Loss of Production of Myoepithelial Cells and Basement Membrane Proteins but Retention of Response to Certain Growth Factors and Hormones by a New Malignant Human Breast Cancer Cell Strain

Philip S. Rudland, Richard C. Hallowes, Simon A. Cox, E. Jane Ormerod, and Michael J. Warburton

Ludwig Institute for Cancer Research (London Branch), Royal Marsden Hospital, Downs Road, Sutton, Surrey SM2 5PX [P. S. R., E. J. O., M. J. W.], and Imperial Cancer Research Fund, P. O. Box 123, Lincoln’s Inn Fields, London, England WC2A 3PX [R. C. H., S. A. C.]

ABSTRACT

Digestion of primary breast cancers and their metastases with collagenase yields cell clusters which can be selectively isolated from stromal cells and from the less malignant-looking epithelium of the primary tumors by their failure to attach as rapidly to collagen gel. Continued passage in culture of one preparation of cell clusters has yielded a continuously growing cell strain, termed Ca2-83. This strain continues to grow mainly as cell clusters with doubling times of 10 to 14 days, although some clusters eventually adhere to plastic substrata. Two morphological extremes of cell were observed, smaller polygonal or cuboidal cells and larger, often multinucleated cells which contain fat droplets. Cell clusters grew in a gland-like pattern similar to those of the original carcinoma and formed small nodules in 50% of recipient nu/nu mice. Both morphological forms of Ca2-83 in culture or in tumor nodules stained immunocytochemically with epithelial cell-specific antisera to epithelial membrane antigens and to human keratins but not to laminin or actin. Cultures of Ca2-83 failed to synthesize laminin under conditions where its synthesis was observed in a rat myoepithelial cell line. Ultrastructural analysis of the cell clusters has identified microvilli coated with epithelial membrane antigens and junctional complexes typical of secretory epithelia in both morphological forms, but no characteristics of myoepithelial cells or basement membranes were observed. The DNA content of the cultures increased in response to serum, a bovine pituitary fraction, and insulin. Numbers of cell clusters were also increased in the presence of culture medium exposed to preadipocytes, myoepithelial- or mesothelial-like cells/stromal cells, or to prostaglandin E2.

MATERIALS AND METHODS

Patient History. Cancer patients were predominantly postmenopausal (23 of 30) women of varied ethnic origin with breast carcinomas covering the range of clinical staging. Histology of the majority showed infiltrating ductal carcinomas. Uninvolved peritumoral tissue from half the patients and malignant pleural effusions from 2 patients were also removed. The remaining noncancerous tissues were from reduction mammoplasties of otherwise healthy women and from lumpectomies of patients with benign fibroadenomas. One particular 50-year-old patient presented with an apparently benign lump in the left breast in June 1982. In December 1982, she represented with a second lump which was removed by lumpectomy. This lump, termed Ca2-83, was 2 cm in diameter and composed of Grade II infiltrating ductal carcinoma (10) of rather undifferentiated appearance (Fig. 1A). At subsequent mastectomy, 3 of 27 axillary lymph nodes also contained metastatic carcinoma. She presented again with a histologically similar tumor nodule in the scar region in April and again in September 1983 and with a right supraclavicular node largely replaced by a similar tumor in November 1983. She was treated by radiotherapy but died of the disease in March 1984.

Tissue Preparation and Digestion. About a sixth of the surgically removed carcinomas and lymph node metastases were transported to the laboratory within 1 h in RPMI 1640 (Grand Island Biological Co., Paisley, Scotland), BSA Fraction V, 2.5 mg/ml (Sigma Chemical Co.). Each tumor was processed by a modification (25) of the procedure described for rat mammary tumors (24, 45) and for human mammoplasty specimens (54). The specimen was sliced manually into 1- to 3- x 1-mm pieces and then suspended in 20 ml of DM in a 50-ml flask fitted with a suspended stirrer. The flask was gassed with a mixture of 5% CO₂-95% air, sealed with an inverted beaker, and stirred at 20 rpm for about 5 h at 37°C. About 10% of the tumor fragments were digested, yielding numerous small clusters of cells. These short-digest clusters were separated from undigested tissue by filtration through Nitex 1000-μm cloth (H. Simon, Stockport, Cheshire, United Kingdom). Cell clusters were obtained from the original carcinoma and formed small nodules in 50% of

INTRODUCTION

Although normal epithelial cells from human milk (7, 28, 48, 56) and from enzymatic digestion of reduction mammoplasties (19, 23, 54) readily yield adherent cell cultures which can be subcultured up to 4 times before they senesce, human malignant mammary tumors are generally much more difficult to culture (9, 23, 30). A few epithelial cell systems have been established from primary solid carcinomas (22, 32, 34, 39), but methods are still required for the isolation and characterization of malignant cells in the primary tumors before the properties of such cells can be analyzed in any detail. We describe in detail the continuous propagation and characterization of an initially nonadherent cell strain, Ca2-83, obtained from a primary adenocarcinoma. We also examine the ability of this cell strain to generate myoepithelial cells and basement membrane proteins, the loss of which are often associated with the transition from benign to malignant breast disease (2, 5, 20, 21, 26). The capability of Ca2-83 cells to respond to systemic (53) and local (47) trophic agents that can stimulate the growth of normal mammary epithelial cells in culture (45, 46, 51, 57, 64) is also described.

1 Supported by internal funding from the Ludwig Institute for Cancer Research and the Imperial Cancer Research Fund.
2 To whom requests for reprints should be addressed.

Received 8/30/84; revised 2/27/85; accepted 4/15/85.
washed twice in warm DM:5% FCS without collagenase and left overnight at room temperature (21°C) in similar medium on a Labquake shaker (Labindustries, Berkeley, CA) inverting 8 times/min. The remaining tissue was redigested overnight in fresh DM at 37°C as before. This procedure resulted in more than 50% of the tissue being dispersed, but the yield of cellular clusters was less than after the short digest. The clusters produced by overnight digestion were selected by filtration and washed at 37°C for 4 h, as for the short digest. Cells from pleural effusions were used directly without any digestion with collagenase, whereas peritumoral tissues, mammaplasties, and fibroadenomas were digested as before (23, 54).

