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Properties of Epithelial Cells Cultured From Human Carcinomas and Nonmalignant Tissues

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Human epithelial cell cultures were examined for expression of plasminogen activator and fibronectin matrix. All of the cells examined showed ultrastructural evidence suggesting their epithelial origin, including microvilli and specialized junctions. The nonmalignant cells were also negative for endothelial cell markers (ie, they lacked factor VIII antigen, a nonthrombogenic surface and Weibel-Palade bodies). The nonmalignant lines all produced large amounts of plasminogen activator, whereas the tumor-derived lines showed a gradation of activities, ranging from lines having as much activity as the nonmalignant lines to lines having little or no activity above background. For both normal and malignant cells, addition of dexamethesone only slightly decreased the levels of plasminogen activator. By immunofluorescence microscopy, normal bladder and fetal intestine epithelial cells showed fibronectin in a globular and fibrillar matrix. In contrast, normal mammary epithelial cells had a much diminished amount of fibronectin with a punctate distribution.

Key words: carcinoma and nonmalignant cells, fibronectin, human epithelial cells, plasminogen activator

Transformation by various agents (ie, chemicals, viruses) or spontaneously has been associated with various in vitro changes, including increased production of plasminogen activator [1–3], loss of fibronectin containing surface matrix [4], abnormal morphology [5], decreased serum requirements [6], acquisition of colony-forming ability on contact-inhibited monolayers [7], increased plating efficiency on plastic, infinite life in culture [8], and acquisition of the ability to produce tumors when inoculated into immunosuppressed mice [9]. Depending on the cell type and/or the trans-

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forming agent, the properties among this list of alterations that are relevant may differ. For example, many of the properties found to correlate with fibroblast transformation such as refractility, piling up of cells, decreased serum requirement, and increased activation of plasminogen, did not apply to chemically induced transformation of rat liver epithelial cells [10]. Two other properties relevant to fibroblast transformation – ie, agglutination by concanavallin A and karyotypic changes – also did not correlate with epithelial cell transformation [11]. In recent studies, plating efficiency on plastic correlated with tumorigenicity for rat tracheal epithelial cells [12] but not for mouse epidermal cells [13]. Anchorage independence appears to be an important marker for epithelial cell transformation [10, 12–14] and for various fibroblast systems [15–17]; however, there seems to be little correlation between growth in agar and transformation for adenovirus transformed cells [18]. Loss of a fibronectin-containing surface matrix is associated with fibroblast transformation in most [4] but not all [19] systems.

Except for human gliomas [20], there is very little information available on which any of the above criteria are applicable for human malignancies. Despite the fact that approximately 90% of human malignancies are carcinomas, ie cancers of epithelial cell origin, there have been almost no systematic comparisons of the in vitro properties of human epithelial cell lines derived from nonmalignant specimens and specimens representing various stages of malignant progression. The major difficulty has been that currently used media favor outgrowth from the normal stromal components; hence most of the epithelial lines* that have been developed [see reference 22 for a summary] represent the rare variant capable of overgrowing normal fibroblasts. Thus, it is not surprising that Marshall et al [23], when examining human carcinomas of bladder and colon origin, found that all 5 of the lines examined had lost topo-inhibition and grew on contact-inhibited fibroblasts.

We have approached the problem of determining which in vitro parameters correlate with malignancy in humans by developing cell lines of various human carcinomas and nonmalignant epithelial tissues and then comparing their properties [24–27]. The cell lines were cultured by techniques that we developed to remove contaminating fibroblasts; hence these lines, which have not been subjected to the selective pressure of overgrowing normal fibroblasts, may represent types of epithelial cells not previously studied in vitro. Among the lines that grew well enough to be extensively characterized were at least a few lines representing stages of malignant progression, including specimens of metastatic vs primary carcinoma origin as well as lines derived from nonmalignant tissue. In addition, there has been a recent report on techniques to routinely culture normal human mammary epithelium for at least one to two subcultures [29]. In this paper we describe some of our studies to characterize these cultures for various transformation parameters.

MATERIALS AND METHODS

Cell Culture

Epithelial cell lines. All of the cell lines used (listed in Table I) were obtained from the Cell Culture Laboratory, Naval Supply Center (Oakland, CA). The growth medium used was Dulbecco's modification of Eagle's minimum essential medium (#H21HG; GIBCO

*Nomenclature used conforms with that of Fedoroff [21]: A "cell line" arises from a primary culture at the first subculture, whereas an "established cell line" is one that has demonstrated the potential to be subcultured indefinitely.

TABLE I. Summary of Source and Nuclear Morphology of Human Epithelial Cell Lines

Cell source (designation)[a]	Nuclear ultrastructure[b]	Morphology[c]
Derived from nonmalignant tissue		
Fetal intestine (74Int)	Smooth contour lacking invaginations,	Normal
Fetal intestine (677Int)	uniform nuclear envelope, dispersed	Normal
Fetal intestine (680Int)	chromatin, few if any nuclear	Normal
Adult bladder (767B1)	bodies or perichromatin granules	Normal
Derived from primary carcinomas		
Colon (675T)	Tortuous contour with extensive	Abnormal
Colon (785T)	invaginations, irregular nuclear	Abnormal
Breast (578)[d]	envelope, condensed chromatin, many	Very abnormal
Transitional cell (761T)	nuclear bodies and perichromatin granules	Abnormal
Derived from metastatic carcinomas		
Stomach to muscle (746T)	Indistinguishable from primary	Very abnormal
Pancreas to lymph node (766T)	carcinoma lines	Very abnormal
Adenocarcinoma to sacrum (696T)		Very abnormal
Adenocarcinoma to hip (700T)		Very abnormal
Breast to pleura (MCF7)[e]		Very abnormal

[a]Unless otherwise indicated, lines are described in references 24, 26–28.
[b]Summarized from reference 28.
[c]Summarized from reference 24.
[d]Described in reference 25.
[e]Described in reference 40.

Grand Island, NY) containing 4.5 gm glucose/liter and supplemented with 10% fetal calf serum and 10 μg/ml insulin (Calbiochem, San Diego, CA).

Primary mammary cell cultures. Primary cultures of breast epithelial cells were prepared as described elsewhere in detail [29] from specimens of reduction mammoplasties. The specimen was washed in Ham's F-12 medium containing 5% fetal calf serum and antibiotics. Skin tissue and any grossly damaged tissue were cut off. The remaining tissue was gently lacerated with opposed scalpels, areas of lobulated fat were removed and placed directly into Ham's medium containing 5% fetal calf serum, antibiotics, 200 units/ml of collagenase (Sigma, St. Louis, MO), and 100 units/ml of hyaluronidase (Sigma, St. Louis, MO). The tissue was incubated at 37°C at room temperature with gentle rotation with fresh enzyme solution being added every 12–24 h, until by light microscopy the mammary organoids (ducts, ductules, and lobules) were without association with stromal elements. The organoids were separated from single cells by filtration through a polyester cloth filter of 95 μm pore (Pekap, Tetko, Inc., Monterey Park, CA), washed off the filter, and plated in tissue cultures vessels in a mixture of Ham's F-12 medium and Dulbecco's modified Eagle's medium (1:1) plus 5% fetal calf serum, mixed with conditioned medium from several human epithelial cell lines, and containing insulin (5 μg/ml), hydrocortisone (5 μg/ml), 17β-estradiol, and 5-dihydrotestosterone (10^{-9} M), triiodothyronine (5×10^{-8} M), epidermal growth factor (5 ng/ml), and antibiotics. Cells were subcultured by exposure to STV (calcium and magnesium free saline, 0.05% trypsin, 0.02% versine).

