Culture of Normal and Malignant Primary Human Mammary Epithelial Cells in a Physiological Manner Simulates in Vivo Growth Patterns and Allows Discrimination of Cell Type

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ABSTRACT

We cultured primary human mammary epithelial cells from five reduction mammoplasties and five breast carcinomas and attempted to improve culture conditions and define cell populations grown. Normal cells cultured on Matrigel basement membrane-like substance formed multicellular three-dimensional structures reminiscent of tissue ducts and alveoli, while malignant cells remained as single cells crawling through Matrigel much as malignant cells separate and invade basement membrane in vivo. This re-creation of normal and malignant breast cell morphology may facilitate studies of breast cancer cell biology and determination of malignant cell authenticity in culture. Growth of cells in a reduced oxygen concentration of 12% improved cell proliferation over room air (21%); however, cells could not proliferate in a completely physiological oxygen concentration of 6%, perhaps because of the medium used. We developed an improved medium for malignant cell growth, which lengthened their life span in culture, and a completely defined medium which supported cell proliferation for six passages. Methods to determine the epithelial nature of mammary epithelial cells are illustrated and discussed. The authenticity of malignant cells in culture was suggested by their proliferation without certain growth factors required for normal cell growth or with transforming growth factor-β, which arrests normal cell proliferation, and by their contact independence.

INTRODUCTION

Studies of breast cancer cell biology have traditionally favored use of a few cell lines primarily derived from human breast cancer metastases (1-4) or rodent primary cells (5-7). More recently, techniques have been developed which enable proliferation of primary human normal and tumor-derived breast epithelial cells in culture (8, 9). Normal cells can be obtained from reduction mammoplasties, and cancer cells can be obtained from mastectomies. In addition, apparently normal cells from cancer patients taken from sites distant from the tumor can serve as intrapatient controls for breast cancer cells or be used to study premalignant changes when compared to cells from cancer-free women.

However, primary HMEC are difficult to cultivate, and growth of cells from breast carcinomas is even more problematic (10, 11). In addition, it is not always clear from which in vivo cell population the cultured cells are derived (i.e., epithelial or mesenchymal, benign or malignant). Methods have been proposed to recognize malignant HMEC in culture, such as their growth in agar/methylcellulose (12), growth factor independence (13), resistance to growth inhibition by serum (14) or transforming growth factor-β (15), and many others (16, 17), but all represent graded differences between normal and transformed cells rather than absolute delineations. It has been difficult to find criteria that clearly distinguish these cell types qualitatively (18, 19). On the other hand, expression of markers currently used to recognize epithelial cells may be influenced by choice of culture medium (20) and other growth conditions.

In most cases primary HMEC have been grown in monolayer culture on plastic in media containing serum (21) or pituitary extract (22). These conditions are not ideal for study of the cell biology of normal and malignant cells. Growth factors and hormones contained in serum and pituitary extract can have profound effects on properties of normal and malignant HMEC. For example, production of proteases (23), protease activators (24, 25), growth factors (26), and basement membrane components (27) are under hormonal control in HMEC, as are cellular interactions with basement membranes (28). The supraphysiological oxygen concentrations in which cells are grown (usually room air) can affect the binding of transcription factors (29), change gene expression (30, 31), and modulate the effects of growth factors (32).

Culture conditions also influence the morphology of HMEC. Normal HMEC in vivo grow as polar cuboidal or columnar cells in glandular structures, and cancer cells have often lost polarity and tissue organization. HMEC in culture, however, form a squamous monolayer regardless of their in vivo growth pattern. This presents certain problems for cell biology studies: (a) cell interactions in vivo may differ from those in culture and (b) discernment of benign from malignant cells may be difficult. Attempts have been made to grow HMEC in a more physiological manner in collagen gels (33), and normal mouse (34) and rat (35) mammary epithelial cells have shown ductal and alveolar morphogenesis on a complex extracellular matrix. (For an excellent review of advances in rodent mammary epithelial cell culture, see Ref. 36.)

In our laboratory we have endeavored to grow normal and tumor-derived primary HMEC. In this paper we characterize the epithelial nature of these cells and discuss problems with markers currently in use. We illustrate the use of methods of distinguishing normal and malignant HMEC previously reported for related systems. We suggest improvements in culture medium for tumor cell growth and describe a completely defined medium devoid of serum or pituitary extract that supports proliferation of HMEC for at least six passages.

We also suggest more physiological means of cultivating HMEC. We have determined the effects of oxygen concentration on cell growth and maintained cells at a more physiological concentration. We have grown normal and tumor-derived HMEC on Matrigel. This complex extracellular matrix re-creates the in vivo tissue architectural differences between normal and malignant cells, which provides a useful means of distinguishing the two cell types, and should allow for more relevant studies of cell biology.

