/ml
Streptomycin	100 µg/ml

Statistical analysis

For statistical analysis, Fisher's exact test and chi-square test were performed using SPSS 11.5 for Windows (SPSS, Chicago, IL, USA). A *P*-value of 0.05 or less was considered statistically significant.

Results

After overnight treatment in 0.1% EDTA solution, the tumour layers could easily be stripped from the submuccosal tissue (Fig. 1a–d). The EDTA treatment and very low-serum concentration of 1% led to a homogenous tumour

cell population almost free of fibroblast contamination (Fig. 1e, f). Initial cultures were attempted in a completely serum-free medium. However, we found that the addition of fibroblast-conditioned media evidently improves the proliferation and survival of the cultures. The urothelial origin of the cells was demonstrated by the immmunohistochemical detection of cytokeratin 7 and 13 (Fig. 1g, h). Superficial low-grade tumours grew best in Epilife Medium supplemented with bovine pituitary extracts, 1% FBS, growth factors and non-essential amino acids (Table 1). Coating of the flasks with culture matrix and supplementing the culture medium with 3 mM glycine dramatically enhanced tumour cell growth. Cell viability analysis demonstrated a 3.6-fold higher growth rate compared to controls cultured in medium without glycine. Furthermore, adding 1% supernatant of bladder fibroblast cultures and simulating hypoxic conditions by using 20 ml instead of 10 ml medium resulted in a remarkable better growth rate (data not shown). Under these conditions, up to three passages of superficial low-grade UC cultures were possible.

Across all stages and grades, the success rate of primary cell cultures was 63%, but the technique appeared not equally well suited for tumours of all grades and stages (Table 2). In particular, tumour cells from superficial low-grade UC (pTa) could be cultured in 86.4% of the cases, whereas only 50% of invasive pT1 and 38% of advanced stage (≥pT2) tumours showed sufficient tumour cell growth in vitro ($P = 0.004$). A major reason preventing establishment of cultures from advanced stage tumours was that tumours cells did not detach from the surrounding stroma during overnight treatment by EDTA in 6 of 16 tumours. Interestingly, tumours graded as G1 grew significantly better than G2 or G3 tumours when the new technique was used (Table 2). There was good correlation between culture success and tumour stage ($P = 0.006$) and grade (G1 + G2 versus G3 $P = 0.034$).

Table 2 Influence of tumour grade and stage on success of primary culture and passage

	n	Successful culture *n* (%)	Successful passage *n* (%)
pTa	22	19 (86.4)*	12/19 (63)
pT1	8	4 (50)	2/4 (50)
≥pT2	16	6 (38)	5/6 (83)
Total (%)	46	29 (63)	19/29 (66)
G1	7	6 (86)**	5/6 (83)
G2	22	16 (72)**	15/16 (94)
G3	17	7 (41)	4/7 (57)
Total (%)	46	29 (63)	19/29 (66)

*$P = 0.004$

**G1 + G2 versus G3 $P = 0.034$

In 34% of the initially successful primary cultures, a satisfactory passage was not possible (Table 2). However, after optimisation of medium and substrate conditions, almost all cultures of superficial low-grade UC grew to the point of possible passage.

Chromosome banding analysis clearly demonstrated the malignant origin of the cultured cells showing an abnormal karyotype in all metaphases analysed. At first passage of a pT1G2 tumour culture, an isochromosome i(5)(p10) and loss of an entire chromosome 9 were observed. In later passages, the abnormal karyotype was duplicated and additional clonal aberrations occurred (Fig. 2).

Discussion

Most studies analysing superficial low-grade UC used animal models, cancer cell lines or short-term cultures of tissue specimens. Because there are specific differences between rodent and human UC [23], findings from animal studies are of limited evidence for the understanding and therapy of human UC. Most human bladder cancer cell lines are aneuploid and display genetic and molecular alterations comparable to those in advanced UC [7–10]. This may partly be due to a requirement for overcoming senescence by inactivation of the *RB1* or *CDKN2A*/p16 genes [24]. To date, all permanent cell lines derived from low-grade UC possess one or more of these alterations, limiting their value for studying superficial UC. Several groups investigated short-term cultures of UC derived from tumour tissue pieces [12–15] or urinary exfoliated cells [16, 17]. The majority of these studies cultured tumour explants from advanced bladder cancer, which exhibit a high-take rate in culture due to their high degree of growth autonomy. However, primary cultures of low-grade superficial bladder cancer are much more difficult to establish. Only a few investigators were able to perform cultures of low-grade superficial UC over periods ranging from a few hours to several days [13]. Due to the very short time frame of the culture systems used, it remained unclear whether the cells were still viable or able to proliferate, making the results hard to interpret. Primary cultures of exfoliated cells from spontaneously voided urine of patients with bladder cancer or washouts during transurethral resections have apparently been established more successfully [16, 17], but the cultures were not well characterised and may contain variable cell admixtures. Three-dimensional culture systems for UC have been reported recently [12], which might be superior to monolayer cultures in evaluating sensitivity to chemotherapeutics. However, the spheroids investigated contained variable cell admixtures as well, so that further development and validation of this in vitro model seem to be advisable.

Fig. 2 Spectral Karyotyping of a cell culture established from a pT1G2 tumour carrying typical genomic alterations of UC: Isochromosome i(5)(p10) and loss of an entire chromosome 9 were observed. The abnormal karyotype was duplicated and additional chromosomal aberrations occurred

Here we present a new culture system allowing the successful culture of superficial low-grade UC of the bladder under almost serum-free conditions and low-calcium concentration. The rationale for developing this culture system was the assumption that the growth requirements of superficial low-grade UC resemble those of normal urothelial cells, rather than those of advanced cancers [18]. Based on the supplemented keratinocyte serum-free medium as described by Southgate et al. [19], modifications for successful growth of superficial UC were developed. Precoating the culture flasks with extracellular matrix resulted in a higher growth rate and significantly better attachment of the cells. This is in agreement with previous findings addressing culture conditions for epithelial tissue [25]. In addition, it is known that bladder cancer cells are adapted to a hypoxic environment [26]. Simulating more hypoxic growth conditions in our culture system by using more medium per culture flask resulted in a higher growth rate of the tumour cells. Another important step was the addition of glycine to the culture medium. Glycine can promote integrin-mediated cell adhesion [27], and it is particularly protective against hypoxia/reoxygenation [28], which is a typical exposure during cell culture. Before we used glycine as a medium supplement, establishing the first passage of the tumour cells was evidently more difficult. Growth and survival of urothelial carcinoma cultures were also improved by addition of fibroblast-conditioned media. This is in accord with the idea that normal urothelial cells are induced largely by autocrine mechanisms to proliferate, but significant support is given by paracrine factors from the mesenchyme, especially FGF7/KGF [29].

To our surprise, the success rate for establishing cultures from advanced UC using our culture technique was low. A reason for this phenomenon could be that tumour cells from EDTA-treated papillary tumour pieces detached more easily from the surrounding tissue than those from invasive UC. The latter might be more deeply imbedded in the tissue and extracellular matrix in particular. Moreover, the bad success rate supports our previous findings suggesting fundamental differences in the tumour growth of invasive versus superficial UC [18].

The culture system described may be helpful for testing chemotherapeutics and for functional studies about tumour biology. In the future, it may provide a tool to determine individualised therapy approaches for patients with superficial UC.

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