Electron Microscopy

Cells were glutaraldehyde fixed in situ (2.5% glutaraldehyde in 0.1 M cacodylate buffer pH 7.3), post-fixed with 1% buffered osmium tetroxide followed by 2% ethanolic uranyl acetate. The specimens were dehydrated in graded ethanol and embedded with Epon. After polymerization, cells were sectioned parallel to the substrate surface with a diamond knife, were mounted on grids, examined, and photographed with a Philips EMZ01 electron microscope operated at an accelerating voltage of 50 V.

Immunofluorescence Studies

Cells were trypsinized and plated onto coverslips at approximately 10^5 cells per 60 mm diameter dish. When the cells reached confluence the coverslips were rinsed with phosphate-buffered saline, fixed in cold acetone, air dried, and kept frozen until assayed.

The indirect fluorescent antibody procedure used in this study has been described [30]. Briefly, dilutions of the antisera were reacted with the fixed cells on coverslips for approximately 1 h at 37°C in a moist chamber. The coverslips were then washed with phosphate-buffered saline (PBS) and reacted with goat anti-rabbit globulin that had been conjugated with fluorescein isothiocyanate [31] for an additional hour at 37°C. The coverslips were washed again and mounted in Elvanol mounting medium on a microscope slide.

Antibody to plasma fibronectin was prepared in rabbits as previously described [32]. The specificity of the antiserum for tissue and plasma forms of fibronectin has been well established [33–35]. Rabbit antiserum to human factor VIII antigen was obtained from Berring Diagnostics (Calbiochem Co.).

Plasminogen Activator

Multi-well linbro dishes coated with ^{125}I-labeled fibrinogen were prepared as described [36]. Each well was incubated with 2.0 ml of medium containing 10% fetal calf serum plus 0.5 NIH units of thrombin for 24 h and then washed once with TD buffer (0.024 M Tris, ph 7.4; 0.14 M NaCl; 0.05 M KCL; 0.0037 M Na_2HPO_4). Unless otherwise indicated, cells that had been trypsinized 4 to 5 days prior to the experiment and that were nearly, but not completely, confluent were trypsinized, and 10^5 cells were added to each well in 2.0 ml of medium. The cells were initially allowed to settle for 5 h in medium containing 2.5% fetal calf serum; subsequently they were incubated with medium containing 2.5% dog serum; since fetal calf serum has been found to possess high levels of plasmin inhibitors. Plasminogen-free dog serum was prepared as described elsewhere [3]. For each of these experiments, one human embryo lung fibroblast line, HeLu, which was positive for plasminogen activator, and one human embryo skin fibroblast line, HeSk, which did not produce plasminogen activator [37, 38], were assayed as controls.

For studies using dexamethasone, cells were grown for 3 days prior to the experiment and maintained throughout the assay in the indicated concentration of dexamethasone, which was added to the medium from a 5×10^{-5}M stock solution in ethanol. The culture receiving no dexamethasone was treated in an identical manner with an equivalent volume of ethanol. To perform the assay, cells were replaced on ^{125}I-fibrin-coated dishes in medium containing 2.5% fetal calf serum plus dexamethasone or ethanol.

Binding Of Platelets

Platelets prepared from 60–120 ml human whole blood according to the procedure described by Tollefsen [39] were generously provided by Drs. D. Gospodarowicz and I. Vlodafsky (University of California, San Francisco, CA). To observe platelet binding,

2×10^8 platelets were added to confluent cultures of cells in 35 mm culture dishes containing 1 ml of Dulbecco's modified Eagle's medium with 0.25% bovine serum albumin and incubated for 30 minutes at 37°C in a humidified CO_2 incubator. Following incubation, the cultures were washed 10 times with the same medium and viewed under phase microscopy.

RESULTS

Description Of The Cell Lines

The source of the cell lines used in this study and a summary of their morphologies and nuclear ultrastructures are shown in Table I. The lines derived from nonmalignant tissue were uniform in morphology and ultrastructure and could readily be distinguished from those lines derived from malignant tissues, which were morphologically [24] and ultrastructurally [28] abnormal.

The lines derived from both normal and malignant tissues showed ultrastructural evidence suggesting their epithelial origin, including microvilli and specialized junctions [41]. Microvilli were plentiful on the free surfaces of the cells in areas where the cells did not closely oppose each other (Fig. 1A). Desmosome-like structures and specialized junctions were also characteristic of these cultures. Figure 1B illustrates adjacent cell membranes lying approximately parallel to each other, occasionally bridged by desmosome attachments (small arrows). In this preparation, the desmosomes appear as dense plaques with short filaments on their cytoplasmic faces. In the middle of this picture, one of the cells has a persistent hemidesmosome (large arrow). Although the membranes are somewhat retracted from each other at this site, flocculent material is just visible in the intervening space. Tight junctions were also readily distinguishable in the cell lines. Figure 2 illustrates a tight junction lined by short fibrils that protrude perpendicularly from the junction into the cytoplasm of the opposing cells (inset). An electron-dense material fills the 100 Å space that separates cells in this region.

Since all of the lines showed the cuboidal morphology [24], specialized cell-attachment and microvilli typical of epithelium, it is likely that they were not derived from normal stromal fibroblasts; however, somewhat similar structures have been reported for vascular endothelium [42]. All of the lines derived from malignant tissues also showed extensive morphologic and ultrastructural abnormalities; therefore, it is not likely that they were derived from normal vascular tissue. However, the possibility remained that the lines derived from nonmalignant tissue were vascular in origin. Although endothelial-specific organelles (Weibel-Palad bodies) were never observed in the electron microscope, recent studies suggest that bona fide endothelial cells need not have this marker [43]. To exclude the possibility that the lines derived from nonmalignant tissue were endothelial in origin, three lines (767B1, 74Int, and 680Int) were tested for an endothelial cell marker, antihemophilic factor (factor VIII antigen), by immunofluorescence microscopy. In contrast to human umbilical vein endothelial cells, which were positive in the assay (Fig. 3A), all three cell lines were negative as illustrated for fetal intestine line 74Int (Fig. 3B). Normal endothelial cells in culture also have a nonthrombogenic cell surface and do not bind platelets. Therefore, we have examined the same three nonmalignant lines (767B1, 74Int, and 680Int) for their ability to bind washed human platelets. As illustrated in Figure 4A, cultured normal bovine endothelial cells did not bind platelets. The few platelets remaining after washing the culture adhered only to the plastic surface accessible at intercellular junctions and not to the cellular membranes. In contrast all three lines (74Int, 680Int, and 767B1) tested for bound

