In Vitro Cultivation of Epithelial Cells Derived from Tumors of the Human Urinary Tract

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SUMMARY

Finely minced explants from 54 TCCs of the human urinary tract were cultured in vitro in an attempt to establish cell lines. Cells with epithelial morphology grew out from 48 tumor explants, and long-term cell cultures were established from 10. Six of the cell cultures have been maintained for over 18 months with 50 to 70 transfers and, therefore, are considered permanent cell lines. The epithelial cells in the established cultures are small, exhibit rapid doubling time, and show multilayering. The cells were examined both microscopically and by cultivation techniques, and they were found to be free from contaminating microorganisms, including Mycoplasma. The established cultures grow rapidly in roller bottles and, therefore, can be produced in large quantities. These cells also remain viable after being stored for 3 years in liquid nitrogen.

INTRODUCTION

Several investigators (1, 11, 14) have reported the establishment of cell lines derived from human urothelial tumors. However, these reports emphasize the difficulties encountered in attempting to cultivate the tumors, and no laboratory previously has reported success in establishing more than 1 long-term cell line from TCC. For example, Rigby and Franks (11) attempted to culture cells from 18 human bladder tumors, but only 3 of the cultures were maintained for 7 transfer generations, and all had a fibroblastic morphology and a normal diploid karyotype. A 4th culture (RT4), derived from a papillary tumor of the bladder, possessed an epithelial morphology and was maintained in culture for more than 80 transfer generations. In 1968, Yajima (14) reported attempts to culture cells from 34 human urinary bladder tumors. Cells grew out from 18 of the tumors, and 11 formed monolayers after 1 month in culture. Although 9 of the cultures started by Yajima were of pure epithelial morphology, only 1 of these could be maintained in culture for more than 12 months.

Initially, in 1970, Bubenik et al. (2) reported that epithelial cells grew out from 4 of 8 bladder tumors placed in culture, but no long-term cell lines were established. However, in 1973, Bubenik et al. (1) described the establishment and characterization of a cell line (T14) derived from a human urinary bladder carcinoma. On the basis of the reported success rate for propagating cell lines from TCC, it is not surprising that no laboratory has yet reported the establishment of more than 1 long-term TCC cell line.

Previously, we reported the establishment and characterization of a cell line (253J) derived from a metastatic lymph node from a patient with multiple transitional cell tumors of the urinary tract (4). Utilizing the technique for establishing the 253J cell line, as well as another method, we have established 5 additional long-term TCC cell lines, as reported herein.

MATERIALS AND METHODS

Primary Culture: Method 1. Tumor tissues were obtained at surgery and brought directly to the tissue culture laboratory in RPMI Medium 1640 (Grand Island Biological Co., Grand Island, N. Y.) containing 15% heat-inactivated fetal bovine serum (Armour Co., Kankakee, III.), 100 units of penicillin per ml, and 100 μg of streptomycin per ml (Grand Island Biological Co.).

In most cases, processing of the tumors began within 15 min of the time they were removed from the patient. The tissue was minced very fine (1 mm) with sharp scissors in a sterile Petri dish. The minced tissue was floated in an appropriate amount of outgrowth medium (RPMI Medium 1640 containing 15% heat-inactivated fetal bovine serum, 10% tryptose phosphate broth, 2 mM L-glutamine, 100 units of penicillin per ml, and 100 μg of streptomycin per ml) in a Petri dish, and the small fragments and the medium were drawn up into a 10-ml plastic syringe without a needle. Approximately 1 ml of outgrowth medium containing tumor fragments was placed in each of several 25-cm Falcon flasks, and the flasks were rotated to distribute the fragments evenly over the surface. The flasks were then incubated at 37° without CO2 and not disturbed for 24 hr. It is of primary importance to use only a minimum amount of medium, or many of the tissue fragments will not attach to the surface of the flask. After growth began, 2 to 3 ml of outgrowth medium were added carefully so as not to disturb the attached pieces.

Primary Culture: Method 2. With a technique described by Warren and De La Cruz (13), the tumor tissue was finely minced, and 5 to 6 small fragments (approximately 1 mm) were placed in a group with 4 groups in a 100-mm tissue culture dish. A small amount of sterile silicone grease (Dow...
Corning Corp., Midland, Mich.) was placed near each group with a syringe. A sterile coverslip was placed so that it covered the tissue with 1 edge touching the silicone grease and gently pressed into position. The silicone grease holds the coverslip in position, and it, in turn, immobilizes the tissue fragments against the surface of the plate. Fifteen ml of outgrowth medium were added to each plate, and the plates were incubated at 37° in a humidified atmosphere of 5% CO₂.

