ABSENCE OF FIBRONECTIN AND PRESENCE OF PLASMINOGEN ACTIVATOR IN BOTH NORMAL AND MALIGNANT HUMAN MAMMARY EPITHELIAL CELLS IN CULTURE

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ABSTRACT

Primary monolayer cultures of normal and malignant human mammary epithelial cells were tested for fibronectin by indirect immunofluorescence using antisera specific for fibronectin. This protein was not detectable on either the normal or malignant epithelial cells. Similar results were obtained for normal and malignant mouse mammary epithelial cell cultures. Control normal and transformed fibroblasts exhibited the expected result: the normal cells were positive and the transformed cells were negative. With the use of supernatant fluids from the same cultures or an agar-overlay assay on viable cells, high levels of plasminogen-dependent fibrinolytic activity were detectable in both the normal and malignant mammary cells. Thus, two characteristics that distinguish normal from transformed fibroblasts do not serve as markers of malignancy in mammary epithelial/carcinoma systems.

KEY WORDS fibronectin · immunofluorescence · plasminogen activator human mammary epithelium · hormone

Over the past few years, two putative markers of neoplastic transformation have been extensively studied in a number of laboratories. The first, a large external transformation-sensitive glycoprotein (LETS protein), is lost, or greatly reduced in amount, when normal fibroblasts are transformed in vitro with oncogenic viruses or chemical carcinogens (reviewed in references 14, 42, and 47). This protein, now identified as the major surface glycoprotein of all tested normal fibroblasts and endothelial cells (4, 17, 18, 48, 49), is identical, or very similar, both physicochemically and immunologically, to several glycoproteins previously observed in, or isolated from, sera and cell cultures (i.e., CIg, SFA, CSP, fibronectin; for nomenclature and relationship of these proteins, see review by Yamada and Olden, reference 47). Various biochemical and cellular functions have been proposed for fibronectin (the name to be used in this report), although none of these has yet been clearly demonstrated (47). However, fibronectin has generally been accepted as a (negative) transformation-related marker for fibroblast/sarcoma systems in vitro.

Plasminogen activator is a second marker that is reported to distinguish normal from transformed fibroblast cells. Only low levels of plasminogen-dependent fibrinolytic activities are exhibited by normal cells, whereas high levels are generally associated with transformed cells (30, 31, 41). A number of glycoproteins, including fibronectin, have been suggested to be in vivo substrates for

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plasmin, the product of the plasminogen activator (5, 15, 40). Surface expression of fibronectin and the level of plasminogen activator, however, may not be directly related (25).

Because most human cancers are carcinomas, it is important to establish the relevance of these two cellular characteristics to neoplastic transformation in epithelial systems. To date, there have been few reports in which these characteristics have been examined in epithelial cells (8, 20, 45). We have developed methods that allow normal and malignant human mammary epithelial cells to be routinely grown as monolayers in primary culture: normal cells are isolated from the breast fluids of healthy women, and malignant cells are isolated from primary breast carcinomas (19). We have demonstrated that these cell cultures and a permanent line of human breast cancer cells, MCF-7 (38), are applicable to a number of experimental studies of the cell biology of human breast cancers (10, 19, 35, 43, 50, 51). Using this system and mouse mammary epithelial cells, we investigated the expression of fibronectin and plasminogen activator to determine whether they would serve as markers for transformation of mammary epithelial cells. In addition, several epithelial cell lines not of breast origin were examined to establish whether there is a correlation between these two characteristics and the neoplastic transformation of epithelial cells in general.

MATERIALS AND METHODS

Cell Cultures

Breast milk samples were obtained from normal women who were weaning or had just weaned their infants (10). The human mammary epithelial (HME) cells were pelleted, washed, and seeded onto coverslips in 35-mm tissue culture dishes in milk cell (MC) medium (19), which consists of Dulbecco's modified Eagle's medium (MEM) (pH 6.8), 15% fetal calf serum (FCS), 10 μg/ml insulin, 5 μg/ml ovine prolactin, and 15 mM *N*-2-hydroxyethyl piperazine-*N'*-2-ethane sulfonic acid (HEPES) buffer (pH 6.8). Under these conditions, the cells grew into confluent epithelial islands, which we have previously shown to possess all of the ultrastructural features of normal mammary epithelium (35).

Cells were released from primary human breast carcinomas by enzymatic digestion without agitation for 18 h at 37°C in culture medium supplemented with 5% FCS and containing 0.5 mg/ml collagenase (19). Epithelial cell aggregates and fibroblasts were recovered by centrifugation, washed, and seeded into culture dishes as described for the normal HME cells.

Primary cultures of normal and malignant mouse mammary epithelial cells were established from BALB/c lactating mammary glands and from GR mammary tumors, respectively, as described by Voyles and McGrath (44) and McGrath and Blair (28).

