Differential Isolation of Normal Luminal Mammary Epithelial Cells and Breast Cancer Cells from Primary and Metastatic Sites Using Selective Media

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ABSTRACT

The present studies were aimed at determining if the use of a cell culture medium that supports proliferation of human mammary epithelial cells of the luminal lineage would allow routine isolation of breast cancer cells from primary and metastatic tumor specimens. Results obtained with mammary epithelial cells derived from reduction mammoplasty specimens and primary breast carcinomas indicated that growth of cells on type I collagen-coated dishes in Ham's F-12 medium supplemented with insulin, hydrocortisone, epidermal growth factor, cholera toxin, and 5% fetal bovine serum resulted in the growth and serial passage of cells that stained positively for the luminal cell marker cytokeratin-19. By contrast, growth of mammary epithelial cells in a growth factor-supplemented serum-free medium resulted in the emergence of mammary epithelial cell colonies that were uniformly negative for keratin-19. Filter isolation methods were used to isolate individual keratin-19-positive colonies from primary cultures derived from breast cancer specimens. All of the luminal mammary epithelial cells isolated from breast cancer tissues expressed characteristics of normal cells. Keratin-19-positive colonies isolated from several different tumors all grew rapidly for 30 to 60 days in culture and then senesced. Cells were isolated from one tumor that was known to have undergone a loss of heterozygosity at a specific locus in the p53 gene. All colonies isolated from this specimen contained both p53 alleles, which was consistent with their origin from normal luminal cells. Cells were also isolated from one tumor in which the c-erbB2 protein was drastically overexpressed in the neoplastic cells. Once again, keratin-19-positive colonies isolated from this tumor did not overexpress the c-erbB-2 protein. Experiments were then performed with cells derived from pleural effusions and metastatic lymph nodes. Results obtained with these specimens indicated that the growth conditions that support the growth of normal luminal mammary epithelial cells do not support the growth of neoplastic cells. However, the omission of cholera toxin, epidermal growth factor, and type I collagen substratum resulted in the isolation of two long-term cell lines. Both cell lines have population doubling times of approximately 100 h, are hyperdiploid, and stain positively for cytokeratin-19. Thus, culture conditions that support the growth of normal luminal mammary epithelial cells do not, in general, support the growth of breast cancer cells.

INTRODUCTION

Experimental systems that allow direct comparisons to be made between normal and neoplastic cells provide a powerful tool for the determination of specific phenotypes of neoplastic cells that contribute directly to their neoplastic potential. In addition, determination of the important altered phenotypes expressed by neoplastic cells allows mechanistic connections to be made regarding the molecular (genetic) basis for those altered phenotypes. Over the past several years, we have taken this approach in the study of the biology of rat mammary carcinogenesis by identifying alterations in growth-regulatory mechanisms that characterize the neoplastic cells and studying these alterations at the cellular and molecular level (1–6). A similar approach to the study of human breast cancer biology has been hampered by technical limitations that have precluded the in vitro growth of normal mammary epithelial cells of the lineage that gives rise to HBC and the successful culture of primary human breast cancer cells themselves on a routine basis.

There have been a number of improvements in cell culture technology that have led to the development of culture conditions that support rapid and prolonged proliferation of normal HME cells obtained from reduction mammary specimens. Stampfer et al. (7–10) developed a culture medium for the growth of HME cells that originally consisted of many undefined components and was later refined such that bovine pituitary extract constituted the only undefined component of the medium. The medium developed by Stampfer et al. (11) is a hormone- and growth factor-supplemented medium that supports proliferation of HME cells over many in vitro passages and was used to develop an immortalized cell line following chemical carcinogen treatment of the cells. Subsequently, other groups reported the successful culture of HME cells using similar media. Band and Sager (12) demonstrated extensive proliferation of HME cells in a growth factor- and hormone-supplemented medium that also contained serum and pituitary extract. Petersen and Van Deurs (13) and Ethier et al. (14) reported growth of normal HME cells in serum-free media in the absence of pituitary extract or serum. Despite this apparent success in the development of tissue culture methods for the growth of normal mammary epithelial cells, none of these systems support growth of mammary epithelial cells of the luminal lineage. This is important because Taylor-Papadimitriou et al. (15, 16) demonstrated that HBC cells in vivo express cytokeratin markers consistent with an origin from the luminal cells of the terminal ductal lobular unit. In addition, these workers demonstrated that human breast cancer cell lines retain expression of keratins expressed by luminal mammary epithelial cells even after extended in vitro passage. By contrast, mammary epithelial cells that proliferate rapidly in vitro under culture conditions discussed above are uniformly negative for the expression of luminal cell cytokeratins and are positive for keratins expressed by basal/myoepithelial cells present in the mammary gland. Thus, culture conditions that support rapid proliferation of normal HME cells over many in vitro passages do not support the growth of the normal cell type from which breast cancer arises.