Subculture. When the primary cultures reached a monolayer, the cells were removed from the flask or Petri dish by the technique previously described by Elliott et al. (5).

Cell Freezing and Storage. Cells were removed from the flasks by trypsin treatment, washed 4 times in outgrowth medium, counted, and diluted in storage medium (RPMI Medium 1640 with 30% fetal bovine serum, 10% d-methyl sulfoxide, penicillin, and streptomycin) to a concentration of 5.0 × 10⁶ cells/ml. Three ml of the cell suspension were added to each plate, and the plates were incubated at 37° for 1 hr and then put into the vapor phase compartment of a liquid nitrogen refrigerator (Minnesota Valley Engineering Co., New Prague, Minn.). For revival of the stored cells, the ampuls were removed from the refrigerator and thawed at 40°. The cells were washed several times in outgrowth medium and seeded at a density of 5.0 × 10⁶/ml of outgrowth medium in 75-sq cm Falcon plastic flasks.

Large-Volume Production. Glass roller bottles (No. 7000 Bellico disposable bottles; Bellico Glass Co., Vineland, N. J.) were used to produce large volumes of tumor cells. Before the cells were planted, the surface of each bottle was conditioned by adding 100 ml of outgrowth medium and rotating the bottle at 37° for 30 min. The medium was then removed from the bottle and replaced with 100 ml of fresh outgrowth medium containing 1 × 10⁷ tumor cells. The bottles were placed in a Bellico roller bottle incubator at 37° and rotated at 0.5 rpm.

RESULTS

Method 1. Cultures were observed daily for evidence of cell growth from the tumor explants. In 48 of 54 tumors (89%), epithelial-like cells grew out from the tumor explants within 24 to 48 hr after planting (Fig. 1). In the case of 12 tumors, the epithelial cells continued to grow for 1 to 4 weeks, then began to round up and detach from the surface of the flask. These cultures exhibiting this spontaneous degeneration have been described in detail previously (5).

Each of these cultures showed complete destruction of the cells within 7 days, and attempts to subculture the detached floating cells proved futile. Extensive culture and ultrastructural studies failed to detect bacteria, mold, yeast, Mycoplasma, or ubiquitous viruses.

Cells from 36 tumors grew rapidly and were subcultured. However, 28 of the epithelial cell cultures could not be kept in culture longer than 12 months. At various times between 2 and 12 months after planting, the cells would either grow very slowly or cease to grow altogether. The cells that remained on the flask usually were much larger than the characteristic small TCC cells and were frequently multinucleated. All attempts to keep these cells in culture by increasing the serum concentration, by adding L-glutamine, nonessential amino acids, insulin, cortisone, or testosterone, or by using Eagle’s minimum essential medium, Medium 199, or Earle’s minimum essential medium instead of our usual medium did not prevent the death of these cells. Even when earlier passage level of the same cells were revived from storage in LN₂ and placed in culture, the cells stopped growing after a short period of time.

Eight long-term cell lines, in culture more than 1 year, have been established by this method. Four of these lines (253J, 292W, 192B, and 647V) have been subcultured more than 50 times and continue to grow at a consistent rate. The cells are small (averaging 10 μm in diameter), epitheloid, and usually uninucleate. They grow rapidly in culture, exhibit multilayering, and can be subcultured at a 1:1 to 3 split at weekly intervals (Fig. 2). For practical reasons, the other 4 lines, after more than 20 passages in culture, have been stored in LN₂ for future study. Culture and ultrastructure studies in our laboratory and by HEM Corp. (Rockville, Md.) have failed to detect bacteria, yeast, molds, or Mycoplasma in these cultures.

Method 2. Cells growing out from tumor tissue placed in culture by Method 2 (Fig. 3) took much longer to reach a monolayer than those cultures by Method 1. Production of cells in sufficient quantity to allow subculture required 3 to 8 weeks. In the case of 2 tumors that eventually became established lines (486P and 639V), no epithelial cells were observed until the tumor tissue had been in culture approximately 4 weeks (Fig. 4), and only fibroblasts grew initially from the 639V tissue. As epithelial cells began to grow, the fibroblasts near the tumor tissue began to degenerate, and the epithelial cells slowly replaced the fibroblasts.

