Development and Application of Basic Research Techniques in Bladder Cancer Research

David F. Paulson, Kenneth R. Stone, Don D. Mickey, Robert A. Bonar, and Heidi Wunderli

Division of Urologic Surgery, Duke University Medical Center, Durham 27710 [D. F. P., R. J. S., D. D. M., R. A. B., H. W.], and Veterans Administration Hospital, Durham, North Carolina 27705 [D. F. P., R. A. B.]

Summary

The growth of transitional epithelial cells with different growth media and growth supports was examined. Sephadex G-10, Bio-Gel P-20, Bio-Glas-1000, DEAE-Sephadex A-50, DEAE-cellulose, CM-Sephadex C-50, acid-soluble collagen, and immobilized collagen fibers were used to enhance plating efficiency. Acid-soluble collagen layers optimally increased the plating efficiency of primary cultures of bladder carcinoma. Media alterations with serial combinations of fetal calf, newborn calf, calf, bovine, and bull serum with minimum essential medium, Roswell Park Memorial Institute Tissue Culture Medium 1640, Connaught Medical Research Laboratories Medium 1066, Medium 199, Grand Island Biological, National Cancer Tissue Culture 135, 1415, McCoy's 5A, and National Cancer Institute medium were established. No promotion of cell division was noted with any one of these basic medium formulations.

Control of transitional cancer of the urinary bladder will be achieved by a coordinated program of prevention, early detection, and eradication of established disease. Although the projects within this program have been goal directed, they have served to identify the problems rather than define the solutions. This early period of hypothesis generation has provided much information not before available, and recapitulation of the major problem areas identified should serve as a guideline in the period ahead.

Prevention

The involvement of viruses in the initiation of bladder cancer is largely speculative. While no firm evidence has been produced that would link either the RNA or the DNA viruses to the production of transitional tumors, there is no reason to believe that humans alone should stand immune to virus-induced cancer. Indirect evidence of the presence of virus-specific proteins in bladder tumors has been provided by Mickey et al. (38, 39). Particles resembling C-type RNA tumor virus isolated from urothelial tumor tissue have been reported, but the role of these virus-like particles in the generation of bladder cancer is unknown (22, 23, 27, 28).

Hybridization studies with probes derived from the most promising of the primate viruses and the search for viral-specific RNA-dependent DNA transcriptase have been tantalizing but inconclusive. However, the failure to identify a causative agent does not exclude the possibility that infectious particles or their genomic products are involved in the generation of this tumor. It is highly probable that the methodology available today is not sufficiently sensitive to detect small quantities of virus, and therefore future work in this area probably will depend on the development of new methodology rather than on the laborious study of ever-increasing volumes of tumor tissue to locate minute amounts of virus.

The role of chemical carcinogens in the production of bladder cancer is much more convincing. Careful epidemiological studies have identified many industrial and environmental carcinogens (12, 35, 40, 45, 46, 51, 66). A large number of these have been confirmed in vivo (7, 16, 17, 24, 25) or in vitro (32, 48). Now information on the events that accompany transformation must be acquired. Initiation and promotion are the 2 basic stages of carcinogenesis (6). The initiation step is irreversible, representing a somatic mutation, and may be associated with detectable alterations in membrane proteins similar to those changes demonstrated by Stone et al. (58) in fibroblast membranes after viral transformation. Morphological changes have been identified in the surface membrane of superficial cells in neoplasms provoked by the in vivo administration of carcinogen to Wistar rats, and the presence of these changes correlates well with irreversibility of the carcinogenic transformation (34, 36). Future studies with chemical carcinogens must focus not only on identification of the agent but also on the events surrounding both initiation and promotion. Perhaps it is at this level that the collective influence of virus and carcinogen impact. The development of a reliable methodology for examination of the molecular events accompanying carcinogenesis would do much to provide needed information. Similarly, the generation of methodology for determining the latent period in the human system would permit monitoring of that population during their period of greatest risk.