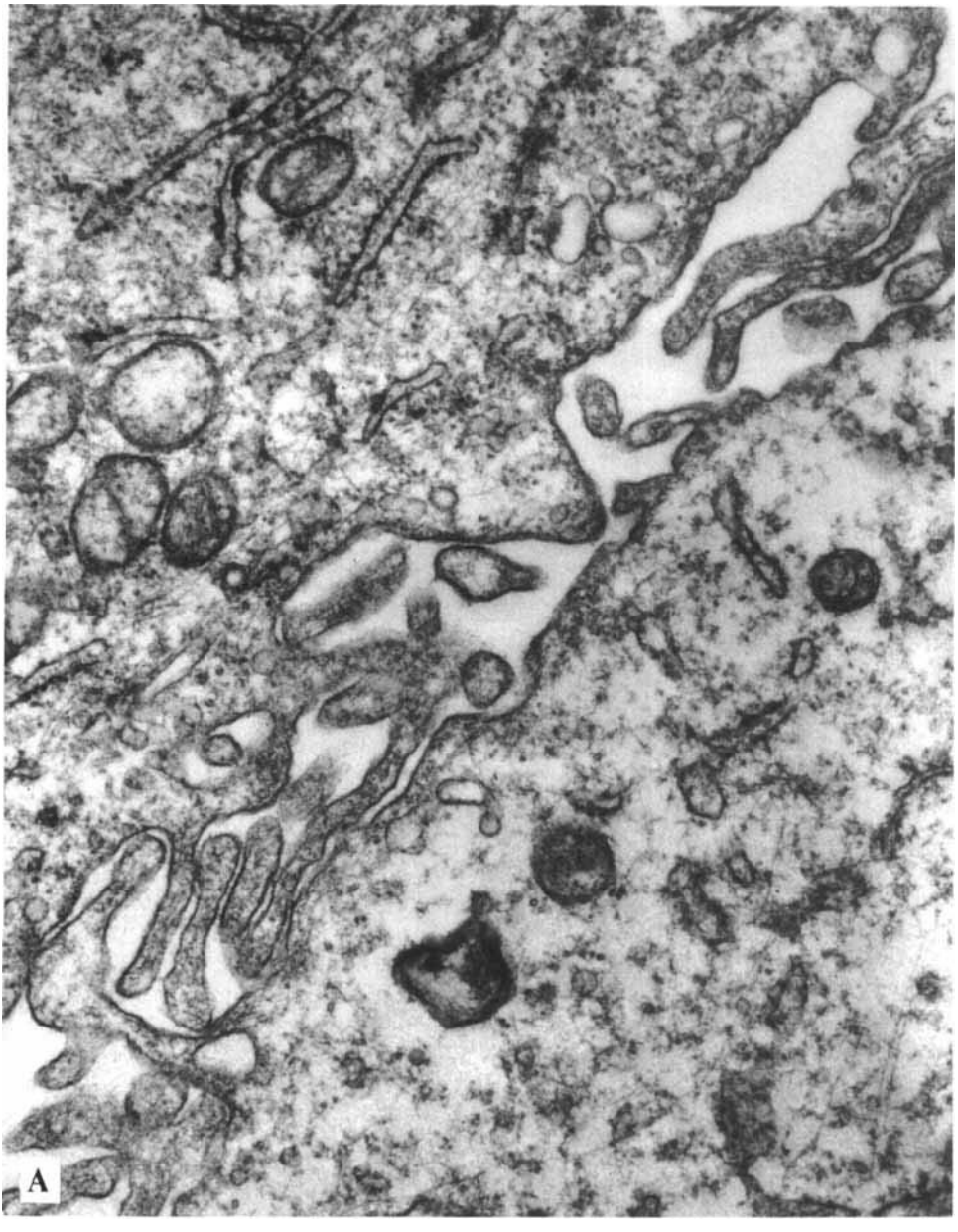
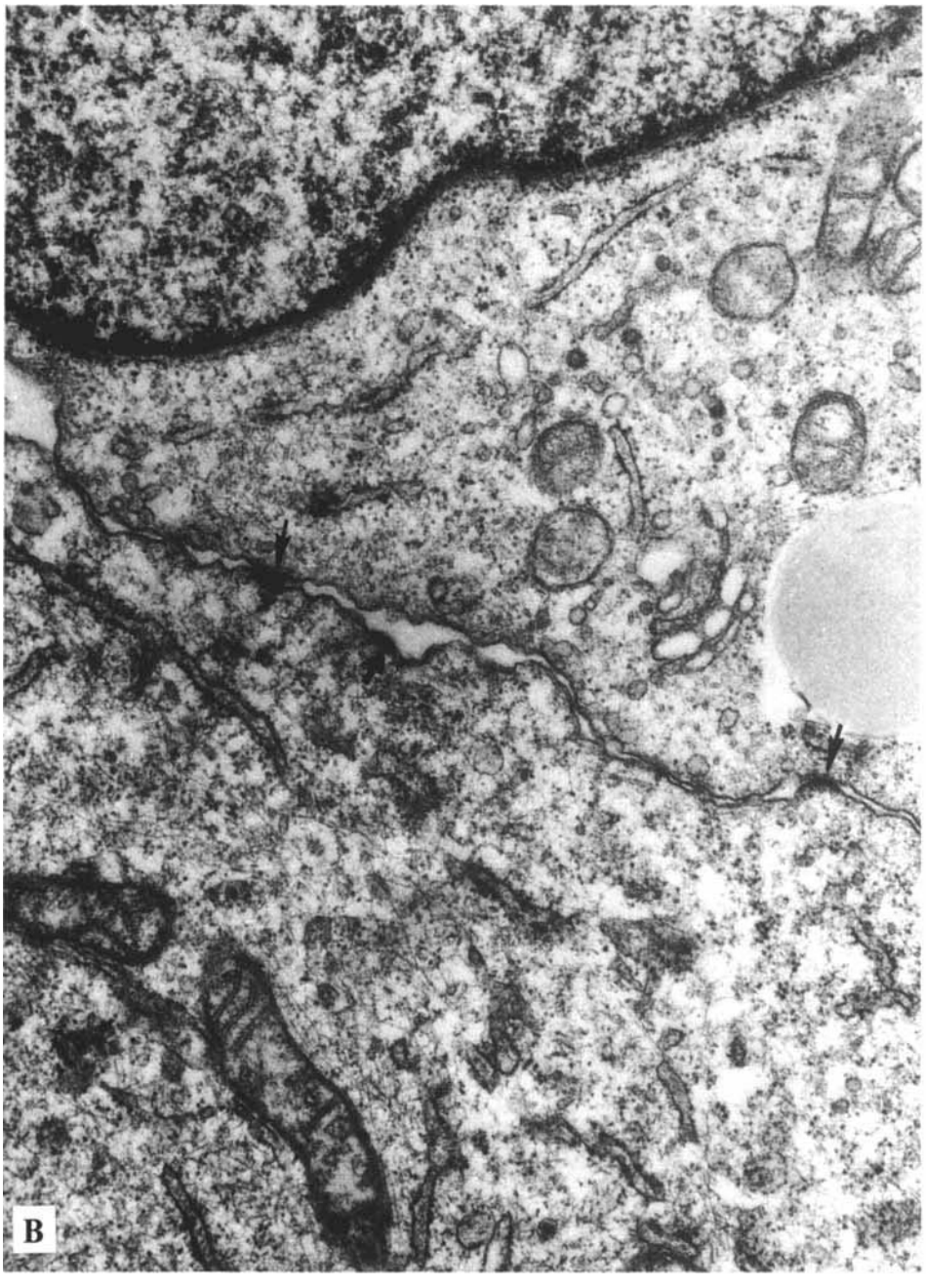