Human Tissue Culture and Establishment of Ca2-83. Cell clusters were suspended in 4 ml of DM minus collagenase plus monothioglycerol, 2 ng/ml, (Sigma) (61), and plated in 2 T25 flasks ( Falcon Plastics, Los Angeles, CA) containing 2.5 ml of polymerized and medium-equilibrated 0.2% (w/v) collagen gel prepared from rat tails (38). The contents were gassed with 5% CO2:95% air and incubated at 37°C in the same water-saturated atmosphere for 24 h. Unattached cells and clusters were removed with the medium and replated in fresh T25 flasks containing gel. After a further 24 h, this replating was repeated, but into one T25 flask with and one T25 flask without collagen gel. This stage corresponded to day 0 of tissue culture. Two ml of fresh medium were added to each T25 flask at weekly intervals. After 28 days, the floating cell clusters were collected by centrifugation at 250 x g for 2 min, suspended in 2 ml of medium, and plated into 2 T25 flasks without collagen gel. Cell clusters that had attached to the plastic surface were released by pipetting, collected by centrifugation, and replated in 2 ml of medium into one T25 flask. Two ml of fresh medium were added at weekly intervals. Three weeks later, each of the 3 T25 flasks were subcultured 1:2. Since then the stock flasks have been subcultured 1:2 at 4- to 5-weekly intervals by gently pipetting to release attached cells, collecting cell clusters at 1 x g for 20 to 30 min, and replating the cultures in fresh cultures. The entire population of cells growing in suspension from the original specimen was cultured for 5 passages, and a fraction of the overall pool was passaged further; the remainder was stored as frozen cell stocks as described previously (54).

One cell strain from the Ca2-83 lumpectomy has been subcultured 23 times in the above way by January 1985, except that after 4 subcultures the monothioglycerol was omitted from the medium, and after the sixth subculture, phosphoethanolamine, 1.4 µg/ml (Sigma) and Batch 5, 70 µg/ml, of a bovine pituitary extract from Dr. R. Ham (University of California, Boulder, CO) (37) were included. The latter extract was the aqueous soluble fraction of a 0.15 m (NH4)2SO4 precipitation step at pH 7 and contained putative glycoprotein and fibroblast growth factor (53). After 9 subcultures, some stocks of Ca2-83 (approximately, 2 x 107 cells) were suspended in 20 ml of GM (RPMI 1640, 2.5% FCS, 1 µg/ml insulin, 10 ng/ml hydrocortisone, 5 µg/ml human transferrin, 5 ng/ml EGF, 1 µg/ml phosphoethanolamine, and an extra 2.4 mg/ml NaHCO3) and grown in T75 Falcon flasks in an atmosphere of 10% CO2:90% air for 2 weeks, and then 20 ml fresh GM were added for a further 2 weeks before transfer as above. Ca2-83 was found to be free of mycoplasma by fluorescent staining with an indicator cell layer and did not contain visible particles visible in the electron microscope.

Routine Tissue Culture. The established cell lines from the rat mammary gland, Rama 29 (6), Rama 401 (59), Rama 351 (47), and the SMT-2A cell feeders (12, 14) were cultured as described previously. For preparation of feeder cells, 80 to 90% confluent cultures were incubated with mitomycin C, 0.5 µg/ml, for 16 to 18 h, and then 4 x 106 cells were transferred into T25 Falcon flasks in 5 ml of GM (6). For preparation of conditioned media, 80 to 90% confluent cultures were washed twice with PBS and incubated with RPMI 1640: NaHCO3, 2.4 mg/ml, for 24 h at 37°C, filtered through 0.22-µm filters and stored at 4°C before use. Growth factors and hormones were then added directly to reconstitute serum-free conditioned GM. FCS was stripped of free steroids by treatment with dextran-coated charcoal as described previously (50).

Morphological appearance of living cultures was observed in a Zeiss ICM 405 inverted microscope fitted with phase-contrast optics and a green filter, and photographs were recorded on Ilford Pan F film.

Production of Biological Material for Histology. The contents of 2 T75 flasks of Ca2-83 (about 106 cells) were collected by centrifugation at 1 x g as before, washed twice in 20 ml PBS and fixed in Methacarn (60% methanol:30% inhibiol:10% acetic acid [v/v]) for 24 h at 4°C. Cells were rinsed with 70% alcohol, embedded in agar before blocking in paraffin wax, and then processed as before (60). Samples were also smeared onto slides after the final collection step, air dried for 30 min, and left unfixed.

To investigate their growth in nude mice, Ca2-83 cells at passage 11 were collected and washed twice in PBS as before. Approximately 0.5 x 106 cells in 0.2 ml of PBS were injected into the right inguinal mammary fat pad of 6-week-old female BALB/c nude (nu/nu) mice (Olac, Banbury, England). After 12 weeks, the animals were sacrificed, and the injected areas plus potential metastatic sites were examined for visible tumor deposits, sliced into pieces less than 0.5 cm thick, and fixed in Methacarn as above.

Histology and Immunocytochemistry. Histological sections of the paraffin-embedded material were stained by hematoxylin and eosin, and the unfixed smears were stained by oil red O, a nonspecific stain for neutral lipids (35).

Immunocytochemical staining of sections was carried out using an alkaline phosphatase-conjugated secondary antibody (3, 60). Primary antibodies raised in rabbits against rat milk fat globule membrane, purified human keratins (Mr: 69,000; 57,000; 47,000; 45,000; and 43,000), bovine smooth muscle actin (Miles Chemical Co.), and mouse laminin have been described previously (21, 59, 60). Rabbit antisera to human EMA (52) was obtained from Dr. M. G. Ormerod (Institute for Cancer Research, Sutton, Surrey, United Kingdom) (52). Purified IgGs (60) were diluted between 1:20 and 1:2,000 with 0.5% BSA in PBS before use. Sheep anti-rabbit IgG conjugated to alkaline phosphatase with glutaraldehyde was purchased from Sigma. Absorbed antisera were prepared by incubating antisera with 1 mg of antigen per ml at 37°C for 3 h. Sections were counterstained with Mayers hemalum and photographed on a Reichert Polysvar microscope with a Kodak Wratten No. 44 filter on Ilford Pan F film.

The specificity of staining for each antiserum was checked by the following controls: (a) sections of normal mammary glands from a 19-year-old woman or from 70-day-old female rats were stained in parallel and compared with previous results for consistency of staining (21, 52, 59); (b) complete abolition of staining was achieved by prior incubation of each antiserum with the requisite antigen(s); (c) tumor cells in nude mice which failed to stain were only recorded as negative, providing that an appropriate normal mouse cellular structure could be identified as staining on the same section; and finally, (d) sections from 2 nude mouse tumor nodules or from 4 separate isolates of cultured Ca2-83 cells were examined with each antibody.