MATERIALS AND METHODS

Cell Culture. HMEC were derived from surgical specimens from normal women who had undergone reduction mammoplasty and from the breast tumors of cancer patients. Epithelial cells were culled and grown by the method

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3 The abbreviations used are: HMEC, human mammary epithelial cells; SFS, serum-free supplements; PBS, phosphate-buffered saline; BPE, bovine pituitary extract; MTT, 3-(4,5-dimethylthiazol)-2-yl)-2,5-diphenyl-tetrazolium; TGF-β, transforming growth factor-β; HMFG, human milk fat globule antigen; ECM, extracellular matrix; MEC, mammary epithelial cells.
of Stamper (37) and Hiratsuka et al. (38). Briefly, tissues were dissected and enzymatically digested into duct-like structures termed organoids, filtered to retain only organoids, and plated or frozen in aliquots. Since many tumor cells do not form ducts, tumor samples were digested for a shorter time. Organoids were plated, and cells crawled off onto the plastic. There was no decrease in viability or alteration in morphology when cells were frozen before plating. After cells were growing in large colonies, fibroblasts were removed with brief exposure to trypsin-versene (differential trypsinization). Cells were serially passed at near-confluence with trypsin-EDTA until senescence. Human mammary fibroblasts removed by differential trypsinization were also maintained.

Cells were grown in MCD2-B170 medium (Irvine Scientific, Santa Ana, CA) + SFS, a complex medium designed for growth of HMEC (37). Hormones and growth factors present included estradiol (Sigma Chemical Co., St. Louis, MO). hydrocortisone (Sigma or Collaborative Research Inc., Bedford, MA), insulin (Sigma), epidermal growth factor (Sigma or Collaborative Research), iso proterenol (Sigma), and BPE (Clonetics Corp., San Diego, CA), at concentrations as published (37).

Immunofluorescence. Cells were plated on glass coverslips in six-well plates at 10⁵ cells/well and grown for 7–14 days. Coverslips were then removed, washed three times in PBS (pH 7.4), fixed for 3 min in methanol at −20°C, and allowed to dry. Fixed cells were incubated with 45 µl primary antibody for 30 min at 37°C in a humidified atmosphere, rinsed three times with PBS, incubated 30 min with 35 µl normal goat serum to block nonspecific staining, rinsed three times with PBS, and incubated 30 min with 35 µl fluorescein- or rhodamine-conjugated secondary antibody. Coverslips were washed three times in PBS and one time in distilled water and then mounted on microscope slides with gelvatol (polyvinyl alcohol 205; Sigma) containing 25 mg 1,4-diazabicyclo[2.2.2]octane (Sigma) per ml gelvatol to reduce fluorescent quenching. Slides were observed and photographed using a fluorescence microscope and TMAX 100 film (Eastman Kodak Co., Rochester, NY). Control slides without primary antibody or with it replaced with nonimmune sera were also prepared. The PBS used contained 0.2 g/liter KCl-0.2 g/liter KH₂PO₄-8.0 g/liter NaCl-1.15 g/liter Na₂HPO₄.

Monoclonal antibody to cytokeratins 8, 18, and 19 and the monoclonal pancytokeratin antibody AE1 + AE3 (ICN Biochemicals, Cleveland, OH) were used at 1:5 and 1:100 dilution, respectively. Polyclonal antibody to vimentin (Sigma) was used at 1:100. Monoclonal anti-desmoplakin 1 and 2 (ICN) was used at 1:10; three polyclonal antibodies to desmoplakin, two to bovine (39, 40) and one to a human fusion protein from the COOH terminus of desmplakin (41), were gifts of Dr. Kathy Green and Dr. Jonathan Jones (Departments of Pathology and Cell, Molecular and Structural Biology, Northwestern University, Chicago, IL) and used at 1:10–1:20. Monoclonal anti-desmoglein (Boehringer-Mannheim Corp., Indianapolis, IN) was used at 1:4. Polyclonal anti-fibronectin (UBI, Lake Placid, NY) was used at 1:75. Antibodies to the HMFG included HMFG-1, HMFG-2 (42), and SM-3 (43), which were kind gifts of Dr. Joyce Taylor-Papadimitriou (Imperial Cancer Research Fund, London, England), used neat; and polyclonal anti-human epidermal growth factors present included estradiol (Sigma Chemical Co., St. Louis, MO), hydrocortisone (Sigma or Collaborative Research Inc., Bedford, MA), insulin (Sigma), epidermal growth factor (Sigma or Collaborative Research), iso proterenol (Sigma), and BPE (Clonetics Corp., San Diego, CA), at concentrations as published (37).

Characterization of Cells as Normal or Malignant. In assays for growth factor independence, cells were plated at 1–2 × 10⁶ or 1–2 × 10⁷ cells/well, depending on the method of cell number determination (see below), in MCD2-B170 + SFS with or without various growth factors, re-fed every 3 days, and terminated when cells were in logarithmic growth phase (6–12 days). The cell number was determined.

In assays for growth inhibition by TGF-β, cells were plated at 1–2 × 10⁶ or 1–2 × 10⁷ cells/well, as described above, in MCD2-B170 + SFS in the absence of TGF-β and were allowed to attach overnight. The next morning they were re-fed MCD2-B170 + SFS ± 1 ng/ml human platelet TGF-β (Sigma). Cells were then re-fed every 2 days with their experimental medium and terminated at various times for cell number determination.

In experiments to determine growth inhibition by serum, cells were plated at 1–2 × 10⁶ or 1–2 × 10⁷ cells/well in MCDB-170 + SFS with or without heat-inactivated fetal bovine serum (Gibco, Grand Island, NY) at concentrations of 10, 5, 2, or 1% serum or no serum and were terminated at 1, 4, 7, and 10 days with feedings on days in between.

