Role of the matrix in modifying the growth of prostatic epithelial cells in vitro[1]

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Preliminary data demonstrate that migration, proliferation, and differentiation of prostatic epithelial cells in vitro is altered when the cells are grown on an extracellular matrix rather than on bare plastic. Clonal plating efficiencies are used to distinguish this growth and to identify ways to provide an in vitro model for further study of prostatic carcinogenesis. Consequently, this method, with suggested refinements, may improve the understanding of prostate cancer and related disorders.

Index terms: Epithelium, cytology • Prostatic neoplasms

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Adenocarcinoma of the prostate is the second most common malignancy and the third largest cause of cancer death in men, primarily affecting those between 50 and 80 years old.[1] The reason why prostatic carcinoma metastasizes to lymph nodes and bone, and rarely to other target organs, is not well understood. Because extracellular matrices have important effects on cellular growth and behavior, we postulated that clues to tissue predilection by metastatic tumor cells may be found in the composition of the matrix formed by those distant tissues. We have recently found that DU-145 cells, as well as cells derived from patients with benign prostatic hypertrophy or prostatic carcinoma, grow at different rates on matrices than on uncontaminated plastic, and the growth rate of these cells is further dependent on the type of cell from which the matrix is derived. We believe that modified growth on extracellular matrices in vitro may serve as a model for studying the behavior of prostatic carcinoma in vivo.

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Materials and methods

Cells: Cells obtained in a primary culture from patients with benign prostatic hypertrophy, carcinoma of the prostate, and existing cancer-cell lines (DU 145 and LNCP) were used for plating efficiency assays and to provide the prostate cellular matrix. Large numbers of plates with an extracellular matrix (ECM) were made at one time and used in batches for any given experiment or for groups of similar experiments to ensure consistency. Prostate tissue was gotten after obtaining informed consent from the patients and represented the collection of cells discarded by the pathologist (S.H.L.). Cell markers were prostatic acid phosphatase and prostate-specific antigen, as demonstrated by stains using the immunoperoxidase technique. Primary cultures of bovine aortic endothelial cells were obtained as a gift from George Melnykovych, Ph.D., and served as additional sources of the ECM.

Culture methods: Needle biopsy tissue and transurethrally resected prostate chips were collected aseptically and placed in a sterile transport medium (RPMI-1640 without fetal bovine serum). These fragments were stored at 4° C until laboratory processing, which occurred within 18 hours of surgery. When sufficient tissue was unavailable, collagenase dispersion was not used and only explants were cultured. In most cases, however, both techniques were employed. Tissue was split to permit mirror image histologic examination in permanent tissue sections.

The basic medium was RPMI-1640 with L-glutamine (DM-319-01, K.C. Biological, P.O. Box 14848, Lenexa KS 66215) and the following additives: fetal bovine serum, 10%; HEPES buffer, 25 mM; epithelial growth factor (EGF), 10 ng/ml (#40001, Collaborative Research, 128 Spring St., Lexington MA 02173); insulin, transferrin, and selenium (ITS), 5 ng/ml each (#40351, Collaborative Research); penicillin, 50 units/ml; streptomycin, 50 μg/ml; and 5α-androsten-17β,Ol-3-one, 10 ng/ml (A-8380, Sigma, P.O. Box 14508, St. Louis MO 63178). Incubation was at 37° C with 5% carbon dioxide; all cells were humidified.

The explant culture technique, borrowed from Clarke and Merchant,[2] was then carried out. In a large, sterile glass Petri plate, all burned areas of tissue were cut away; the tissue was then minced with two sterile scalpels into 1 × 2 × 1mm sections. By using a sterile Pasteur pipette, eight explants were evenly distributed in 25-cm^2 tissue culture flasks (3013, Falcon, American Scientific Products, Division of American Hospital Supply Corporation, 1430 Waukegan Rd., McGaw Park IL 60085) with inside bottom surfaces made sticky by the application and removal of medium. Next, 1.5 ml of medium was gently added to each flask so as not to shake off the explants. Feeding was three times a week (beginning 48–72 hrs after plating) with removal of all old medium and additions of 2 ml of new medium. Cells could be subcultured and the original explants given new medium for another two or three outgrowths if desired.

