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Establishment and characterization of long-term primary mouse urothelial cell cultures

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Summary. Long-term mouse urothelial cell cultures were routinely established from explants of neonatal mouse bladders. Foci of proliferating cells could be observed one week after the initiation of the explant cultures. These persisted throughout the culture period and up to one year. Expression of keratin proteins confirmed the epithelial nature of the cultured cells. Morphologic analysis of nuclei sorted after DNA flow cytometry revealed a population of DNA-tetraploid and octoploid cells with large nuclei and prominent nucleoli in addition to a DNA-diploid cell population. Both cell populations showed DNA replicative activity as reflected by bromodeoxyuridine incorporation studies and mitotic activity. These long-term primary mouse urothelial cell cultures may prove useful for studies on urothelial cell kinetics and bladder carcinogenesis.

Key words: Bladder cell culture – Mouse urothelium – Flow cytometry – Bladder neoplasm

Introduction

The establishment of murine long-term primary urothelial cell cultures would be of great benefit for the study of urothelial cell kinetics, the regulatory activities of growth factors and bladder carcinogenesis in this species. As yet, only a few reports have appeared on the use of mouse bladder explant cultures for in vitro carcinogenesis [4, 18, 19].

The mouse bladder is lined with a three-layered transitional cell epithelium. This urothelium consists of DNA diploid, tetraploid and octoploid cells [1, 21]. Immediately after birth a brief period of rapid proliferation occurs in rats [2] and mice [8] preceded by perinatal shedding of the superficial cell layer. The aim of this study was to establish and characterize long-term primary mouse bladder explant cultures using the rapidly proliferating urothelium obtained at the neonatal period.

Materials and methods

Urothelial cell culture medium

The urothelial cell culture medium (UCM) consisted of a 1:1 mixture of Ham's F10 medium and Dulbecco's modification of Eagle's medium (DMEM) supplemented with 10% foetal calf serum (FCS) (Gibco, Scotland), 5 µg/ml insulin, 5 ng/ml selenite and 5 µg/ml transferrin, as well as 5×10^{-8} M hydrocortisone, 25 µg/ml prostaglandin E1, 2 mM L-glutamine and 4×10^{-5} M spermine tetrahydrochloride. In addition, 100 IU penicillin/ml and 100 µg/ml streptomycin (PS) was added. All additives were commercially obtained from Sigma, USA. Cultures were maintained in disposable Falcon tissue culture flasks of 75 or 150 cm^2. The medium was changed once a week. Passage of confluent cultures was done by vigorous syringing followed by replating of the cells.

Isolation of bladder explants

Bladders were aseptically removed from neonatal BALB/c mice within 48 h after birth. After transfer into a petri-dish with DMEM the bladders were opened with a pair of scissors. After rinsing the bladders were transfered into another petri-dish containing UCM. The specimens were than further sliced until tissue pieces measured approximately 1 mm^3. Four to 8 tissue clumps were plated per culture flask and kept in a humidified atmosphere of 5% CO_2 at 37°C.

Cytology and immunocytochemistry

After removal of cultured cells by vigorous syringing cytospin preparations were made (Shandon Cytospin 2, UK), airdried and Giemsa stained. For immunocytochemistry airdried cytospin preparations were acetone fixed and incubated with the appropriate dilutions of the following monoclonal antibodies: anti-vimentin (DAKO, Denmark), RKSE60 and RGE53 specific for keratin 10,

respectively keratin 18 (Eurodiagnostics, The Netherlands), CAM5.2 directed against a common epitope of keratins 8, 18 and 19 (Becton and Dickinson, CA). Visualization was performed using the indirect conjugated peroxidase method as described previously [6]. DNA replicative activity was assessed by immunocytochemical staining of incorporated bromodeoxyuridine (BrdU) commercially obtained from Sigma, USA. To that purpose cell or explant cultures were exposed to 40 μg/ml BrdU for 2 h enabling S-phase labeling. Subsequently, the cells were harvested and cytospin preparations were made. Alternatively, explants were fixed in situ and immunocytochemistry was performed as described previously [6].

