Development of Tissue Culture Procedures for Predicting the Individual Risk of Recurrence in Bladder Cancer

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Summary

We are using three correlated approaches in tissue culture to develop procedures for distinguishing between histologically similar tumors and to develop distinctions that we hope can be correlated with a favorable outcome or with recurrence of more serious disease. Our procedures involve study of the growth of resected tumor tissue in a three-dimensional matrix of collagen-coated cellulose sponge. Using bladder cancer cell lines we are also studying the patterns of cytotypic zonation that appear in response to prolonged exposure to continuous gradients of oxygen tension and of temperature. Finally, we are using vitamin A and modifiers of cyclic adenosine 3':5'-monophosphate as molecular probes to alter the morphological expression of tumors in matrix and in gradient cultures.

We have studied over 80 specimens of clinical cancer in matrix culture. Tumors of similar histopathology grow with distinctly different architecture in the matrix of collagen-coated sponge. We must now determine whether these patterns in vitro can be correlated with the course of individual patients.

Introduction

At the core of surgical pathology is the axiom that resected tissue can provide clues to the future biological course of the related residual tissue in the patient. Unfortunately, proliferative growths that are indistinguishable to the surgical pathologist occur in patients whose subsequent courses differ very much. There are many routes that investigation can take to account for these discrepancies. Differences that may reside in the host can be immunological or hormonal or may involve other homeostatic systems. Alternatively, differences may be in the tumors themselves even though they are of similar histopathology. These differences, which are not evident by evaluation with classical histopathological techniques, may be recognizable when the biological behavior of the tissue is observed in the laboratory. Tumor tissues of similar histopathology have been evaluated by their transplantability into a laboratory animal host and in 1 of several organ sites. For human malignant melanoma, Greene (6) made a good case for the prognostic value of transplantation by determining whether or not tumor fragments would grow in the anterior chamber of the guinea pig eye.

Our laboratory has concentrated on the proposition that tissue culture techniques, because of their versatility, offer the best potential for identifying clinically important differences between similar-appearing tumors. Our goal has been to develop assay systems that will distinguish between early, similar-appearing, proliferative abnormalities of uroepithelium, and that will enable us to classify them in ways that can be correlated with later clinical behavior. In a preliminary stage of our work we observed that urinary bladder tumors of similar histopathology from several patients grew in very different patterns in vitro (1).

We are converging from 3 directions on the problem of characterizing early bladder cancer with tissue culture. With the 1st approach, we grow clinical tumor tissue in a matrix of collagen-coated cellulose sponge and study the morphogenetic responses of the cultured tissue with routine histological techniques (7-10, 12). In the 2nd approach, cells are grown as a monolayer and exposed to a continuous gradient of oxygen tension, i.e., meniscus-gradient culture (13, 14, 16), or to a range of temperatures from 46-28°. In the 3rd approach we are studying the response of cancer cells to several molecular probes, such as vitamin A and modifiers of cyclic 3':5'-AMP (19, 21).

Materials and Methods

For tissue culture study of clinical material, by whatever culture technique, certain conditions are essential. Resection of tumor must be accomplished by the surgeon with minimal risk to the viability of the tissue, while not compromising the patient's welfare. Since the use of hot surgical instruments severely damages viability, unheated cutting instruments are required. The tissue must be placed promptly in an environment conducive to maintaining viability, i.e., a modified culture medium with pH between 7.2 and 7.6 and supplemented with appropriate antibiotics. The antibiotics and concentrations used must not be significantly toxic to the tumor tissue.

When the surgery is conducted in one city and the tissue culture study in another, conditions for maintenance of viability are of particular importance. This matter merits independent future research. In the tissue culture laboratory the specimen must be handled in such a way that it can be used readily for the preparation of viable inocula, whether they consist of fine tissue particles of several sizes.

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1 Presented at the National Bladder Cancer Conference, November 28 to December 1, 1976, Miami Beach, Fla. This work was supported by Research Grants CA 14137 and CA 17772 from the National Cancer Institute through the National Bladder Cancer Project.

2 Presenter.
or of completely dissociated cells. The regular preparation of viable cells in suspension from a consecutive series of solid specimens of bladder carcinoma has not been accomplished, and this is another problem for future research.

For meaningful correlations between observations in vitro and the clinical course of individual patients, the continued collaboration between urologist and laboratory investigator is essential. In our experience and in the experience of others, this collaboration runs an uncertain course. The problem may require a professional person in the laboratory group whose primary concern is the integration of laboratory and clinical data.

The last methodological essential concerns the techniques by which the research team hopes to develop clinically important indices of discrimination between tumors of similar histopathology. The possibilities include identification of antigenic features, synthesis of particular enzymes or of other metabolites, specific abnormalities observable with surface scanning electron microscopy, and growth in culture in which interaction is examined between cells at several levels of integration.