Electron Microscopy. Clusters of Ca2-83 cells were fixed and processed for electron microscopy as described previously (40). Immunolocalization of EMA at the ultrastructural level (41) was accomplished by incubating the cell clusters with 1:500 anti-EMA serum in PBS and 0.5% BSA for 1.5 h at room temperature. Cell clusters were washed in PBS, then with PBS plus 0.5% BSA three times, before being incubated with 1:100 sheep anti-rabbit serum conjugated to horseradish peroxidase for 1.5 h. Cell clusters were rewarshed as above and incubated with 0.05% diaminobenzidine and 0.06% H2O2 in PBS for 5 min at room temperature. Cells were fixed and processed as described previously (40), except that the staining with uranyl acetate and lead citrate was omitted, and viewed directly in the electron microscope.

Determination of Laminin Synthesis. Approximately 106 Ca2-83 passage 14 cells were plated in 10 ml of 90% Dulbecco's modified Eagle's medium without proline:5% Dulbecco's modified Eagle's medium:5% FCS dialyzed extensively against PBS: hydrocortisone, 50 ng/ml: insulin, 50 ng/ml: L-[3H]proline 10 µ Ci/ml (Amersharm Int., United Kingdom) and grown for 7 days. Cell clusters were collected by centrifugation and suspended in 20 ml of DM. The cells were then washed twice with fresh DM minus collagenase and left overnight in fresh DM at 37°C as before. This procedure resulted in more than 50% of the tissue being dispersed, but the yield of cellular clusters was less than after the short digest. The clusters produced by overnight digestion were selected by filtration and washed at 37°C for 4 h, as for the short digest. Cells from pleural effusions were used directly without any digestion with collagenase, whereas peritumoral tissues, mammaplasties, and fibroadenomas were digested as before (23, 54).

Cancer Research Vol. 45, August 1985

3865

Downloaded from cancerres.aacrjournals.org on January 3, 2018. © 1985 American Association for Cancer Research.
ugation, lysed, and the dialyzed lysate and medium were immunoprecipitated with rabbit anti-mouse laminin serum or normal rabbit serum as described previously (59). Quantitation of laminin released into GM was carried out by enzyme-linked immunosassay as described by Rennard et al. (44).

**Determination of Growth Rates of Ca2-83 Cells.** For determination of total DNA in cultures, cell clusters equivalent to about 2 μg of DNA were suspended in 3 ml of the appropriate medium in each of 10 T25 flasks, and 2 similar samples were retained for 0-time estimation. Further additions of appropriate medium were made as follows: 2 ml immediately; 2 ml on Day 7; 3 ml on Day 14; and 4 ml on Day 21. Duplicate cultures were removed at each time point and harvested separately by first jetting off any adherent colonies and then displacing any remaining cells with a silicone-rubber wedge in 5 ml PBS. Cells were collected by centrifugation at 500 × g for 3 min, washed in 10 ml of PBS by centrifugation, and stored at -20°C. DNA was estimated by the binding of DABA-2HCl (Aldrich Chemical Co., Gillingham, United Kingdom) to its purine deoxyribose portion as reported by Hinegardener (27), except that the DABA-2HCl was purified in aqueous suspension with activated charcoal and dried slowly at 37°C instead of at 60°C, and 0.22-μm filtered glass-distilled water was used throughout. DNA standards in the range 0.4 to 2.0 μg and thawed-cell pellets were evaporated to dryness at 37°C and incubated with 0.25 ml of freshly purified DABA-2HCl, 0.4 g/ml, at 60°C for 60 min in sealed tubes. They were cooled, 5 ml 1 M HCl was added to each, and the fluorescence was recorded in a modified Aminco Bowman spectrophotometer at 495 μm using excitation at 405 μm.

For determination of the number of cell structures, duplicate cultures were seeded at 500 to 1000/T25 Falcon flask in either regular or Primaria flasks in 5 ml of the specified medium. The average number of adherent and total cell structures was determined by counting 20 randomly selected microscopic fields in an Olympus CK inverted microscope (×40, 14 sq mm microscopic field). Adherent colonies were assessed by their ability to remain attached to the substratum after 2 shakes of the culture flasks. For determination of the total protein content, the adherent and suspended cells were collected by centrifugation at 200 × g for 5 min and assayed by the method of Lowry et al. (36).

For autoradiography, 2-day-old cultures in GM were incubated with 3 μCi/ml [3H]thymidine, at 1 μM, and duplicate flasks were removed after 48 and 72 h. Cell clusters were collected by centrifugation and processed as described previously (45) before being smeared onto slides. The slides were air dried for 30 min, coated with Nuclear Immulsion, exposed, developed, and fixed as described by Williams (62), and finally counterstained by hematoxylin and eosin. They were viewed and photographed in a Reichert Polyvar microscope as above. Results for the percentage of cells with radioactively labeled nuclei were the average of duplicate smears, 10 fields of not less than 100 cells being counted.

**RESULTS**

**Culture of Human Breast Cancer Cells and Establishment of Ca2-83.** Collagenase digests of primary and secondary tumors from patients with infiltrating ductal carcinomas behaved differently on collagen gels in our general GM (see "Materials and Methods"). Digestion of the primary tumors usually yielded organoids which adhered to the collagen gel after 24 to 48 h. These fast-adherent structures grew rapidly for the next 7 to 21 days to produce cellular islands or colonies containing polygonal, epithelial-like cells and at their peripheries, elongated, presumptive myoepithelial-like cells (Table 1). Three-dimensional tubes were also seen to protrude below some of the colonies into the gel. These cellular colonies bore a striking morphological resemblance to those obtained from digests of reduction mammaplasties of otherwise healthy women, benign fibroadenomas, and uninvolved peritumoral tissues of the breast (Table 1) (23, 25, 54). In these cases, both the polygonal epithelial-like cells and the elongated presumptive myoepithelial-like cells were cytologically normal in that there was no marked nuclear pleomorphism, few if any bi- or multinucleated cells, and no aberrant mitotic figures. In addition, variable numbers of cell clusters were also seen that rarely attached to the collagen gel within 72 h (Table 1), and those that eventually did were easily released by gentle pipetting (Table 1). Any cellular colonies that formed from these clusters contained only polygonal epithelial-like cells (Fig. 1C); no elongated cells were observed. Cells in these slow-adherent colonies and nonadherent clusters were cytologically malignant in that they showed a marked nuclear pleomorphism, usually high nuclear to cytoplasmic ratios, and were often bi- or multinucleated with some aberrant mitotic figures. Only a few of these cells proliferated and then at a rate slower than those in the fast-adherent colonies. In contrast, cultured lymph node metastases and malignant pleural effusions yielded only the nonadherent cell clusters which occasionally formed the slow-adherent colonies; no fast-adherent colonies containing elongated cells were observed (Table 1). In all cases, the stromal cells attached readily to the gel so that in 3 days transfers their total number was greatly reduced. The same pattern of finding only fast-adherent colonies of epithelial and elongated presumptive myoepithelial cells from normal mammary glands, benign breast lesions and peritumoral but pathologically "normal tissues," and of finding only nonadherent or loosely adherent epithelial cell clusters devoid of elongated myoepithelial cells from secondary breast cancers has now been observed for many more tissue samples.4 Although a few of the nonadherent cell clusters appeared to multiply initially and at least to double their cellular volume, only 5 of the 30 primaries gave rise to short-term cell strains that could be subcultured up to 2 times during the following 6 months, but even these were eventually lost. Most of the primary cultures were eventually lost to fungal infections. However, nonadherent