This inability to culture the appropriate cell lineage has had important implications for experiments in which similar culture conditions were applied in an attempt to culture HBC cells from primary tumors. Wolman et al. (17) demonstrated that cells cultured from primary tumors were uniformly diploid even when obtained from tumors that were known to consist of aneuploid breast cancer cells. This was one of the first indications that culture conditions that support the growth of normal HME cells were not suitable for the growth of breast cancer cells obtained from primary tissues. Evidence obtained by Taylor-Papadimitriou et al. (16) is consistent with that notion, since they

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3 The abbreviations used are: HME, human mammary epithelial; HBC, human breast cancer; FBS, fetal bovine serum; IN, insulin; HC, hydrocortisone; EGF, epidermal growth factor; CT, cholera toxin; K-19, cytokeratin-19; LOH, loss of heterozygosity; PCR, polymerase chain reaction.
demonstrated that HME cells cultured from primary breast cancer specimens expressed the same cytokeratin profile as cells cultured from reduction mammoplasties.

We have worked to develop and refine cell culture methods that support the proliferation of normal HME cells of the luminal lineage and bona fide HBC cells obtained from primary and metastatic tumor tissues. These tissue culture methods support primary culture and serial passage of luminal mammary epithelial cells derived from reduction mammoplasties and primary breast cancers. In addition, we have developed methods to selectively isolate luminal cell colonies in order to study their proliferative potential in the absence of the more rapidly growing basal/myoepithelial cells. Finally, we have used these methods to isolate HBC cells from pleural effusions, metastatic lymph nodes, and primary tumors and have detected significant alterations in growth phenotypes between these cells and normal luminal mammary epithelial cells.

MATERIALS AND METHODS

Preparation of Normal and Neoplastic Mammary Epithelial Cells. Mammary epithelial cells were obtained from normal mammary tissues, primary breast cancers, and metastatic lymph nodes by enzymatic dissociation as described previously (14) but with minor modifications. Briefly, tissues were minced with scalpels and incubated overnight in Medium 199 containing type III collagenase (Worthington Biochemical Corp., Freehold, NJ) at a concentration of 200 units/ml and Dispase (Boehringer Mannheim, Indianapolis, IN) at 1 mg/ml. Twenty ml of media/g of tissue were used. The tissues were agitated gently in a shaking water bath at 37°C. The cells are then washed extensively in Medium 199, and an aliquot of the cells is counted by isolating nuclei and counting nuclei with a Coulter counter as previously described (18).

Cells were isolated from pleural effusion specimens by the centrifugation of 1 liter of fluid at 800 × g for 5 min. The pellets were resuspended in 500 μl of Medium 199 and layered onto a 2-ml bed of Percoll (1.09 g/ml) and centrifuged at 800 × g for 5 min. Under these conditions, erythrocytes form a pellet below the Percoll, and the cells that form a layer between the medium and the Percoll are collected, washed with Medium 199, enumerated, and seeded into culture.

When more cells than were used in primary culture experiments were obtained from patient specimens, the excess cells were cryopreserved for future use. Cell aggregates obtained by enzymatic dissociation or collection from pleural fluids freeze well, and most samples yield viable cell cultures upon reactivation. To freeze cells obtained from patient samples, cells are suspended in Medium 199 supplemented with 20% FBS and 5% dimethyl sulfoxide at a concentration of 5 × 10⁶ cells/750 μl of freezing medium. The cells are cooled slowly to −80°C using a step freezer and then stored over liquid nitrogen. Over the past 3 years we have established a cell bank of mammary epithelial cells from normal and neoplastic tissues from over 30 patients.

Cell Culture. Mammary epithelial cells isolated by the methods described above were seeded onto collagen-coated 60-mm or 35-mm culture dishes at densities ranging from 10² to 5 × 10⁶ cells/dish. The complete serum-containing medium consists of Ham’s F-12 supplemented with IN (5 μg/ml), HC (1 μg/ml), EGF (10 ng/ml), CT (100 ng/ml), 5% FBS, gentamycin, and Fungizone. The complete serum-free medium is the same as just described, except that the serum is replaced with ethanolamine (5 μM), transferrin (5 μg/ml), bovine serum albumin (1 mg/ml), sodium selenite (50 ng/ml), and triiodothyronine (50 ng/ml).