Early Detection

Early detection of transitional cancer remains the domain of the cystoscopist and cytologist. No immunological or biochemical markers that permit either early detection or careful monitoring of disease under treatment have been
identified in urine or serum. CEA, a glycoprotein found in fetal gut and in various endodermal carcinomas, was once felt to be a promising marker (30, 31). Early studies indicated that elevated plasma CEA levels were specific for endodermal cancer; however, later studies showed that plasma CEA levels were elevated in nonendodermal cancer and even in some nonmalignant diseases (42, 60, 61). The apparent nonspecificity of CEA may result from antigenic heterogeneity, which exists among CEA molecules (21, 49). Thus, the development of early detection methodology may rest on isolation and identification of CEA-like neoantigens associated with the transforming event.

The lack of a reliable method for in vitro propagation of benign and malignant transitional cells of human origin in either small or large quantity has hindered progress in etiology and early detection (54, 57). Our own early efforts to develop a satisfactory methodology for in vitro culture were marked by a conspicuous lack of success.

Transitional epithelial cells have poor plating efficiency on Falcon tissue culture plasticware (Bioquest, Inc., Cockeysville, Md.). This observation initiated study of the adhesive character of these cells, as compared with prostate and kidney epithelial cells, and involved the use of chromatography supports carrying different surface charges. Those with a net positive charge, such as DEAE-Sephadex and DEAE-cellulose (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) demonstrated marked affinity for the bladder explants so strong that the explants were never able to attach to the culture vessel and, instead, became surrounded by adhered beads or fibers (Table 1). There was little evidence of outgrowth of the cells onto these supports. Experiments with cultured cells indicated that the strong affinity for bladder tissues was to the stromal cells and not to the malignant epithelial cells. These growth supports were felt to offer no distinct advantage in the in vitro growth of bladder carcinoma cells.

Petri dishes were coated with a variety of charged molecules [polyamines (spermidine, spermine, polylysine), polyanions (polyglutamic acid), and acido-soluble collagen (Calbiochem, San Diego, Calif.)] by incubation with 1-mg/ml solutions of the charged molecules followed by distilled water rinse, drying, and UV sterilization. Collagen coating of the Petri dishes was done by the method of Haukshke and Konigsberg (33). Acid-soluble collagen layers on the culture dishes increased the plating efficiency of primary cultures of bladder carcinoma. Neither polyamines nor polyanions could be substituted for this collagen layer. Immobilized whole collagen fibers offered no distinct advantage over acid-soluble collagen.

A large percentage of the highly anaplastic bladder carcinoma tissues can be successfully initiated into culture in vitro under the conditions used for their cultivation. The life cycle of the average bladder cell culture involves attachment of the explants to the culture dish followed by rapid spreading of the cells onto the dish and increase in the cell and colony diameter as the cells spread out. Within 7 to 10 days, however, the cells from the majority of the tumors begin to round up and come off the dish, and by 2 weeks most of the cells have eluted off the dish and the culture is lost. If the cultures are passaged in the early phases of culture (Days 2 to 4), a number of the cells attach to the secondary Petri dishes; these secondary cells also exhibit rapid degeneration. If the primary cells are passaged into secondary culture at the late stages of culture (Days 7 to 10), very few cells attach to the secondary dishes. Colcemid blockade followed by Giemsa staining fails to demonstrate cell division during the attached and spreading phase of the bladder cell cultures.

The failure to identify cell division was felt to be an indicator that the growth medium was deficient in some unknown but necessary ingredient. A number of liquid growth media were obtained from Grand Island Biological Co., Grand Island, N. Y., for comparison. All were supplemented with 20% fetal calf serum (Grand Island Biological), 0.005% streptomycin sulfate (El Lilly and Co., Indianapolis, Ind.) 62.5 units of penicillin G (E. R. Squibb and Sons, New York, N. Y.) per ml, 0.5 µg of Fungizone (Squibb) per ml, and 0.075% sodium bicarbonate. The media tested included minimum essential medium, RPMI 1640, Connaught Medical Research Laboratories Medium 1066, Medium 199, Grand Island Biological, National Cancer Tissue Culture Institute, Dulbecco’s minimum essential medium, RPMI 1415, Ham’s F10, Ham’s F12, McCoy’s 5A, and National Cancer Institute medium. Serumless medium was examined and found to be inadequate for growth of the bladder cultures. No promotion of cell division was noted using any one of these basic medium formulations. Alterations in a variety of supplementary factors, including vitamins, made little difference in the growth of the bladder cells. In addition to alterations in the basic medium formulation, RPMI 1640 and Medium 199 were supplemented with 0.3% tryptose phosphate broth (Difco Laboratories, Inc., Detroit, Mich.) with no observed effect. The growth media also were supplemented with vitamin A (10 ng/ml to 10 µg/ml) with little effect. No promotion of cell division was noted with the addition of the growth factor glycyl-L-histidyl-L-lysine acetate (2 to 20 ng/ml) (Calbiochem No. 363951).