DNA flow cytometry

Urothelial cells were collected from the culture flasks and either frozen in fresh medium, containing 10% FCS or fixed in buffered formalin. Nuclei were prepared from the frozen samples using the method described by Vindelov et al. [21] and filtered through a nylon mesh with a pore size of 40 μm. The nuclei were stained with propidium iodide (PI). The final PI concentration was 125 μg/ml. The nuclei of the fixed cells were isolated by a modification of the procedure described by Hedley et al. [7, 8]. The fixed cells were centrifuged (800 g, 5 min) and resuspended in saline, centrifuged again and resuspended in saline containing 0.5% pepsin (pH 1.5). The cells were subsequently incubated in a waterbath (37°C) for 45 min with intermittent vortex mixing. After the digestion the nuclei were centrifuged, resuspended in Hanks' balanced salt solution containing 50 μg/ml ethidium bromide and filtered through a nylon mesh (see above). The nuclei were analyzed on a fluorescence activated cell sorter (FACS II, Becton & Dickinson, Sunnyvale, CA). The laser was tuned to 488 nm with an output of 0.4–0.5 W and red fluorescence was measured with the use of two colored glass filters transmitting light above 600 nm (Ditric). Data were fed into a PDP11 computer and analyzed. Morphometry was performed on Giemsa stained preparations of sorted nuclei, using the interactive image analysis system IBAS 2000.

Results

Morphology of neonatal bladder explant cultures

A total of 20 experiments were performed. Explants usually adhered to the plastic culture flasks between 2 and 4 days. Subsequently, large cells spread out from the majority of the adhered explants forming sheets with an epithelial appearance. From day 7 onwards variable numbers of small round cells could be observed overlying these sheets of larger cells. These small cells gradually formed three-dimensional foci of closely packed cells (Fig. 1A). The surface covered by these sheets of epithelioid cells gradually increased and after 3 months the entire surface was covered by a layer of largely polygonal cells. Some areas consisted of rather irregularly shaped cells lying apart from each other without signs of multiplication. Occasionally, fibroblast proliferation was observed during the first month of culture, but this used to be very limited and fibroblast overgrowth was actually never encountered. The original tissue clump detached after about 2 months of culture. The foci of small round cells overlying the larger cells remained in place during the entire culture period. After passage into new culture flasks, achieved by mechanical dissociation of the adhered cells, again focal accumulations of these small cells appeared. Trypsinization and subsequent passage did not result in successful replating. Even after one year of continuous culture no signs of senescence were seen.

Table 1. See text

Designation of antibody	Antigen	Long-term cell culture				
		L1	L2	L3	L5	L6
RKSE60	keratin 10	5[a]	5	70	10	70
RGE53	keratin 18	0	0	0	0	0
CAM5.2	keratins 8, 18, 19	5	10	80	0	5
RV202	vimentin	100	100	100	100	100

[a] Figures represent the percentage of immunoreactive cells

Cytology and intermediate filament immunocytochemistry

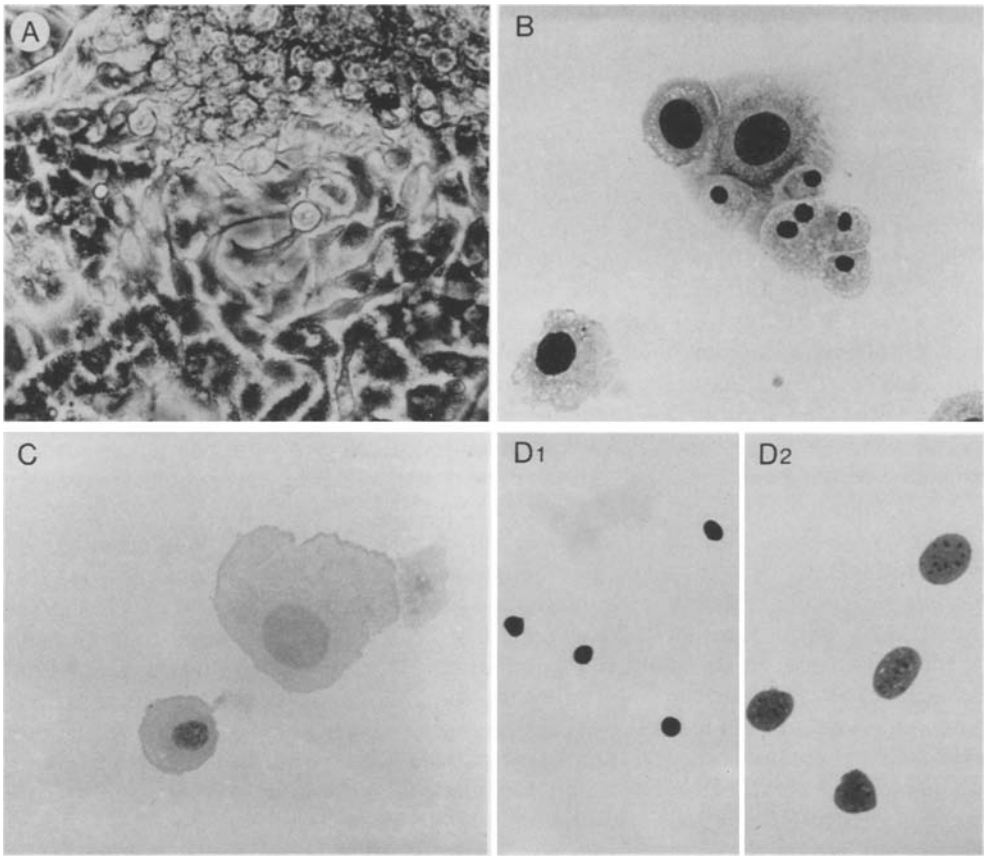
Cytospin preparations made at several intervals after initiation of the culture contained cells with varying sizes and somewhat eccentric situated nucleus (Fig. 1B). Some larger cells were binucleated. Two cell populations could be distinguished mainly on basis of the size of their nucleus. One population consisted of cells with a small sized nucleus and the other consisted of cells with a large nucleus and one or more conspicuous nucleoli. Mitoses were rather infrequent, but were present in both cell populations. The cytoplasm of both cell types was basophilic and sometimes vacuolated. Immunocytochemistry of 5 different primary cultures generally revealed paranuclear staining of varying proportions of cells with monoclonal antibody CAM5.2 specific for a common epitope of keratin 8, 18 and 19, but no staining with RGE53 (keratin 18) was detectable. Varying numbers of cells expressed keratin 10 mostly along their cytoplasmic membrane. Expression of this keratin chain suggests squamous cell differentiation. Strong expression of vimentin was always observed (Table 1).

