Growth in Semisolid Agar of Prostate Cancer Cells Obtained from Bone Marrow Aspirates

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ABSTRACT

Thirty-one bone marrow aspirations were performed on patients with prostatic carcinoma metastatic to bone. After separation over a Ficoll-Hypaque gradient viable nucleated cells were cultured in semisolid agar. Colony formation occurred in 14 of 27 (52%) nonbacterially contaminated cultures. Characterization of cells from the colonies showed them to be consistent with malignant prostate cells. After staining, these cells were periodic acid-Schiff positive, prostatic acid phosphatase positive, and prostatic specific antigen positive. Other studies demonstrated the cells to be karyotypically abnormal, ultrastructurally similar to epithelial cells, and capable of secondary colony formation. Three bone marrow aspirate specimens did not have metastatic prostatic carcinoma detected by standard methods but did demonstrate colony formation. However, colony formation was most frequently seen when a radionuclide scan was positive at the aspiration site and when tumor cells were microscopically detectable by Wright staining of a smeared aspirate. The potential utility of colony forming cultures in prostate cancer is discussed. In working with bone marrow aspirates, additional cell separation procedures may be required to calculate and maximize plating efficiencies.

INTRODUCTION

Several characteristics set human prostatic carcinoma apart from other solid tumors. Based on autopsy studies and evaluations of prostate tissue obtained from transurethral resections of benign prostatic hypertrophy, the incidence of a man having prostate cancer rises from 10% at age 60 to 50% at age 80 (1, 2). Yet, less than 1% of these cases are even manifested clinically (3). Prostate cancer is also unique in that the clinical course of 80% of cases can be modulated by androgen suppression (4). Despite these features, prostate cancer has uncommonly been the subject of biological studies.

In part, this lack of research efforts is due to the difficulty in applying many traditional laboratory techniques to study prostate cancer. There are no completely satisfactory animal models for prostate cancer (5, 6), and information obtained from such models with other tumors has inconsistently correlated with the same disease in humans (6). There have been only a few human prostate cancer cell lines developed and characterized (7), and the growth of human prostate cancer in athymic nude mice has met with only limited success (8).

A productive laboratory method over the past two decades has been an in vitro technique using semisolid medium to grow clonogenic cells (9, 10). The application of such methods to the study of human hematopoietic cells has led to major insights into an understanding of normal hematopoietic cell physiology and abnormalities which exist in various hematopoietic disorders, including chronic myelogenous leukemia, acute myelogenous leukemia, asplastic anemia, myelofibrosis, polycythemia vera, and preleukemia (10–13). Similar culturing methods have now been successfully applied to human solid tumors (14). Such experiments in solid tumors have defined cytogenetic abnormalities, new tumor markers, cellular interactions, and a number of pharmacological tumor-host growth modifiers including clinically useful anticancer compounds (15–22).

Metastatic prostatic carcinoma has the clinical characteristic of frequently metastasizing diffusely to bone. We report here our experience of culturing, in semisolid medium, prostate cancer cells obtained from bone marrow aspiration.

MATERIALS AND METHODS

Patient Selection. All patients were followed at the University of Arizona Cancer Center Prostate Cancer Clinic or at the Tucson VA Oncology Clinic and had histologically proven prostatic carcinoma. The malignancy was required not to be in a clinical remission and each patient had to have multiple (>15 sites) bony metastases on technetium radionuclide bone scan. This criterion was an arbitrary one and was present in 70% of all patients seen with metastatic prostate cancer during the last 2 years. Informed written consent for a bone marrow aspiration was obtained under the guidelines of a protocol approved by the Human Subjects Committee of the University of Arizona Health Sciences Center and the Tucson VA Research and Development Committee.

Biopsy Procedure, Site Selection, and Pathological Studies. Bone marrow aspirations were performed via standard technique (23) from either the left or right posterior superior iliac crest or from the sternum at the fourth intercostal space. In patients found to have a nonaspirable iliac crest, a sternal aspiration was performed. Bone marrow biopsies were not studied as adequate numbers of single cells cannot be harvested from this material. When available, a recent radionuclide bone scan was utilized to select from among these sites the one physically closest to a bony site positive on the imaging study. The initial 0.5 ml of marrow aspirate was immediately utilized to prepare smears and clot sections for examination after Wright staining. An additional 5 to 7 ml of marrow were drawn into a 10-ml syringe containing 0.5 ml of preservative-free heparin and promptly delivered to the research laboratory.