<table>
<thead>
<tr>
<th>Mammary tissue</th>
<th>Total no. of patients</th>
<th>Fast-adherent colonies</th>
<th>Slow-adherent colonies</th>
<th>Nonadherent clusters</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal (reduction mammaplasty)</td>
<td>40</td>
<td>40</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Benign tumor (fibroadenoma)</td>
<td>10</td>
<td>10</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Peritumoral (uninvolved)</td>
<td>15</td>
<td>12</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Primary carcinoma</td>
<td>30</td>
<td>25</td>
<td>12</td>
<td>27</td>
</tr>
<tr>
<td>Secondary carcinoma in axillary lymph node</td>
<td>10</td>
<td>0</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>Secondary carcinoma as pleural effusion</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

4 R. C. Hallowes, unpublished results.
cell clusters from the primary tumor of Ca2-83 (Fig. 1A) continued to proliferate in culture with a doubling time of 10 to 14 days without any such infections, and this cell strain has now been passaged routinely 23 times (see "Materials and Methods"). Many of these cells continued to grow in suspension as cell clusters, some of which possessed bulbous projections (Fig. 1B). Some clusters eventually adhered to the plastic substratum, and polygonal epithelioid cells spread out as a monolayer forming small compact islands (Fig. 1C). However, transfer of slow-adherent colonies alone to fresh medium yielded the same proportion of slow-adherent and suspended cell clusters, and vice versa. Ca2-83 cells did not attach to 0.15 to 0.2% collagen gels and failed to produce tubular protrusions when embedded in similar gels, although expanding cell clusters were observed in 0.05 to 0.1% gels. Two morphological extremes of cell were observed in the slow-adherent colonial cells at all passage numbers, small (10 to 20 μm), dark, isometric cells with a large pale nucleus, often with more than one prominent nucleolus (Fig. 1E), and large (>40 μm), more irregularly shaped, often multinucleated cells with a very granulated or foamy cytoplasm (Fig. 1D). There were also morphological intermediates between these 2 extremes (Fig. 1C). No permanently elongated or fibroblastic cells were observed. Ca2-83 cells were aneuploid with a mean chromosomal number of 63 ± 11 (SD). Stocks of Ca2-83 were slowly frozen to −70°C as described previously (54) and then stored in liquid nitrogen. These stocks were successfully thawed, grown up, and found to be indistinguishable from nonfrozen Ca2-83.

**Histology and Immunocytochemical Staining of Cultured Cells.** Histological examination of the nonadherent cell clusters revealed circular structures of tightly compact, cuboidal cells of varying sizes with a tendency to multilayering. They thus formed a gland-like pattern of a rather undifferentiated adenocarcinoma (Fig. 2A) similar to that of the original tumor (Fig. 1A). The cultured cells were cytologically malignant with a marked nuclear pleomorphism, usually high nuclear-to-cytoplasmic ratios, often bi- or multinucleated with some aberrant mitotic figures. The larger cells often possessed a granulated cytoplasm which stained intensely with oil red O (Fig. 2B), a stain for neutral lipids (35). Even some of the smaller cuboidal cells possessed finely staining lipid droplets (Fig. 2B).

Antiseras to human EMA and to human keratins were used to identify the epithelial cells. Antibodies to EMA stained epithelial cell surfaces lining the ducts and lobules of the human mammary gland (52) (Fig. 2C). The myoepithelial and stromal cells failed to stain. Antiseras to human keratins stained the human glandular epithelial cells strongly but heterogeneously and the myoepithelial cells more weakly; the stromal cells failed to stain (21) (Fig. 2D). The majority of Ca2-83 cells in the circular structures could be stained specifically by antisera to both EMA (70 to 80% of cells) (Fig. 2, E and F) and human keratins (45 to 55% of cells) (Fig. 2, G and H), yielding appreciably higher percentages of cell staining than in the human mammary gland (Table 2). The strongest staining for EMA was observed at the cellular peripheries, although weaker cytoplasmic staining was also observed; staining for keratins was confined to the cytoplasm.

Antiseras to actin and to the basement membrane protein laminin were used to identify any myoepithelial cells. In the normal mammary gland, antisera to actin stained the myoepithelial cells and smooth muscle cells which lined blood vessels; the epithelial cells and fibroblasts failed to stain, whereas antisera to laminin stained the basement membrane adjacent to the myoepithelial cells as reported previously (21). However, no cellular staining with either antibody could be detected in any cultured cell clusters (Fig. 3, A and B). To confirm that laminin was not synthesized, Ca2-83 cells were labeled with [3H]proline, and the culture medium was immunoprecipitated with antilaminin serum. No radioactive peptide corresponding to laminin was detected in the immunoprecipitate, although laminin was detected in the immunoprecipitate, although laminin was detected in the culture medium of the rat myoepithelial cell line Rama 401 (not shown). Similarly, although Rama 401 cells secreted 1.4 to 5.1 μg of laminin/24 h/mg cell protein as measured by an enzyme-linked immunosay, that secreted by Ca2-83 cells was not detectable, the lower limit of detection being 0.02 μg/24 h/mg protein.