Our collagenase dispersion technique was modified from that of Webber.[3] Under aseptic conditions, burned areas were dissected away and prostatic tissue was cut into 3–4mm fragments. These fragments were added to 2 ml of RPMI-1640 plus 200 units of collagenase (Gibco, 3175 Staley, Grand Island NY 14072) per millimeter of medium and stored at 37° C for 36–48 hours. Vigorous pipetting was performed to break up fragments, and then the preparation was re-incubated at 37° C for two hours. After a second vigorous pipetting, the suspension was transferred to 50-ml centrifuge tubes and spun at 1,200 rpm for 10 minutes. The sediment, enriched with epithelial cells (acinar clusters), was washed several times in medium plus 10% fetal bovine serum and suspended in Puck's saline G (Gibco). Vigorous pipetting was once again performed, and the cells were then allowed to settle for 15 minutes. The supernatant, containing mostly fibroblastic elements, was discarded, and the sediment was re-suspended in Puck's saline G. After repeating the maneuver several times, the supernatant was fairly clear. The last sediment was then suspended in culture medium, counted, adjusted to a density of 5 × 10^5 cells/ml, and plated in T-flasks (2 ml/25-cm^2 T-flask or 6 ml/75-cm^2 T-flask). The feeding schedule was identical to that of the explant culture technique.

After removal of medium and several washings with serumless medium, 4 ml of trypsin ethylenediaminetetra-acetic acid (EDTA) 1× solution (Gibco) was placed at a ratio of 4 ml/25-cm^2 T-flask and incubated at 37° C for five to 10 minutes. The suspension was then poured into a centrifuge tube containing RPMI-1640 plus 10% fetal bovine serum, and the cells were spun at 1,200 rpm for 10 minutes. Next, the cells were washed once in culture medium, counted, and resuspended at 5 × 10^5 cells/ml. In each 25-cm^2

T-flask, 2 ml of cells was then plated and fed as done previously. Transfers were performed on confluent or nearly confluent cultures only.

After trypsinization, cells were pelleted and resuspended in culture medium containing 10% dimethyl sulfoxide (2×10^6 cells/ml). Next, they were placed in Nunc tubes (Intermed Corp., supplied by Gibco) and then in Styrofoam wrap within a small cardboard mailing cylinder. When placed in a freezer at −70° C, cooling was roughly 1° C/min. After overnight freezing, the tubes were immediately transferred to permanent storage in liquid nitrogen. To confirm preservation of cell viability, one of the tubes was thawed three to five days later by placing the Nunc tube in a 37° C water bath with gentle agitation. The cell suspension was added to 4 ml of regular culture medium for four to five hours. The medium was then removed gently and replaced with 4 ml of fresh medium and not disturbed for four days. Regular feeding was then resumed according to the explant technique.

As described by Stone et al,[4] the DU-145 cells were maintained in standard culture medium minus the EGF and ITS. Transfer was done every three or four days with a 1:5 or 1:10 split. Cells were not fed between transfers. This culture provided comparative data and positive controls (such as the prostate antigen stain).

As detailed by Horoszewicz et al,[5] LNCaP cells were cultured on medium lacking EGF and ITS. Transfer was done every two or three weeks with cold medium and a sharp tap to the flask; pipetting was carried out to break up clumps. No trypsin-EDTA was used. Cells were fed once each week rather than three times a week.

Confirmation of cell types: At early stages, cells were compared morphologically by light and electron microscopy with epithelial cells in permanent sections of the tissue of origin. Later, prostatic acid phosphatase and prostatic specific antigen immunoperoxidase stain kits (Immulok Incorp., 1019 Mark Ave., Carpinteria CA 93013) were tested with deparaffinized tissue sections cut from formalin-fixed prostatic tissue with appropriate positive and negative controls. Cells in culture were either grown on coverslips until confluent or were plated on slides using the cytocentrifuge and immediately placed in Bouin's fixative, stained, and examined. Karyotypes for chromosomal examination were obtained in the clinical laboratory in selected cases.