Fig. 1. Ultrastructure of epithelial cells. Fetal intestine line (680Int), passage 16. A) Microvilli; bar = 0.2 μ; B) desmosomes (small arrow) and persistent hemidesmosome (large arrow); bar = 0.5 μ.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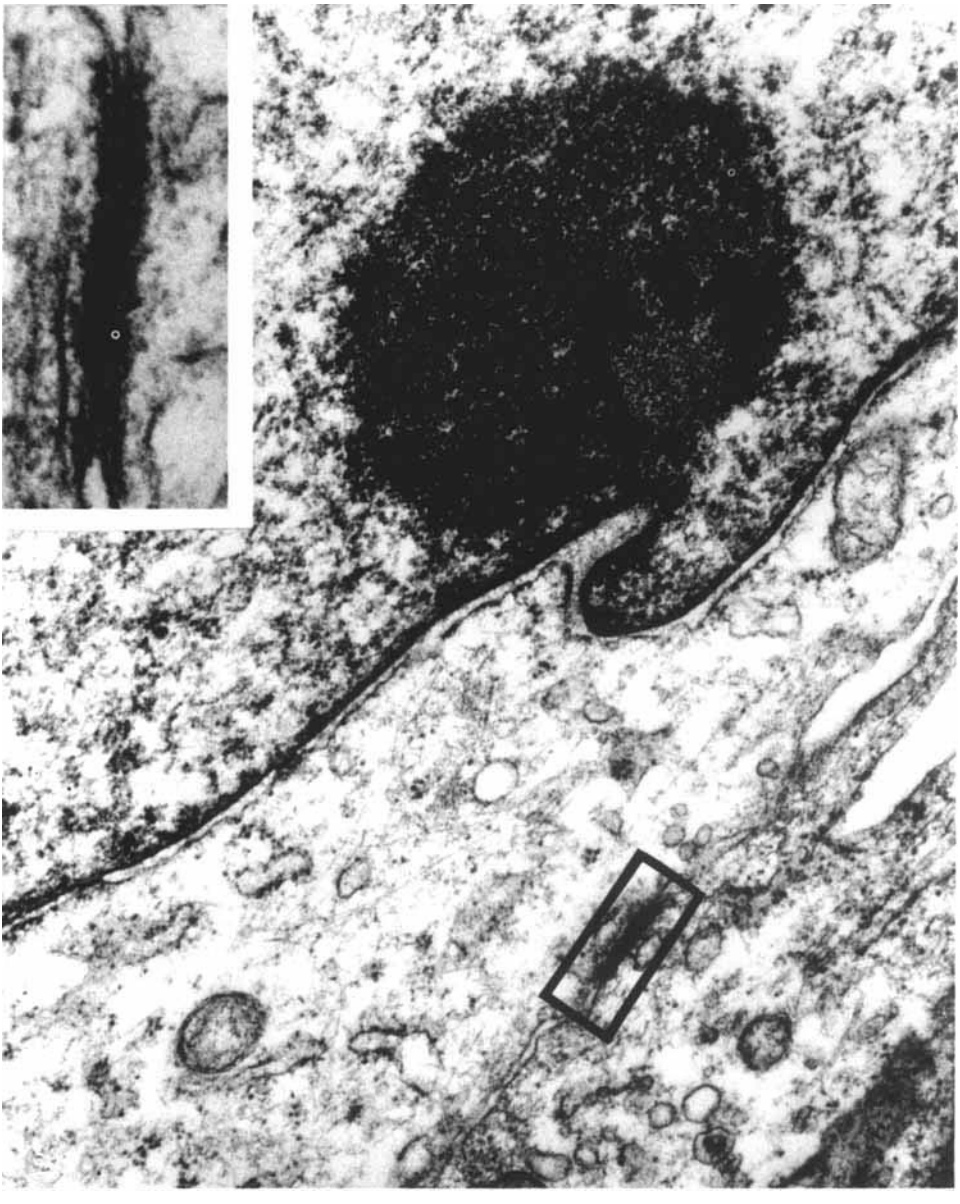

Fig. 2. Ultrastructure of epithelial cells. Fetal intestine line (680Int), passage 16. Figure illustrates a tight junction (arrow); bar = 0.2 μ. Inset shows tight junction at high magnification; bar = 0.1 μ.