**Induction of Tumor Nodules in Nude Mice.** Injection of Ca2-83 cells in PBS into the right inguinal fat pad of female nude mice yielded no palpable tumors after 12 weeks. However, 50% (4 of 8) of the animals contained a small nodule of cells, less than 1 mm diameter, at the point of injection (Fig. 3, C to F). Nude mice injected with PBS alone formed no nodules. No additional tumor deposits were detected on inspection or in histological sections of the left inguinal mammary fat pad, lungs, liver, spleen, kidneys, heart, lymph nodes, or gonads of any of the 4 mice with tumor nodules. Tumor nodules were well circumscribed by condensed connective tissue elements and were composed of a relatively solid cord of cells occasionally surrounding small, lumen-like structures. The cells varied in size, were cytologically malignant, and were often bi- or multinucleated (Fig. 3, C to F).

The majority of tumor cells in the nodules stained immunocytochemically with antisera to human EMA, whereas no cells of the nude mouse host stained with this antibody (Fig. 3C). Moreover, tumor cells failed to stain with antisera to rat milk fat globule membrane, whereas the majority of the luminal epithelial cells of the nude mouse mammary gland stained intensely (Fig. 3D). Approximately one half of the tumor cells and most of the mouse mammary gland cells also stained with antihuman keratins serum (Fig. 3E). No staining of the tumor cells for laminin (Fig. 3F) or for actin (not shown) was observed under conditions suitable for immunocytochemical procedures.

<table>
<thead>
<tr>
<th>Antiserum used</th>
<th>Normal human gland</th>
<th>Cultured cells</th>
<th>Normal nude mouse gland</th>
<th>Tumor nodules</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human EMA</td>
<td>40–60°C</td>
<td>70–80</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Rat milk fat globule membrane</td>
<td>0</td>
<td>0</td>
<td>40–70°C</td>
<td>0</td>
</tr>
<tr>
<td>Actin</td>
<td>20–30°C</td>
<td>0</td>
<td>25–35°C</td>
<td>0</td>
</tr>
<tr>
<td>Laminin</td>
<td>25–35°C</td>
<td>0</td>
<td>25–40°C</td>
<td>0</td>
</tr>
</tbody>
</table>

* Histological sections were immunocytochemically stained with rabbit antisera to the requisite antigens (see "Materials and Methods"); but only the staining of the mammary parenchymal cells were scored in the normal mammary glands. All results are expressed as a range of percentages from determinations made upon 6 microscopic fields from each of 2 sections of 2 separate specimens.

* Only the epithelial and not the myoepithelial cells stained.

* Only the myoepithelial and not the epithelial cells stained.

* Mouse myoepithelial cells stained intensely, although some epithelial cells were also stained rather more weakly.

**Table 2**

**Immunocytological staining pattern of cultured cells and tumor nodules in nude mice**

---

**CANCER RESEARCH VOL. 45 AUGUST 1985**

3867
where basement membranes and myoepithelial cells of the adjacent host mouse mammary glands were stained. The percentages of tumor cells staining with these 5 antisera were virtually identical when growing in culture or as nodules in nude mice (Table 2).

Ultrastructure of Cultured Cells. When observed in the electron microscope, the cells in clusters of Ca2-83 were predominantly cuboidal, although they varied in size, and were closely apposed with interdigitating membranes between adjacent cells (Fig. 4A). Where cells were exposed to the medium, some junctional complexes typical of epithelia were seen at apical-lateral borders, although their 3 characteristic elements were not always distinguishable. Desmosomes containing a recognizable intercellular contact layer were also present, and typical tonofilaments issued from both halves (Fig. 4B). Where cells were exposed to the medium, microvilli were also seen, and these varied from being short and sparse to being long and in parallel arrays (Fig. 4, A and C). The nuclei varied from having a rounded and smooth morphology to an indented shape associated with prominent heterochromatin. Prominent, sometimes multiple, nucleoli were seen. Mitochondria, rough endoplasmic reticulum, polyribosomes, and Golgi apparatus were present to varying degrees in the cytoplasm. Some cells contained large numbers of fat droplets, either concentrated in the perinuclear region or scattered throughout the cytoplasm. Some fat droplets were as large as 2 to 3 μm diameter and could occupy up to 70% of the cytoplasm (Fig. 4A). Individual filaments, 10 to 12 nm diameter and probably of the intermediate type (43), were abundantly scattered throughout the cytoplasm of some cells (Fig. 4B). However, features characteristic of myoepithelial cells such as myofilaments, pinocytotic vesicles, hemidesmosomal connections to basement membranes (43), and even basement membranes themselves were absent.

Ultrastructural localization of the binding of anti-EMA serum to the cultured cell clusters revealed that this antigen occurred mainly on the surfaces of cells that were exposed to the medium, particularly in areas where there were microvilli (Fig. 4D). In these areas, the reaction product coated the whole of the outer surfaces of the microvilli (Fig. 4C). The large, fat-droplet-containing cells also possessed extensive arrays of microvilli on their surfaces that were coated with EMA (Fig. 5).

Effect of Different Medium Components on Growth in Culture. The total DNA content of cultures of Ca2-83 cells increased throughout the 28 days tested, provided that additional volumes of media were added to feed the increasing numbers of cells. In A, the medium consisted of RPMI 1640, BSA alone, 2.5 mg/ml (•). With pituitary extract, 70 μg/ml and insulin, 1 μg/ml (★); controls with RPMI 1640 and 2.5% FCS (●) were also included. In B, the medium consisted of RPMI 1640 and 2.5% FCS alone (♦) or with the following additives: V, EGF (5 ng/ml); O, hydrocortisone (10 ng/ml); △, insulin (1 μg/ml); ◄, pituitary extract (70 μg/ml); ▲, pituitary extract (70 μg/ml) plus insulin (1 μg/ml).

Table 3

<table>
<thead>
<tr>
<th>Additions to medium</th>
<th>Fold increase in DNA in cultures containing FCS</th>
<th>untreated FCS</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>5.3</td>
<td>12.1</td>
</tr>
<tr>
<td>Progesterone</td>
<td>7.5</td>
<td>15.2</td>
</tr>
<tr>
<td>Estradiol</td>
<td>10.1</td>
<td>16.5</td>
</tr>
<tr>
<td>Pituitary extract</td>
<td>11.3</td>
<td>18.0</td>
</tr>
</tbody>
</table>

* Cultures of Ca2-83 cells were set up as described in "Materials and Methods" in a medium consisting of RPMI 1640, 2.5% FCS, insulin (1 μg/ml), hydrocortisone (10 ng/ml), EGF (5 ng/ml), plus one of 1 nm progesterone, 1 nm estradiol, or pituitary extract (70 μg/ml) as indicated.