MCF-7 cells derived from a pleural effusion of a female patient with a scirrhous carcinoma of the breast (38) exhibit human, breast, epithelial, and malignant cell characteristics (2, 29, 36). D-549, a normal fibroblast cell line derived from human foreskin, and D-562, a human carcinoma cell line derived from the pleural effusion of an adenocarcinoma of the throat (32), were obtained from Dr. W. Peterson, Children's Hospital of Michigan. HBL-100, a line of putative normal HME cells isolated from colostrum (33), was obtained from Dr. E. Gaffney, Pennsylvania State University, University Park, Pa. Normal human fetal kidney cells were provided by Dr. W. Peterson, Children's Hospital of Michigan. The latter cells grow in continuous culture, exhibit epithelial-like morphology, but have not been designated as a permanent cell line. Fibroblastic cell cultures derived from normal human liver, testis, and lung were also provided by Dr. W. Peterson. All of the above cell lines or continuous cultures and the primary cultures of GR and BALB/c mouse mammary epithelial cells, were grown in Dulbecco's MEM supplemented with 15% FCS. 3 d before fixation for the immunofluorescence (IF) assay, cultures were replenished with MC medium and grown under the same conditions as the HME cells in primary culture (19). In some co-cultivation experiments, D-549 fibroblast cells were treated for 18 h with 2 μg/ml mitomycin C in medium, washed, trypsinized, and added to the HME cell cultures.

IF Microscopy

Rabbit antisera against human plasma cold-insoluble globulin and against LETS protein purified from hamster cells were generously provided by Drs. L.-B. Chen (8) and R. O. Hynes (27), respectively. Goat antiserum against chick embryo fibroblast fibronectin was the gift of Dr. K. M. Yamada (46). Cells on glass coverslips were washed twice with serum-free Dulbecco's MEM and five times with phosphate-buffered saline (PBS), fixed for 30 min at 4°C in 10% phosphate-buffered formalin, and then washed five times with PBS and five times with distilled water. To facilitate detection of intracellular antigens, some cells were fixed for 10 min at −20°C with acetone. Fixed cells were air-dried and stained by the indirect IF technique. The primary antifibronectin sera prepared by Chen, Harvard University, Cambridge, Mass., Hynes, Massachusetts Institute of Technology, Cambridge, Mass., and Yamada, National Institutes of Health, Bethesda, Md., were used at dilutions of 1:80, 1:25, and 20 μg/ml, respectively. The secondary antisera, fluorescein isothiocyanate- (FITC) conjugated goat anti-rabbit IgG (Antibodies Inc., Davis, Calif.) or FITC-conjugated rabbit anti-goat IgG (Miles Laboratories, Inc., Elkhart, Ind.), were used at dilutions of 1:30 and 1:10, respectively. Cells were incubated in the primary antiserum for 30 min at 37°C in a humidified chamber, washed with PBS, and incubated for an additional 30 min with the secondary serum. Stained cells were washed five times with PBS and twice with distilled water, air-dried, and mounted with glycerol:PBS (1:1). As a control for each tested antiserum, cells were incubated in normal rabbit serum or preimmune goat serum at the same dilutions as the antisera.

IF-stained cells were examined with a Leitz photomicroscope equipped with epi-illumination and a dark-field condensor. Photographs were taken with Kodak Tri-X film.

Plasminogen-dependent Fibrinolytic Activity

Qualitative measurement of plasminogen activator production by individual epithelial colonies was made using the casein-agar-overlay assay described by Goldberg (11). Cells were washed in two changes of serum-free MC medium and then overlaid with serum-free Dulbecco's MEM containing 0.5% agar, 2% nonfat

milk as a source of casein, and 1% dog serum as a source of plasminogen. Before use, the dog serum was acid treated as described by Ossowski et al. (30) to destroy the naturally occurring serum inhibitors of plasmin. The overlay mixture was allowed to solidify at room temperature, and the cultures were incubated for 18–20 h at 37°C in a humidified CO_2 incubator. Plasminogen activator production was detected as cleared zones of caseinolysis superimposed over individual epithelial colonies. The plasminogen dependence of the caseinolytic activity was established by omitting plasminogen from the overlay mixture; under these conditions no lysis was observed.

Extracellular plasminogen activator activity elaborated by the cells in culture was determined by adding aliquots of conditioned media to [^{3}H]fibrin-coated Petri dishes and measuring the release of [^{3}H]fibrinopeptides essentially as described by Barret et al (3). Acid-treated dog serum was used as a source of plasminogen in all assays. The number of HME cells that grow out in replicate primary cultures is variable. We have found that the most accurate method for determining the expression of plasminogen activator in response to steroid hormones is to initially measure the activity in the absence of hormone and subsequently to measure the activity generated by the same culture in the presence of test hormones. Exponentially growing cells in 35-mm culture dishes were washed five times with serum-free MC medium and then incubated for 24 h in 1.0 ml of serum-free MC medium containing 0.001% ethanol. This medium was clarified by centrifugation, and the supernatant fluid was stored at −20°C. An additional 1.0 ml of serum-free MC medium containing the desired hormone(s) dissolved in 0.001% ethanol was added to the same culture dishes, and the cultures were incubated for an additional 24 h. Conditioned media were cleared and stored until testing. Under these serum-free conditions, no change in cell number per culture was observed during the 48-h experimental period, as determined by direct microscopic counts of cell number per colony, and, if no hormones were added during the second incubation, the amount of plasminogen activator released was the same as during the first incubation (Table I).

