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Short-term culture of exfoliated cells from the urine of patients with bladder tumors

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Summary. This report concerns the short-term culture of urothelial cells from the urine sediment of over 100 patients with bladder tumors. Primary cell outgrowth was obtained in approximately 60% of the cultures initiated. Culture outcome was not related to tumor grade, patient age, or volume of the urine sample. Around 85% of the proliferating cultures were successfully transferred into multi-compartment chamber/slides. These results suggest that the culture system may be a useful tool for the study of urothelial cells using patient material obtained by non-invasive means.

Key words: Bladder neoplasm – Cultured cells – Urine

Voided urine from normal adults contains viable cells that can be propagated in vitro [8]. The cultured cells form desmosomes and tight junctions typical of epithelial cells [13], and the presence of cells lined by the characteristic asymmetric unit membrane [11] confirmed their urothelial origin [13]. In addition, from flow cytometric DNA and chromosome analyses it was established that the cultures were composed of non-synchronously proliferating normal diploid cell populations [9]. Based on the results obtained with one patient, it has recently been proposed that short-term cultures from urine could be utilized for the detection of early karyotypic changes in bladder cancer [15]. Moreover, the cultures could also be used to study potential immunocytochemical bladder tumor markers [6] and to detect gene mutations [2, 14] on patient material obtained by non-invasive means. However, before the culture system can be applied to such investigations it was considered necessary to test urine from a large number of bladder cancer patients, to assess the influence of urine parameters that may affect culture outcome, and to determine whether the proliferating cells can be transferred into glass chamber/slides, a culture modality that allows the concurrent analysis of cell populations with various antibodies [3]. Here we present the results of such a study, which to our knowledge has not been performed previously.

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Materials and methods

Urine from bladder cancer patients was obtained either by spontaneous micturition or through the cystoscope or by catheterization. Clinical data, including the results of the most recent cytological examination of the urine, were retrieved from the hospital files. Specimens, collected in sterile containers, were stored for 1 h at 4°C and then centrifuged in (several, if necessary) 50-ml tubes at 850 *g* for 10 min. During this time the few drops of urine remaining in the original container were tested with a dipstick for leukocytes, glucose, ketones, proteins, blood, and pH. The sediments were washed with 10 ml Eagle's miminum essential medium (MEM) containing 20% fetal bovine serum (FBS), penicillin (100 U/ml), streptomycin (100 µg/ml), and amphotericin B (0.25 µg/ml), transferred into a 12-ml conical tube, and centrifuged again. Sediments of male urine containing inordinate numbers of leukocytes and those of female patients were washed twice with medium containing a five times greater concentration of antibiotics. After discarding the supernatant, the urine sediments were resuspended in 3 ml regular medium and inoculated into a plastic T25 culture flask [8].

The cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air and were not disturbed for 48 h. Subsequently they were examined every other day for cell attachment and outgrowth. Large numbers of erythrocytes did not interfere with the microscopic examination of cultures from patients with gross hematuria. Flasks contaminated by bacteria and/or fungi were discarded as soon as noticed. To remove the unattached cells and other urine sediment components, the cultures were washed several times with, and then fed, fresh medium 5–7 days after their initiation. The medium was changed twice a week and cell growth monitored for 10–15 days. Where indicated, the number of cells in a given colony was counted with the inverted microscope.

The following procedure was used to transfer cells of proliferating cultures into chamber/slides. After discarding the medium, the colonies were washed with Ca^{2+}- and Mg^{2+}-free Earle's balanced salt solution, rinsed briefly with 3 ml of a mixture of 0.05% trypsin and 0.02% EDTA, and incubated for 3 min at 37°C with 2 ml of the same mixture. The dispersed cells were suspended in 5 ml medium, transferred into a conical tube, and centrifuged at 350 *g* for 5 min.