The serum concentration was varied from 5 to 30%. Very little effect was noted in the range of 10 to 25% serum, while decreased cell survival occurred below 10% and above 25%. The type of bovine serum used was of little importance, since fetal calf serum, newborn calf serum, calf serum, adult bovine serum, and bull serum all produced similar responses.

---

### Table 1

<table>
<thead>
<tr>
<th>Type of support*</th>
<th>Size</th>
<th>Affinity for explant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sephadex G-10</td>
<td>40–120 µm</td>
<td>None</td>
</tr>
<tr>
<td>Bio-Gel P-20</td>
<td>50–150 mesh</td>
<td>None</td>
</tr>
<tr>
<td>Bio-Glas-1000</td>
<td>120–200 mesh</td>
<td>Weak</td>
</tr>
<tr>
<td>DEAE-Sephadex A-50</td>
<td>40–120 µm</td>
<td>Strong</td>
</tr>
<tr>
<td>DEAE-cellulose</td>
<td>Fine mesh</td>
<td>Strong</td>
</tr>
<tr>
<td>CM-Sephadex C-50</td>
<td>40–120 µm</td>
<td>None</td>
</tr>
</tbody>
</table>

* All supports were washed with and autoclaved in 0.85% NaCl solution. The supports were then washed extensively with growth medium before use. Bio-Gel and Bio-Glas are trade names of chromatography supports marketed by Bio-Rad Laboratories. The rest were sold by Pharmacia Fine Chemicals, Inc.

---

* The abbreviations used are: CEA, carcinoembryonic antigen; RPMI, Roswell Park Memorial Institute tissue culture medium.
Results. This also would indicate that the sex hormone level of the serum was unimportant in the growth of these cells (Table 2).

Finally, the osmolarity of the culture media was varied between 250 and 350 mOsmoles/kg. Although there were drastic effects on morphology and growth properties of some other cell types, no obvious effect on the growth of bladder carcinoma cells was noted.

The continuing difficulty in establishing transitional epithelium in vitro led to consideration of an immunologically suppressed host for production of human tumors in large volume. Neonatal or unweaned hosts (with relatively immature immune systems), when treated with antilymphocyte serum or antithymocyte serum, supported the growth of malignant human cells in nearly 100% of the cases (14, 15, 41). These animals, however, uniformly rejected the tumor with time. In order to circumvent this problem, we chose to examine the athymic nude mouse as an in vivo culture system. The nude mouse is a mutant, with the homozygous nude gene (nu/nu) resulting in an immune systems), when treated with antilymphocyte serum or antithymocyte serum, supported the growth of malignant human cells in nearly 100% of the cases (14, 15, 41). These animals, however, uniformly rejected the tumor with time. In order to circumvent this problem, we chose to examine the athymic nude mouse as an in vivo culture system. The nude mouse is a mutant, with the homozygous nude gene (nu/nu) resulting in an immunologically deficient (26, 47). Benign and malignant tissues have been heterotransplanted into these mice, and the resultant growths were serially transplanted (50). Histological identity is maintained between the original tissue and that growing in the mouse.

Male and female 6-week-old weanling nude mice from an outbred NIH Swiss colony have received s.c. implants of human bladder carcinomas. Ten tumors have been implanted; 3 were implanted as a fine mince and did not take. The remaining 7 were implanted as small s.c. tumor nodules. Two of the 7 have produced growth; 5 are too early to determine whether growth has occurred. One of these 2 bladder carcinomas has been serially transferred 4 times in 6 months. Histological identity has been maintained during in vivo passage.