For the subculture of cells grown in primary culture, cells were rinsed with calcium and magnesium-free Hanks’ balanced salt solution and incubated with trypsin-EDTA for 2–5 min. The cells were resuspended at split ratios of 1:3 to 1:10. For filter isolation of individual mammary epithelial cell colonies, cells were rinsed with Hanks’ balanced salt solution as above, and colonies were covered with pieces of Whatman 3-mm paper that had been soaked in warm trypsin-EDTA. After two min, the filter was removed from the colony with gentle downward pressure and then placed, cells down, in a 35-mm dish containing fresh growth medium. The plates were agitated gently and incubated overnight to allow cells to detach from the filter and attach to the substrate. For the subculture of cells obtained from metastatic lymph nodes, confluent lawns of stromal cells containing growing epithelial colonies were rinsed with Hanks’ balanced salt solution as described above and then incubated in calcium and magnesium-free Hanks’ alone or in the presence of 10 mM EDTA. The cells were observed under the phase-contrast microscope until the epithelial cells detached. The plates were then rinsed gently, and the cells were transferred to a new dish.

Immunocytochemistry. For immunocytochemical analyses of cultured cells, 35-mm dishes were rinsed with phosphate-buffered saline and fixed with methanol at −20°C for 10 min. The fixed cells were then rinsed three times with phosphate-buffered saline and incubated in primary antibody in phosphate-buffered saline for 30 min at room temperature. Primary antibodies used in these experiments include AE1/AE3 for broad-spectrum keratin recognition, Ks 19.1 for cytokeratin 19 recognition (ICN Flow, Costa Mesa, CA), and Tab 259 (kindly provided by Dr. Beatrice Langton, Berlex Biosciences, Alameda, CA) for c-erbB2 protein detection. Following the primary antibody step, cells were rinsed three times with phosphate-buffered saline and then incubated with biotinylated anti-mouse IgG for 30 min. The cells were then rinsed and processed using Vectastain ABC reagents (Vector Laboratories, Burlingame, CA) and visualized using diaminobenzidine as the substrate.

PCR Analysis of the P53 Gene for Loss of Heterozygosity. For these experiments, tissues obtained from histological sections of breast cancer specimens and cells cultured from breast cancer specimens were examined for LOH using a BstU-I restriction fragment length polymorphism in the P53 gene. To determine which patients were heterozygous at the BstU-I restriction site and which of those patients tumors had an LOH at that locus, new histological sections were cut from breast cancer specimens of patients whose cells were cryopreserved in our cell bank. Areas consisting of neoplastic cells and normal cells were microdissected separately from these specimens, digested with proteinase K, and PCR amplified using nested primers (see below). The PCR-amplified DNA was then digested with BstU-I (New England Biolabs, Beverly, MA) and analyzed in 3% NuSieve agarose gels. Similarly, cultured cells derived from the same patients were isolated by filter selection and washed five times with 5% dextrose in water and suspended in 10 mM Tris (pH 8.3) containing 5.0 mM potassium chloride, 1.5 mM magnesium chloride, 20 μM dihydrothreitol, 1.7 μM sodium dodecyl sulfate, and 10 μg Proteinase K/μl of cell suspension. The Proteinase K digestion was carried out for 1 h at 37°C before initiating PCR amplification.

Oligonucleotide primers were designed to amplify DNA sequences flanking the BstU-I restriction site in codon 72 of the p53 gene (19). Primers were designed to optimize annealing at a common temperature and with discordant 3’ ends to prevent formation of primer dimers. Oligonucleotide primers were synthesized by the University of Michigan DNA Synthesis Core Facility. Primers used in the first round of nested PCR had the sequences 5’-TGGATGATTTGATGCTGTC-3’ (upstream primer) and 5’-CGTG-CAAGTCACAGACTT-3’ (downstream primer), which produced an amplified segment of 257 base pairs corresponding to bases 23–279 of the p53 gene. Primers used in the second, “nested” round had the sequences 5’-CCCCGAC-GATATTGAAACA-3’ (upstream primer) and 5’-GCTGTCACAGACTT-3’ (downstream primer), resulting in a PCR product of 219 base pairs corresponding to bases 42–260 of the 4th exon.

After the addition of 31 μl of crude DNA, the final 50-μl reaction mix contained 200 μM of each deoxynucleotide triphosphate, 2.5 μg/ml (approximately 380 nm of each) first-round primers, 2.5 units of Taq polymerase (Promega, Madison, WI), and 1 μl Taq polymerase buffer. PCR parameters for both first- and second-round amplifications consisted of an initial 4-min denaturation step at 94°C coupled to a repeating cycle of 1 min at 94°C, 2 min at 48°C, and 2 min at 72°C for 35 cycles followed by a 7-min “completion” step at 72°C. Five μl of first-round PCR product were transferred to a 45-μl pre-mix solution with deoxynucleotide triphosphate, oligonucleotide, and Taq polymerase concentrations as above, and amplification was repeated for 35 cycles. After amplification, 15 μl of each PCR mixture were digested with 5 units of BstU-I at 60°C for at least 4 h in a 30-μl volume using buffer provided with the enzyme, followed by electrophoresis through a 3% NuSieve (FMC Bioproducts, Rockland, ME)/1% agarose (BRL) gel containing 0.5 μg/ml ethidium bromide. The BstU-I site, when present, results in the cleavage of the 219-base pair nested PCR product into fragments 78 and 141 base pairs in length.
RESULTS