TABLE I

Presence of Plasminogen Activator in Human Mammary Cell Cultures and Cell Cultures Not of Breast Origin

Cells tested	Plasminogen-dependent fibrinolytic activity/ 100 μl medium*	
	^3H *cpm*	*equivalent urokinase units* × 10^2
Primary or early passage cells		
Normal human mammary epithelial cells (5–10 × 10^4 cells/culture)		
Culture A	30,615	7.3
Culture B	58,069	8.7
Culture C‡		
1st 24 h incubation	71,464	9.3
2nd 24 h incubation	68,775	9.2
Malignant human mammary epithelial cells (5–10 × 10^4 cells/culture)		
Culture A	82,424	10.1
Culture B	26,759	6.9
Cell lines		
MCF-7, human breast carcinoma (1 × 10^4 cells)	37,142	7.7
D-562, human throat carcinoma (1 × 10^4 cells)	51,456	8.4
HBL-100, human colostrum (1 × 10^4 cells)	17,495	5.9
D-549, human foreskin fibroblast (1 × 10^5 cells)	<200	<0.1
Control Medium§	0	0
Background	1,240	

* The 0.1-ml aliquots of 24-h conditioned media of the cultures were assayed for fibrinolytic activity as described in Materials and Methods. Urokinase equivalents were obtained by extrapolation and normalization from standard curves of urokinase activity included with each assay.

‡ Two consecutive 24-h incubations were performed for culture C to test the continuity of release of plasminogen activators within a 48-h culture period.

§ Fresh medium was incubated at 37°C for 24 h in a Petri dish without any cells.

RESULTS

Surface-exposed Fibronectin

The presence or absence of fibronectin was tested in IF assays using specific antifibronectin antisera (see Materials and Methods). The efficacy of these methods was first established on fibroblast (or endothelial) control cells. Under our experimental conditions, 3T3 cells expressed a high level of fibronectin (Fig. 1 *a*), whereas SV40-transformed 3T3 cells expressed a much reduced level (Fig. 1 *b*). These data agree well with previous reports on surface expression of fibronectin by these cells (12, 16). Fig. 1 *c* shows that normal human foreskin fibroblasts D-549 exhibit readily detectable surface fibronectin in culture. We also observed (data not shown) that fibroblastic cells cultured from normal human liver, testis, amniotic fluid, and lung also express high levels of fibronectin.

No IF reactivity was detected when these cells were stained with normal rabbit serum or preimmune serum. Three different preparations of antifibronectin serum, shown to be highly specific for fibronectin (see Materials and Methods and references 8, 27, and 46), were tested for their reactivity on these cells, and the same results were obtained.

Normal HME cells were examined to determine whether they also express this surface protein. A