In contact inhibition assays, cells were plated at 2 × 10⁶ cells/well in six-well plates. Two wells were terminated at near-confluence to determine whether cells grew at the same rate prior to confluence. After confluence, cells were grown for an additional 30 days, removed by trypsin-EDTA, and counted with a hemocytometer.

Three types of assays for growth in semisolid media were used. First, in two experiments, 10¹ and 10² cells in 0.3% Bactoagar (DIFCO, Detroit, MI) were plated per 60-mm plate on top of 0.5% bottom agar. Control plates contained 200 cells in MCD2-B170 + SFS. Feedings were by addition of medium only, two times/week. Second, in two experiments, cells were plated in 1.25% methylcellulose (Fisher, 4000 centipoise) at 10⁴ cells/60-mm plate on top of 0.5% DIFCO bottom agar. Refeeding was done by addition of 1 ml 1.25% methylcellulose containing 2× growth factors one time/week. Control plates contained 200 cells re-fed by addition of medium only, with 1 ml MCD2-B170 + 2× growth factors. Third, in one experiment, cells were grown in 0.3% SeaPlaque low-melt agar (FMC, Rockland, ME) on 0.5% bottom agar. Cells were plated at 10⁴ cells/60-mm plate and re-fed one time/week with 1 ml MCD2-B170 + 2× growth factors. Control plates were described above. In all experiments, six plates per condition were observed regularly for 1 month before concluding whether formation of colonies occurred.

Table 1 Defined medium components

<table>
<thead>
<tr>
<th>Component</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin</td>
<td>5 µg/ml</td>
</tr>
<tr>
<td>Hydrocortisone</td>
<td>500 ng/ml</td>
</tr>
<tr>
<td>Epidermal growth factor</td>
<td>10 ng/ml</td>
</tr>
<tr>
<td>Ethanolamine</td>
<td>100 µM</td>
</tr>
<tr>
<td>Phosphorylcholine</td>
<td>100 µM</td>
</tr>
<tr>
<td>Transferrin</td>
<td>10 µg/ml</td>
</tr>
<tr>
<td>Glutamine</td>
<td>290 µg/ml</td>
</tr>
<tr>
<td>Iso proterenol</td>
<td>1 µM</td>
</tr>
<tr>
<td>β-Estradiol</td>
<td>2 ng/ml</td>
</tr>
<tr>
<td>Triiodothyronine</td>
<td>200 ng/ml</td>
</tr>
<tr>
<td>Progesterone</td>
<td>1.25 ng/ml</td>
</tr>
<tr>
<td>Linoleate:albumin, 3:1</td>
<td>10 µg/ml</td>
</tr>
<tr>
<td>Growth hormone</td>
<td>2 ng/ml</td>
</tr>
<tr>
<td>Proline</td>
<td>1 µg/ml</td>
</tr>
<tr>
<td>Luteinizing hormone</td>
<td>45 µg/ml</td>
</tr>
<tr>
<td>Follicle-stimulating hormone</td>
<td>4 ng/ml</td>
</tr>
<tr>
<td>Thyroid-stimulating hormone</td>
<td>3 µU/ml</td>
</tr>
<tr>
<td>Adrenocorticotropic hormone</td>
<td>1 µg/ml</td>
</tr>
<tr>
<td>Oxytocin</td>
<td>50 ng/ml</td>
</tr>
<tr>
<td>Thymotropin-releasing hormone</td>
<td>2 ng/ml</td>
</tr>
<tr>
<td>Growth hormone-releasing hormone</td>
<td>1 ng/ml</td>
</tr>
<tr>
<td>Luteinizing hormone-releasing hormone</td>
<td>1 ng/ml</td>
</tr>
<tr>
<td>Corticoropin-releasing factor</td>
<td>1 ng/ml</td>
</tr>
</tbody>
</table>

Table 1 Defined medium components

A defined medium was designed using MCD2-B170 + SFS (35) and replacing bovine pituitary extract with hormones and growth factors it might contain.
set up and cells were terminated every few days until confluence; cell number was determined at each time. Media that could sustain short-term growth of cells were tested in long-term assays. HMEC were seeded in flasks and serially passaged at confluence until they died out or senesced. The number of passages they survived was recorded, and their percentage of confluence at each feeding was noted and graphed as an approximation of their growth pattern. Third, media in which cells could grow for several passages were tested on primary explants. HMEC were plated from organoids in various media, differentially trypsinized at 1 week, then passed every 2 weeks in a stringent manner regardless of confluence, and seeded at approximately 10% confluence. The percentage of confluence and number of passages until cell death or senescence was noted.

Cell Number Determination. Cell number was determined in two ways: directly by hemocytometer and indirectly using an MTT assay. For the former, cells were plated at 1–2 × 10^4 cells/well in 24-, 12-, or 6-well plates, terminated with trypsin-EDTA, washed once with PBS, and counted in a hemocytometer. MTT assays were performed by the method of Mosmann (50) with revisions by Romijn et al. (51). Briefly, cells were set up in quadruplicate in 96-well plates at 1–2 × 10^4 cells/well. On the day of termination, 10 μl of 5 mg/ml sterile MTT (Sigma) in PBS was added per well. After 5 h at 37°C, medium was removed, 200 μl dimethyl sulfoxide (Fisher, Itasca, IL) were added, and the plate was shaken for 15 min. Plates were read at 595 nm, and values at 700 nm were subtracted. Cell number was calculated from counts by performing an MTT assay on the day of plating at which time cell number plated had been determined by hemocytometer. Wells without cells were also run as a control. The optimal concentration of MTT and the time of incubation were determined experimentally (data not shown).