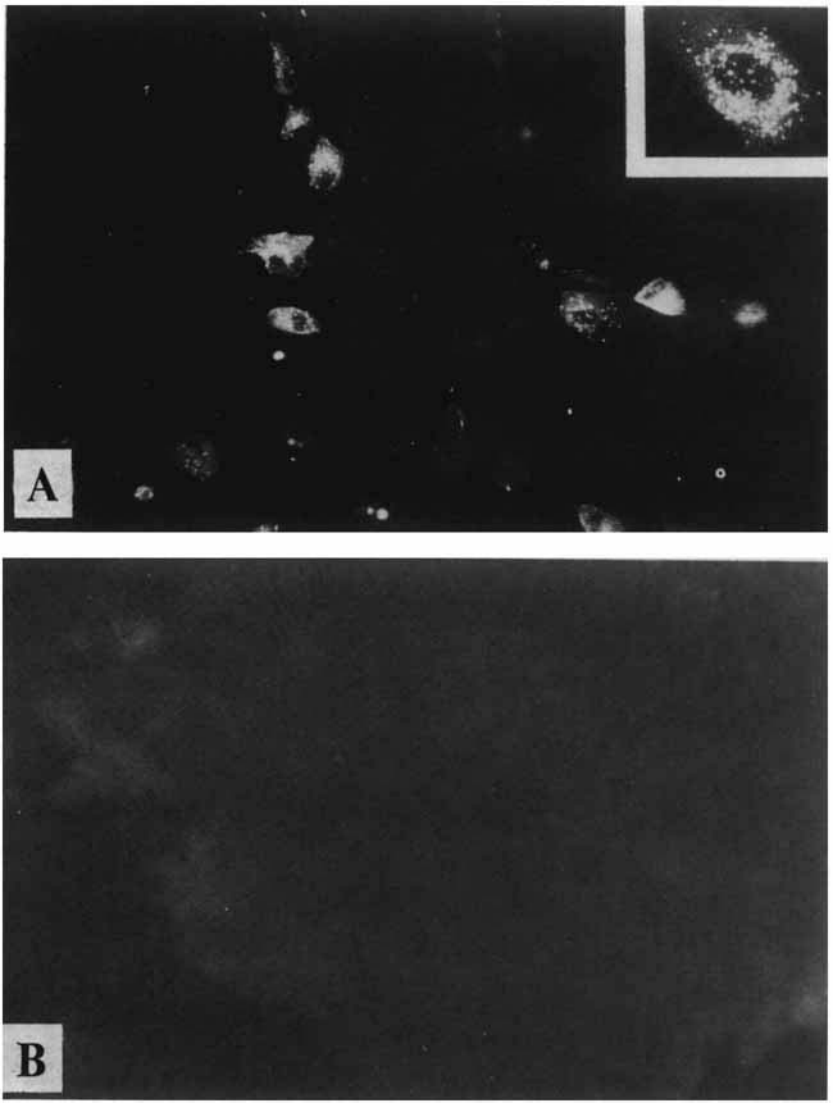

Fig. 3. Expression of antihemophilic factor (factor VIII antigen). A) Subconfluent human umbilical vein endothelial cells with approximately 20% of the cells showing bright fluorescence. Inset shows higher magnification of positive cell with discrete punctate pattern of fluorescence. B) Confluent monolayer of fetal intestine cells (74Int), with no positive cells.

platelets. Both lines derived from fetal intestine caused the platelets to coalesce into large aggregates (Fig. 4B) while the line derived from adult bladder (767B1) bound the platelets singly and did not cause platelet aggregation (data not shown).

Characterization Of The Cells For Properties Associated With Transformation

All of the epithelial lines derived from nonmalignant tissue showed extensive aibility to activate plasminogen. Figure 5 illustrates a typical experiment showing extensive solubili-

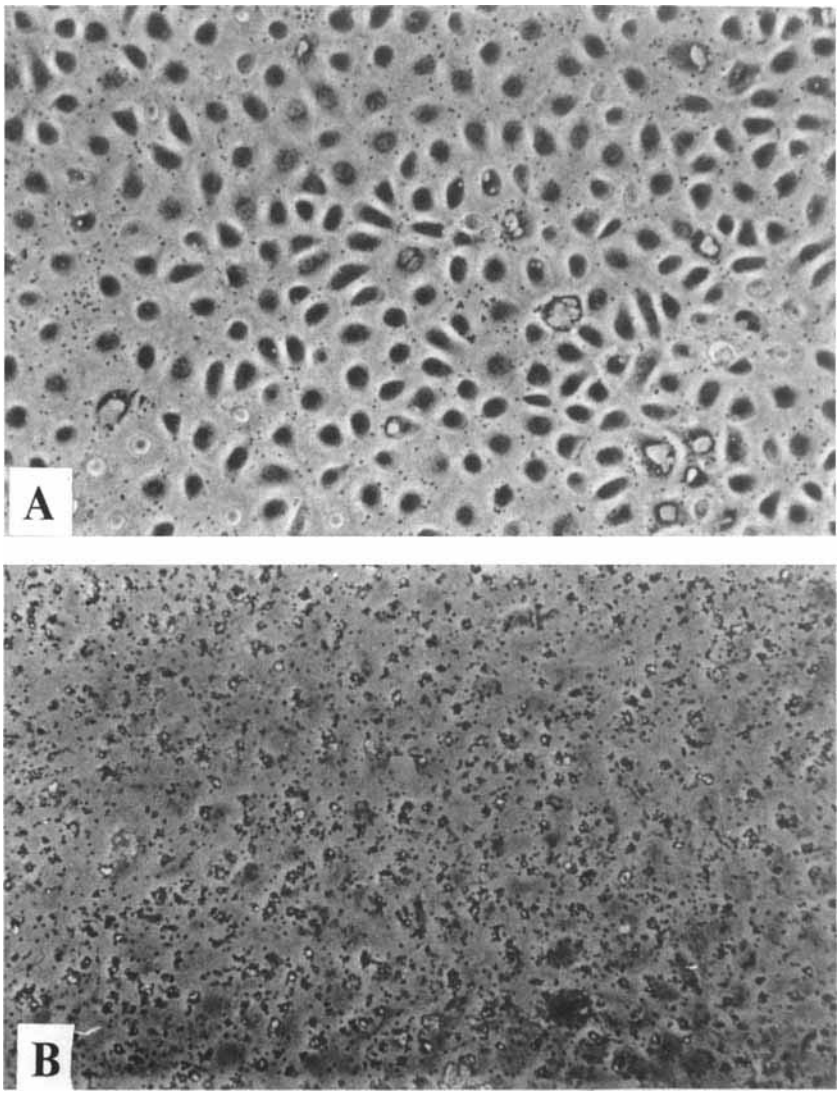

Fig. 4. Adherence of platelets. A) Bovine aorta endothelial cells; Note few platelets adhering only at intercellular junctions. B) Fetal intestine cells (74Int). Note extensive adherence and aggregation of platelets.

zation of ^{125}I-fibrin by an epithelial line derived from normal adult bladder. This activity was plasminogen dependent since it was absent when plasminogen was removed from the medium.

The carcinoma-derived lines varied in their production of plasminogen activator. Figure 6 illustrates the extremes of activity observed among the carcinoma-derived lines. The transitional cell carcinoma line, 761T, showed even more activity than the positive control line, HeLu, whereas the metastatic carcinoma line, 696T, showed little if any activity above background. In the experiment illustrated in Figure 2, the positive control,

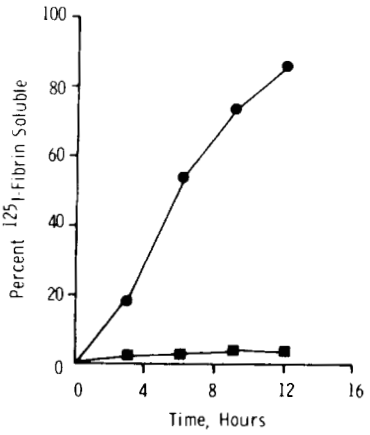

Fig. 5. Fibrinolytic activity of an epithelial cell line derived from nonmalignant adult bladder (767B1). Cells at passage 13 were plated on ^{125}I-fibrin-coated dishes in medium containing 2.5% plasminogen-free dog serum. After 12 hours, the assay was initiated by replacing the original medium with fresh medium containing either 2.5% dog serum (●——●), or 2.5% plasminogen-free (■——■) dog serum.

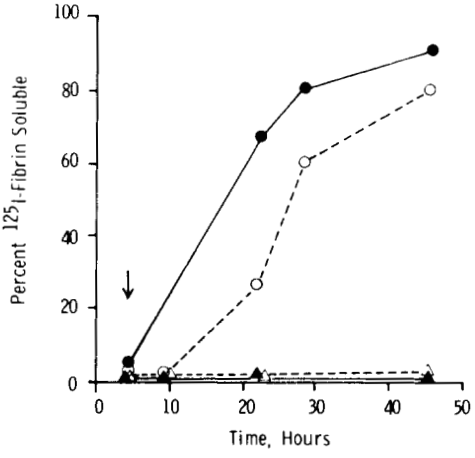

Fig. 6. Fibrinolytic activities of epithelial cell lines derived from carcinomas. Cells were plated on ^{125}I-fibrin-coated dishes in medium containing 2.5% fetal calf serum. Arrow indicates time of shift to medium containing 2.5% dog serum. Transitional cell carcinoma (761T), passage 24, ●——● ; metastatic carcinoma (696T), passages 21 ▲——▲ ; control human embryo lung (HeLu), - - ○ - - ○; control human embryo skin (HeSk), △ - - △ - - .