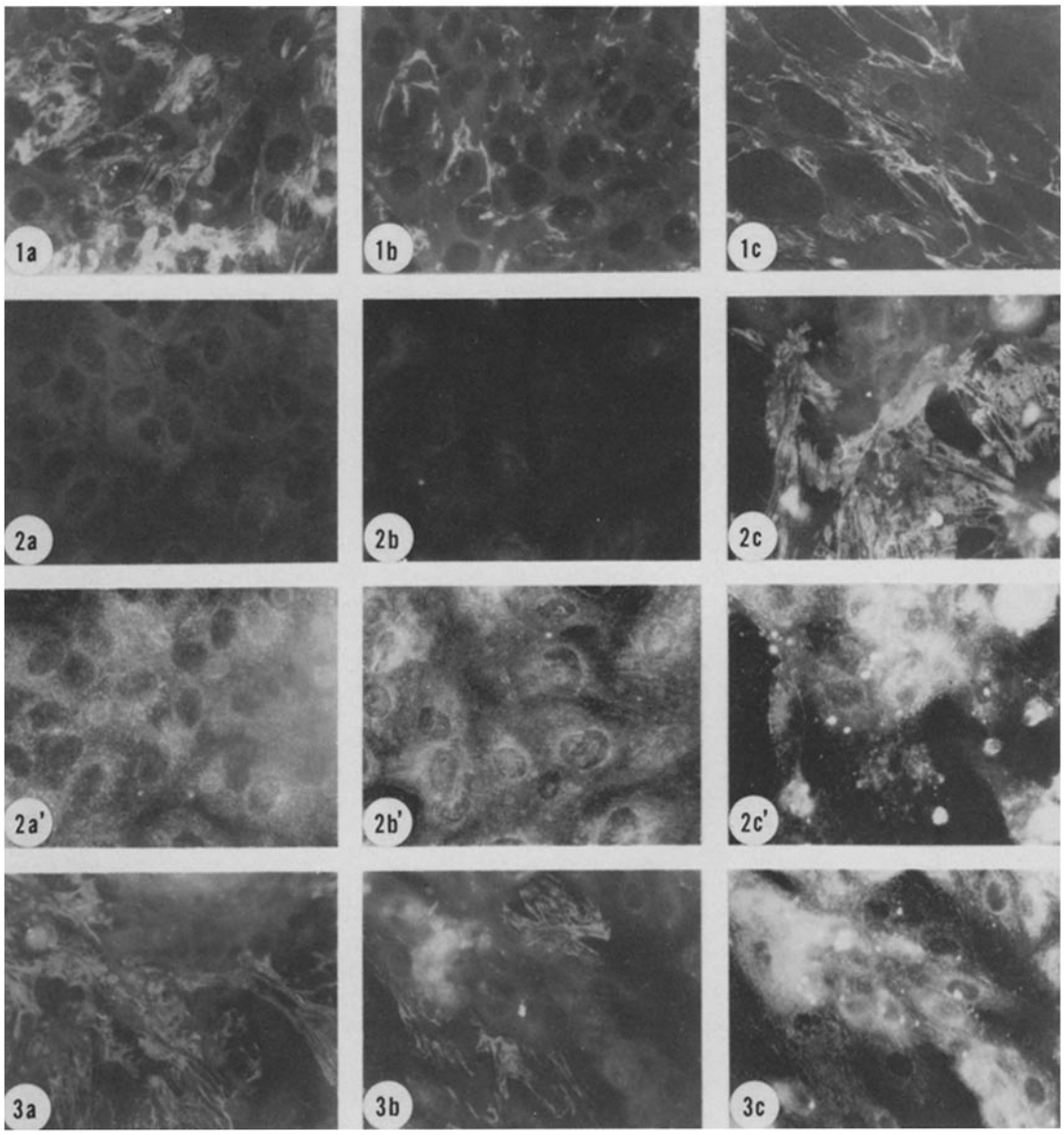

FIGURE 1 Indirect IF detection of fibronectin: (*a*) normal 3T3 cells; (*b*) SV-40 transformed 3T3 cells; (*c*) normal human foreskin fibroblast cells, D-549.

FIGURE 2 Indirect IF detection of fibronectin on normal HME cells in primary culture: (*a*) 22 d in culture; (*b*) 80 d in culture; (*c*) normal HME cells co-cultivated with normal human foreskin fibroblasts, D-549, for 30 d. *a'*, *b'*, and *c'* show the cell morphology of the same fields as in *a*, *b*, and *c*, respectively, viewed using a dark-field condensor. Because the D-549 fibroblasts are much flatter than the normal HME cells, the abundant peripheral cytoplasm of the D-549 cells is not in the plane of focus in (*c'*).

FIGURE 3 Indirect IF detection of fibronectin on malignant human mammary epithelial cells in primary culture: (*a*) 17 d in culture; (*b*) 26 d in culture; (*c*) cell morphology of *b* viewed using a dark-field condensor. The more flattened, less light-refracting cells in *c*, which exhibited fluorescence reactivity in *b*, are stromal fibroblast cells.

total of 11 primary cultures derived from 6 donors were tested. No IF reactivity was detectable on these normal HME cells (Fig. 2 *a*). Cells fixed with either formalin or acetone were tested and the results were identical, showing that both extracellular and intracellular fibronectins were absent from the normal HME cells. Fibronectin could not be demonstrated even at concentrations of antiserum 3- to 10-fold higher than the concentration sufficient to show maximal staining of the control cells (data not shown).

Cultures of normal HME cells consist of individual islands of contiguous cells (19). It has been reported (7) that surface expression of fibronectin on some fibroblasts is dependent upon cell-to-cell contact and time in culture. Therefore, to determine whether fibronectin expression by contiguous normal HME cells is a function of the time the cells have been in culture, large islands of confluent normal HME cells (>5,000 cells/colony) were maintained in primary cultures for 30, 45, and 80 d before IF testing. Again, no reactivity was detectable on these cells (Fig. 2 *b*), suggesting that expression of fibronectin is not induced on the contiguous epithelial cells by prolonged cellular contact.

To establish that the lack of fibronectin staining of normal HME cells is specific and not the result of an artifact of the culture conditions used, we co-cultivated positive control cells with normal HME cells. D-549 cells, which express fibronectin reactivity (Fig. 1 *c*), were treated with mitomycin C (to prevent overgrowth of the culture with the fibroblasts) and grown together with the normal HME cells. Fig. 2 *c* shows that after 30 d of cocultivation, the fibroblast feeder cells surrounding the epithelial islands showed readily detectable IF reactivity. No reactivity was observed within the HME islands.