Growth at Varying Oxygen Concentrations. Cell proliferation in various concentrations of oxygen was measured in short-term and long-term assays. Incubators were set up with oxygen concentrations of 18, 12, and 6% with the difference between these and room air composed of nitrogen and 0.2% CO₂. For long-term assays, cells were explanted from organoids in flasks at a particular oxygen concentration and serially passed at near-confluence. Morphology, growth rate, and life span in culture were noted. In short-term experiments, cells initially grown in a long-term experiment were seeded in wells and returned to the same oxygen concentration in which they had been explanted. Wells were then terminated, and cell number was determined every few days to generate a growth curve.

Growth in Matrigel. Matrigel (Collaborative Research) is a substance produced by the Engelbreth-Holm-Swarm murine sarcoma in vivo, which when gelled in tissue culture resembles the lamina lucida portion of the basal lamina by electron microscopy (52). It contains laminin, collagen IV, heparan sulfate, entactin, and other uncharacterized substances (53). Assays were performed in 35-mm culture dishes or six-well plates coated with 500 μl undiluted cold Matrigel according to the manufacturer’s instructions and then placed at 37°C for at least 30 min. Cells were plated at 1–5 × 10^4 cells/well on top of gelled Matrigel. Cells were fed two times/week with MCDB-170 + SFS and observed.

In some experiments, cells were removed from Matrigel by treatment with dispase (Collaborative Research) according to the manufacturer’s instructions and counted with a hemocytometer. Viability both in situ on Matrigel and after termination was determined by trypan blue exclusion.

Statistics. t tests were performed using the SPSS statistical package on an IBM PC. Cell number or percentage of control was compared between the mean of the values from normal cells from five women and cells from each tumor patient for Fig. 3 and Table 3 or between cells grown in different media but from the same woman in Table 2.

RESULTS

Growth and Characterization of HMEC. Primary HMEC from five women without cancer and the tumors of five patients with cancer were grown in long-term culture. After subculture and removal of fibroblasts by differential trypsinization, cells exhibited a typical epithelial cobblestone morphology with some dome and ridge formation. Cells from different women grew at different rates, but most exhibited a doubling time of approximately 48 h. Cells underwent a selection (“crisis”) at about passage 2 and then grew for 7–25 passages before senescing. There was no consistent difference in morphology between normal and tumor-derived cells (Fig. 1); however, tumor cells tended to senesce at earlier passages than normal cells. Both normal and tumor-derived cells varied in size and shape in culture.

One hundred percent of cells in the HMEC cultures appeared epithelial by morphology after differential trypsinization, whereas the cells removed by differential trypsinization and replated were a mix of fibroblastoid and epithelioid morphologies. However, to be sure that HMEC cultures contained no fibroblasts, they were characterized as follows.

a. Cells were stained with antibodies to cytokeratins, which are usually expressed only in epithelial cells, and vimentin, which is not usually expressed in epithelial cells. Both normal and tumor-derived HMEC showed immunofluorescent staining with two cytokeratin antibodies which varied from cell to cell: 100% of confluent cells stained with a pancytokeratin antibody and 50% stained with antibody to keratins 8, 18, and 19; small actively proliferating cells showed keratin staining in only some cells with either antibody; and 100% of single nonproliferative cells stained with both antibodies. Therefore, there were some cells which were epithelial by other criteria (e.g., morphology and staining for desmosomal proteins) that did not stain for cytokeratins. No human mammary fibroblasts cultured after differential trypsinization from the same specimens showed keratin staining (Fig. 2). All fibroblasts exhibited staining with antibodies to vimentin (data not shown). Staining of many epithelial cells with vimentin antibodies was also seen, however.

b. Cells were stained with antibodies to the HMFG, a mucin on the luminal surface of MEC (54). Each of four antibodies to the HMFG stained some epithelioid cells in the post-differential trypsinization HMEC cultures but none of the fibroblastoid cells removed by differential trypsinization (data not shown).

c. Cells were stained with antibody to smooth muscle actin which is usually found in breast myoepithelial cells but not in luminal epithelial cells, fibroblasts, or other nonmuscle cells. All epithelioid cells stained with antibody to smooth muscle actin, whereas fibroblastoid cells did not (data not shown).

d. Cells were stained for fibronectin which has been suggested to discriminate between epithelial cells and fibroblasts by its pattern of staining and is not expressed by macrophages or mast cells. All epithelioid cells exhibited a punctate pattern of cell-associated fibronectin, while staining of fibroblastoid cells was lacy and fibrillar (data not shown).

e. Because both epithelial cells and fibroblasts exhibit fibronectin staining, because expression of cytokeratins or vimentin in intermediate filaments has been suggested to change in culture (20), and because not all epithelioid cells in our experiments expressed HMFG, we used an additional method to show that our HMEC were 100% epithelial. Cells were stained with a battery of antibodies to desmosomal proteins: four desmoplakin antibodies from three sources and...
one desmoglein antibody. One hundred percent of normal and tumor-derived HMEC stained at areas of cell-cell contact with all five antibodies, while no cultured fibroblastoid cells removed by differential trypsinization stained (Fig. 2). Thus, staining of desmosomal proteins appeared to discriminate best between epithelial cells and fibroblasts and also confirmed that our HMEC cultures were not contaminated by fibroblasts.

