Polyamine-stimulated Growth of Cultured Rat Urinary Bladder Epithelial Cells

James A. Roszell, Carolyn J. Douglas, and Charles C. Irving
Veterans Administration Hospital and Department of Urology, University of Tennessee Center for the Health Sciences, Memphis, Tennessee 38104

SUMMARY

A technique for isolating and establishing long-term cultures of rat urinary bladder epithelium has been devised. Cells isolated and cultured by this method have been grown for 12 weeks without subculturing. Rat bladder epithelial cells require the addition of putrescine, spermine, and spermidine to attain maximum growth and long-term survival. Monolayer cultures have been subcultured and carried through five passages.

INTRODUCTION

At least 3 methods of inducing predominantly transitional cell carcinoma of the rat urinary bladder epithelium are available: N-butyl-N-(4-hydroxybutyl)nitrosamine administered in drinking water (12, 13); N-methyl-N-nitrosourea administered intravesically (9, 10); and N-[4-(5-nitro-2-furyl)-2-thiazoly]formamide fed in the diet (15, 19). Studies on mechanisms of carcinogenesis and on in vitro carcinogenesis using these model systems have been hindered because no methods for the long-term culture of rat urinary bladder epithelium were available. Reported methods for culturing urinary bladder epithelium of other species (5, 6, 16) have not been successful with rat bladder epithelium.

We report a method for isolating and culturing rat bladder epithelial cells with long-term growth potential and subculturing capability. Our method involves the stimulation of growth by the addition of the polyamines, putrescine, spermine, and spermidine to the culture medium. The availability of these cells will be an asset in the study of bladder carcinogenesis.

MATERIALS AND METHODS

Animals used in these experiments were 3- to 6-week-old male Sprague-Dawley rats supplied by Charles River Breeding Laboratories, Inc., Wilmington, Mass. Nembrual (sodium pentobarbital) was from Abbott Laboratories, North Chicago, III. Trypsin and McCoy's Medium 5A were from Grand Island Biological Co., Grand Island, N. Y. Calf serum was purchased from Worthington Biochemical Corp., Freehold, N. J. Horse serum was from K. C. Biological, Lenexa, Kans. Hydrocortisone, insulin, putrescine, spermine, and spermidine were purchased from Sigma Chemical Co., St. Louis, Mo. Thermox coverslips were supplied by Microbiological Associates, Bethesda, Md. [5-3H]Thymidine (40 to 60 Ci/m mole) was from New England Nuclear, Boston, Mass. NBT2 nuclear tract emulsion was from Eastman Kodak Co., Rochester, N. Y. All other chemicals were from Fisher Scientific Co., St. Louis, Mo.

Bladders were removed from the rats under light anesthesia with Nembutal and collected in PBS. Bladders were everted on an 18-gauge needle from which the bevel had been removed, tied with sterile 3-0 silk suture, and inflated with 0.3 to 0.5 ml PBS. Inflated bladders from 6 animals were placed in a 15-ml solution of 0.3% collagenase:0.25% trypsin dissolved in McCoy's Medium 5A. The bladders were then placed in the cold at 4° for 4 hr as suggested by Bonar. After treatment in the cold, the bladders were incubated with shaking at 37° for 5 to 7 min. The bladders were removed with forceps, and the cell suspension was centrifuged at 1000 rpm in a tabletop International Clinifuge for 5 min. The cell pellet was resuspended in McCoy's Medium 5A containing 15% horse serum, hydrocortisone (5 μg/ml), insulin (5 μg/ml), 1 μM putrescine, 4 μM spermine, and 4 μM spermidine. Cell counts and viability were determined in 0.05% Trypsin B. Cells were plated at a density of 1 to 3 × 10⁶ cells/ml in plastic tissue culture dishes and incubated at 37° in an atmosphere of 5% CO₂ and air. The cultures were left undisturbed for 24 to 48 hr after which the medium was changed 3 times per week. Monolayers were obtained in 2 to 4 weeks.

Monolayer cultures were passaged by harvesting with a solution of 0.25% trypsin dissolved in 0.1 M NaCl:0.01 M trisodium citrate, pH 7.8. The cells were centrifuged as described above and plated at 1 to 2 × 10⁴ cells/ml in growth medium. First-passage cells monolayered in 1 to 2 weeks.