Culture on matrices: Cells were inoculated at serial dilutions (10^2, 10^3, 10^4) into plastic 60mm Petri plates with or without the ECM. The medium used was RPMI-1640 with the usual additives (EGF, ITS, etc.). A total of 2 ml of medium was used with each inoculum. The medium was not changed or disturbed during each seven-day experiment. Incubation was at 37° C in 5% carbon dioxide with humidification. All cultures were done in triplicate.

Preparation of the ECM: This technique was similar to that described by Gospodarowicz et al[6] and worked well for us in preliminary experiments. Briefly, cells to produce the ECM were grown to confluency and then washed gently with distilled water and immediately covered with 0.1% ammonium hydroxide for five to 10 minutes. The remaining matrix was then gently washed with phosphate-buffered saline and stored until ready for use.

Plating efficiency assays: These were based on methodology as desribed by McKeehan and Ham.[7] Colonies grown on the matrix or plastic were stained with 0.1% crystal violet and counted. Colonies must have had a diameter of at least 0.1mm to be counted. The number counted divided by the number of cells inoculated resulted in the efficiency, expressed as a percentage. Plating was compared for the ECM and plastic (control) and for the ECM derived from the different cell types as described. Additional controls included the medium on the matrix to confirm that the original matrix-forming cells were not viable.

Statistics: Student *t* test, two-sample t test, and chi-square analysis were the statistical methods employed for this study.

Results

Cells cultured in both primary cultures and subcultures routinely exhibited morphologic characteristics of epithelial cells when seen through the electron microscope (*Fig. 1A*), although by light microscopy, one could not always differentiate an epithelial from mesenchymal appearance (*Fig. 1B*). The gross cell shape often seemed dependent on cell density (i.e., when a sparse number of cells was present, cells were more elongated and spindle-shaped, while closely spaced cells became rounder as cultures grew to confluency). The stains of cells in primary and subcultures were positive for acid phosphatase and prostate-specific antigen, again confirming the epithelial origin of the cells (*Fig. 2*). Carcinoma cells from our patients showed less contact inhibition (cultures tended to choke off and die