Development of Culture Conditions That Support the Growth of Luminal Mammary Epithelial Cells. It is now clear that technical limitations that preclude the growth of luminal mammary epithelial cells have had a significant impact on progress in breast cancer cell biology. Therefore, we set out to modify our culture conditions in ways that would support the growth of luminal mammary epithelial cells. Previous studies performed by Taylor-Papidamitriou et al. (20, 21) indicated that luminal cells obtained from human milk could be grown in culture in media supplemented with growth factors and serum. The original medium that we developed for the growth of normal rat mammary epithelial cells was supplemented with growth factors and 5% FBS (18) and is similar to the medium described by Taylor-Papidamitriou. Therefore, we performed experiments in which the growth of HME cells in medium supplemented with growth factors and 5% FBS was compared to that obtained in our hormone and growth factor-supplemented serum-free medium. The results of these experiments indicated that the growth of HME cells in the growth factor- and serum-supplemented medium was not significantly different from that obtained with the serum-free medium on a quantitative basis (14); however, the morphology of the cells that emerged in the presence of serum was altered. Whereas, in the serum-free medium, all of the epithelial cells that grew were highly refractile and polygonal, two distinct populations developed in the presence of serum. One population retained the refractile appearance of the cells observed in serum-free medium, and the second population had a more cuboidal morphology and granular appearance. Moreover, in cultures derived from normal tissues, these dense granular-appearing cells were predominant in the center of colonies that emerged from the attachment of mammary organoids. The location and appearance of these cells suggested that they were derived from the luminal lineage (13). To test this hypothesis, cells from normal mammary tissues were cultured either in the serum-free medium or the growth factor- and serum-supplemented medium for 10 days, fixed, and processed for immunocytochemical analysis with antibodies that were specific for the luminal cell marker K-19. The results of these experiments confirmed our hypothesis and indicated the presence of K-19-positive cells in cultures grown with growth factors and 5% FBS but not in cultures grown in the serum-free medium (Fig. 1, a and b). Thus, HME cells of both the luminal and basal/myoepithelial lineages proliferate well in Ham’s F-12 medium supplemented with IN, HC, EGF, CT, and 5% FBS on plates coated with type I collagen.

Next, experiments were performed aimed at using the culture conditions described above to obtain cultures of luminal cells from primary breast cancer tissues. Growth of primary tumor-derived cells in the growth factor- and serum-supplemented medium yielded the emergence of three types of colonies that were morphologically distinguishable. One type of colony consisted of both K-19-positive and K-19-negative cells, with the positive cells present in the center of the colonies. These two-cell-type colonies were similar to those that arose in cultures derived from normal tissues. In addition, one-cell-type colonies emerged that consisted uniformly of either K-19-positive or K-19-negative cells (Fig. 1, c and d). Petersen and van Deurs (13) provided compelling evidence for the notion that two-cell-type colonies that develop in breast cancer-derived cultures represent normal HME cells and that breast cancer cells yield one-cell-type colonies in these cultures. Furthermore, Petersen’s evidence suggests that the breast cancer cells represent the most slowly growing population of cells and grow with doubling times of more than 5 days. Our immunocytochemical observations are consistent with the findings of Petersen in that one-cell-type, K-19-positive colonies were typically the most slowly growing colonies that developed in primary cultures initiated from breast cancer specimens. Therefore, subsequent experiments were aimed at developing a method that would allow direct isolation of individual K-19-positive, one-cell-type colonies in a manner that would allow their growth potential to be characterized in the absence of more rapidly growing cells.

In these experiments, we chose to use filter-selection methods to isolate individual colonies from primary cultures derived from normal or neoplastic tissues. Cells were seeded at low density (≤10^3 cells/60-mm dish) so as to allow individual colonies to grow well separated from each other. The cells were grown for 10 days, at which time colonies were chosen for filter selection. Every attempt was made to choose colonies that consisted of one morphological cell type, and colonies of various sizes were chosen in order to isolate cells with different growth rates. Individual colonies were isolated by overlaying