Control of Established Disease

Carcinoma of the bladder will account for 30,000 new cancer diagnoses in 1976. Treatment of disease demonstrating muscle invasion has routinely been either surgery or radiotherapy. Either treatment modality alone seems to provide less control than treatment by combination of the 2 modalities (3, 13, 19, 29, 53, 63-65, 68). Survivorship in certain patients with invasive disease seems improved at 3 and 5 years if they undergo preoperative radiotherapy. This enhanced survivorship is noted particularly in those patients who experience total ablation or a downstaging of their disease (53, 63-65). Preoperative radiotherapy reduces local recurrence rates; however, many patients then die of metastatic disease, presumably from distant microscopic metastases undetectable prior to surgery. It seems reasonable to attempt control of micrometastatic disease by postoperative adjunctive chemotherapy.

There are relatively few studies of chemotherapy trials of bladder carcinoma (10). Responses have been noted in at least 4 patients treated with 5-fluorouracil, Adriamycin, methotrexate, or mitomycin C (5, 9, 11, 20, 52). There have been a few responses with Cytoxan and hydroxyurea. In general, the numbers of patients studied have been small and the criteria for response were neither clear nor consistent.

Recent experience urges the development of postsurgical adjuvant chemotherapy (4, 59). Selection of the appropriate drugs for human use is based primarily on the results derived from Phase 2 trials of randomly chosen agents.

While animal models have been promoted as an aid in drug selection, there is a hazard, in that the models do not always closely approximate the human system (18, 56). Development of methodology for the rapid screening of chemotherapeutic agents active against transitional cell carcinoma on an individualized basis would permit effective selection of adjuvant agents. In vitro screening procedures have been developed (1, 2, 37, 62), and they correlate well with the in vivo response (55, 67). Preliminary studies with a modification of the procedures based on inhibition of cellular dehydrogenase activity by prospective chemotherapeutic agents in microtiter plates should allow assay of multiple human tumors and agents with relatively few tumor cells and should permit quantitation, as serial dilutions of drug can be used.

Future efforts to control established disease must adjust basic research techniques to the patient care problem. Until that transition can be accomplished, we will be forced to sort through cumbersome clinical tools and to laboriously dissect from our armamentarium those concepts and methods of control that are relatively ineffective. Prevention may prove to be a biological impossibility; early detection and control of established disease are a prospective reality.

Table 2

<table>
<thead>
<tr>
<th>Serum</th>
<th>Testosterone* (ng/100 ml)</th>
<th>Estrogens* (pmoles/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fetal calf*</td>
<td>22</td>
<td>349.3 ± 27.6</td>
</tr>
<tr>
<td>Newborn*</td>
<td>12</td>
<td>223.9 ± 19.9</td>
</tr>
<tr>
<td>Calf*</td>
<td>17</td>
<td>55.3 ± 1.7</td>
</tr>
<tr>
<td>Bovine*</td>
<td>10</td>
<td>64.8 ± 3.2</td>
</tr>
<tr>
<td>Bull*</td>
<td>671</td>
<td>&lt;10</td>
</tr>
</tbody>
</table>

* Assayed by Bio-Science Laboratories, Van Nuys, Calif. Average value for mature human male, 300 to 1200 ng/100 ml.

* Assayed by Dr. D. W. Schomberg, Duke University. Assay is predominantly directed at a measure of estradiol level but also measures the presence of estrone and estriol.

* Pooled sera obtained from slaughterhouse by Grand Island Biological Co.

* Pooled sera obtained from controlled herd by K. C. Biologicals.

* Obtained by bleeding a single animal at North Carolina State University, Raleigh, N. C.

References
4. Bonadonna, G., Brusamolino, E., Valagussa, P., Rosso, A., Brugnatelli,
D. F. Paulson et al.


Development and Application of Basic Research Techniques in Bladder Cancer Research

David F. Paulson, Kenneth R. Stone, Don D. Mickey, et al.


**Updated version**
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/37/8_Part_2/2969

**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/37/8_Part_2/2969.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.