Table 1. Culture outcome in relation to gender, tumor grade, age, and urine cytology

	Cultures initiated	Cont.[a]	No growth	Growth	Ch./slides[b]
Males					
Grade I–II	25	4	5	16	14
Grade II–III	49	8	11	30	27
Unknown	9	2	1	6	4
Females					
Grade I–II	12	1	4	7	5
Grade II–III	20		9	11	9
Unknown	2			2	2
Age					
< 60 years	15	1	3	11	8
61–70 years	29	4	5	20	18
71–80 years	33	5	8	20	17
> 81 years	40	5	14	21	18
Urine cytology					
Negative	27	4	8	15	13
Suspicious	11	2	5	4	4
Atypical	32	4	3	25	22
Positive	27	3	7	17	14
No recent report	20	2	7	11	8
Total	117	15 (13%)	30 (26%)	72 (62%)	61 (53%)

[a] Cont., number of contaminated cultures
[b] Number of cultures transferred into chamber/slides

The sedimented cells were resuspended in 250–300 µl medium and 20–30 µl were inoculated onto the center of each compartment of 8-well glass chamber/slides. Following incubation for 24 h at 37°C, 0.4 ml medium was added; the procedure was considered successful when 4 days after transfer each chamber had a similar number of attached cells.

Results

We have obtained primary cell outgrowth with 63% of 83 cultures initiated from urine sediments of male patients with bladder cancer and 59% of 34 cultures from female patients (Table 1). There was no relationship between outgrowth or lack thereof and tumor grade or age of the patient. Cell growth was obtained with 56% of sediments from patients in whom the results of urine cytology were negative and 37% from those in whom the results were suspicious. By comparison the proportions of successful cultures from patients with atypical and positive urine cytology results were 78% and 63%, respectively (Table 1). No outgrowth was seen with 26% of the specimens and 13% were discarded because of microbial contamination.

Cell outgrowth was observed as early as 3 days and as late as 2 weeks after culture initiation. The multiplying cells had epithelial morphology (Fig. 1) resembling that of cultured cells from normal urines [8, 13], in which the presence of desmosomes, tight junctions, and urothelium-specific asymmetric unit membrane has been demonstrated [13]. Outgrowth of fibroblasts was never seen. In many instances the number of cells in a given colony could easily be counted. As shown in Fig. 2, there was an initial exponential increase (log phase) in cell number, followed by the typical decrease in growth rate. During log phase the number of cells doubled approximately every 48 h (Fig. 2), but the variability within and between different cultures was not determined. Cells of approximately 85% of the proliferating cultures were successfully transferred into chamber/slides (Table 1). Because of the nature of the study, no attempts were made to establish continuous cell lines.

There are several potential variables that may influence the outcome of cultures initiated from urine. The effect of these variables was assessed with 111 cultures. Our data indicate that the volume of the urine sample was not a limiting factor in obtaining cell outgrowth (Table 2). Indeed, growth has been seen in cultures initiated from the sediment of as little as 5 ml urine, whereas in other instances no colony formation was observed with sediments of 200-ml specimens. Since the washed urine sediments were inoculated directly into the culture flasks, culture outcome could not be related to the total number of cells in a given sample, nor could plating efficiency estimates be obtained. With respect to pH, the percentage of successful cultures was somewhat greater with urines of pH > 7 (86%) than with those of pH < 6 (55%) (Table 2). There was essentially no difference in outcome between cultures initiated from specimens with minimal, moderate, or no hematuria, but the proportion of proliferating cultures was reduced when urines contained large

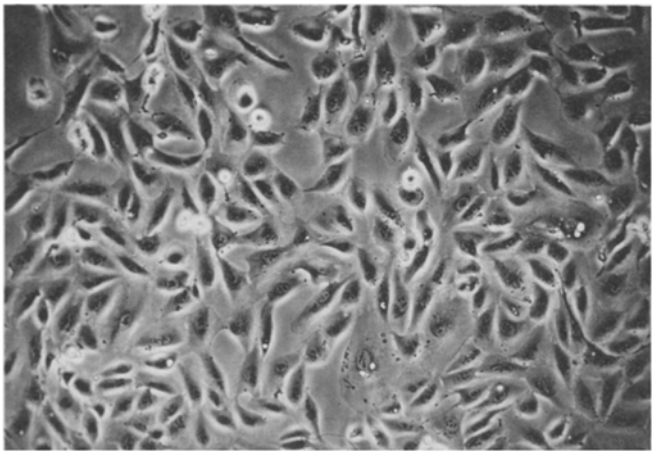

Fig. 1. Phase-contrast photomicrograph of cultured, urine-derived cells from a patient with bladder tumor grade II. The picture, taken 8 days after culture initiation, shows several round mitotic cells. (×100)