**Discrimination of Normal from Malignant HMEC.** Many methods have been proposed to distinguish normal from transformed MEC. It has been shown that tumor cells have a reduced requirement for certain growth factors for proliferation in culture (7, 13), that their growth is not as inhibited by TGF-β as is normal cell growth (15, 55), that they exhibit decreased contact inhibition of proliferation, that they can form colonies in methylcellulose (12, 16), and that they can survive in bovine serum, whereas normal cells cannot (14). Since most of these properties were illustrated in rodent systems, human cell lines, or in vitro transformed cells, we decided to test their ability to discriminate primary cultures of normal from malignant HMEC.

### Table 2 Growth of HMEC in further refined defined media

<table>
<thead>
<tr>
<th>Cell strain</th>
<th>Defined medium A (BPE (%))</th>
<th>Defined medium B (BPE (%))</th>
<th>Defined medium C (BPE (%))</th>
</tr>
</thead>
<tbody>
<tr>
<td>N1</td>
<td>60 ± 3</td>
<td>88 ± 8e</td>
<td>80 ± 10</td>
</tr>
<tr>
<td>N10</td>
<td>39 ± 3</td>
<td>82 ± 4c</td>
<td>86 ± 7e</td>
</tr>
<tr>
<td></td>
<td></td>
<td>77 ± 10f</td>
<td></td>
</tr>
</tbody>
</table>

*Defined medium A contains MCDB-170 without BPE and with the additions listed in Table 1.
*Defined medium B is defined medium A less thyrotropin-releasing hormone, growth hormone-releasing hormone, luteinizing hormone-releasing hormone, and corticotropin-releasing hormone.
*Defined medium C is defined medium B less luteinizing hormone, follicle-stimulating hormone, thyrotropin-stimulating hormone, and adrenocorticotropic hormone.

We grew normal and tumor-derived HMEC in MCDB-170 + SFS with or without one of the following growth factors usually present: BPE, insulin, or hydrocortisone. In six experiments, proliferation of HMEC from five women without cancer was inhibited by a mean of 28% in medium without BPE, 41% without insulin, and 24% without hydrocortisone. Some tumor-derived cell strains exhibited reduced or no inhibition of proliferation under these conditions (Table 3). We grew normal and tumor-derived HMEC in their usual medium ± 1 ng/ml TGF-β for 5 days. Whereas normal cell proliferation of five cell strains was reduced an average of 36% in three experiments, proliferation of cells from both tumor-derived cell strains tested was not inhibited by TGF-β (Table 3). A 1-ng/ml concentration was chosen after a concentration curve from 0.25–2.5 ng/ml showed that it discriminated maximally between normal and tumor; 5 days was chosen after a time course from 1–15 days showed this to be during the time of logarithmic proliferation of control cells (data not shown).

To study the release of tumor cells from restraint of proliferation by contact with neighboring cells, we plated normal or tumor-derived HMEC at equal cell numbers, grew them for 30 days beyond confluence, and determined the cell number. Tumor-derived cells proliferated to an average final cell density twice that of normal cells (Table 3). This was not due to an increased preconfluent rate of division; wells containing normal and tumor-derived cells terminated several days preconfluence contained about the same number of cells and, therefore, exhibited similar growth rates.

In our hands, in five experiments, neither normal nor tumor-derived primary HMEC could grow beyond a nine-cell stage in methylcellulose or soft agar (data not shown).

As little as 1% heat-inactivated fetal bovine serum added to our culture medium resulted in the death of both normal and tumor-derived HMEC in 2 days (data not shown).

In Fig. 3 data from one of the five tumor cell strains are compared to the mean of the five normal cell strains with regard to growth factor independence, release from growth inhibition by TGF-β, and contact.
Normal or tumor-derived HMEC were tested for their ability to proliferate in the absence of certain growth factors BPE, insulin, or hydrocortisone, or with the addition of TGF-β. The growth of tumor cells from a different breast cancer patient and is designated "T" and numbered in the order in which the samples were received. No normal cell strains showed normal growth in the absence of a growth factor or in the presence of TGF-β.

**HMEC Cell Culture Refinements.** We have been working to improve culture conditions for HMEC toward three goals: to improve the growth of tumor cells, to maintain cells in media which contain no unknown components, and to grow cells under more physiological conditions. Tumor HMEC are difficult to grow and do not proliferate for as many passages in MCDB-170 + SFS as do normal HMEC (average, seven versus 15 passages for our 10 cell strains). Therefore, we have worked to improve growth of tumor HMEC through refinements in culture conditions. Addition of triiodothyronine, cholera toxin, sodium selenite, and ascorbate to MCDB-170 + SFS results in more rapid proliferation of tumor HMEC and an increase in life span by an average of two passages (data not shown).