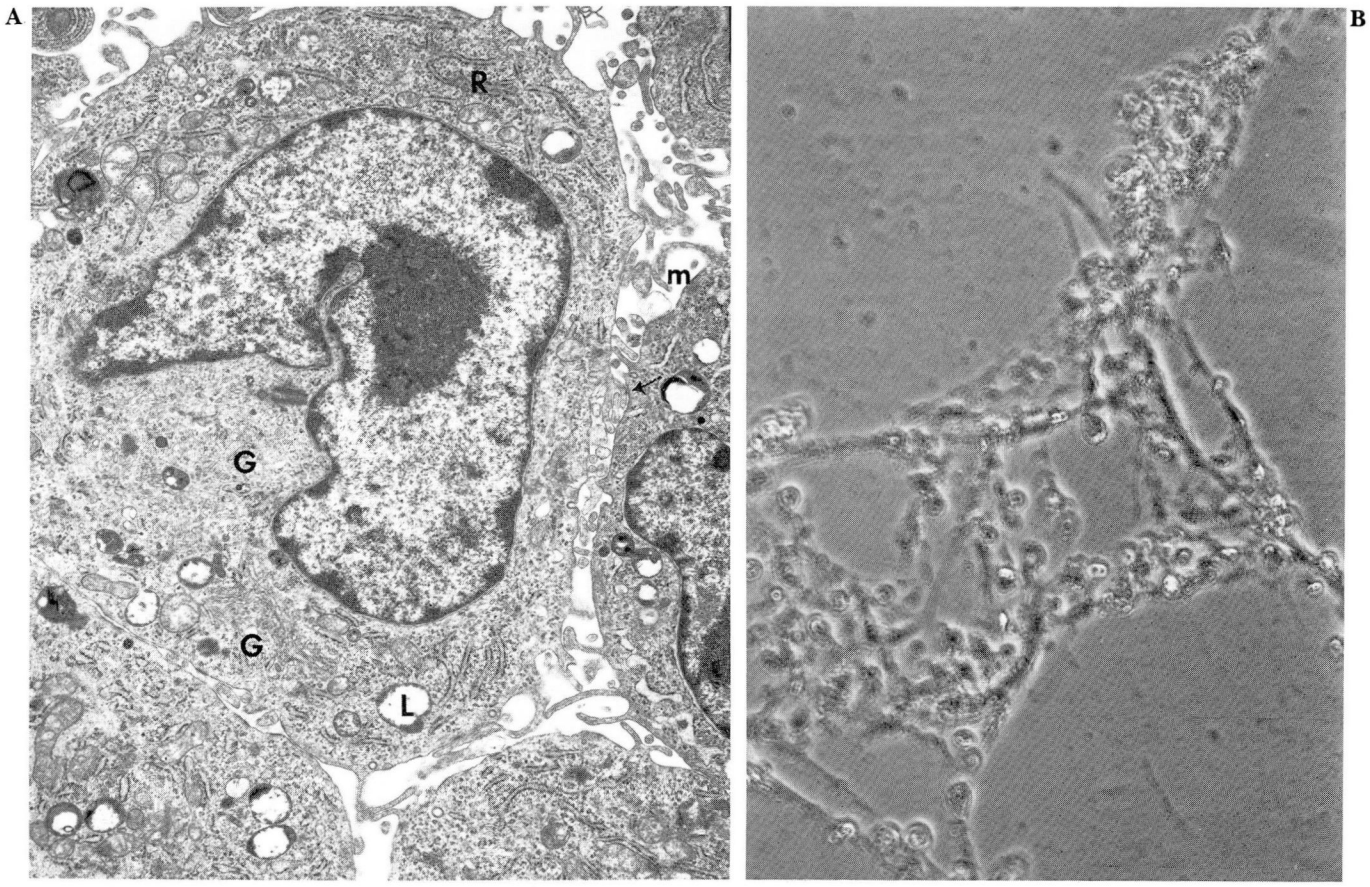

Fig. 1. A. Abundant well-formed microvilli (*m*), a prominent Golgi apparatus (*G*), moderate amounts of rough endoplasmic reticulum (*R*), occasional tight cell junctions (*arrow*), and at times, secretory vacuoles (not shown) are characteristics of epithelial cells examined with transmission electron microscopy (×10,000). *L* denotes a lysosomal vacuole.

B. Both benign and malignant prostatic epithelial cells in a monolayer culture occasionally spindle, pile up, float, or take on bizarre forms. These cells were derived from a patient with high-grade prostatic carcinoma. (Phase contrast, ×100)

unless transferred before confluency) than did the cells derived from benign prostates. More polypoid (as identified by the karyotype) and multinucleated cells were present as well. Still, we could not absolutely prove that the prostatic epithelial cells were cancerous except from prostate glands that were replaced totally by cancerous epithelial cells. By exclusion then, all epithelial cells in the culture were derived from malignant cells.

In preliminary observations, we have examined plating efficiencies (*Fig. 3*) of cells passaged from cultures of both benign and malignant tissue origin, and we also used established malignant prostate cell lines (DU 145 and LNCaP). DU 145 did not form much of an ECM as assayed by crystal violet staining after dissolution by diluted ammonium hydroxide, while LNCaP cells, bovine aortic endothelial cells (BAC-MAC), as well as many of our patients' prostate cells of benign and malignant origin, formed an abundant ECM.

The *Table* depicts results of a number of trials using various types of inoculated cells and matrices. Note that DU-145 cells grew no better on their own matrix than on bare plastic, but they grew relatively better on a matrix derived from cells of the LNCaP line. The plated benign prostate cells did not have observably enhanced growth on their own matrix after a short observation period (1–2 wk), at least not when plated at clonal density, yet the BAC-MAC cells had dramatic clonal growth on a matrix as compared to bare plastic. LNCaP cells and cancer cells from one of our patients exhibited intermediate levels of enhanced growth. In trials with other cells, we sometimes observed unexpected but reproducible results (more diffuse growth of cells within clones plated on ECM compared to plastic [*Fig. 4, A and B*]), and occasionally, we seemed to observe growth inhibition by the matrix.