Matrix Cultures. The use of a matrix of cellulose sponge for studying 3-dimensional growth in vitro was introduced in 1951 (7). The matrix in current use, a collagen-coated cellulose sponge, is a decided improvement, since many cells adhere, migrate, and proliferate on collagen-coated substrates. In our procedure with clinical bladder cancer, a fine mince of tumor tissue is placed in the slice of sponge, and the combination is embedded in a fibrin clot. Details of this technique have been published (10). At least 1 culture is fixed for histological study at zero time, as soon as the fibrin clot has formed. The others are incubated in tubes with 20% fetal calf serum in Waymouth's medium supplemented with antibiotics. After 1 week the cultures are fixed for histological study.

When the surgical specimen is adequate for inoculating several sponges, a 2nd series is prepared in which tumor mince and a mince of chick embryonic tissue are combined. This permits histological examination of the interaction of the outgrowth of carcinoma with embryonic tissue. Several embryonic tissues, including heart and liver, have been used.

Gradient Cultures. Gradient culture involving oxygen tension was first described by Osgood and Krippahlne (15). We have developed the model with particular emphasis on the study of neoplastic epithelial cells. Two gradients have been examined. In the 1st model a monolayer, growing on a slide inside a culture tube, is changed from the usual horizontal position to a vertical position. A column of medium covers the lower part of the monolayer while the upper part is exposed directly to the gas phase in the culture tube.

In the 2nd model a monolayer is arranged on a plexiglass platform and exposed to a continuous gradient of temperature ranging from 46–28°. Studies thus far have been limited to rat bladder cancer cell lines NBT II (18) and R 4909 (17) and to a line derived from normal dog kidney, MDCK (4).

Molecular Probes. Many investigators have observed in tissue culture that various chemicals modify cell function, morphology, and even differentiation. Several of these substances may serve as bioassay reagents when added to cultures of similar-appearing tumors from different patients. They may induce microscopic changes in the cultures from some patients and not others, and thereby enable us to recognize differences that may be clinically important. The chemicals under study in our laboratory as bioassay reagents are known to modify keratinization and transport activities of epithelia in vitro.

Results

Matrix Cultures. Early in this study we observed different patterns of growth from tumors of similar histopathology. Figs. 1 to 3 illustrate one patient's tumor; Figs. 4 to 6 are from another patient. Figs. 1 and 4, zero-time specimens, show transitional cell papillary carcinomas of similar grade. Compare the 1-week-old cultures of these 2 tumors (Figs. 2 and 5). In Fig. 2 the tumor is seen to be migrating extensively into the collagen-coated cellulose sponge as monolayers and multilayers. In Fig. 5 the tumor appears as many small nodules in a sponge interstice, with almost no migration into the matrix. When combined with chick embryonic heart, the patterns differed markedly too. In Fig. 3 there is extensive migratory interaction of tumor and chick connective tissue. In contrast, in Fig. 6 the tumor cells are arranged as a circular cluster separated from surrounding connective tissue by a distinct plane of cleavage. Comparisons of this kind make it clear that tumors of similar histopathology may be distinctly dissimilar in their biological potential.

Our experience with early and late human bladder cancer in matrix culture includes 81 cultures. We have observed and identified a number of histological qualities in matrix culture that may serve eventually as indices for discriminating between patients with different future clinical courses. A review of the histology of 40 cases has been completed and provides the basis for the following tabulation and comments.

I. Tumor tissue only in matrix culture
   A. migration into the matrix
      1. as monolayers
      2. as multilayers with stratification
   B. no migration; nodular rearrangement of the explant
   C. in the case of an explant consisting largely of fibrous tissue, formation of a coat of carcinoma cells around the explant
   D. polarization of epithelium
   E. formation of glands or cysts
   F. squamous change and keratinization
   G. frequency and distribution of [3H]thymidine-labeled nuclei
   H. frequency and distribution of mitoses
   I. appearance of tumor giant cells
   J. degree of fibrin clot lysis

II. Tumor plus embryonic heart in culture
   A. isolation of large clusters of viable carcinoma cells, with discrete planes of cleavage between tumor and chick heart fibroblasts
   B. infiltrative intermingling of carcinoma cells and fibroblasts

III. Uniformity of pattern for a single surgical specimen
   A. high degree of uniformity
   B. marked variation from one field to another

The patterns illustrated in Figs. 1 to 6 were the first to draw our intense interest, since they suggested the possi-
bility of a simple means of discriminating in culture between tumors that were otherwise virtually indistinguishable. We have seen 1 of these 2 patterns in nearly pure form in all cultures of some specimens. For others, however, the patterns were "mixed" or "intermediate," i.e., the tumor tissue in some microscopic fields of the same slide was nodular, and in others it was migrating.