Cell Separation. Separation of RBC and most granulocytes from the bone marrow aspirates was carried out with a Ficoll-Hypaque gradient (24). In brief, the bone marrow "buffy coat" was diluted 1:1 with CMRL medium (Gibco), layered over Ficoll-Hypaque (Pharmacia) in a 50-ml test tube, and centrifuged at 80 x g for 40 min. The cells concentrated to a bony site positive on the imaging study. The initial 0.5 ml of marrow aspirate was immediately utilized to prepare smears and clot sections for examination after Wright staining. An additional 5 to 7 ml of marrow were drawn into a 10-ml syringe containing 0.5 ml of preservative-free heparin and promptly delivered to the research laboratory.

Culture Technique and Colony Scoring. The colony forming culture method used was essentially that described by Hamburger et al. (15). Petri dishes (35 mm) were prepared by plating a 1-ml underlayer made up of CMRL plus 20% FCS in 0.5% agar. One million nucleated cells were visible in the central area of the underlayer. The underlayer was supplemented with insulin, sodium pyruvate, 2-mercaptoethanol, 1-glutathione, penicillin, and streptomycin. All cultures were incubated at 37°C in 95% air, 5% CO2 with continuous shaking at 100 rpm.

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The abbreviations used are: FCS, fetal calf serum; CFU-GM, colony forming unit-granulocyte-macrophage; PAS, periodic acid-Schiff.
ture plates were incubated at 37°C in a humidified 7.5% CO₂ environment. After 24 h, plates were examined for clumps and a control plate was preserved by fixation with glutaraldehyde and refrigeration.

All culture plates were examined serially with an Olympus inverted-phase microscope. Colony formation was defined as an aggregate growth encompassing >30 cells, with cluster formation defined as aggregates of >4 but <30 cells. Colony scoring was recorded at 7-day intervals for 35 days.

Characterization Techniques and Morphological Studies. Semisolid culture material was prepared for staining by one of three methods. The "dry slide" method was used to fix an entire platting layer on a glass slide; staining reagents were added directly to the culture plate; or colonies were "plucked" individually from the plates and placed directly onto a glass slide or processed in paraffin (25-27). Staining markers included the following: peroxidase, periodic acid-Schiff, acid phosphatase with and without tartrate, Wright-Giemsa, nonspecific esterase, and prostate specific antigen (28, 29).

Cytogenetic Analysis. Cells cloned in agar were removed from the plates along with the entire upper layer of agar. Samples were then transferred to 16 ml conical centrifuge tubes where they were treated with 0.075 M KCl hypotonic solution prewarmed to 37°C. Finally, cells were fixed repeatedly in methanol:acetic acid (3:1), air dried slides were prepared, and specimens were stained by G-banding as previously described (30).

Ultrastructural Analysis. High resolution morphological studies of the prostatic tumor colonies were accomplished by plucking individual colonies from the agar and placing them directly into plastic conical centrifuge tubes (Corning Glass Works, Corning, NY) filled with Karnovsky fixative (1% paraformaldehyde, 1.5% glutaraldehyde, pH 7.4; 560 mosmol) (31). The colonies were fixed for 90 min at room temperature and then rinsed in 0.1 N cacodylate buffer. Specimens were then postfixed in 2% OsO₄ buffered in 0.072 N cacodylate buffer, pH 7.4, for 1 h in the dark on ice. Colonies were rinsed in cacodylate buffer, stained en bloc with uranyl acetate, dehydrated in increasing concentrations of ethanol, followed by 100% propylene oxide, and finally embedded in Spurr low viscosity resin. Thin sections of the colonies were cut on a Porter-Blum MT2 ultramicrotome with a DuPont diamond knife, mounted on uncoated 200 mesh copper grids (Fullum, NY), and then stained with uranyl acetate and lead citrate. Grids were examined in a Philips 300 transmission electron microscope, operating at 60 kV.

Self-Renewal Studies. Assessment of secondary colony formation (self-renewal) was performed according to the technique of Thomson and Meyskens (27). Briefly, individual tumor colonies were plucked from the agar overlay, passed through small gauge needles to produce a single cell suspension, and then plated into microtiter wells in CMRL plus 20% FCS, an admixture of growth factors, and 0.3% agar. Serial observations were then recorded using the same scoring criteria for colony formation described above.