Fig. 3. Evidence for the malignant nature of cells. Summary of evidence from five types of assays that cells grown from the tumor of one breast cancer patient are malignant. Assays were performed for independence from each of three growth factors, ability to grow in the presence of TGF-β and lack of inhibition of growth by contact with other cells as described in “Materials and Methods.”

<table>
<thead>
<tr>
<th>Cell strain</th>
<th>-BPE (%)</th>
<th>-Insulin (%)</th>
<th>-Hydrocortisone (%)</th>
<th>+TGF-β (%)</th>
<th>Contact inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean of five normal</td>
<td>72 ± 4</td>
<td>59 ± 7</td>
<td>76 ± 7</td>
<td>64 ± 12</td>
<td>(13.5 ± 1.5) x 10⁴</td>
</tr>
<tr>
<td>T3</td>
<td>108 ± 8b</td>
<td>88 ± 8b</td>
<td>146 ± 99</td>
<td>22.0 x 10⁴</td>
<td></td>
</tr>
<tr>
<td>T4</td>
<td>225 ± 75</td>
<td>112 ± 21b</td>
<td>124 ± 10b</td>
<td>41.0 x 10⁴</td>
<td></td>
</tr>
<tr>
<td>T12</td>
<td>88 ± 9c</td>
<td>67 ± 1</td>
<td>150 ± 6c</td>
<td>111 ± 10c</td>
<td></td>
</tr>
<tr>
<td>T16</td>
<td>68 ± 2</td>
<td>49 ± 4</td>
<td>117 ± 6c</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T17</td>
<td>85 ± 5b</td>
<td>158 ± 32c</td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

Values from cell strains from five women without cancer are averaged to represent the normal reaction of HMEC to these growth conditions. Each tumor cell strain contains primary tumor cells from a different breast cancer patient and is designated “T” and numbered in the order in which the samples were received. No normal cell strains showed normal growth in the absence of a growth factor or in the presence of TGF-β.

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- P < 0.01 compared to normal.
- P < 0.05 compared to normal.
- P < 0.001 compared to normal.

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- Values from cell strains from five women without cancer are averaged to represent the normal reaction of HMEC to these growth conditions. Each tumor cell strain contains primary tumor cells from a different breast cancer patient and is designated “T” and numbered in the order in which the samples were received. No normal cell strains showed normal growth in the absence of a growth factor or in the presence of TGF-β.

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Fig. 4. Growth of normal HMEC in a defined medium. Cells were seeded in 96-well plates at 2000 cells/well in MCDB-170 + SFS containing BPE (control medium), without BPE (−BPE), or MCDB-170 plus the components listed in Table 1 (defined medium). Wells were fed twice/week and terminated in quadruplicate at 6, 13, and 20 days. Cell number was determined by MTT assay. This is an experiment with one cell strain (representative of three performed with three cell strains). Points, means; bars, ±SD.

more than half the rate of cells in their usual medium or for more than three passages. Since this was no improvement over published defined media, this strategy was abandoned.

With the second strategy, we developed a defined medium for HMEC by replacing many components of BPE en masse (Table 1). In short-term growth curves, cells in the defined medium reached a cell number approximately 85% of that in medium containing BPE at 20 days (Fig. 4). Cells also show some growth in MCDB-170 + SFS even in the absence of BPE, but they cannot be passaged (removed by trypsin and replated to yield proliferating cells). In our defined medium, in long-term growth assays, cells that were not begun from explants could be maintained for six passages, and those explanted directly into our defined medium grew well for three to four passages. Cells grown in defined medium did not differ in appearance from those grown with BPE.

We have begun to simplify our defined medium. When all hypothalamic hormones were removed from the mix (defined medium B), proliferation of cells in short-term culture was not decreased compared to the original defined medium (defined medium A). When these hormones plus all pituitary hormones except prolactin, oxytocin, and growth hormone were removed (defined medium C), proliferation was slowed, although growth was not statistically different from that in defined medium A for either cell strain. In Table 2 growth in these three media is illustrated at 9 days for two cell strains and expressed as a percentage of growth in medium containing BPE. Note that, although growth in defined medium A is significantly different from that without BPE for both cell strains, growth in media B and C is not statistically different from that without BPE for cell strain N1.

Most investigators maintain cells in tissue culture at room oxygen concentrations (approximately 21% oxygen), exposing cells to levels of oxygen tension much higher than in vivo. We attempted to reduce oxygen stress by growing HMEC at a more physiological oxygen concentration. Fig. 5 shows growth of normal HMEC at 12 and 6% oxygen at two different passages expressed as a percentage of their growth in 18% oxygen (close to room air). Early passage cells tended to grow better in 12 than in 18% oxygen (Fig. 5A), whereas at later passages they grew equally well in 12 and 18% oxygen (Fig. 5B). HMEC did not grow well in 6% oxygen. Cells in long-term culture senesced at approximately the same passage regardless of oxygen concentration. Because of the improved early growth and more physiological condition, we currently maintain our cells in 12% oxygen.