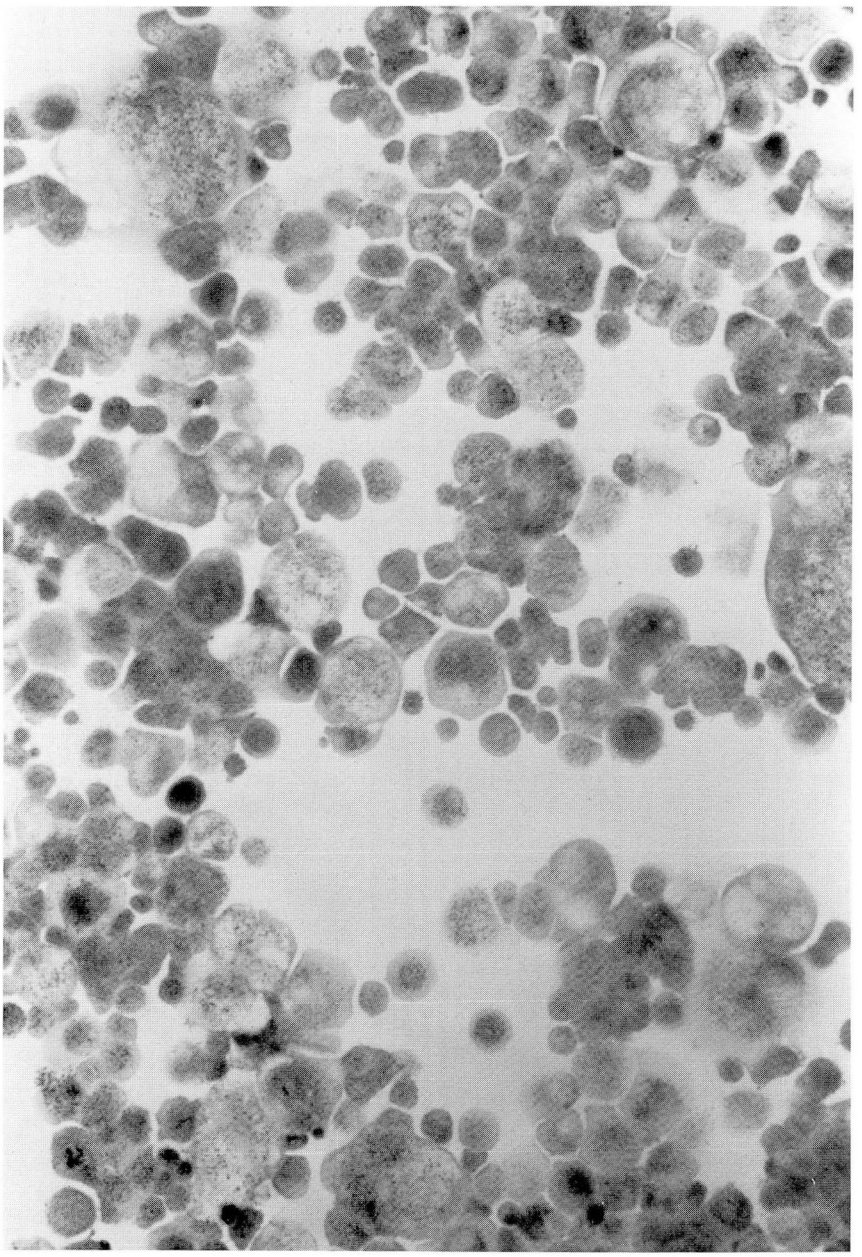

Fig. 2. Fine granules within cultured prostatic epithelial cells depict an immunoperoxidase-stain deposition when a prostatic acid-phosphatase staining technique is used. The stain for the prostatic specific antigen has a similar appearance. (Light microscopy, ×100)

Whether substances accumulated which played a role in contact inhibition or which acted like waste products could not be determined from these early observations, but further studies are pending which may resolve these and other questions.

Discussion

The ECM seems to closely resemble the natural substrate upon which cells migrate, proliferate, and differentiate in vivo. Most cells which grow well in tissue culture retain their ability to produce the ECM, and we believe that the ECM plays a role in modulating cellular morphology and function both in vitro and in vivo.