Table 1 summarizes the results of attempts to establish cell lines from TCC of the human urinary tract by the 2 culture methods used in this study.

Revival of Cells from LN₂ Freezer. Cells retrieved from LN₂ storage settled onto the surface of the flasks and began to grow within 1 hr after planting, often forming a monolayer in 4 to 7 days. Cells have been revived from LN₂ storage of early passages of each of the 10 long-term cultures with no apparent damage or change in growth pattern.

Large-Volume Cell Production. TCC cells grew better on plastic flasks than on disposable glass roller bottles, but after several passages of the cells on plastic flasks, tumor cells seeded in roller bottles at a high cell density (1 × 10⁹ cells/bottle) would grow and form a heavy multilayered cell sheet. Good cell attachment and outgrowth were not obtained if the surface of the bottles was not exposed to outgrowth medium before the cells were seeded. The cells grew out more slowly on glass and required 10 to 14 days to form a monolayer. When confluent, the cells continued to grow in multilayers, and after 3 to 4 weeks in culture, 1 × 10⁹ cells could be recovered from a single bottle. This procedure has allowed us to produce larger numbers of tumor cells in early passages and to store them for future studies.
DISCUSSION

The difficulties encountered by others (1, 3, 10–12, 14) in attempts to establish epithelial cell lines from TCC are also reflected in our work. Eight long-term epithelial cell cultures were established in 48 attempts using cell cultivation Method 1, and 2 additional (3 total) long-term cultures were established in 12 attempts using Method 2. Long-term cell cultures were established by both methods from Tumor 647V. The overall success rate using both methods is 10 of 54, or approximately 18%.

The first method of in vitro cultivation had the advantages of (a) rapid cell outgrowth; (b) not requiring CO2; (c) ease of subculture of cells directly off flasks, and (d) production of large numbers of primary tumor cells in culture. One of the lines (253J) has been in culture more than 3 years and has been characterized. This line, after more than 70 cell transfers, continues to have an epithelium-like morphology, produces tumors in immune-depressed hamsters, and exhibits multilayering. Three other cell lines (647V, 192B, and 292B) have undergone characterization studies and have been shown to possess properties similar to those found in 253J cells.3 Four other cell lines were stored in liquid nitrogen and await further studies.

The second method of in vitro cultivation had the advantages of (a) holding tissue fragments in position for months, which prevents interruption of cell outgrowth resulting from the detachment of the tissue fragments during laboratory manipulation; (b) allowing growth of cells both on the surface of the plate and on the underside of the coverslip; and (c) permitting the culture of minute biopsy specimens that are too small for use in the first method. Using this method of cultivation, cells can be obtained from the coverslip without disturbing the layer of cells growing on the plate. This is of particular importance when fibroblasts also grow out from the tissue, because only epithelium-like cells will frequently grow on the underside of at least 1 coverslip if several tissue culture dishes are seeded with tumor tissue. However, because the amount of tumor tissue that can be placed in each plate is much less than that used in Method 1, the growth of epithelium-like cells in sufficient quantity to allow subculture could take 1 to 2 months. Although different methods of cultivation were used, the cell lines established from Tumor 647V appear to be identical in morphology, growth characteristics, and medium requirements. As mentioned previously, 2 other cell lines, 636V and 486P, were also established using the second cultivation method. Long-term cell cultures from these 2 tumors were not obtained using Method 1. One reason for this may be the slow growth rate of both these cell lines when compared with the cell lines established using the first method. As shown in Table 2, the success rate for establishing long-term lines was slightly higher with the second method.

With the roller bottle technique described in this report, it was possible to produce large volumes of cells from each of the 10 TCC cell lines. This technique provided large numbers of cells from both early and late tissue culture passages so that we could compare the results of cytogenetic, immunology, biochemistry, ultrastructural, oncogenicity, and growth studies. It was also possible to store large numbers of these cells in LN2 and later revive them for tissue culture passage and study.