Bromodeoxyuridine incorporation

After pulse-labeling of the cultures with BrdU for 2 h a proportion of both the smaller cells (Fig. 1C) and the larger cells were labelled. In situ labeling in the culture flask at 15 days after initiation showed that the focal accumulations of smaller cells were preferentially labeled.

DNA flow cytometry and cell sorting

Suspensions of cell cultures obtained at various intervals from 2 weeks to 1 year after initiation of culture were tested for the cellular distribution of DNA content. In all experiments a clear bimodal frequency distribution was observed with a first DNA-diploid peak and a second DNA-tetraploid peak (Fig. 1E). In some experiments an additional minor DNA-octoploid peak could be distinguished. It was not exceptional that the DNA-tetraploid peak was higher as compared to the DNA-diploid peak. Sorting and subsequent morphometric study of the sorted nuclei in cytospin preparations revealed that the DNA tetraploid nuclei were larger (average diam 13.0 μm) as compared to the DNA-diploid nuclei (average diam 7.5 μm). The DNA tetraploid nuclei contained one or more prominent nucleoli (Fig. 1D).



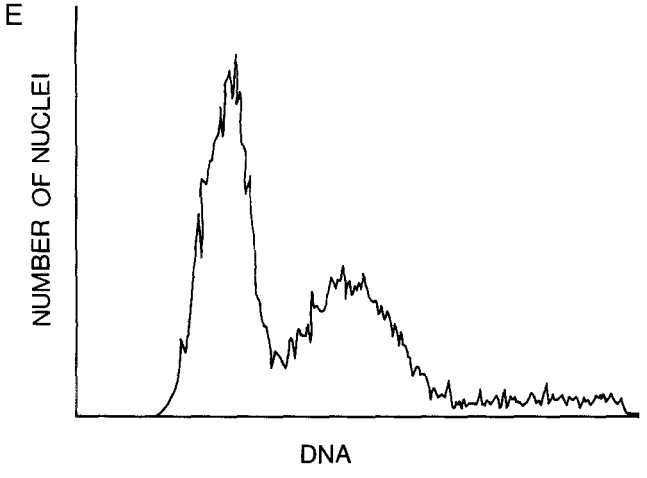


Fig. 1 A–E. Phase-contrast picture of an area of contiguous polygonal cells focally covered with an accumulation of smaller round cells. **B** Giemsa stained cytospin preparation showing cells with larger and smaller nuclei (×250). **C** Positive nuclear staining of the smaller cell after 2 hours of BrdU incorporation (immunoperoxidase staining and slight nuclear counterstaining with haematoxylin). **D** Giemsa stained cytospin preparation of DNA-diploid (D1) and DNA-tetraploid (D2) nuclei after nuclear sorting. **E** Bimodal DNA histogram of urothelial cells cultured for 6 months showing an DNA-diploid and DNA-tetraploid peak