We could not locate a published work dealing with the growth of prostatic cells on different ECMs as a means to possibly model the behavior of metastatic prostate cancer. Some investigators, however, are growing prostatic epithelial cells on

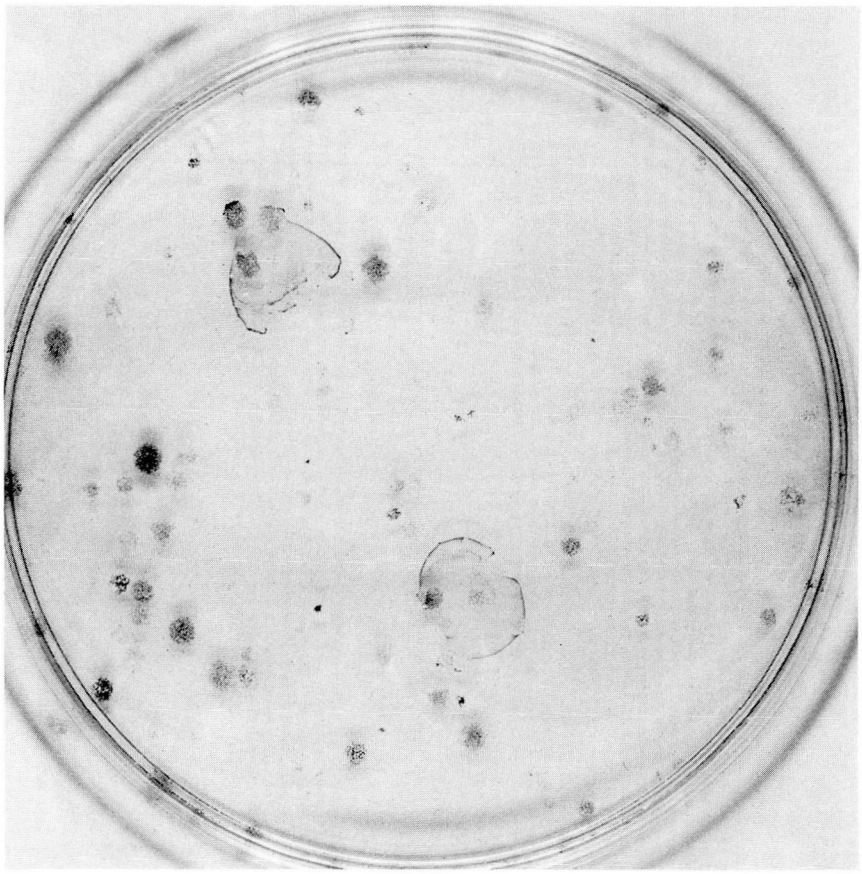

Fig. 3. Clonal growth on the ECM resembles that for a bacterial culture and enables calculation of plating efficiency. Prostate-carcinoma colonies were fixed, stained with crystal violet, and photographed.

Table: Trial results

Cell type inoculated	Matrix derivation	No. of cells inoculated	No. of clones after 7 days on ECM	No. of clones after 7 days on Plastic	% plating on ECM	% plating on Plastic
DU 145	DU 145*	10^2	1	0	1	0
DU 145	DU 145	10^3	15	10	1.5	1
LNCaP	LNCaP	10^2	28	0	28	0
LNCaP	LNCaP	10^3	240	2	24	0.2
BAC-MAC	BAC-MAC	10^2	75	0	75	0
BPH_1†	BPH_1	10^2	0	0	0	0
BPH_1	BPH_1	10^3	0	0	0	0
BPH_1	BPH_1	10^4	120	0	1.2	0
Ca_1‡	Ca_1	10^2	10	0	10	0
Ca_1	Ca_1	10^3	130	0	13	0
DU 145	LNCaP	10^2	8	0	8	0
DU 145	LNCaP	10^3	60	12	6	1.1
LNCaP	Ca_1	10^2	55	0	55	0
Ca_1	LNCaP	10^2	40	0	40	0
LNCaP§	Ca_1	10^2	55	0	55	0
Ca_1	LNCaP	10^2	40	0	40	0

* The matrix was nearly not discernible after staining; all others resulted in an abundant matrix.
† The cells of one patient with benign prostatic hypertrophy passed three times 2½-mo after the initial cultures were obtained.
‡ One patient had carcinoma of the prostate.
§ This group was assayed after two weeks since only scanty growth was evident after one week.