To determine whether malignant HME cells also lack fibronectin, we tested cells in primary culture derived from four breast carcinomas (19). Fig. 3 shows that, whereas the stromal fibroblasts present in these cultures exhibit a high level of fibronectin, the malignant epithelial cells are completely unreactive. Similar data, obtained using paraffin sections of human breast carcinomas, were recently reported by Linder et al. (21), although these investigators did not test for expression of fibronectin on the counterpart normal HME cells.

In addition to examining primary cultures of human breast carcinoma cells, we also examined the expression of surface fibronectin on cells of a well-characterized malignant human mammary epithelial cell line MCF-7 (38). Fig. 4 shows that MCF-7 cells that have maintained many of the characteristics of breast carcinoma cells through several years of serial passage (36, 43, 50, 51) also do not express detectable levels of fibronectin.

Our data demonstrate that both normal and malignant HME cells lack surface-exposed fibronectin. To determine whether this is unique to human mammary cells, we tested mouse mammary epithelial cells isolated either from normal lactating glands or from spontaneous mammary tumors. Figs. 5 and 6 show that, like the human cells, mouse mammary epithelial cells exhibit no surface-exposed fibronectin in primary culture.

Other investigators have reported that some epithelial-like cells have detectable levels of surface fibronectin (8, 9, 37), but it has not been established whether this expression is tissue or organ specific, or whether it is related to the developmental origin of the cells tested. A number of other epithelial cell lines were, therefore, tested for their expression of this cell-surface protein under our experimental conditions. Fig. 7 shows that normal human fetal kidney cells, D-562, a cell line derived from a human throat adenocarcinoma (32), and HBL-100 cells derived from human colostrum (33) all express readily detectable levels of surface fibronectin. The distribution of fibronectin was pericellular on the human kidney cells and cellular on the D-562 and HBL-100 cells. D-562 and HBL-100 cells were found to deposit fibronectin only between the cell surface and substratum or between cells, and not on the upper cell surface. Although the exact embryonic origin of some of these cells has not been clearly established (43), all the cells assume a polygonal morphology and exhibit an epithelial-like growth pattern in culture.

Plasminogen Activators

Fibrinolytic activity was determined by two methods. As shown in Fig. 8, we found that both normal and malignant human mammary epithelial cells express readily detectable fibrinolytic activities as determined by the casein-agar-overlay technique (11). These activities were totally dependent on the presence of plasminogen. Clearing of the casein overlay was observed only over the islands of epithelial cells; macrophages or histiocytes in the MC cultures and stromal fibroblasts in the breast tumor cultures (19) were totally in-