Fig. 1. Photomicrographs of human mammary epithelial cells cultured from normal and neoplastic tissues and immunostained using antibodies against K-19; a, normal tissue-derived cells grown in growth factor supplemented serum-free medium; b, parallel culture from the same specimen grown in growth factor and 5% FBS-supplemented medium; c and d, primary breast cancer-derived cultures growing in serum and growth factor-supplemented medium. Note the K-19-positive colony growing adjacent to a K-19-negative colony in d.
the colonies with filter discs that were soaked with trypsin:EDTA and then transferring the discs to 35-mm wells containing fresh growth medium. Cells isolated in this way were then grown to confluence and split at a ratio of 1:3. At the end of the second passage, cells from one well were subcultured, and the cells in the remaining two wells were tested for keratin expression. In all cases, the cultures derived from individual colonies reacted positively with broad-spectrum anti-keratin antibodies confirming the epithelial nature of the cells. However, certain colony-derived cultures were uniformly positive for K-19, indicating the luminal origin of the cells, whereas others were uniformly K-19-negative, indicating the basal or myoepithelial nature of those cells. The filter isolation methods were used to analyze the growth rate and proliferative life span of approximately 50 colony-derived cultures originating from normal tissues and 5 different primary breast cancers. The proliferative life span of colonies derived from tumor specimens was consistently greater than that of cultures derived from normal cell colonies. Whereas colonies isolated from normal tissues typically senesced in the second or third passage, colonies derived from tumor tissues proliferated for three to five passages before senescing. One slow-growing colony isolated from a tumor specimen proliferated for 10 passages or approximately 6 months in culture before senescing. In no case did we isolate an immortal cell line from a primary tumor using these methods to selectively grow K-19-positive cells.

The ability to isolate individual K-19-positive colonies from breast cancer-derived primary cultures allows genetic analysis to be carried out on these cultures to determine unequivocally if they are normal or neoplastic. To perform the genetic analysis, a marker is required that will distinguish normal from neoplastic cells from an individual patient and which can be detected with a small number of cells. We chose to examine breast cancer specimens for the presence of a polymorphism in the BstU-I restriction site of the p53 gene using nested PCR (19). The p53 gene is an important genetic locus in human breast cancer (22, 23), and the presence or absence of the restriction site can be examined by PCR analysis. The first step in our approach was to determine, for patients whose cells are frozen in our cell bank, which are constitutionally heterozygous at this locus in normal cells and which have lost one allele in their cancer cells. To do this experiment, new histological sections were cut from formalin-fixed tissue blocks from a series of patients. Next, normal and neoplastic cells were isolated from different areas of the sections, and these samples were prepared for PCR analysis of the p53 gene. The samples were amplified using a nested PCR approach, incubated with the restriction enzyme BstU-I, and analyzed on Nuiseve/agarose gels. From our initial series, we identified four patients that were heterozygous at this locus and one that had a LOH at that locus in the cancer cells (Fig. 2). Thus, this LOH can be used as a genetic signature that distinguishes normal from neoplastic cells from that patient. Accordingly, cells from the patient found to have lost an allele in the tumor cells (SUM-43; Fig. 2, Lanes 4 and 5) were reactivated from frozen stocks and cultured at low density, and individual colonies were isolated by filter selection. Cultures that arose from the isolated colonies were analyzed by PCR as described above. Fifteen separate colonies were analyzed in this way, and all of the colonies had the genetic signature of normal cells; i.e., both alleles present (Fig. 2, Lanes 6–8). It must be noted that the presence of any normal cells in a colony that was composed predominantly of cancer cells would be read as normal in this assay because of the sensitivity of the PCR method. Our current data indicate that we were not able to isolate pure colonies of neoplastic cells from SUM-43 by filter selection of colonies proliferating in medium that supports the growth of luminal mammary cells.

A second approach that would allow the detection of neoplastic cells in primary cultures derived from tumor tissues involves the identification of specimens in which the neoplastic cells overexpress a specific protein as a result of a genetic alteration in the tumor cells. We screened a series of histological sections of primary breast cancer specimens of patients whose cells were frozen in our cell bank, for overexpression of the erbB-2 protein. This protein is dramatically overexpressed in cells that have an amplified c-erbB-2 gene. Although a number of the specimens analyzed expressed moderate levels of the c-erbB-2 protein, in one specimen the protein was dramatically overexpressed by the neoplastic cells (SUM-33; Fig. 3a). Tumor-derived cells from this patient were then reactivated from frozen stocks and cultured for 7 days in the medium that supports the growth of luminal mammary epithelial cells and then tested immunocytochemically for erbB-2 expression and K-19 expression. For this experiment, SKBr-3 cells were used as positive controls for immunocytochemical detection of erbB-2 protein. K-19-positive colonies did emerge in primary cultures derived from this specimen (Fig. 3c). However, none of the colonies stained positively for erbB-2 protein (Fig. 3b). By contrast, SKBr-3 cells yielded strong positive cell surface and cytoplasmic staining under identical conditions (Fig. 3d). The results of this experiment are consistent with the results described above and indicate that primary HBC cells do not proliferate well in a growth medium that supports the growth of luminal mammary epithelial cells.