Crude quantification is possible for this histological index and for the others listed above in the same way that semi-quantitative observations may be made in other contexts of surgical pathology. We have compared the frequency with which we found nodular, mixed, or migrating patterns in bladder carcinomas of low and of high grade. For low-grade tumors the incidence was 50% nodular, 5% mixed, and 45% migratory. For high-grade tumors the incidence was 42% nodular, 42% mixed, and 16% migratory.

In zero-time sponges of some tumors, the donor tissue fragments were surrounded by many mononuclear cells. We thought that such donors would give rise to migrating patterns in the matrix. We found, however, that patterns of exfoliation at the outset did not correlate either with the grade of the original specimen or with any particular pattern of growth after 1 week in culture. A number of patterns were seen when different tumors were combined with chick embryonic heart. The pattern that developed when tumor and embryonic heart were combined could not, however, be predicted on the basis of the behavior of the tumor grown alone. The migratory pattern was not regularly accompanied by an infiltrating pattern when presented with stroma. The nodular pattern was not regularly correlated with cleavage between tumor and chick embryonic heart.

A review of the 40 cases studied to date makes it clear that there is marked biological heterogeneity of morphological expression in 3-dimensional matrix culture. The cultures are being carefully reviewed to semiquantify them with regard to the indices presented in the previous table. The donor patients’ records are being studied to determine the subsequent course. Some of our specimens were resected as early as 1971. We will be comparing those who are doing well with those doing badly to determine whether any constellation of indices of matrix culture has distinctly favorable or unfavorable prognostic correlation.

**Gradient Cultures.** In meniscus-gradient cultures the quality of growth on the glass slide immersed in medium changed after several weeks, with densest growth appearing on the glass surface just below the meniscus of the liquid column. Several reports have been published on this meniscus-gradient system (13, 14, 16). In studies with cell lines we reported that each of several bladder cancer cell lines responded to the gradient with a distinctive pattern of banding transverse to the axis of the gradient. One aspect of the banding was the presence of mononucleated tumor giant cells in 1 zone, a zone that was characteristic for each cell line. When NBT II cells were fed 2 or 3 times weekly, giant cells in mitosis were almost never seen. With daily replacement of medium, however, a dramatic change took place, and numerous NBT II giant cells underwent mitoses, many with highly abnormal figures.

In thermal-gradient culture, the zone of growth at the upper end of viability, 38-42°C, was of particular interest. For cell line NBT II, mononucleated giant cells became numerous in 1 week, and in many of them we observed very abnormal divisions with 3 or more metaphase plates. In the same zone 1 week later giant cells were less common, and there were, instead, many plaques of proliferating small cells (5).

We have not been able to apply the meniscus-gradient or the thermal-gradient models to clinical bladder cancer because of our present inability to regularly obtain dispersions of cells that yield monolayers, even though the specimens are obviously viable as evaluated in matrix culture. When the methodology for obtaining viable monolayers from clinical bladder cancer has been perfected, we will examine such cells in both models. In meniscus-gradient culture we will then be able to examine the differing sensitivity of carcinoma cells to potential chemical agents along the gradient of decreasing oxygen tension. Combinations of chemical agents might provide rational “broad spectrum” chemotherapy for the individual patient. In thermal gradients, we will try to identify patients whose cells express a distinct sensitivity to hyperthermia, since these persons could be candidates for treatment with hyperthermia.

A gradient system that is presently on the drawing board will expose monolayers of bladder cells to an increasing dosage of radiotherapy. As in the case of hyperthermia, we hope to identify individuals whose tumors are particularly sensitive to radiation. Furthermore, both for heat and for radiation, compounds that are putative enhancers of these physical modes of therapy could be tested for individual patients.

**Molecular Probes.** In matrix cultures of clinical cancer we have observed stratification, squamous differentiation, and sometimes foci of keratinization. In other cultures, we have seen the formation of multicellular cystic structures of 3 distinct types. In work with cell lines we have found that vitamin A inhibited keratinization of a squamous cell carcinoma of the rat bladder (19). We are currently studying other effects of vitamin A on this carcinoma line and have found that vitamin A enhanced both the migration and the DNA synthesis of these cells. With cell lines of columnar epithelial type we have observed the formation of multicellular epithelial hemicysts (11, 20). Formation of hemicysts by cell lines was inhibited by ouabain (2) and enhanced by dibutyryl cyclic 3′:5′-AMP or papaverine (21).

These examples, one where keratinization was modified and the other where cyst formation may be enhanced or blocked depending on the chemical added to the tissue culture system, have been defined with the use of cell lines. We are now ready to apply these probes to matrix cultures of the same cell lines to obtain a view of their effect in a defined 3-dimensional setting. Dosages and durations of exposure, when worked out on matrix cultures of cell lines and transplantable rat tumors, will then be used to identify indices of distinction in matrix cultures of clinical bladder cancer.