Not only has it been difficult to establish long-term cell cultures from human transitional cancers of the urinary tract, but similar problems have been encountered by investigators attempting to culture epithelium-like cells from other solid human neoplasms. For example, Giard et al. (7) summarized the results obtained when a series of 200 human tumors was cultivated in vitro in an attempt to establish cell lines. Having obtained only a 6% success rate, the authors concluded that, in contrast to human fibroblasts, epithelium-like cell cultures from human tumors were indeed difficult to develop into the long-term lines.

However, the studies reported herein suggest that a reasonable success rate can be achieved with TCC if a certain

### Table 1

<table>
<thead>
<tr>
<th>Tumor Site</th>
<th>No. of specimens studied</th>
<th>Initial cell growth after tumor attachment</th>
<th>Growth of cells in vitro 1–12 mos.</th>
<th>Establishment of long-term cell lines in cultures longer than 12 mos.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Renal pelvis</td>
<td>17</td>
<td>15</td>
<td>12</td>
<td>4</td>
</tr>
<tr>
<td>Ureter</td>
<td>29</td>
<td>28</td>
<td>19</td>
<td>4</td>
</tr>
<tr>
<td>Bladder</td>
<td>7</td>
<td>6</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Urethra</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Totals</td>
<td>54</td>
<td>48 (89%)</td>
<td>36 (67%)</td>
<td>10 (18%)</td>
</tr>
</tbody>
</table>

* Percentage of transitional cell tumors successfully cultivated in primary and long-term cell culture from different anatomical sites in the human urinary tract.

### Table 2

Comparison of tissue culture results by methods 1 & 2

<table>
<thead>
<tr>
<th>Cell cultures</th>
<th>Method 1</th>
<th>Method 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of tumors cultured</td>
<td>54</td>
<td>12</td>
</tr>
<tr>
<td>No. where cells grew out from tumor</td>
<td>48 (89%)</td>
<td>4 (33%)</td>
</tr>
<tr>
<td>No. where cell lines were established</td>
<td>8 (15%)</td>
<td>3 (25%)</td>
</tr>
</tbody>
</table>

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Cancer Research, 1976, Vol. 36, No. 1
Protocol is followed. First, tumors should be placed in culture as soon as possible. We believe that it is important to process the tumors and place them in culture within 1 hr of their removal from the patient. Second, TCC may be obtained either at open surgery or, more frequently, by transurethral resection of the bladder. We have had our best results in tissue culture with surgical specimens or with tumor removed mechanically through the resectoscope using biopsy forceps. Specimens obtained by transurethral resection with an electrically activated cutting loop often are so badly damaged by the electrical current that they are not satisfactory for cultivation. Third, it is imperative to use an outgrowth medium that provides the proper nutrients for rapid outgrowth of cells from tumor explants. The outgrowth medium described herein appears to meet this requirement for TCC cells. Fourth, it is desirable to use more than 1 method for starting tumor explants in tissue culture. As mentioned previously, both of the methods described were used successfully to establish long-term cell cultures from tumors. Depending on the size and condition of the tumor, one method may prove more successful than the other for establishing cultures.

Cell lines established from human TCC have been used in various immunology and biochemical studies. It was previously shown that humoral complement-dependent cytotoxic antibodies directed against 253J cells were present in the sera of TCC patients (8). Also, lymphocytes cytotoxic to this cell line were obtained more frequently from patients with TCC than from patients without TCC (9). In addition, both 253J and 292W cell lines are being used in biochemical studies to characterize particles concentrated from supernatant culture fluids (6).

Transitional cell cancer of the urinary tract has been studied in the laboratory as comprehensively as any other solid human tumor. There has been special interest in both the immunobiology and virology of these tumors. Almost all of these studies have utilized an established TCC cell line, usually either T24 or RT1. If studies on the basic biology of TCC are to continue and to be expanded, additional cell lines will be required in order to establish or refute the accuracy of data gathered on TCC in the above assay.

REFERENCES
Figs. 1 to 4. Phase contrast photographs of tumor cells in culture. × 400.
Fig. 1. Epithelial cells growing out from tumor (192B) piece (arrow) placed in culture by Method 1.
Fig. 2. Monolayer culture of 192B cells in Passage 25.
Fig. 3. Epithelial cells growing out from tumor (486P) piece (arrow) placed in culture of Method 2.
Fig. 4. Monolayer culture of 486P cells in Passage 16.
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