Isolation and Growth of HBC Cells from Metastatic Sites. Since experiments with cells obtained from primary human breast cancer specimens indicated that normal mammary epithelial cells are the predominant cell type to emerge in these cultures and that these cells proliferate rapidly, yielding confluent monolayers of normal cells, experiments were initiated aimed at isolating neoplastic HBC cells from metastatic sites. In the first experiment performed with pleural effusion-derived cells (SUM-44PE), we made use of the culture conditions that we had shown previously support the growth of HME cells of the luminal lineage. SUM-44PE cells were isolated from 1 liter of pleural effusion fluid, seeded onto type I collagen-coated tissue culture plates, and grown in Ham’s F-12 medium supplemented with IN, HC, EGF, CT, and 5% FBS. In addition, attempts were made to culture these cells in similar media in which individual factors had been deleted. The results shown in Fig. 4 indicate that SUM-44 PE cells grew poorly in the fully growth factor- and serum-supplemented medium that supports the growth of normal luminal HME cells. However, deletion of CT resulted in improved proliferation. In this experiment, the best growth was obtained in media supplemented with

Fig. 2. LOH at the BstU-I restriction site in patient sample designated SUM-43. Lane 1, size markers. Lanes 2 and 3 were obtained from DNA prepared from a patient that was heterozygous for the BstU-I site in both normal (Lane 2) and neoplastic (Lane 3) cells. Lanes 4 and 5, heterozygosity for the site in normal cells (Lane 4) and loss of an allele in the cancer cells (Lane 5) of specimen SUM-43. Lanes 6–8, LOH analysis of three representative colonies isolated from cultures obtained from SUM-43. Upper band, 219-base pair PCR fragment that does not contain the BstU-I site; two lower bands, 141- and 78-base pair PCR fragments that result from cleavage with BstU-I.
5% FBS and IN plus HC, and under these conditions the cells grew with a population doubling time of approximately 12 days. The presence or absence of EGF had no effect on the growth of these breast cancer cells. In the next experiment, SUM-44 PE cells were thawed and grown in media supplemented with 5% FBS, IN, HC, and other hormones and growth factors to determine if the culture conditions could be improved further. Progesterone, estradiol, acidic fibroblast growth factor, and basic fibroblast growth factor had no significant effect on the growth of these cells when added to the medium supplemented with IN, HC, and 5% FBS (data not shown). However, this experiment did indicate that SUM-44 PE cells grew better in serum-free medium supplemented with IN plus HC than in the serum-containing medium supplemented with the same factors (Fig. 5). When grown in serum-free medium, omission of IN in primary culture had a significant effect on cell growth with little or no proliferation taking place in the absence of this factor (Fig. 6). Next, cells that grew to confluence in primary culture were subcultured and tested for their ability to grow on tissue culture plastic versus tissue culture plates coated with type I collagen. In this experiment, the cells exhibited improved attachment and viability and appeared to grow better on tissue culture plastic than on the collagen-coated plates. These experiments suggested that the growth of SUM-44PE cells could be improved by the omission of serum and by growing cells on tissue culture plastic. To test those observations, cells were reactivated from frozen stocks, and a primary culture growth curve was obtained for the cells growing under the improved culture conditions. The data in Fig. 7 indicate that SUM-44 PE cells grown in serum-free medium with IN and HC on tissue culture plastic proliferate with a population doubling time of approximately 5 days. This is significantly faster than the 12-day doubling time observed in the original experiments with cells grown in the presence of serum and on collagen-coated plates. Thus, metastatic breast cancer cells obtained from the patient designated SUM-44 PE exhibit a number of cellular phenotypes that distinguish them from normal luminal mammary epithelial cells. SUM-44 PE cells are positive for the luminal cell marker K-19, yet, unlike K-19 positive normal HME cells, these breast cancer cells do not require serum factors for growth in culture. Whereas normal HME cells respond to EGF and CT by rapid proliferation, SUM-44PE cells do not respond to exogenous EGF and are growth inhibited by CT. These cells do exhibit a requirement, however, for exogenous IN and HC for growth in culture. SUM-44 PE cells grow better on tissue culture plastic than on a collagen substratum. Finally, SUM-44PE cells grow very slowly in culture and have not exhibited signs of senescence. This characteristic is dramatically different from that expressed by normal cells, which grow very rapidly in culture (population doubling times of 24 to 36 h) and then uniformly senesce after 30 to 60 days. SUM-44PE cells have now been in culture for over 15 months and are in their 17th passage (split ratios 1:3). Thus, we estimate that these cells have undergone approximately 30 population doublings. Preliminary karyotypic analysis indicates that SUM-44PE
cells have a model chromosome number of 60 and exhibit several marker chromosomes that are being characterized further (Figs. 8a and 9A).