Histology specimens were prepared from cultures grown on 25-mm round coverslips and fixed in 2% glutaraldehyde buffered with 0.05 M phosphate, pH 6.8. The coverslips were then stained with either hematoxylin or eosin or toluidine blue according to the method of Trump et al. (20) and mounted on slides with Permount.
Autoradiographs were prepared from coverslip cultures that were labeled with [5-^3H]thymidine (0.4 \mu Ci/ml) for 60 min. The labeled cultures were washed in 0.9% NaCl solution and then fixed with four 250-ml changes of 2% glutaraldehyde in 0.05 M phosphate buffer, pH 6.8; one 250-ml wash of distilled water; and one 250-ml wash of 95% ethanol. The coverslips were air dried and mounted on acid-cleaned slides for autoradiography.

Autoradiography was performed essentially as described by Boren et al. (3). Slides were dipped in melted NTB2 nuclear track emulsion at 46°. Slides were drained, cooled, and stored for 2 weeks at 4° in taped Bakelite slide boxes containing Drierite. Exposed slides were developed in D-19 at 15° for 6 min and fixed with acid hardening fixer for 10 min at 15°. Fixer was removed with 20 changes of distilled water, and slides were stained with hematoxylin and eosin. A total of 1000 labeled and unlabeled cells were counted. Labeling index was calculated as (labeled cells/total cells) \times 100.

For electron microscopy, cells were scraped from monolayer cultures and suspended in PBS. Cells were pelleted at 2000 \times g and fixed in 5% glutaraldehyde. The cell pellet was postfixed with 1% osmium tetroxide and embedded in Spurr. Electron micrographs were taken on a Zeiss EM 10 transmission electron microscope.

RESULTS

The average yield of cells from 1 bladder was 5 \times 10^6 cells. Viability of our preparations, measured by Erythrosin B dye exclusion, was 95 to 99%. The plating efficiency determined at 48 hr was approximately 1%; this resulted in very few cells remaining attached to the surface after the 1st medium change. Small colonies were apparent in 3 to 4 days.

Our initial efforts to obtain monolayer cultures of rat bladder epithelial cells capable of long-term survival were unsuccessful. A number of different media were tried. The best results were obtained with McCoy's Medium 5A with 10% fetal calf serum supplemented with hydrocortisone (5 \mu g/ml) and insulin (5 \mu g/ml). Cells in these cultures grew slowly but died after 3 to 4 weeks in culture. Attempts to passage these cells into secondary cultures failed. However, when rat bladder epithelial cells were cultured in McCoy's Medium 5A supplemented with horse serum, hydrocortisone, insulin, and polyamines, monolayers with only minor contamination by fibroblasts were obtained in 2 to 4 weeks, and the cells remained viable without subculturing for periods up to 12 weeks.

Table 1 shows the effects of adding polyamines on the labeling index of rat bladder epithelial cells measured at 1 week of culture. The best and most consistent growth was obtained with medium supplemented with putrescine, spermine, and spermidine in \mu M concentrations of 1:4:4; higher concentrations of polyamines showed lower growth rates and increased toxicity. Light and electron microscopy of rat bladder epithelial cell cultures showed cells of epithelial morphology with fine-structure characteristics of epithelial cells (Figs. 1, 5, and 6). The culture-doubling time between 4 days and 1 week was 27 hr. During the 2nd and 3rd weeks of culture, the cell density had increased to such a level that contact inhibition caused the doubling time to increase to 460 hr (Fig. 2). Similarly, the labeling index was 18% at 1 week, 1.79% at 3 weeks, and 0.65% at 5 weeks of culture. Older cultures showed increasing numbers of large, flattened cells (Fig. 3).

Table 2 shows the effects of horse serum concentration on the labeling index of cultured rat bladder epithelial cells at 1 week of culture. The highest labeling index of 53.1% was obtained with 15% horse serum. Additions of polyamines to cultures containing fetal calf serum resulted in the death of cultures within 2 days.

Rat bladder epithelial cell cultures have been carried through 5 passages without any changes in morphology (Fig. 4). Passaged cells showed more rapid initial growth than primary cultures and monolayered in 7 to 10 days, but they did not lose their ability for contact inhibition of growth.