** Fold increase in DNA = DNA content in cultures after 21 days + DNA content after 0 days for a given set of additives. Results are the average of duplicate cultures which agreed to within 10%.

FCS was stripped by incubating with charcoal (see "Materials and Methods") prior to its addition to the medium.

without effect. When FCS was replaced by BSA, 2.5 mg/ml, the DNA content failed to increase with time, although the pituitary extract and insulin still stimulated a 5-fold increase by 28 days (Chart 1A). Growth of Ca2-83 cells with charcoal-stripped FCS resulted in a lower DNA content per culture than with nonstripped serum, and in both cases, 1 nm estradiol or 1 nm progesterone increased slightly the DNA content of the cultures (Table 3). Their combination, however, was inhibitory (not shown).

The total number of Ca2-83 cells growing in culture was linearly related to the number of cell clusters, since the number of cells in a cluster was approximately constant at 100. Hence, the progress of the cultures could be followed readily by counting such structures. The total number of structures in GM without...
serum declined slightly over a period of 32 days. With increasing concentrations of serum up to 10%, the number of structures was dramatically increased by up to 5-fold for the first 10 days of culture. Beyond 32 days, however, the optimum serum concentration for producing the maximum number of structures and for the highest cellular protein content was 5% (Chart 2B). The structure doubling time was about 10 days in GM. That the cells in GM were synthesizing DNA was confirmed by autoradiography (Fig. 6). Exposure of cells for 48 or 72 h to [3H]thymidine yielded 43 ± 3% or 64 ± 1% radioactively labeled cell nuclei, respectively, corresponding to a cell division time of about 10 days. Increasing concentrations of FCS caused an even more dramatic increase in the number of adherent structures, rising to almost 50% of the total number in 10 or 20% FCS after 10 days (Chart 2A).

Incubation of Ca2-83 cells with nondividing rat feeder cells, the mesothelial-like/stromal mesothelial-like/stromal feeder SMT-2A feeder cells (13, 14), the myoepithelial-like Rama 29 cells (6), and the mammary preadipocytic Rama 351 cells (47) also stimulated the total number of structures and protein content by about 2-fold over that observed in GM alone. However, whereas the SMT-2A feeders reduced the number of adherent structures of Ca2-83, Rama 29 and Rama 351 cells stimulated their appearance by 3- to 4-fold (Table 4). When serum-free medium was exposed to any one of the feeder cells for 24 h (see "Materials and Methods") and then incubated with Ca2-83 cultures in GM without serum, the total number of cell structures was increased by about 2-fold after 10 days, and thereafter declined (Chart 3B). This increase in 10 days was approximately

![Chart 2. Effect of different concentrations of serum on the number of cell structures in culture. Cultures were seeded at 550 cell structures/standard 26-sq cm tissue culture flask in GM without serum, and various concentrations of serum were added back. The average number of structures ± SE (bars)/14-sq mm microscopic field from 2 flasks, 20 fields/flask, were recorded. A, medium with different concentrations of serum: O, no PCS; T, 2.5% PCS; A, 5% PCS; and •, 10% FCS. The total number of structures adhering to the plastic substratum ± SE (bars)/14-sq mm microscopic field from 2 flasks, 20 fields/flask, were recorded. B, 10% PCS: 24 ± 3, 320 ± 30, 420 ± 50, 360 ± 30, and 390 ± 6 µg of total protein, respectively.](chart2.png)

![Chart 3. Effect of growth-promoting agents released by different cultured cells on the number of cell structures in culture. Cultures were seeded at 550 cell structures/standard 26-sq cm tissue culture flask in GM without serum and with various additives. The average total number of structures (both floating in the medium and attached to the substratum) ± SE (bars)/14-sq mm microscopic field from 2 flasks, 20 fields/flask, were recorded. A, 10% FCS; B, medium with different concentrations of serum: O, no PCS; T, 2.5% PCS; A, 5% PCS; and •, 10% FCS. The total cellular protein content (±SE) of the flasks after 28 days was for 0, 2.5, 5, and 10% FCS: 750 ± 40, 700 ± 40, and 680 ± 30 µg of total protein, respectively.](chart3.png)

### Table 4

<table>
<thead>
<tr>
<th>Feeder cells</th>
<th>Number of structures/field</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>6.3 ± 0.8</td>
</tr>
<tr>
<td>SMT-2A feeders</td>
<td>11.1 ± 0.4</td>
</tr>
<tr>
<td>Rama 29 (myoepithelial-like)</td>
<td>27 ± 2</td>
</tr>
<tr>
<td>Rama 351 (preadipocytes)</td>
<td>19 ± 3</td>
</tr>
</tbody>
</table>

4 Near confluent (80 to 90%) cultures of rat feeder cells were inactivated by treatment with mitomycin C. C, and 4 × 10⁶ feeder cells were plated per 26-sq cm normal tissue culture flask.

5 Approximately 1000 cell structures/26-sq cm flask were in 5 ml of GM, and after 10 days an extra 5 ml of fresh GM was added. The average number of cell structures ± SE (bars)/14-sq mm microscopic field from 2 flasks, 20 fields/flask, was recorded after a total of 28 days. Both the total number of structures and the number of structures sticking to the surface of the flask (adherent structures) were recorded. The total cellular protein content (±SE) of the flasks after 28 days was for no feeders, SMT-2A feeders, Rama 29 cells, and Rama 351 cells: 350 ± 10, 750 ± 40, 700 ± 40, and 680 ± 30 µg of total protein, respectively.

CANCER RESEARCH VOL. 45 AUGUST 1985

3869

Downloaded from cancerres.aacrjournals.org on January 3, 2018. © 1985 American Association for Cancer Research.
the same as that achieved by 2.5 to 5% FCS, although the effects of serum were maintained over longer periods of time (Chart 3A). To obviate the possibility that trace amounts of serum were released from the feeder cells, so causing the stimulatory effects, the conditioned media were added separately to cultures of Ca2-83 cells in GM with the usual 2.5% FCS. However, all 3 conditioned media still stimulated the number of cell structures by about 2-fold (not shown). The trophic agent for rat mammary epithelial cells that is released by Rama 351 preadipocytes is PGE$_2$ (47). PGE$_2$ at 0.5 to 5 ng/ml also stimulated the number of cell structures by 2-fold or more after 10 days in GM without serum; no further stimulation was achieved at 50 ng/ml, whereas 500 ng/ml was slightly inhibitory (Chart 3C).