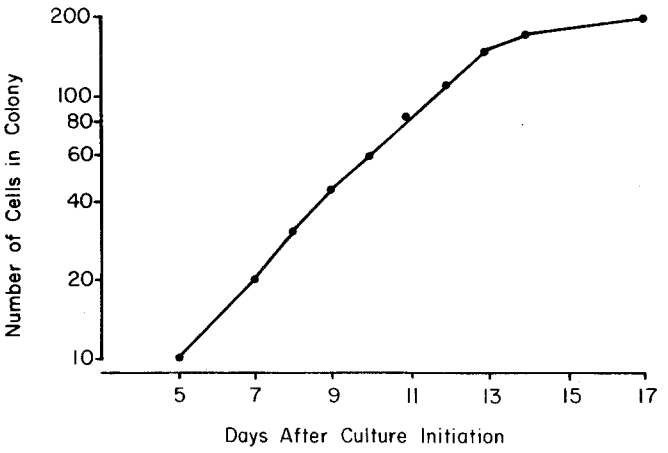

Fig. 2. The number of cells in a colony was counted through an inverted microscope

Table 2. Culture outcome in relation to urine variables

	Cultures initiated	Cont.[a]	No growth	Growth	Ch./slides[b]
Volume					
< 25 ml	13	0	5	8	8
25–75 ml	44	5	11	28	23
> 75 ml	54	7	13	34	30
pH					
< 6	44	5	15	24	21
6–7	45	7	11	27	23
> 7	22	0	3	19	17
Hematuria					
None	34	2	9	23	21
Minimal	29	1	6	22	21
Moderate	13	3	0	10	9
Gross	35	6	14	15	10
Proteinuria					
No	20	0	5	15	15
Yes	91	12	24	55	46
Total	111	12 (11%)	29 (26%)	70 (63%)	61 (55%)

[a] Cont., number of contaminated cultures
[b] Number of cultures transferred into chamber/slides

amounts of blood (Table 2). The influence of glucose could not be investigated systematically because there were only three patients with glycosuria.

Discussion

From the data presented it is evident that the urine of patients with bladder tumors contains viable cells that can be grown in vitro. Although the malignant nature of the proliferating cells has not been established, the results obtained would indicate that the culture system may indeed be useful for the study of certain clinically relevant cellular parameters. Thus, as shown by us with cultures from normal subjects [9] and by others with a culture from one patient with bladder cancer [15], metaphases for chromosome analysis of the cells in primary culture can readily be collected after colcemid arrest. The demon-

stration that the cells can be transferred into chamber/slides would suggest that the concurrent assessment of several potential immunological markers of bladder tumor behavior [6, 10] can be carried out on the same slide. Although cultured cells from normal urine express the asymmetric unit-membrane-associated urothelial membrane antigen [9], it remains to be determined whether the cells from bladder tumor patients retain their in vivo antigenic characteristics. On the other hand, as shown with continuous human cancer cell lines, the chamber/slide modality is well suited for quantitating epitope expression by computer-assisted image analysis [3].

Moreover, the cultured cells may also be useful in gene analysis. This is of special interest since we [2] and others [1] have recently demonstrated that the frequency of Ha-*ras* gene codon 12 mutations in bladder cancer is significantly greater than that previously recorded [5, 7, 16]. Our study also showed that mutation correlates with DNA ploidy [2] and that Ha-*ras* gene analysis may be of value for identifying aggressive variants of grade II tumors, the prognosis of which is not predictable by conventional histopathological methods [12]. Although polymerase chain reaction (PCR)-based tests [4] for Ha-*ras* gene abnormalities can be done on urine sediments (unpublished results from this laboratory), the use of cultured, urine-derived cells could enhance the efficiency with which mutated genes are detected since the non-urothelial cells, including white blood cells that have only the normal Ha-*ras* gene, would be excluded from analysis. The same considerations may also apply to the study of p53 alterations, recently detected in urine sediments of three patients with invasive bladder cancer [14].

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