RESULTS

Cell Yield and Culture Results

Thirty-one bone marrow aspirations were processed and cultured in semisolid agar (results are summarized in Table 1). In the first 12 patients, iliac crest aspirations were attempted and were unsuccessful in 6 of them. All subsequent patients had sternal aspirations done initially which were successful in all patients. The median yield of nucleated cells after separation with Ficoll-Hypaque gradients was 4.52 x 10⁷ cells (range, 8.0 x 10⁶ to 2.0 x 10⁸). The culture plates from four specimens became overgrown with contaminating bacteria within 72 h and were discarded and not assessed for tumor colony formation. There was no of clumping of plated cells in vitro after 24 h.

In the remaining 27 culture experiments, significant CFU-GM occurred in 12 (44%). The CFU-GM growth was documented between days 7 and 14. The CFU-GMs were easily identified based on the cellular components being loosely aggregated (secondary to autodigestion of the agar) and positive peroxidase staining. Scoring of the CFU-GMs in these 12 cases yielded a mean maximum count of 106 colonies (range, 10 to 226 per plate). By days 17 to 21 of incubation, the CFU-GMs began to deteriorate and their counts were markedly reduced. There was no clear correlation between tumor colony formation and CFU-GM formation.

Tumor colony formation occurred in 14 (52%) of 27 evaluable cultured bone marrow aspirates. Counts of tumor colony forming units were maximal in number after 21 to 28 days of culture, and while colonies frequently increased in size after that time, no new colonies were observed to appear. Growth of tumor cells to the cluster stage without colony formation occurred in seven (26%) experiments. No cultured specimens which had not grown aggregates of cells consistent with colony formation by day 21, subsequently did so after 2 additional weeks of observation. The median colony count per plate among those forming colonies was 6 with a range of 1 to 181 colonies per plate. Only 3 specimens had colony counts greater than 30 per plate.

In 12 specimens, an analysis was made of the following factors potentially correlating with the diagnosis of prostate cancer in the bony site aspirated: recent (within 30 days) radionuclide bone scan positivity at the bone marrow aspiration site, assessment of a Wright stained bone marrow aspirate for tumor cells by a hematopathologist, and the presence of tumor colonies forming in semisolid agar culture. Bone scans were "positive" at the aspiration site in 9 of the 12 patients scanned within the preceding month; tumor cells were identified in the aspirates in 7 of the 12; and tumor colonies formed in the cultures in 9 of the 12. Tumor colony formation (mean colony counts per plate of 1, 3, and 16) occurred in 3 bone marrow cultures in which no tumor cells had been detected in smears. In two of these three cases, the bone scans were positive in the area sampled. In two cases where there was no evidence of bone scan activity at the aspiration site, tumor colony formation occurred. Tumor colony formation occurred in all five cultured aspirations where the aspiration contained identified tumor cells and where the bone scan was positive at the aspiration site. Colonies from two of the three cases in which no tumor cells were identified from a direct bone marrow aspirate smear were histochemically characterized and the findings were consistent with cells of prostate cancer origin.

Prostate Cancer Colony Characterization

Staining. Morphological staining was carried out on the tumor colonies grown from eight cases (Fig. 1). All eight tumor colonies studied stained positive with PAS displaying cytoplasmic collections of PAS positive material. All eight tumor colonies did not stain positive for peroxidase and nonspecific esterase. Three tumor colonies were stained with and without tartrate for the presence of acid phosphatase and all three
displayed positive staining for acid phosphatase that was inhibited by tartrate. Immunohistological staining for the presence of prostatic specific antigen was positive in three of three cases studied.

Ultrastructural Studies. Ultrastructural studies revealed intracellular and extracellular components of the tumor colonies. The predominant morphological feature observed in the tumor cells was the accumulation of lipid droplets (Fig. 2). Low magnification views of the cells present in the tumor colonies showed depositions of extracellular matrix in the form of striated and nonstriated fibrils, as well as amorphous material (Fig. 2A). Higher magnification revealed mitochondria, secondary lysosome-like structures, epithelial type cell junctions, rough endoplasmic reticulum, and intracytoplasmic filaments (Fig. 2B). In general, the cisternae of the rough endoplasmic reticulum appeared dilated, indicative of highly productive cells. In addition to the predominant tumor cell type observed in the colonies, occasional cells resembling macrophages morphologically were seen (data not shown). These cells were larger than the tumor cells and typically contained numerous secondary lysosomes and elongated cytoplasmic processes, and they formed no cell junctions with other cells.