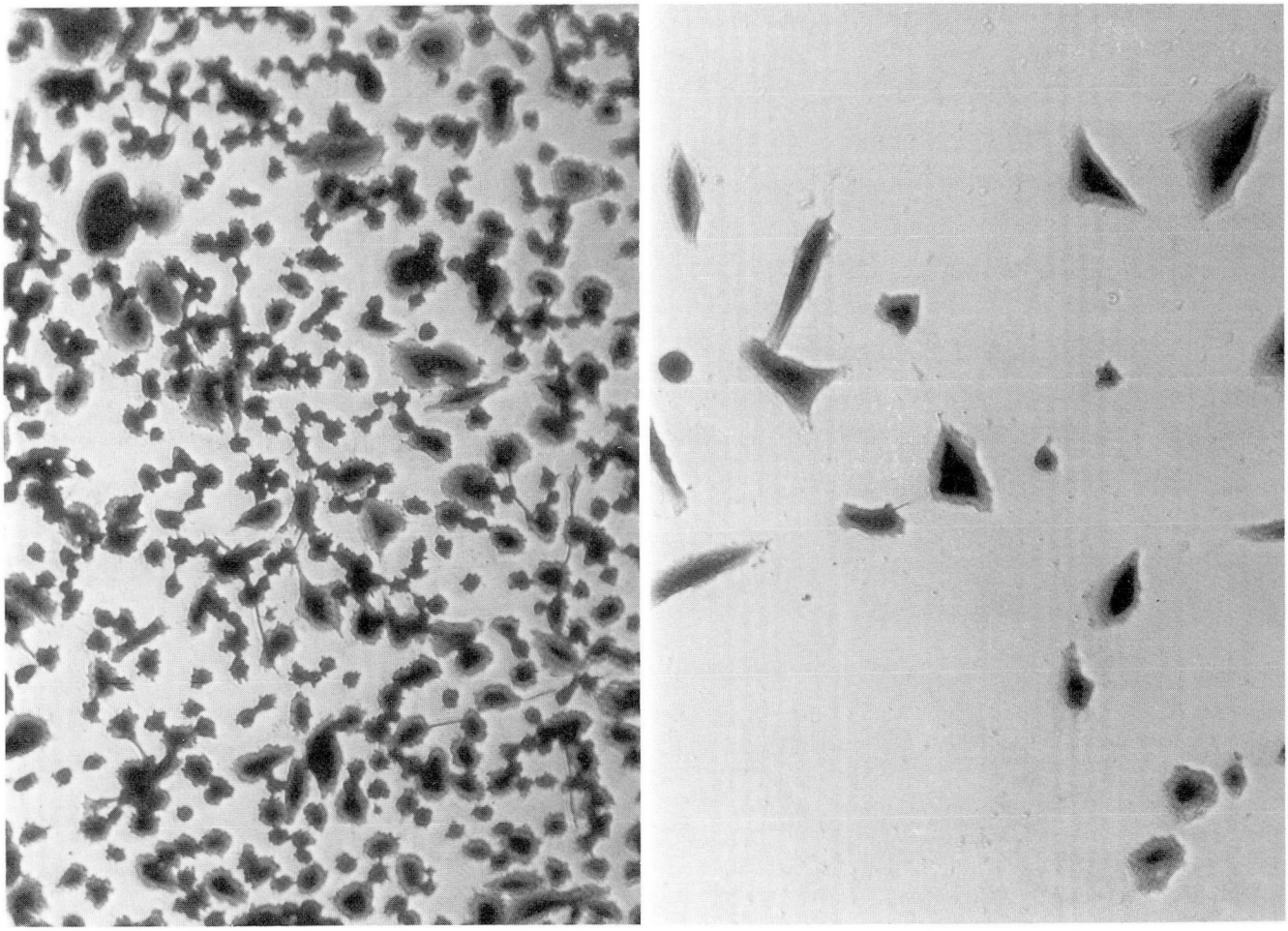

Fig. 4. Note the closer spacing between cells grown on the bare plastic (**A**) compared with those grown on the matrix (**B**) under clonal growth conditions. The cells were fixed and stained with crystal violet in T-flasks. (Light microscopy, ×100)

different substrates (Merchant DJ, personal communication). In general, the work that exists with respect to growth of cells on matrices has been accomplished with other types of cells.