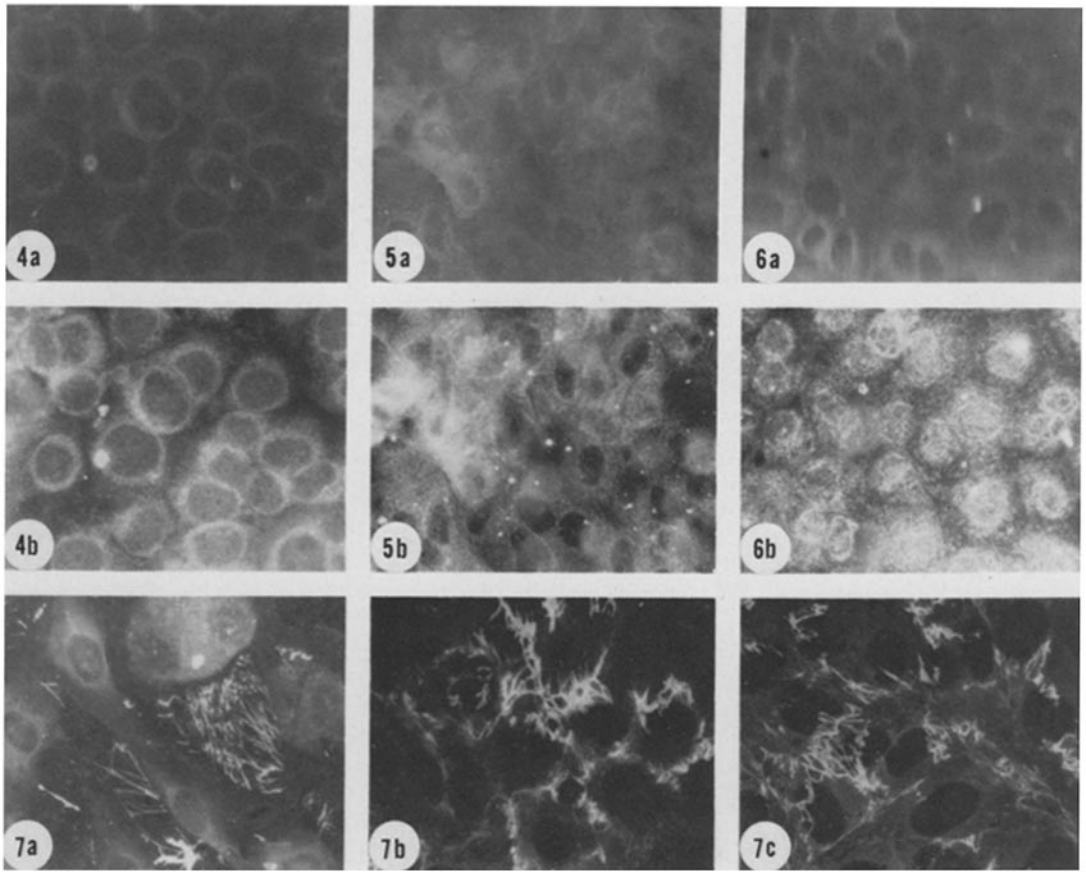

FIGURE 4 (*a*) Indirect IF detection of fibronectin on human breast carcinoma cells, MCF-7; (*b*) cell morphology of the same field viewed using a dark-field condensor.

FIGURE 5 (*a*) Indirect IF detection of fibronectin on normal BALB/c mouse mammary epithelial cells in primary culture; (*b*) cell morphology of the same field viewed using a dark-field condensor.

FIGURE 6 (*a*) Indirect IF detection of fibronectin on malignant GR mouse mammary epithelial cells in primary culture; (*b*) cell morphology of the same field viewed using a dark-field condensor.

FIGURE 7 Indirect IF detection of fibronectin on (*a*) normal human kidney epithelial cells in continuous culture; (*b*) human throat carcinoma cells, D-562, and (*c*) human HBL-100 cells.

active in this assay. The malignant HME cell line MCF-7 expressed a similar level of plasminogen activator in culture, as did the primary cultures of normal and malignant HME cells.

Using an insoluble ^{3}H-labeled fibrin layer as the substrate and serum-free medium conditioned by the cells as the enzyme source (3, and see Materials and Methods), we quantitated the plasminogen-dependent fibrinolytic activities released by cells in culture. A total of six normal HME cell cultures and four malignant HME cell cultures were tested. Table I shows that high levels of plasminogen activator are consistently observed in culture fluids conditioned by both normal and malignant HME cells. Because the number of epithelial cells may vary from one primary culture to another, and because cell clumping and the presence of non-epithelial cell types in these cultures makes conventional cell counting by trypsinization inapplicable (19), it has not been possible to determine the exact specific activity of plasminogen activator (i.e., enzyme units per cell) in either the normal or malignant HME cells. However, on the basis of microscopic estimation of epithelial cell number