DISCUSSION

Breast cancer cells from the primary tumors or metastases have been grown in culture only with difficulty (17, 23, 30, 33). Several reports, however, have indicated that unlike the epithelium and fibroblasts derived from normal or near-normal human mammary glands, clusters of slow-adherent or nonadherent epithelial cells are often obtained both from collagenase digestion of solid tumors (31, 32, 34) and from pleural effusions of malignant disease (11, 15, 49, 58, 61), and these results are consistent with our findings reported here. The slow or lack of adherence of epithelial cell clusters obtained from secondary breast carcinomas does not necessarily prove that epithelial cells from normal breast tissues adhere, whereas those from secondary carcinomas do not, since the adherent properties of the epithelial cell clusters may depend more on the presence or absence of myoepithelial cells and/or their products (54). Normally, most breast cancer cells eventually die out on repeated transfer in vitro (16, 39, 42). Those rare instances where epithelial cells emerge as permanently growing cell strains usually involve their passage through a period of crisis which is often characterized by a switch from very slow-growing, nonadherent cell clusters to more rapidly growing cultures of adherent cell sheets (15, 34, 49). In the case of Ca2-83, there has been little change in adherent, morphological, or growth rate properties throughout its entire period of culture. Indeed, even the estrogen receptor content of the primary tumor and of early and late-passage cells was roughly constant at 22, 14, and 17 fmol/mg cell protein respectively. The reason why this particular cell strain grew continuously in culture, whereas the remainder of the isolates did not, may be due in part to the relatively aggressive behavior of the original cancer.

The small polygonal cells of cultures of Ca2-83 have been characterized as cells of an epithelial origin by their staining with epithelial cell-specific antiserum to keratin (18) and to EMA (52) and the occurrence of luminal-like cells in the clusters which display microvilli and junctional complexes typical of glandular epithelial cells (9, 43). Although large, fat-containing cells are seen in these cultures, they also have an epithelial ultrastructure and possess microvilli coated with EMA. Further characterization of the epithelial nature of Ca2-83 cells comes from their possession of estrogen receptors. That Ca2-83 cells are of human origin is confirmed by their staining with antisera to EMA, which is specific for human epithelial cells (52), and their failure to stain with antiserum to a similar rat determinant (60). No evidence for the other major cell type of the mammary parenchyma, the myoepithelial cell, can be found at the light or electron microscopic level in cultures or in tumor nodules of Ca2-83 in nude mice (43). That Ca2-83 cells are the malignant cells from the original lumpectomy specimen is suggested by the following findings: (a) the cells are cytologically malignant; (b) the histology of the cell clusters in culture is similar in many respects to clusters in the original specimen; (c) the 2 major cellular pleomorphic variants seen in culture can be identified in the original lumpectomy specimen and in the recurrent metastases of the patient; and (d) there is little or no synthesis of laminin or deposition of basement membrane by the cultured cells, consistent with the loss of the basement membrane being a marker for human mammary carcinomas (5, 21).

The ability to form small tumor nodules of Ca2-83 in nude mice is not necessarily a property of most malignant breast carcinoma cells (29). However, immunocytochemical analysis has been undertaken to confirm the results found in vitro where full expression of the differentiation potential of cells, particularly myoepithelial cells (6), is more limited. Despite the various cellular pleomorphic forms of Ca2-83, there is still a complete absence of any elongated, myoepithelial-like cells that are in any way equivalent to those generated by the normal and benign tumor stem cells of rat mammary epithelial origin. In this respect, Ca2-83 parallels our malignant metastasizing rat mammary tumors and cell lines (12–14, 63). This absence of myoepithelial-like cells has been noted previously in other cultures of malignant breast cancer cells (22, 23, 30) and is also consistent with their virtual absence in histological sections of infiltrating ductal carcinoma in humans (1, 4, 20, 21, 26).

Infiltrating ductal carcinomas are composed of cells that are highly heterogeneous, and their relationship to the malignant/metastatic progression is not known (4). Whether Ca2-83 is a good representative of the original tumor cells of the patient is not entirely clear. However, all the cells growing in suspension from the original specimen of the patient have been retained up to passage 5, and further cultures have been made from such pooled stocks. Furthermore, the main pleomorphic variants of the epithelial cells in the primary tumor and secondary metastases of the patient, particularly the fat-containing epithelial cells, can also be distinguished in the cultures of Ca2-83, indicating that Ca2-83 can reconstitute the major epithelial cell types observed in the primary tumor and recurrent metastases of the patient. Since Ca2-83 has been developed from one of 30 tumors cultured, its relationship to tumor cells occurring in other primary carcinomas and their metastases is also not entirely clear. However, the vast majority of epithelial cells from secondary carcinomas grow only as loosely adherent or nonadherent aggregates, whereas those from normal mammary tissues or benign mammary tumors grow only as fast-adherent sheets of epithelium. In this respect, Ca2-83 is similar to the epithelial cells obtained from the malignant metastatic lesions of infiltrating ductal carcinomas of the human breast.

Often, the growth of malignant human mammary epithelial cells is relatively unaffected by changes in the concentrations of serum or of growth factors (particularly EGF) and hormones in the medium (30, 55, 57). In the case of Ca2-83, it is moderately responsive to changes in the serum concentration but still shows little response to EGF. However, the major growth stimulator
test is contained in pituitary extracts, together with insulin and possibly estradiol and progesterone. This pituitary activity may be related to the new pituitary mammary growth factor, which stimulates normal and benign rat mammary epithelial cells to grow in culture (53). However, the apparent lack of response to other hormones and factors may merely reflect the design of the assays or the combinations tested. For example, although the Ca2-83 cells maintained for 1 month in serum-free medium containing BSA can be stimulated to grow somewhat with the pituitary extract, if the cultures are left for 2 months or more in BSA the cells will not respond. Ca2-83 cells can also be stimulated to grow by media from fibroblasts-adipocytes and from myoepithelial cells that surround the epithelium within the normal rat mammary gland and by medium from rat mesothelial-like/stromal cells that are required for the growth in culture of the metastasizing cell strain SMT-2A (12, 13, 14, 29). One of the major mitogenic components synthesized by the fibroblasts-adipocytes is PGE2 (47). That some of the components released by the SMT-2A cell line are different from those released by the myoepithelial-like or predipocyte cells can be seen from their differential effects in stimulating attachment of the cell clusters to plastic substrata (Table 4). This result, however, does not necessarily prove that the growth-stimulating agents themselves are different.