In the model by Gospodarowicz et al,[8] bovine corneal endothelial cells produce an ECM in the presence of certain growth-promoting substances (fetal bovine serum, fibroblast growth factor [FGF]). The cells are then dissolved with diluted ammonium hydroxide or triton X-100, and the matrix is washed. Cells are then re-inoculated and grow normally even without the FGF, while in flasks lacking the matrix the cells grow in disorderly fashion and do not reach proper densities. Cells grown on an ECM in this way exhibit "postponed senescence." Substituting for a matrix with known components in the medium (collagens, fibronectin) does not intensify the growth-enhancing effects seen with a matrix.[8]

Another area of study involves the behavior of cells grown on matrices in the presence of high- and low-density lipoproteins.[9] The FGF is not required with high-density lipoproteins, yet it provides a protective effect against apparently toxic low-density lipoproteins.

Human umbilical vein endothelial cells exhibit reduced proliferative rates, but form crude tubular arrangements when grown on fibronectin matrices.[10] Reduced growth is not completely reversed by addition of the EGF to the medium, a result which may be difficult to interpret with respect to other models.

Although the composition of an ECM has not been completely characterized, it consists primarily of glycosaminoglycans, proteoglycans, and glycoproteins (including collagens, fibronectin, and laminin).[11–14] Collagen has perhaps been the most studied component, and though present in all its isotypes, it is type IV which comprises basement membrane and is synthesized in vitro by endo-

thelial and epithelial cells. How the various components of an ECM interact to improve adherence and growth of cells is not completely clear. Nevertheless, the matrices derived from one cell type may be used to study these and other factors affecting growth of cells from other tissue origins.[15]

One of the important steps involved in metastatic spread of neoplastic cells involves extravasation into target organ tissue and subsequent survival and growth at that site.[16] Until neovascularity develops, the survival of small clones of metastatic cells, indeed the implantability of one or more cells, may depend on characteristics of the ECM present at the chosen locale. Gospodarowicz et al[6] and Gospodarowicz and Ill[8] have demonstrated that growth of cells in tissue culture may be enhanced, even when certain substances are lacking in the nutrient medium, by the presence of an appropriate matrix substance, and in preliminary work, we have observed enhanced plating efficiency on matrices with LNCaP and DU-145 prostate cancer line cells and with benign and malignant prostate cells derived from patients in our hospital. Currently, we are using matrices made from prostate cells and bovine aortic endothelial cells. As work progresses, murine osteoblasts, human cervical carcinoma cells, and possibly, human organ primary cultures (bone, lymph nodes, lung, liver, etc.) can be used for production of additional matrices for study. We are hopeful that, in the near future, plating efficiencies for a variety of malignant prostatic cells will be enhanced more by one type of matrix than by another in a reproducible fashion.

Ultimately, matrices identified as promoting or retarding growth can be prepared by including (^{3}H)- or (^{14}C)-labeled precursor (proline, leucine, mannose, glucosamine, and fucose) in growth media and proteins separated by gradient acrylamide slab-gel electrophoresis followed by fluorography. Comparison can then be made between matrices which promote growth and those which do not to characterize differences in composition which may be subjected to further study.

While present research in this area has just begun, we believe great potential exists to use the ECM-modified cell culture system for in vitro modeling of tissue predilection by metastatic cancer cells. Although there is presently little understanding of why prostate cancer metastasizes to bone and lymph nodes but rarely to other organ sites, it may be that extracellular matrices play a demonstrable role in defining such tissue preference as the target-organ cellular matrices either promote or inhibit growth. We hope that study of the ECM effects on growth of cultured prostatic carcinoma cells, and ultimately, study of promoting substances within the matrices, may enhance understanding of prostate cancer while furthering development of in vitro models which avoid direct human experimentation.

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