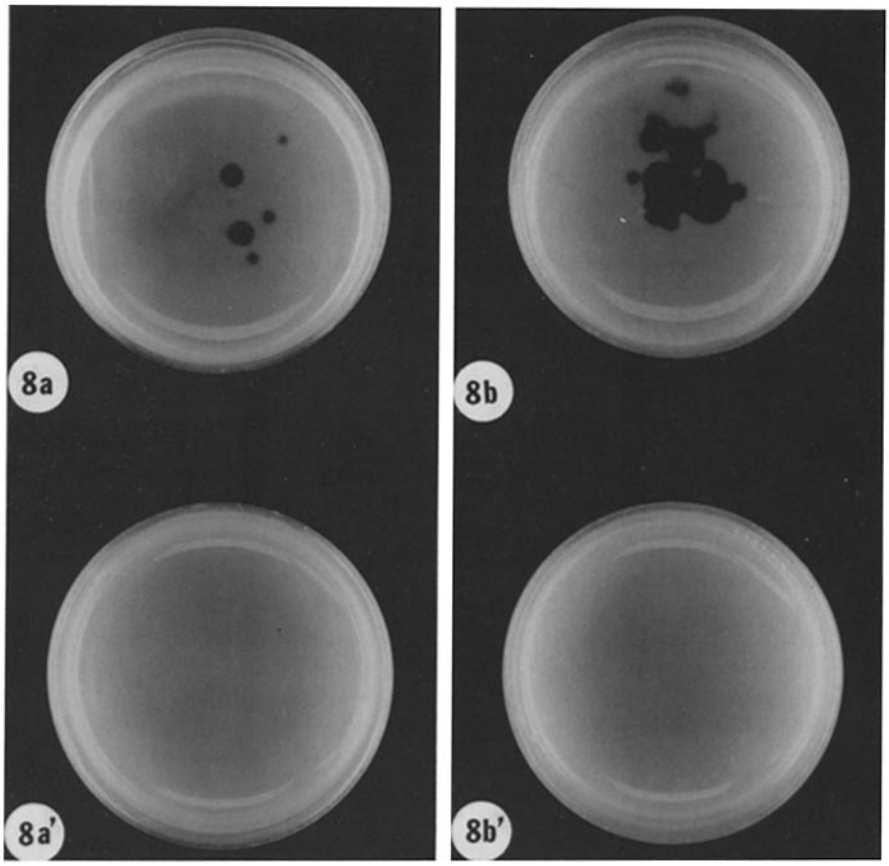

FIGURE 8 Agar-overlay assay of plasminogen-dependent fibrinolytic activities present in normal (*a* and *a'*) and malignant (*b* and *b'*) HME cells in primary cultures. *a* and *b* were assayed in the presence of plasminogen; *a'* and *b'* in the absence of plasminogen. The cleared zones demonstrate the presence of activity directly over each of the epithelial colonies in the culture.

per culture (19), we conclude that the specific activities of the plasminogen activator in the normal and malignant cell cultures are similar, although smaller quantitative differences cannot be excluded at this time.

In addition to HME cells, a number of epithelioid cell lines not of breast origin and one fibroblast cell line were also examined for production of plasminogen activator. All of the epithelial-like cell cultures tested, including D-562 cells, HBL 100 cells, and normal human fetal kidney cells, were found to exhibit high levels of plasminogen-dependent fibrinolytic activity (Table I). However, essentially no reactivity was observed for the normal human fibroblast cells, D-549. These data demonstrate that, in contrast to many fibroblast/sarcoma systems (30, 31, 41), expression of plasminogen activator in vitro does not distinguish normal from malignant epithelial cells.

Mammary epithelial cells are known to be target cells for polypeptide hormones and sex steroids (34). Because several characteristics of the human breast cancer cell line MCF-7 (13, 22–24, 51), including plasminogen activator production (6), have been found to be responsive to progesterone and estradiol, the possible effects of these hormones on the expression of plasminogen activator of HME cells in primary culture were examined. Table II shows that a combination of progesterone and β-17 estradiol (each at 1×10^{-8} M) caused up to a 10-fold increase in the plasminogen-dependent fibrinolytic activity of normal HME cells. Hormonal stimulation of these activities in primary cultures of malignant HME cells was also observed but appeared to be less pronounced. Normal HME cells treated with progesterone, estradiol, or dexa-

TABLE II

Hormonal Stimulation of Plasminogen-dependent Fibrinolytic Activities in Human Mammary Epithelial (HME) Cell Cultures

Cell tested*	Treatment	Relative activity‡	
		equivalent urokinase units × 10^2/100 μl medium	
Normal HME cells			
Culture 1	None	3.01 ± 0.11	$P < 0.01$
	+ progesterone and estradiol	5.25 ± 0.16	
Culture 2	None	0.81 ± 0.05	$P < 0.01$
	+ progesterone and estradiol	8.42 ± 0.47	
Culture 3	None	1.62 ± 0.38	$P < 0.01$
	+ progesterone and estradiol	9.57 ± 0.33	
Malignant HME cells			
Culture 1	None	1.81 ± 0.21	$P < 0.05$
	+ progesterone and estradiol	2.76 ± 0.12	
Culture 2	None	5.18 ± 0.22	$P < 0.05$
	+ progesterone and estradiol	6.57 ± 0.31	

* Number of cells in the cultures tested was 5×10^3 to 5×10^4.

‡ Relative levels of plasminogen activator were determined in 100 μl aliquots of control (no hormone) and hormone-treated conditioned media from the same cell cultures, as described in Materials and Methods and Table I.

methasone alone exhibited similar or slightly augmented levels (<30%) of plasminogen activator when compared with normal HME cultures that received no hormones (data not shown). The data shown in Table II thus demonstrate that levels of plasminogen activator released by HME cells in culture are stimulated by progesterone and estradiol, and that this hormonal regulation is likely to be a characteristic of HME cells, irrespective of whether they are derived from normal or malignant tissues.

Our observations of the expression of fibronectin and plasminogen activators in human cells are summarized in Table III.

TABLE III

Expression of Surface Fibronectin and Plasminogen Activator in Human Cell Cultures

Cells	Fibronectin*	Plasminogen activator
Primary cultures		
Normal human mammary epithelial cells	−	+
Malignant human mammary epithelial cells	−	+
Cell lines		
MCF-7, human breast carcinoma	−	+
HBL-100, human colostrum	+	+
D-549, human foreskin fibroblast	+	−
D-562, human throat adenocarcinoma	+	+
Continuous culture		
Human fetal kidney cell	+	+

* Surface fibronectin and plasminogen activator were assayed by indirect immunofluorescence staining and agar-overlay assays, respectively.