In conclusion, we have established in culture a cell strain, Ca2-83, which, although not yet single-cell cloned, still retains some of the properties of the original epithelial cells from a human breast cancer. We also show that Ca2-83 cells fail to produce myoepithelial-like cells and basement membrane proteins and that they are primarily responsive to novel growth factors for the mammary gland.

ACKNOWLEDGMENTS

We thank Christine Hughes and Sharon Ferns for expert assistance, Linda Lovell for looking after the nude mice, Dr. M. J. O'Hare for the mycoplasma tests (all Ludwig Institute for Cancer Research), Dr. M. G. Ormerod (Institute for Cancer Research), Dr. M. J. O'Hare for the mycoplasma tests, and Dr. M. J. O'Hare for the mycoplasma tests. We also show that Ca2-83 cells fail to produce myoepithelial-like or preadipocytic cells can be seen from their differential effects in stimulating attachment of the cell clusters to plastic substrata (Table 4). This result, however, does not necessarily prove that the growth-stimulating agents themselves are different.

In conclusion, we have established in culture a cell strain, Ca2-83, which, although not yet single-cell cloned, still retains some of the properties of the original epithelial cells from a human breast cancer. We also show that Ca2-83 cells fail to produce myoepithelial-like cells and basement membrane proteins and that they are primarily responsive to novel growth factors for the mammary gland.

REFERENCES

Fig. 1. Morphology of Ca2-83 cells. A, histological section of the lumpectomy specimen of the primary breast tumor from which Ca2-83 cells were cultured showing areas typical of a rather undifferentiated adenocarcinoma with cells arranged in cords and circular structures. Some of the cells were multinucleated with a granulated, foamy cytoplasm (g). H & E ×300. B, living Ca2-83 cells growing in plastic flasks were photographed with phase-contrast optics. Cells growing in suspension in clusters, some of which possessed bulbous projections (b). × 190. C, cells spreading and growing from a slow-adherent colony (d) on the surface of the flask giving rise to a cohesive islet of isometric cells, some of which possess a rather undifferentiated adenocarcinoma of the human mammary gland in culture. H & E, ×300; and 364–376, 1984.

Fig. 2. Histological and immunocytochemical staining of Ca2-83 cells. Cells growing in suspension were harvested by centrifugation and embedded in paraffin wax. A, histological section showing circular arrays of cells surrounding a central cavity; some of the cells are multinucleated (m), and some possess a lighter, granulated, foamy, or speckled region within their cytoplasm (g). H & E, B, similar sections stained with oil red O, a stain for neutral lipids, showing accumulation of lipids in an intracytoplasmic droplet (d) within a multinucleated cell (m). C, sections stained with antiserum to a specific antibody to keratin, and only weakly stained the myoepithelial cells (m). Antisera absorbed with the requisite antigens yielded no immunocytochemical staining (not shown). In E and F, sections of Ca2-83 cells were stained with antiserum to EMA and with the same antiserum preabsorbed with EMA, respectively. Strongest specific staining was observed at the cellular peripheries (arrows), although weaker cytoplasmic staining was also observed. In G and H, sections of Ca2-83 cells were stained with antiserum to keratin and with antisera preabsorbed with keratin. Only specific staining of the cytoplasm was observed, mainly in the perinuclear regions. All sections, ×300.

Fig. 3. Immunocytochemical staining of Ca2-83 cells in culture and in tumor nodules in immunodeficient mice. In A and B, sections of Ca2-83 cells were incubated with antiserum to actin and laminin, respectively, but no specific staining was observed (×300). C to F are histological sections of inguinal mammary glands of 18-week-old female nude mice at the site of inoculation of the Ca2-83 cells. In C, sections were stained with antiserum to human EMA, and the majority of the tumor cells in the nodules were stained positively (arrows), whereas adjacent mouse mammary gland ducts were unstained (insert). In D, sections were stained with antiserum to rat milk fat globule membrane, and all the tumor cells in the nodules were stained positively, and almost all the luminal epithelial cells of an adjacent mouse mammary gland duct were stained intensely (arrows in insert). In E, sections were stained with antiserum to human keratins, and about half the tumor cells in the nodule were stained (arrows). All the myoepithelial cells of an adjacent mouse mammary gland duct were stained intensely (arrows in insert) while the luminal epithelial cells were stained more weakly, if at all (insert). In F, sections were stained with antiserum to laminin, and no tumor cells were stained, nor were the adjacent mouse mammary gland ducts stained (not shown). In sections C to F and insert, antisera preabsorbed with the requisite antibodies abolished completely any immunocytochemical staining (not shown). All in C to F and inserts, ×240.

Fig. 4. Electron micrograph of cultured Ca2-83 cells. A, portion of a cell cluster with microvilli (m) on the cell surface lining the medium-filled cavity (c). An occasional larger, distended cell with fat droplets (f) was also observed. Cells were closely apposed with interdigitating membranes and apical junctional complexes (j), and desmosomes (d) were present between adjacent cells. ×3,400. B, higher magnification showing a desmosome (d) with a thickening of the cell membranes, an intercellular contact layer (i), and associated tonofilaments (t). Individual filaments, 10 to 12 nm diameter, were also scattered throughout the cytoplasm (f). ×53,600. C, and D, immunoperoxidase staining with anti-EMA serum. In the higher-power micrograph (C), EMA was located on the surface of the cells, particularly on the outer surfaces (arrows), ×11,500. The lower-power micrograph (D), EMA was found on the surface of the cells exposed to the medium and was particularly concentrated where there were microwells (arrows), ×4,410.

Fig. 5. Immunoperoxidase localization of EMA on fat-containing cells. Spherical cell containing large numbers of fat droplets was stained with antisera to EMA and observed in the electron microscope. The cell surface exposed to the medium was coated with reaction product (arrows), particularly in those areas with extensive number of microwells, ×3,530.

Fig. 6. Autoradiography of [3H]thymidine-labeled Ca2-83 cells. Cultured cells were exposed to [3H]thymidine for 48 h in normal growth medium, then harvested by centrifugation. The cells were smeared onto slides, exposed to photographic emulsion, developed, and also counterstained with H & E as described in "Materials and Methods." Those cells which were synthesizing DNA in the 48-h period are black (arrows), ×300.
PROPERTIES OF A NEW BREAST CANCER CELL STRAIN

1A

1B

1C

1D

1E
PROPERTIES OF A NEW BREAST CANCER CELL STRAIN
Loss of Production of Myoepithelial Cells and Basement Membrane Proteins but Retention of Response to Certain Growth Factors and Hormones by a New Malignant Human Breast Cancer Cell Strain