HeLu, showed a lag before any activity was detected. Similar lags were also observed for some of the carcinoma lines; the length of these lags varied from one experiment to another. Table II illustrates the data gathered from typical experiments on each cell line. The data are presented as time required for 20% solubilization of the ^{125}I-fibrin; hence, the longer the time, the lower the amount of plasminogen activator produced. All of the lines derived from nonmalignant tissues produced large amounts of plasminogen activator, even at the earliest passage tested, passage 5. The tumor-derived lines varied in activity;

TABLE II. Plasminogen Activator in Human Epithelial Cells

Source of cells (designation, passage)	Time for 20% solubilization of ^{125}I-fibrin
Derived from nonmalignant tissue	
Fetal intestine (74Int, 19)	3
Fetal intestine (677Int, 6)	5
Fetal intestine (680Int, 5)	6
Adult bladder (767B1, 13)	3
Derived from primary carcinomas	
Colon (675T, 11)	5
Colon (785T, 6)	5
Breast (578T, 13)	75[a]
(578T, 40)	27
Trans. cell (761T, 22)	5
Derived from metastatic carcinoma	
Stomach to muscle (746T, 15)	19
Pancreas to lymph node (766, 15)	9
Adenocarcinoma to sacrum (696T, 20)	BKG
(696T, 50)	11
Adenocarcinoma to hip (700T, 16)	13
(700T, 50)	18

[a]Extrapolated from last data point at 42 hours.

6 cell lines had as much activity as the nonmalignant lines; 1 carcinoma-derived line was negative, and 2 lines produced intermediate amounts of plasminogen activator. There was no consistent difference in growth rate between the lines with low or high levels of plasminogen activator. In some cases an increase in plasminogen activator was observed at higher passages. The metastatic carcinoma line, 696T, which showed little activity at passage 15, showed extensive activity at passage 50. The breast carcinoma-derived line, 578T, also produced more plasminogen activator at passage 40 than at passage 13. The time for 20% solubilization of ^{125}I-fibrin at passage 40 was 27 hours, in contrast to an extrapolated 75 hours at passage 13. Another metastatic line, 700T, produced similar levels of plasminogen activator from passage 16 to passage 50. None of the other cell lines were tested beyond passage 25.

Several studies have shown that the plasminogen activators produced by certain nonmalignant cells can be suppressed by corticosteroids [37, 44]. To determine whether corticosteroids could inhibit the plasminogen activator activity found in these epithelial cells, an adult normal epithelial cell line (767B1), a normal fetal line (680Int), and a colon carcinoma line (675T) were incubated for 3 days in various concentrations of dexamethasone prior to assaying for plasminogen activator. A slight diminution in the amount of fibrinolysis was observed in the presence of 10^{-6} M dexamethasone; however, the response was similar for all 3 lines. No further decrease was found when 3×10^{-6} M dexamethasone was used (data not shown).

For most of the lines, including the control lines, HeLu and HeSK, the fibrinolytic activity did not vary when monkey serum rather than dog serum was used as the source of plasminogen. The metastatic stomach carcinoma line, 746T, was unique in that the fibrinolytic activity in dog serum was consistently higher than in monkey serum. Two other lines (578T and 696T) that had little detectable fibrinolytic activity in dog serum did show some activity when monkey serum was used as the source of plasminogen.

The same cell lines have been characterized for a number of other properties associated with transformation, including growth on contact-inhibited monolayers and in methocel, tumorigenicity in immunosuppressed mice [27], and loss of an external fibronectin containing matrix (H.S. Smith, J.R. Riggs, M.W. Mossesson, Production of Fibronectin by Human Epithelial Cells in Culture, Cancer Research, in press). A summary of these results is presented in Table III. For the growth properties, cell lines derived from normal tissue were negative, whereas the carcinoma-derived lines varied in their expression, with some lines being more abnormal than others. In most cases, the lines derived from metastatic lesions had more abnormal properties than did those derived from primary carcinomas. The metastatic adenocarcinoma line, 696T, was a notable exception. At low passages the line was negative for growth on monolayers, in methocel, and in mice. After extensive subculture, the cells gained the ability to grow on monolayers but were still negative for growth in methocel or in immunosuppressed mice. These same cells also increased production of plasminogen activator as a function of passage (Table II). The altered properties at high passage were not the result of an inadvertent contamination of the culture with a different cell line since both high and low passage cells contained identifying marker chromosomes upon giemsa banding (personal communication, Dr. W. Nelson-Rees, Cell Culture Laboratory, Naval Supply Center, Oakland CA). In most cases where cells grew both on monolayers and in methocel, the efficiency of plating was much higher on the monolayers than in the methocel. The highest plating efficiency observed in methocel was only 2.5%.

All of the lines derived from normal tissue as well as primary carcinomas were positive for a fibronectin matrix by immunofluorescence microscopy. In contrast, cell lines from metastatic carcinomas showed little if any fibronectin, including low passages of metastatic adenocarcinoma (line 696T), which showed no other abnormal growth properties. The 696T line also altered in expression of fibronectin as a function of passage; a small amount of fibronectin in an extracellular matrix was observed after extensive subculture.

Further Studies On Fibronectin Synthesis By Human Mammary Epithelial Cells

Recently, Stampfer et al [29] developed a conditioned media that reproducibly supports extensive growth of human mammary epithelial cells in primary and secondary culture. These cells have been shown to have typical epithelial-type specialized junctions and microvilli [29]; in addition, they bind antibodies raised against the human milk fat globule, a property specific to human mammary epithelium (J. A. Peterson, J. C. Bartholomew, M. Stampfer, R. L. Ceriani. Quantitative Changes in Expression of Human Mammary Epithelial Antigens in Breast Cancer as Measured by Flow Cytometry, data to be published). Within these epithelial cultures is also a small proportion of cells with a morphological appearance suggestive of myoepithelium. In addition, with the separation techniques described by Stampfer et al [29], pure cultures of fibroblastic stromal cells can be obtained from the same specimens. We have examined cultures of these various cell types from two reduction mammoplasty specimens for presence of fibronectin by immunofluorescence