DISCUSSION

Attempts by other investigators (8, 14) to maintain normal rat bladder epithelial cells for periods greater than 4 to 5 weeks have been unsuccessful. On the other hand, culture of urinary bladder epithelium from other species has

<table>
<thead>
<tr>
<th>Additions to control medium</th>
<th>Labeling index (% at 1 wk)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>8.2 ± 2.6^e</td>
</tr>
<tr>
<td>Putrescine (1 \mu M)</td>
<td>6.2 ± 0.5</td>
</tr>
<tr>
<td>Spermidine (4 \mu M)</td>
<td>5.0 ± 0.9</td>
</tr>
<tr>
<td>Spermine (4 \mu M)</td>
<td>8.4 ± 3.9</td>
</tr>
<tr>
<td>Putrescine (1 \mu M), spermidine (4 \mu M), and spermine (4 \mu M)</td>
<td>18.0 ± 4.4</td>
</tr>
</tbody>
</table>

^e Mean ± S.E.

Table 2

Effects of serum concentration on labeling index of rat bladder epithelial cells

Rat bladder epithelial cell cultures were plated at an initial density of 1 \times 10^6 cells/ml in 35-mm dishes with 25-mm coverslips in McCoy's Medium 5A supplemented with hydrocortisone (5 \mu g/ml), insulin (5 \mu g/ml), 1 \mu M putrescine, 4 \mu M spermidine, and 4 \mu M spermine. Horse serum was added at the concentrations indicated. Cultures were labeled for 1 hr with [5-^3H]thymidine (0.4 \mu Ci/ml), and autoradiographs were prepared as described in "Materials and Methods." Labeling index, calculated on triplicate cultures, is defined as (labeled cells/total cells) \times 100.

<table>
<thead>
<tr>
<th>% horse serum</th>
<th>Labeling index (% at 1 wk)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>35.0 ± 1.8^e</td>
</tr>
<tr>
<td>10</td>
<td>37.6 ± 2.8</td>
</tr>
<tr>
<td>15</td>
<td>53.1 ± 1.7</td>
</tr>
<tr>
<td>20</td>
<td>48.3 ± 1.4</td>
</tr>
</tbody>
</table>

^e Mean ± S.E.
ACKNOWLEDGMENTS

We feel our methods offer the means to obtain large numbers of uniform primary cultures of long-term growth potential for studies that involve carcinogenesis in rat bladder epithelium. These cells have a high growth capability, undergo contact inhibition, and can be subcultured. Monolayers of rat bladder epithelium have been kept for up to 12 weeks from the initiation of the cultures. This is 2 to 3 times as long as times previously reported for cultures of rat bladder epithelium. The increase in life-span of our cultures is attributed to the use of horse serum supplemented with polyamines rather than fetal calf serum.

REFERENCES


Fig. 1. Photomicrograph of a 2-week-old rat bladder epithelial cell culture grown on McCoy's Medium 5A supplemented with 15% horse serum, hydrocortisone (5 µg/ml), insulin (5 µg/ml), 1 µM putrescine, 4 µM spermine, and 4 µM spermidine, as described in "Materials and Methods." The epithelial morphology is shown. Toluidine blue, × 100.

Fig. 2. [5-³H]Thymidine autoradiography of a 3-week-old culture of rat bladder epithelial cells. A larger number of labeled cells are near the periphery (lower left) of a colony rather than at the center (upper right) of the colony shown. H & E, × 40.

Fig. 3. Photomicrograph of a 2-week-old rat bladder epithelial cell culture showing an area containing larger cells (compare with Fig. 1) that become more common with increasing age of the culture. Toluidine blue, × 100.

Fig. 4. Photomicrograph showing rat bladder epithelial cells 10 days after the 1st passage. The darker staining areas are more densely packed epithelial cells, but the cells are not piled up in these areas. Toluidine blue, × 25.

Fig. 5. Electron micrograph showing a single cultured rat bladder epithelial cell from a 2-week-old culture. × 17,200.

Fig. 6. Electron micrograph showing portions of 2 adjoining cells from a 2-week-old culture of rat bladder epithelial cells. Tight junctions and moderately well-developed Golgi apparatus are apparent. × 40,870.
Polyamine-stimulated Growth of Cultured Rat Urinary Bladder Epithelial Cells

James A. Roszell, Carolyn J. Douglas and Charles C. Irving


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/37/1/239

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link http://cancerres.aacrjournals.org/content/37/1/239.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.