Cytogenetic Analyses. Chromosome analyses were successfully accomplished from the colonies grown from four patient samples. All four tumors demonstrated a hypodiploid chromosome number. Detailed banding analysis was performed in one case on cells taken from agar. Results revealed a modal number of 44 (range, 28–50), with several structural alterations, including deletions involving chromosomes 12q and 3p, as well as the presence of a single double minute (Fig. 3). These results (especially hypodiploidy) are consistent with previous reports of metastatic prostate carcinoma and provide strong support for the origin of the colony forming units from a malignant population.

Self-Renewal. One of three attempts was successful for secondary colony formation. In this case, a fourth generation of colonies was accomplished by repetitive replating.

DISCUSSION

Our results demonstrate that prostate cancer colonies can be grown in semisolid agar medium from metastatic cells present in bone marrow aspirations. A characterization of the cells comprising the colonies showed them to be morphologically distinct from normal bone marrow cells and consistent with malignant cells of prostatic origin. Staining patterns for the colony cells were PAS positive, prostatic acid phosphatase positive, and prostatic specific antigen positive. Ultrastructurally, the cells were epithelial in character; cytogenetically the cells contained a hypodiploid number of chromosomes with additional clonal alterations; and the cells were capable of secondary colony formation. These prostate cancer colonies were cultured in some patients from bone marrow aspirates not identified as containing malignant cells by routine pathological study. However, aspirations which did not contain detectable tumor cells and/or were obtained from aspiration sites imaging abnormally on radionuclide bone scanning had the highest probability of demonstrating colony formation. Our characterization studies revealed the potential utility of this culture method in providing fresh tumor cells for cytogenetic and ultrastructural studies.

Colony forming cultures have been shown to have many potential advantages over other culture techniques. In solid tumors cytogenetic abnormalities, new tumor markers, cellular relationships, and clinically applicable growth modulators have been described with these assays (15–22). In prostate cancer little information in these areas is available from standard culture methods of fresh tumor cells (7, 8). The identification of substances which inhibit prostate cancer colony formation would be particularly useful to aid in designing clinical trials, as there are no clearly effective systemic therapies for patients with metastatic prostatic carcinoma progressing, despite primary androgen suppression.

Other research studies in prostate cancer with a colony forming assay are possible. Bone marrow aspirations can be performed safely and repeatedly with minimal patient morbidity. Successful serial colony forming cultures in the same patient could provide an important source of clinical materials to evaluate evolving chromosomal abnormalities, hormone receptor changes, tumor markers, and the development of drug resistance.

At the present time a shortcoming of culturing bone marrow aspirates from patients with prostate cancer metastatic to bone
Fig. 2. A, transmission electron micrograph of cells within a prostate cancer colony. Note the abundance of lipid droplets (L) intracellularly and the amount of extracellular matrix (arrows) deposited between cells. × 7095. B, higher magnification transmission electron micrograph of two cells in a prostate cancer colony. The epithelial-like cell junctions are obvious (closed arrows), as well as intracytoplasmic organelles: mitochondria (M), structures resembling secondary-like lysosomes (SL), nucleus (N), cytoplasmic filaments (F), and rough endoplasmic reticulum (open arrow). × 20233.
is that it is not possible to quantify the actual number of tumor cells in a bone marrow aspiration. Plating efficiencies cannot be calculated or estimated accurately. Our method of plating 1 million cells resulted in a median colony count of only 6 and in only three specimens were colony counts above 30 per plate, the amount of growth needed to reliably perform growth modulating experiments. Few of the experimental applications discussed above could repetitively be successfully carried out with such low plating efficiency. Plating of more than $10^6$ cells per 35-mm Petri dish would not easily permit single cell dispersal. Improved cell separation techniques or the identification of growth stimulators may be necessary to allow the routine study of metastatic prostatic cancer cells in bone marrow.

REFERENCES

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