The results obtained with SUM-44 PE cells prompted experiments to determine if cells that express similar altered phenotypes could be detected in cells from other metastatic breast cancer specimens. In one experiment, cells from a metastatic lymph node were reactivated from frozen stocks (SUM-16LN) and tested for their ability to grow either in the growth factor- and serum-supplemented medium required by normal cells or in the medium supplemented with IN, HC, and 5% FBS that was used originally to isolate SUM-44PE cells. After 6 weeks in primary culture, plates cultured in the medium supplemented with 5% FBS, IN, HC, EGF, and CT were completely overgrown by stromal cells, and there was no sign of HBC cell growth. By contrast, the plates cultured in the 5% IN, HC medium contained a confluent lawn of density-arrested stromal cells in which slowly growing epithelial colonies emerged. After 6 weeks in primary culture, the neoplastic epithelial cells were harvested by treatment of the cultures with 0.1 M EDTA in calcium- and magnesium-free Hanks' balanced salt solution. These cells reattached in the first passage and gave rise to slowly growing epithelial colonies that grew to confluence after 4 weeks in passage one. These cells proliferate slowly in culture like SUM-44PE cells and have now been in culture for over 6 months. Preliminary karyotypic analysis indicates that SUM-16LN cells have a model chromosome number of 62 and exhibit several marker chromosomes (Figs. 8b and 9B). The results obtained with SUM-16LN cells coupled with those obtained with SUM-44PE cells indicate that a medium supplemented with 5% FBS, IN, and HC, which does not support the growth of normal mammary epithelial cells, can be used to isolate HBC cells from some metastatic breast cancer specimens.

We have now tested the 5% FBS, IN, HC medium and the serum-free IN, HC medium described above with several primary and metastatic HBC specimens to determine if these media can be used for the routine isolation of breast cancer cells from patient specimens. Our results to date indicate that only a subset of tumor specimens can be cultured in these media. Among metastatic specimens, we have initiated cultures from 6 additional pleural effusion specimens and 5 additional metastatic lymph node specimens. Of these specimens, only one additional lymph node-derived specimen and two additional pleural effusion-derived specimens gave rise to subculturable HBC cell colonies. Growth of primary tumor-derived cells in the media used to isolate SUM-44PE and SUM-16LN often resulted in the slow emergence of epithelial colonies that were morphologically distinct from normal mammary epithelial cell colonies. The tumor designated SUM-55 consisted of normal mammary epithelial cells that grew rapidly in the fully growth factor- and serum-supplemented medium. In addition, this specimen consisted of cells that gave rise to many tightly packed epithelial cell colonies in the medium supplemented with IN, HC, and 5% FBS (Fig. 8c). These colonies continued to proliferate in primary culture for 5 months, yet none of the colonies
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Fig. 8. Photomicrographs of cultured HBC cells. a, SUM-44PE cells growing in serum-free medium with insulin and hydrocortisone on tissue culture plastic; b, SUM-16LN cells growing in similar culture conditions; c, typical colony that arose in cultures from SUM-55 after 2 months in selective medium.

yielded viable cultures upon passage, despite numerous attempts using several different methods to remove the cells from the primary dish. We have now attempted to isolate HBC cells from more than 10 primary tumors using the media described above. Although we have detected long-lived colonies in these media in at least three specimens, we have yet to isolate subculturable HBC cells from any primary tumor using these growth conditions.

In summary, our results indicate that selective growth media can be used to isolate human breast cancer cells from primary and metastatic sites; however, only a subset of these tumors consists of cells that can proliferate continuously in these media.

DISCUSSION

The routine isolation and culture of neoplastic mammary epithelial cells from primary breast cancer specimens has still not been accomplished. One hypothesis regarding the inability to culture these cells centers around the observation that the culture media developed for the growth of normal mammary epithelial cells does not support the growth of mammary epithelial cells of the luminal lineage. Since breast cancer arises from luminal mammary epithelial cells, development of a culture medium that supports the growth of these cells should allow the growth of neoplastic cells from breast cancer specimens. The results of our experiments do not support this hypothesis.

In our studies, we used a culture medium that supports the primary culture and serial passage of luminal mammary epithelial cells. In addition, we used a filter isolation method that allowed for the isolation of individual mammary epithelial cell colonies. The use of these methods did not result in the isolation of HBC cells from primary tumor specimens. Rather, primary tumor-derived cells yielded rapidly growing epithelial cell cultures that expressed all the features of normal cells. The cells grew rapidly and underwent a limited number of population doublings before senescing. The limited growth potential of K-19-positive mammary epithelial cells obtained from breast cancer tissues was uniformly observed in mass cultures and in cultures started from colonies isolated by filter selection. Finally, the use of genetic and protein markers that defined the neoplastic versus the normal cell compartment from their respective tissues demonstrated that the cells cultured from these specimens were normal and not neoplastic. In the filter isolation experiments, we consistently observed the extended proliferation of keratin-19-positive cells obtained from breast cancer specimens relative to cells obtained from normal tissues. The mechanistic significance of this observation is not clear; however, it is noteworthy that we previously made similar observations regarding the growth factor requirements of normal cells derived from primary tumors (14). Thus, it is possible that the tumor microenvironment affects many aspects of the proliferative capacity of normal mammary epithelial cells within the tumor.