TABLE III. Summary of Various Growth Properties of Human Epithelial Cell Lines

Source of cells (designation, passage)	Efficiency of plating (%) [a,b] Epithelial cell monolayers	Fibroblast monolayers	Large colonies in methocel	Tumorigenicity [b,c]	Fibronectin matrix [d]
Derived from nonmalignant tissue					
Fetal intestine (74Int, 12–15)	<0.01	<0.01	<0.005	-	+
Fetal intestine (677Int, 7–9)	<0.01	<0.01	<0.005	-	+
Fetal intestine (680Int, 12–13)	<0.02	<0.02	<0.005	-	+
Adult bladder (767B1, 10–17)	<0.02	<0.02	<0.005	-	+
Derived from primary carcinoma					
Colon (675T, 8–10)	2.6 [e]	<0.02	<0.005	-	+
Colon (785T, 9–17)	0.06 [e]	<0.02	<0.005	-	+
Breast (578T, 10–28)	50	48	0.03	- [f]	+
Trans. cell (761T, 18–22)	<0.02	<0.02	<0.005	-	+
Derived from metastatic carcinomas					
Stomach to muscle (746T, 11–14)	46	36	0.05	+	-
Pancreas to lymph node (766T, 15–16)	5.4	3.4	1.0	+	-
Adenocarcinoma to sacrum (696T, 16)	<0.05	<0.05	<0.005	-	-
(696T, 50–52)	57	70	<0.005	-	±[g]
Adenocarcinoma to hip (700T, 9–15)	12	33	0.04	+	-
(700T, 50)	25	90	2.5	NT	NT

[a]Efficiency of plating = (number of colonies formed/number of cells plated) multiplied by 100.
[b]Summarized from reference 27.
[c]Mice immunosuppressed with antithymocyte serum.
[d]Summarized from Smith, Riggs and Mosesson (Cancer Research, in press).
[e]Visible microscopically only.
[f]These cells have been reported to produce tumors in congenitally athymic-asplenic (lasat) mice [45].
[g]Very scanty, dust-like pattern.

microscopy. In contrast to normal human fetal intestine cells, which produce a fluorescent pattern consisting of globular material attached to long fibrous-like structures (Fig. 7a), the normal mammary epithelial cells show a diminished, punctate and dust-like pattern (Fig. 7B) in both primary and secondary cultures. In contrast, both the putative myoepithelial cells (Fig. 7C) and the fibroblasts (Fig. 7D) distribute the fibronectin as an extensive fibrillar lacy network.

DISCUSSION

We have attempted to define how some of the properties associated with in vitro transformation apply to human carcinoma induction. Figure 8 diagrams the progression to malignancy of a typical mammary duct, illustrating the in vivo situation [46, 47], for which we would like to develop a model in culture. In the normal duct (Fig. 8A) a uniform layer of epithelial cells with a regular and normal morphology is separated from the blood vessels and stroma by a basement membrane. As these cells progress to an atypical, premalignant state, they lose growth control and proliferate extensively; morphologically, the atypical cells can become as irregular and pleomorphic as frank carcinoma cells (Fig. 8B). The notable difference between this extreme atypia (sometimes described as "carcinoma in situ") and frank carcinoma is the relationship of the epithelial cells to the basement membrane. In contrast to the premalignant state, where the normal relationship of the various cellular components is maintained, the carcinoma cells invade the basement membrane and grow in the surrounding stromal matrix (Fig. 8C). This progression from normality to carcinoma can follow alternate paths of development; it can occur by either gradual or abrupt changes, and it continues beyond the stage of primary carcinoma to metastases.

Associated with each step in malignant progression, there must be changes in cellular physiology which could potentially be measured in vitro. According to the principles of malignant progression defined in vivo [46, 47], a particular change might appear early on some pathways to malignancy and late on others, whereas another change may be present and important for some pathway to malignancy but not all pathways. Ideally, there may be properties that always and only appear when cells become malignant, but this alternative may not necessarily be true. Finally, there may be "enhancing" properties, which are themselves not necessarily responsible for the malignant phenotype but which confer on malignant cells an increased growth advantage.

We have approached the problem of determining which in vitro parameters correlate with malignancy in humans by culturing cells from tissues representing various stages of malignant progression. Although all of the lines cultured from malignant tissue were morphologically [24, 26] and ultrastructurally [28] abnormal (in contrast to those derived from nonmalignant tissues, which appeared normal), we cannot say for certain that all of the tumor-derived cells are capable of invasive growth. Because of the nature of malignant progression, the frank carcinoma is comprised of a heterogeneous population, only some cells of which may be invasive. Although a metastatic lesion is likely to be enriched for more invasive cells, it also may be heterogeneous, possibly having arisen from a clump of primary tumor cells only some of which are capable of initiating growth at the metastatic site. Once proliferation has been initiated by these more aggressive cells, the noninvasive cells may again commence proliferation.

Because of the central role that invasion of the basement membrane plays in carcinoma induction, we examined the lines for synthesis of fibronectin, a protein found associated with basement membranes as well as connective tissue in various normal human tissues