**Discussion**

**Potential for Better Treatment.** Management of bladder cancer will be significantly improved as we learn to individualize our approach. Bioassay techniques offer promise of
advances by 2 general means. The first is the realm of more effective treatment. If we can identify the optimal therapeutic regimen for the individual patient when the choices are surgery, radiation, chemicals, hyperthermia, or any combination of these, the results for some patients will be better. The 2nd direction of advance is one in which we learn to identify early which patients can expect serious recurrence and which are highly unlikely to have recurrent disease. The 2nd goal, intellectually equally exciting, may be remote for reasons inherent in our present level of understanding of the natural history of carcinoma.

**Significance of Giant Cells in Carcinomas.** The usual view of giant cells in a carcinoma is that they are end-stage cells, reflections of anaplasia that do not, in themselves, play a role in the subsequent course of the disease. Our observations that tumor giant cells formed readily in prolonged culture of several cell lines in meniscus gradient, and that they formed readily at the upper end of viability in a thermal gradient, suggest that this view should be reevaluated. This suggestion is strengthened by our observation that in the thermal gradient, the early prominence of giant cells, many with highly abnormal mitoses, was followed in a few days by fewer giant cells and by many plaques of proliferating small cells. Perhaps giant cells in division are 1 of the cytological units where cells of increased malignancy arise from a lesion of minimally aggressive cancer.

**Fundamental Problems for Laboratory Exploration.** Too often we think of bladder cancers and other carcinomas, as well, as if the entire replicating cell population were identical. There is good reason to doubt this simplistic assumption on the basis of the appearance of histological sections from a single bladder cancer, and of experimental data of several investigators supporting the view that bladder cancer, even at its inception, may be multiclonal. Our knowledge of the regulatory interaction between monoclonal cells in the tissue of a carcinoma is rudimentary. For tumors of multiclonal character, candidor requires us to recognize that almost nothing is known about the regulatory interaction between cells. We are impressed by the fact that the growth of some tumors in matrix is of a relatively uniform pattern, whereas others vary from one field to another. Whether the 2nd situation is the expression of a multiclonal character or of different degrees of differentiation is not known.

Of equal importance, and more elusive than the multiclonal character of tumors, is the matter of the mechanisms operating as tumors change from one level of malignancy to another (6). Two kinds of explanations have been suggested. Burnet has stated that the only road to novelty is error and selection.3 Foulds suggested that changes in the aggressiveness of tumors may be the expression of a program for neoplastic development, a hypothetical phenomenon that he called “progression” (3). We suggest that the tumor giant cell in division is the place where the change to increased malignancy sometimes occurs. This conference is not the place for an extended discussion on this matter, but we must recognize that our understanding of the increasing aggressiveness of bladder cancer in some patients and not in others may not be effectively dealt with until we confront and better understand some of these fundamental problems.

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**References**

Two bladder carcinomas of similar initial microscopic appearance compared in 3 tissue culture settings. Figs. 1 to 3 are all from one surgical specimen, and Figs. 4 to 6 are from another. H & E.

Fig. 1. Fragment of tumor at zero time in collagen-coated cellulose sponge matrix. The explant is part of a cystectomy specimen from a 69-year-old woman. Beside the distinct area of papillary carcinoma, there are many loose cells in the interstices of the matrix. A trabeculum of the matrix is seen in the lower left. × 165.

Fig. 2. Seven-day-old culture of tumor only. Tumor cells have adhered to the collagen-coated sponge and migrated widely on the complex surface, producing both monolayer and multilayer growth. There are frequent small cystic structures lined by several cells. No such cysts were found in the donor tissue. × 68.

Fig. 3. Seven-day-old culture of tumor in association with chick embryo heart from a 10-day-old embryo. The dense connective tissue is outgrowth from the embryonic tissue. There is a diffuse intermingling of carcinoma and chick fibroblasts. The irregular stippled structure extending diagonally across the field is a trabeculum of sponge. × 165.

Fig. 4. Fragment of tumor at zero time obtained by transurethral resection from a 75-year-old man. The tumor is papillary, and there are numerous single cells in the surrounding interstices. × 165.

Fig. 5. Seven-day-old culture of tumor alone. The tissue has developed foci of anchorage on the collagen-coated sponge, and in 1 small area there is a partial monolayer on the matrix. Most of the tumor appears without attachment to the matrix as small connected papillary-nodular structures in which there is extensive remodeling. × 68.

Fig. 6. Seven-day-old culture of tumor in association with chick embryo heart from a 10-day-old embryo. The tumor appears as a discrete nodule separated from the surrounding connective tissue, which is derived from the embryonic heart. × 165.
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