Growth of HMEC on Matrigel. Growth of cells on tissue culture plastic is not physiological. In vivo normal and carcinoma cell growth patterns differ tremendously; in culture normal and tumor HMEC grow as a virtually identical monolayer of flattened cells (Fig. 1). Matrigel allowed HMEC to grow in a more physiological manner. As in vivo, cells grew on a basement membrane-like substance, and normal cells were stimulated in some way to form structures reminiscent of the ducts and alveoli they form in vivo (Fig. 6, N, and Fig. 7). Tumor cells, on the other hand, remained separate and migrated through Matrigel much as invasive carcinoma cells invade the basement membrane and extracellular matrix in vivo (Fig. 6, T, and Fig. 8).
The development of the three-dimensional structures over time is illustrated at low power in Fig. 6 and at high power in Fig. 7. Structures formed by normal cells on Matrigel were initially composed of few cells (Fig. 6, 24 h and 5 d, Fig. 7A) but by 12 days were multicellular and several cell layers thick (Fig. 6, 12 d); after several weeks large structures made of hundreds of cells and many cell layers thick had formed (Fig. 6, 20 d, Fig. 7B).

That the multicellular structures were formed by the migration and congregation of cells rather than by cell proliferation was suggested by two observations. First, the cells were initially distributed evenly over the Matrigel surface, and as three-dimensional structures formed, bare areas of Matrigel appeared. Second, when cells were released from Matrigel with dispase and were counted, the cell number was virtually unchanged from the time of initial plating, even after several weeks.

Tumor-derived cells never congregated or formed three-dimensional structures on Matrigel; they did not elongate or send out cellular processes but remained rounded (Fig. 6, T). Tumor-derived cells did exhibit motion in Matrigel, however. After 1–2 weeks, they began to crawl through the Matrigel, leaving trails in the gel (Fig. 8A). This movement became more active and extensive with time (Fig. 8B); but despite movement of cells quite close to one another, they never congregated.

Normal and tumor-derived cells were viable by trypan blue exclusion and motility and could be replated on plastic after release from Matrigel by dispase. Neither normal nor tumor-derived cells actively proliferated on plastic after growth on Matrigel, however. All Matrigel experiments were performed on all 10 cell strains, and figures shown are composed of photographs of cells from different patients as noted.

DISCUSSION

In this paper we have presented data concerning the growth of normal and tumor-derived primary human mammary epithelial cells. We have described means of determining whether cultures contain 100% epithelial cells and whether tumor-derived cells are truly malignant. We have altered published culture conditions through medium additions that increase tumor cell proliferation, elimination of undefined components from the media, and culture of cells in a more physiological oxygen concentration and on a basement membrane-like substance.

Characterization of Cells as Epithelial. The normal breast contains epithelial cells, fibroblasts, macrophages, mast cells, etc. Because we wanted pure cultures of epithelial cells, we tested our HMEC (which were 100% epithelioid by morphology) to determine whether they were truly epithelial.

Fig. 6. Morphology of HMEC on Matrigel over time. Normal (N) or tumor-derived (T) HMEC were grown on Matrigel in MCDB-170 + SFS for the times indicated and photographed through a phase contrast microscope. Normal cell strain N10 is shown at 24 h and 20 days, and N8 is shown at 5 and 12 days. Tumor cell strain T12 is photographed at 24 h and 12 days, while T17 is at 5 and 20 days. Original magnification, × 40.
Although in vivo expression of keratin intermediate filaments is restricted to epithelial cells, whereas vimentin intermediate filaments are found in fibroblasts, some of our normal and tumor-derived HMEC were positive for keratins, others vimentin, and some both. Other researchers have also seen expression of vimentin and keratin in the same cell at the same time (56) and vimentin in breast epithelial cells in culture (20, 57, 58). In fact, cell expression of intermediate filament proteins in culture may depend more on medium (59), rate of proliferation (60), or cell density (61) than cell lineage. Since a keratin-negative, vimentin-positive cell could be either an epithelial cell or a fibroblast, we conclude that intermediate filament protein expression in culture is not a reliable marker of cell lineage.

b. Macrophages do not express fibronectin, but fibroblasts and normal and malignant HMEC do (62), and epithelial cells and fibroblasts may be distinguished by their different immunofluorescence pattern of fibronectin staining (63). All of our epithelioid cells exhibited the epithelial pattern of fibronectin staining. However, others have reported human milk epithelial cells with an extracellular fibroblast-like fibronectin network (57). Therefore, this is not always a reliable marker for cell type.

c. An M, 400,000 mucin, the HMFG, or epithelial membrane antigen is found on the luminal surface of HMEC (54). When we used antibodies to four different HMFG epitopes in immunofluorescence assays, no antibody stained all of the epithelioid cells. However, exposure of different epitopes of the HMFG can vary by epithelial cell strain (64–66) and growth medium (67) and is often heterogeneous within a population of epithelial cells (68, 69).

d. We suggest that desmosomes are better markers for epithelial cells because they are not seen in nonepithelial cells, and all of our HMEC stained with five antibodies to desmosomal proteins regardless of patient, malignancy, passage number, or medium, while no fibroblastoid cells stained with any of the antibodies.