The results obtained with metastatic HBC specimens are consistent with the results obtained with primary tumors. Thus far, we have attempted to isolate HBC cells from 8 metastatic breast cancer specimens using the culture conditions that support the growth of luminal mammary epithelial cells. Thus far, this medium has not resulted in the isolation of breast cancer cells from these specimens. However, we have isolated two HBC cell lines from the metastatic specimens using a selective medium from which growth factors required by normal cells were omitted. Indeed, we found that omission of these factors was critical for the selective isolation of these breast cancer cells. Thus, the results of our studies suggest that HBC cells have growth factor requirements distinct from those of normal human mammary epithelial cells and express dramatically different growth phenotypes in culture. The inability to isolate HBC cells from all metastatic specimens or from primary tumors using our selective culture conditions suggests further that these cells have requirements for exogenous growth factors that are still to be defined.

The most common phenotype that we have observed in HBC cells obtained from primary and metastatic sites is their slow proliferation rate. Our observations are consistent with the findings of other groups. Many cell lines have been developed from human breast cancers, and virtually all of these cell lines grew very slowly in culture in early passages and acquired rapid growth rates after months or years in
Fig. 9. Karyotypes of SUM-44PE and SUM-16LN cells. A, near-triploid karyotype of SUM-44PE displaying the following representative clonal structural abnormalities: add(1)(p22); i(2)-(q10)x2; del(7)(q32)x2; t(11;10)(q21;q24); dic(11;16)(p13;q22); +8–14 UMARS. B, representative hypotriploid karyotype from SUM-16 LN displaying multiple clonal structural alterations, including del(7)(p11.2), add(13)(p11), add(20)(p13), and +10–16 UMARS.
culture (24, 25). Thus, the rapid growth rates that characterize many human breast cancer cell lines currently in use are a characteristic acquired by the cells over many years in culture and do not reflect the original growth potential of the cells. In experiments with primary tumor-derived cells, Petersen and van Deurs (13) used an enzymatic method for detecting breast cancer cells growing in primary culture in an enriched growth medium while growing in the midst of normal mammary epithelial cells. The results of their growth rate studies also indicated that primary human breast cancer cells grow very slowly in culture (doubling times > 120 h), even when grown in a well-defined, highly growth-factor-enriched medium. It is important to note that the observed growth rates of primary breast cancer cells are entirely consistent with the rate at which these tumors grow in vivo (26–28).

Thus, given the consistency of the experimental observations made by many workers, the fact that breast cancer cells grow slowly despite the presence of many exogenous growth factors, and the agreement with clinical observations of breast cancer growth rates, the slow proliferation of breast cancer cells in culture is likely to reflect the true growth potential of the cells and is unlikely to be an in vitro artifact. Indeed, it is the slow rate at which HBC cells grow in culture that precludes the use of enriched media for the growth of neoplastic cells obtained from primary tumors. Media that support the growth of normal mammary epithelial cells yields confluent monolayers of cells within 1–2 weeks of the initiation of cultures. Thus, even HBC cells that do proliferate in fully growth factor-supplemented media are likely to be rapidly overgrown by normal cells under those culture conditions. It appears that improved success in isolating HBC cells from primary and metastatic sites will require selective culture conditions that provide appropriate growth factor stimulation for the neoplastic cells while at the same time not supporting the rapid proliferation of normal cells.

Some human breast cancer cells can be isolated in highly enriched culture media. In recent years, Petersen et al. (29), Band et al. (30), and Meltzer et al. (31) have developed HBC cell lines from primary tumor specimens using enriched growth media. However, in each of these cases, the lines were established from 1% to 2% of the specimens tested. Thus, HBC cells that can be isolated under these conditions are relatively rare. It is interesting to note that the cell lines developed by Band et al. (30) and Meltzer et al. (31) both have an amplified ERBB-2 gene, and both expressed requirements for EGFR in culture. Thus, HBC cells that overexpress ErbB-2 protein and the EGFR receptor may represent the subset of primary breast cancers most amenable to isolation using enriched culture media.

In conclusion, the results of our studies indicate that a subset of HBC cells can be isolated and cultured under selective conditions with minimal growth factor supplementation. Our results also suggest that breast cancer cells that do not proliferate under the selective culture conditions have requirements for exogenous growth factors that have yet to be determined and which are distinct from the growth factors required by normal human mammary epithelial cells.

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Differential Isolation of Normal Luminal Mammary Epithelial Cells and Breast Cancer Cells from Primary and Metastatic Sites Using Selective Media


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