Discussion

Here we report on the routine establishment of long-term urothelial cell cultures from bladder explants of neonatal mice. No signs of degeneration or senescence of these cultures were observed throughout the culture period. This is in contrast to earlier in vitro studies which had been performed on bladder explants derived from adult mouse donors. Degeneration of these cultures was generally observed by 150 days of culture [19]. Dissociated mouse urothelial cells could be maintained in vitro for only 3 weeks [13]. Similarly, in adult rat bladder explant cultures degeneration became already apparent from 1 month of culture onwards, although culture periods could often be extended for 5 to 6 months [11, 12]. Treatment of these rat bladder explant cultures with carcinogens at an early phase led to the development of proliferating "preneoplastic" epithelial foci and the culture periods could be prolonged significantly. Such rapidly proliferating foci were rarely seen in non-treated rat bladder explant cultures [12]. Similar to the studies on adult rat bladder explants, spontaneous occurrence of "transformed" foci was not observed in explant cultures of 5 to 7 months old mice [19]. Our observation, as early as 1 week after initiation of culture, of proliferating foci

persisting for more than one year stands in clear contrast to the findings of the above mentioned authors. Since mice do not spontaneously develop bladder cancer [17] it is highly unlikely that the epithelial proliferations in our explant cultures represent a preneoplastic phenomenon. Although different culture conditions may account for the discrepancies in culture periods and morphology the use of rapidly proliferating neonatal urothelium instead of slowly replicating adult urothelium as starting material might offer an explanation of the observed differences.

The cultured neonatal bladder cells expressed several keratins consistent with their epithelial nature (Table 1). The expression of vimentin is not surprising as in vitro growing epithelial cells generally express this marker more commonly associated with mesenchymal differentiation [4]. Reactivity of cultured urothelial cells with the monoclonal antibody CAM5.2 directed against a common epitope of keratins 8, 18 and 19 fits with its expression in both adult and neonatal mouse bladder epithelium (data not shown). The absence of reactivity with RGE53 (specific for keratin 18) is surprising as this antigen can generally be found in the umbrella cell layer of both neonatal and adult mouse bladder (own observations) as well as human bladder [15]. The lack of detectable expression of keratin 18 in our cultures suggests a failure of in vitro differentiation into umbrella cells. Expression of keratin 10 in a varying proportion of the cells indicates the induction of squamous metaplasia under in vitro conditions as this particular keratin is exclusively expressed by human [14] and mouse squamous epithelia, and no expression of keratin 10 was found in normal mouse bladder be it adult or neonatal (data not shown). Squamous metaplasia is frequently encountered in inflammatory conditions and in human and murine bladder cancer. The molecular biologic mechanisms leading to squamous metaplasia of urothelium are not yet understood [16].

The DNA polyploidy characteristic for normal mouse urothelium was also reflected in the long-term urothelial cell cultures. Since the DNA ploidy level of urothelial cells increases from the basal cell to umbrella cell layer [2], polyploidy can be considered as a differentiation-related phenomenon. This aspect of cellular differentiation appeared to be preserved during the entire long-term culture period of neonatal mouse urothelium. This phenomenon of polyploidization was, however, not described in a previous report on short-term culture of human urothelial cells [18]. Flow cytometry studies on non-neoplastic human bladder epithelial cells demonstrated the presence of a variable proportion of DNA-tetraploid urothelial cells which became particularly prominent during inflammation [10]. Also a proportion of human bladder cancers is DNA-tetraploid and on the basis of their more favourable prognosis it has been suggested that in bladder carcinogenesis DNA tetraploidy might represent a transitional stage to the development of DNA-aneuploid tumors with less than 4n DNA [1]. Thus, the ability of polyploidization might be preserved during the initial steps of bladder carcinogenesis.

Morphologically, 2 cell populations, i.e. a population of cells with small nuclei and another of larger cells with large nuclei and prominent nucleoli, could be distinguished in the urothelial cell cultures. Sorting of DNA-diploid, tetraploid and octoploid nuclei and subsequent cytologic and morphometric study revealed that the DNA tetraploid and octoploid populations corresponded to the cell population with larger nuclei and prominent nucleoli. Both cell populations clearly demonstrated mitotic activity. Thus, in vitro proliferation was not restricted to the cell population closely resembling basal cells, but this property was also conferred upon the DNA-tetraploid large cell population. Similarly, BrdU incorporation was found in both cell populations. In regenerating mouse urothelium after cyclophosphamide mediated necrosis as well as after the physiologic perinatal shedding of urothelium DNA synthesis was also found without accompanying mitotic activity thus generating tetraploid cells from diploid cells [2, 9]. The same mechanism of polyploidization might be effective in our in vitro cultures.

It is expected that the establishment of primary long-term mouse urothelial cell cultures preserving several properties of normal mouse urothelium will contribute to a better understanding of urothelial cell kinetics and bladder carcinogenesis.

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