DISCUSSION

We have observed that neither normal nor malignant mammary epithelial cells in primary culture express fibronectin. This is characteristic of both human and mouse systems. Control experiments confirm that the lack of IF reactivity observed for these cells is not the result of insensitivity of the IF assay nor does it result from the lack of sufficient cellular contact in the cultures. Normal fibroblasts stain positively for fibronectin; co-cultivation of fibroblasts with the epithelial cells does not alter the reactivity of either cell type. The well-characterized permanent line of human breast carcinoma cells MCF-7 also exhibits no reactivity in the indirect IF assay for fibronectin.

Fibronectin is a component of basement membrane (39). The adjacent epithelium is considered by many to be responsible for basement membrane synthesis, and one might predict that normal epithelial cells, including the mammary epithelial

cells examined in this study, would be positive for fibronectin expression. Our data suggest that this may not be the case. The breast fluid cells tested in this study were collected as exfoliated cells and it is possible that these cells represent terminally differentiated cells that may have lost the capacity to synthesize basement membrane products such as fibronectin. However, it is unlikely that these cells are terminally differentiated, because the majority of these cells isolated from breast fluids eventually grow into large epithelial colonies that exhibit characteristics of mammary epithelium in vivo (35). These cells can be successfully grown as secondary and tertiary cultures under appropriate conditions (19), and yet they consistently remain negative for fibronectin expression. Furthermore, the mouse mammary epithelial cells examined were not exfoliated cells but were derived from the mammary gland of a pregnant mouse, and they were also negative for fibronectin expression.

Another possibility is that mammary epithelial cells may in fact be synthesizing fibronectin but secreting it into the culture fluid. This seems unlikely because negative results were obtained when acetone-fixed cells were tested, indicating that no detectable intracellular pool of soluble fibronectin is present. However, a more direct test of this possibility, by immunoprecipitation of metabolically radiolabeled culture fluids, for example, was not done.

In addition to HME cells, a number of other human epithelial cell lines were also tested for fibronectin expression. We found that HBL-100 cells, normal human fetal kidney cells, and D-562 human throat adenocarcinoma cells expressed detectable fibronectin. The HBL-100 cell line was originally derived from human colostrum and is proposed to be a normal HME cell line (33), although the HBL-100 cells do not possess characteristics normally associated with mammary epithelium (43). It is possible, judging from their morphology and expression of fibronectin, which, as we have shown here, is absent from bona fide normal or malignant mammary epithelial cells, that HBL-100 is derived from vascular endothelial cells.

With the exception of newborn hamster kidney cells and some rat liver cells (8), all primary cultures of normal and malignant (or transformed) epithelial cells previously tested, including those of pig periodontal ligament (20), mouse salivary gland and bladder (45), and mouse mammary tissue (1), lack the distinctive fibrillar or patch distribution of surface fibronectin. In contrast, some tumorigenic animal carcinoma cell lines may exhibit appreciable amounts of fibronectin, as shown by Wigley and Summerhayes (45) and Asch (1).

Our data are in general agreement with those of others (20, 45) and suggest that there is no direct correlation between malignancy or neoplastic transformation and loss of fibronectin in epithelium/carcinoma systems, including mammary epithelium of both mouse and human origin. Whether the presence of fibronectin on some of the epithelial-like cells examined by us and others (1, 8, 37, 45) is (*a*) tissue specific, (*b*) the reflection of an endothelial origin of some of these cells, or (*c*) the result of the cells' dedifferentiation or selection through long-term passage of the cell lines in culture, has yet to be determined. Should future studies support the latter two alternatives, absence of fibronectin might serve as a useful marker for differentiated epithelial cells in culture. Marceau et al. (26) have reported that the major surface protein of rat liver epithelial cells is a glycoprotein of 80,000 daltons, which is clearly distinct from the 225,000-dalton fibronectin isolated from rat liver fibroblast cells.

Using two independent assay methods, we found that both normal and malignant HME cells exhibit readily detectable levels of plasminogen activator in primary cultures. The enzyme activities can be augmented in these cultures by a combination of progesterone and estradiol at physiological concentrations, irrespective of whether the epithelial cells are derived from normal or malignant breast tissues. These results strongly suggest that the presence of plasminogen activator is not a marker for HME cell transformation, nor does the hormonal stimulation of this protease activity appear to be functionally related to malignant transformation of these cells.

The absence of surface fibronectin in HME cells does not necessarily result from the release of plasminogen activators from these cells in culture. This is supported by the observation that co-cultivation of either normal or malignant HME cells with human fibroblast cells did not inhibit fibronectin expression on the fibroblast cells. Furthermore, there is no apparent direct correlation between the presence of fibronectin and the presence of plasminogen activator in the various cells tested (Table III), and cells that express plasminogen activator may, or may not, express fibronectin.

In conclusion, two cellular characteristics, the

expression of fibronectin and the expression of plasminogen activator, which may distinguish normal from transformed or malignant fibroblasts in culture, do not serve as markers for malignancy of mammary epithelial cells. This observation underscores the need to identify additional markers for malignancy and the need to test these markers in epithelium/carcinoma systems, which comprise the vast majority of human cancers.

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