**Discrimination of Normal from Malignant HMEC.** Controversy exists regarding whether tumor-derived HMEC in culture are malignant cells or contaminating normal cells (19). We examined a few of the many methods that have been used to try to distinguish normal from malignant or in vitro transformed MEC in culture. Morphology is not useful because cells change morphology in monolayer culture (30) and because normal and malignant HMEC look alike. Demonstration of tumorigenicity in nude mice is problematic for HMEC because of requirements for placement of cells in the mammary fat pad (70) and supplemental estrogen and low tumor yield with long latency (14, 71). Several groups (12, 16) have used growth in agar or methylcellulose to discern transformed cells. In our hands, in five experiments with five primary normal and tumor cell strains, we saw no growth in agar or methylcellulose. On the other hand, Stampfer et al. (72) found that both normal and tumor-derived HMEC grew in methylcellulose.

Others have used growth properties to distinguish normal and malignant HMEC. Although their population doubling times are not different (73), malignant and transformed cells may maintain their doubling rate in the absence of growth factors and hormones required for normal cell proliferation (7, 13, 57, 74). Our normal HMEC were substantially growth inhibited by the absence of individual growth factors, while tumor cells from different patients had become independent of one or another growth factor.

Reports suggest that serum or TGF-β inhibit proliferation of normal HMEC, whereas transformed cells are relatively unaffected.
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75, 76). Although serum killed our cells outright, TGF-β did show selectivity. However, cancer cell strains varied in their acquisition of resistance to TGF-β.

The clearest distinction found between normal and malignant HMEC in this study was the formation of three-dimensional duct-like structures by normal HMEC on Matrigel basement membrane-like substance and the invasion of Matrigel by tumor-derived HMEC as single rounded cells, as is discussed further below. Because all normal cell strains formed large and extensively interconnected “ducts” on Matrigel, and no congregation of tumor-derived cells occurred, we think this clear-cut difference may be used in the future to determine whether primary cultures contain normal or malignant HMEC.

Cell Culture Refinements. We extended the life span of malignant HMEC in culture through medium additions. Unfortunately, these additions reduced the life span of normal cells by approximately five passages. Band and Sager (21) stressed the importance of growing normal and malignant HMEC in the same medium for purposes of comparison, and in light of the known effects of media on protein expression (see “Introduction”), this emphasis is well placed. Although we maintain normal and malignant cells in slightly different media for some purposes, all direct comparison experiments are performed in the same medium, usually our defined medium.

Other groups have developed defined media for HMEC growth, resulting in limited life span in culture. Among the best growth reported was three to four passages in medium with prolactin and prostaglandin E1 replacing BPE (22). In our hands, cells proliferated for only one passage when explanted in this medium.

Our laboratory is interested in effects of oxygen stress on cells; we have, therefore, tried to limit oxygen stress on our HMEC. The normal pO2 in the breast is 65 mm Hg or 8.6%, while the average pO2 in breast cancer is 30 mm Hg or 3.9% (77). We found that HMEC could grow well at 18 or 12% oxygen. In fact, cells grew better in 12% at low passages. At later passages cells grew as well in 18% oxygen, implying that cells could adapt to higher oxygen or were selected in culture for their ability to grow in higher oxygen.

HMEC did not proliferate in 6% oxygen in MCDB-170. This may reflect that the medium, which was developed for growth in room air (approximately 21% oxygen), might not be optimal for cell growth at physiological oxygen concentrations. Alternatively, the cells may have undergone some adaptation soon after removal from the patient since they are in room air for a variable length of time. This subject has been addressed by Lin et al. (78) in rat MEC in which processing and deoxygenation of bet2 and double minute chromosomes. Genes Chromosomes Cancer, 1: 48—58, 1989.

As interesting as was the histogenesis of normal HMEC on Matrigel, it is actually surprising, because collagen I is not found in the normal breast development (85), and developing ducts grow along connective tissue septa (86).

As interesting as was the histogenesis of normal HMEC on Matrigel was the lack of it by tumor-derived HMEC. Cells from five different patients’ tumors remained as single separate cells on Matrigel, even when left for 60 days. There have been a few previous reports of tumor-derived MEC growth on ECM-like substances. On collagen I, human breast cancer cell lines remained as single cells, whereas SV40 immortalized “normal” HMEC formed “balls” (87), and rat tumor cells formed “spherical” colonies, whereas normal rat MEC formed branching colonies (88); however, a variety of rat breast cancer cell lines did form branching structures (89). On Matrigel, mouse breast cancer cell lines (90) and MCF7 human breast cancer cell line (91), formed “clusters” but no ducts.

It is unclear from our research to date why normal and malignant HMEC assumed different morphologies on Matrigel; however, it is possible that they respond differently to the ECM due to different receptors, and differential expression of laminin receptors has been reported (92, 93). Alternatively, they may secrete different motility factors (94) or chemoattractants, have different receptors for such factors, or have different signal transduction mechanisms to respond to such factors. Finally, they may secrete different proteases to degrade or remodel the matrix (95—97). The differential growth patterns described in this paper may prove useful in the study of these biological differences.

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13. Band, V., Zajchowski, D., Kulesa, V., and Sager, R. Human papilloma virus DNAs immortalize normal human mammary epithelial cells and reduce their growth factor stimulation of morphogenesis is not surprising. The role of ECM in vivo in histogenesis has been suggested by many researchers (83, 85). A variety of changes in the composition of the ECM accompanies breast development (85), and developing ducts grow along connective tissue septa (86).

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Culture of Normal and Malignant Primary Human Mammary Epithelial Cells in a Physiological Manner Simulates *in Vivo* Growth Patterns and Allows Discrimination of Cell Type

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