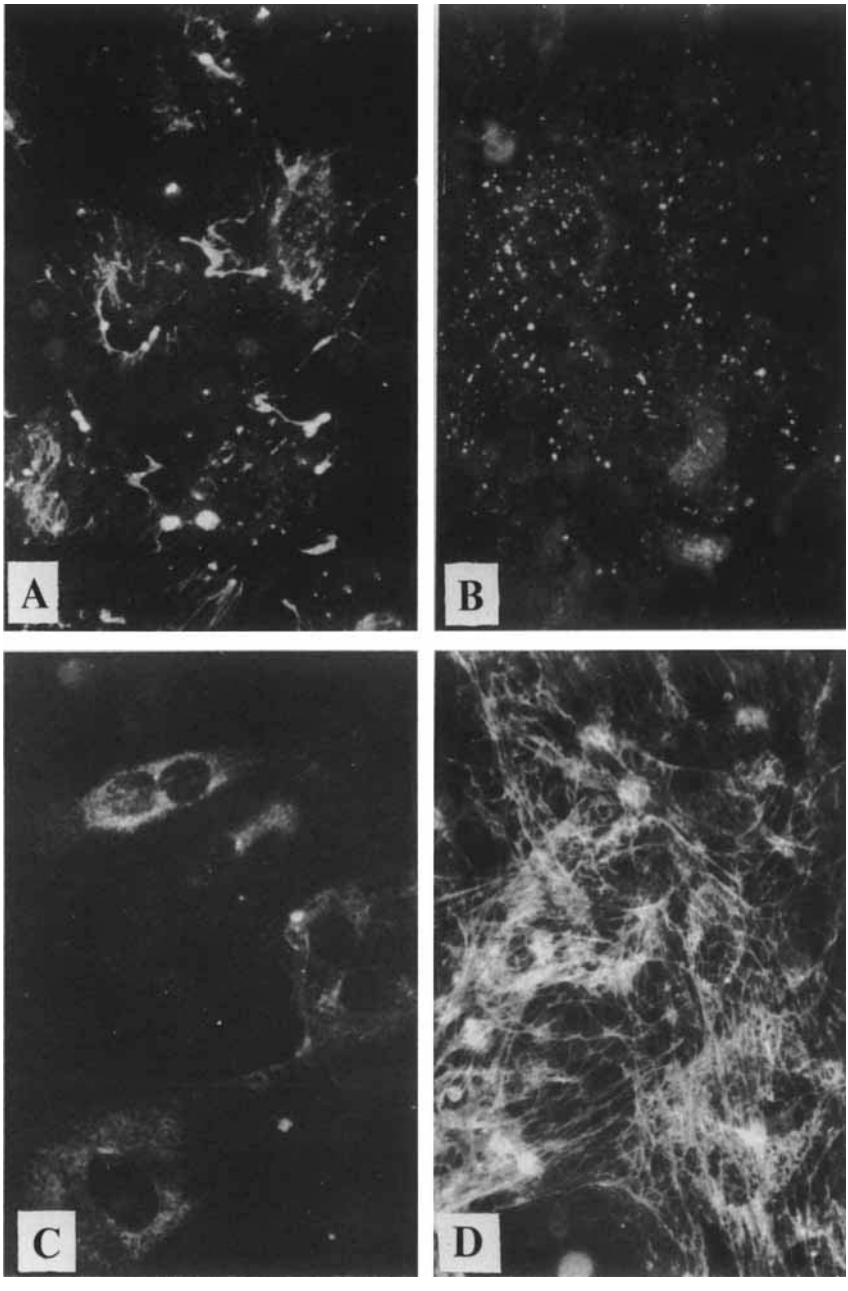

Fig. 7. Expression of fibronectin by various human cells in culture. A) Normal fetal intestine line 680Int showing globular and fibrillar pattern. B) Normal mammary epithelial cells H48RE in primary culture showing punctate and dust-like pattern. C) Putative myoepithelial cell in same epithelial culture as B), showing extensive fibrillar matrix. D) Normal fibroblasts in secondary culture from same patient as B), showing extensive fibrillar matrix.

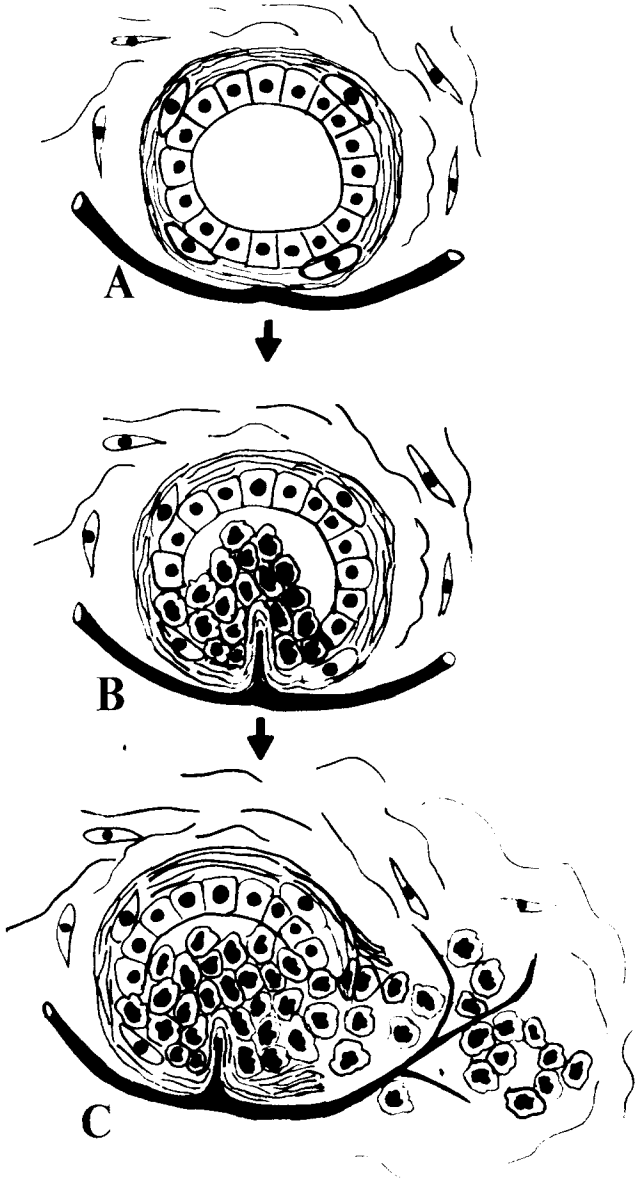

Fig. 8. Diagram of progression to malignancy of a typical mammary duct. A) Normal mammary duct. Note uniform polar epithelial cells and myoepithelial cells surrounding the lumen separated by basement membrane from the blood vessels and stroma. B) Atypical hyperplasia. Note the abnormal pleomorphic cells and intact basement membrane. C) Frank carcinoma. Note the disruption of basement membrane and invasion of epithelial cells into stromal area.

[48]. Absence of fibronectin in an external cellular matrix has been associated with transformation in many [4] but not all [19] systems. We have found that all the cell lines derived from metastatic tissues were negative for fibronectin, whereas those derived from nonmalignant tissues of primary carcinomas all produced fibronectin in an extracellular matrix. These observations suggest that absence of a fibronectin matrix in vitro may be a marker of invasive growth for human epithelial cells. However, this conclusion must be considered only tentative. When we extended these studies to primary and secondary cultures of normal mammary cells, we found that they produced a much diminished amount of fibronectin with a punctate and dusty cellular distribution rather than the extensive matrix produced by the nonmalignant lines derived from adult bladder and fetal intestine. These differences may be related to the fact that mammary ducts contain, in addition to the epithelial cells, myoepithelial cells, which may be involved in basement membrane synthesis. That putative cultured myoepithelial cells produced an extensive fibronectin matrix supports this hypothesis. In contrast, there are no myoepithelial cells in either bladder or intestine; therefore, the epithelial cells may be responsible for basement membrane synthesis in these organs. These observations point out the need to distinguish organ-specific properties from tumor-specific properties. Therefore, it will be important to compare the in vitro properties of cells cultured from specimens representing different stages of malignant progression in a single organ system.

How do the various other properties associated with in vitro transformation fit into the scheme of malignant progression? For properties such as growth on contact-inhibited monolayers, in methocel, or in immunosuppressed mice, the nonmalignant lines were negative, whereas the carcinoma-derived lines varied in expression, with some lines being more abnormal than others. The lines derived from metastatic lesions tended to have more abnormal properties than those derived from primary carcinomas, with the exception of one metastatic line, 696T, which was negative in these assays. Inasmuch as the tumor-derived lines were not consistantly positive in the assays tested, these in vitro parameters probably do not represent changes essential to the malignant state. The fact that the nonmalignant lines were negative for these properties suggests that the markers are somehow related to malignancy even if they are not essential. Therefore, with the reservation that many more studies are needed, we suggest that such properties may be "enhancing" and confer on malignant cells an increased growth advantage.

Finally, increased production of the protease, plasminogen activator, has been associated with transformation in many [1–3, 36–38, 49] but not all systems [10, 19, 50, 51]. For example epithelial cells derived from normal rat liver produced extensive amounts of plasminogen activator [10, 51]. Among the human epithelial lines that we studied, the level of plasminogen activator in culture also did not correlate with malignancy. The control nonmalignant epithelial cell lines all produced large amounts of plasminogen activator, whereas the tumor-derived lines showed a gradation of activities, ranging from lines having as much activity as the nonmalignant cells to lines having little or no activity above background. Since some of the nonmalignant lines were derived from fetal tissue, expression of plasminogen activator might be related to their fetal origin. However, nonmalignant adult bladder also produced extensive plasminogen activator, which suggests that adult human epithelial cells, like rat liver epithelial cells, produce plasminogen activator.

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