PROCEDURE NO. 41408

ISOLATION AND GROWTH OF HUMAN MAMMARY EPITHELIAL CELLS

Martha R. Stampfer

Lawrence Berkeley Laboratory, Building 934, University of California, Berkeley, California 94720

SUMMARY: Techniques for the isolation and culture of human mammary epithelial cells are described. The isolation procedure consists of dissection followed by partial enzymatic digestion with collagenase and hyaluronidase and subsequent filtration to separate the epithelial clusters from the digested stromal elements. Culture procedures utilizing two different growth media are presented. A serum-free medium, MCDB170, permits long-term growth (45 to 60 population doublings) of a pure epithelial population; a less defined medium, MM, yields fewer population doublings but increased expression of some mammary-specific properties.

Key words: mammary; epithelial; human.

I. INTRODUCTION

Human mammary epithelial cells (HMEC) represent a valuable cell culture resource because: (a) large quantities of normal, atypical, and malignant tissues are readily available as discard material from commonly performed surgical procedures, e.g., reduction mammoplasties, biopsies, mastectomies; (b) breast epithelial cells are the origin of the most commonly occurring cancer of women in this country; (c) these epithelial cells perform a variety of type-specific functions in vivo, with modulation by hormonal stimuli, and thus possess numerous differentiated properties which may be examined in response to different culture environments; and (d) markers exist that allow for definitive identification of HMEC in culture.

The first efforts to develop normal HMEC culture systems employed lactation fluids (1,6,10,18). Only small numbers of epithelial cells were available from each specimen donor, and growth potential was limited. The early studies utilizing surgically derived breast tissue employed various combinations of dissociation techniques: mechanical dissection and separation, gradient density separation, and enzyme digestion (4,5,9,17). However, most of these methods did not yield pure epithelial populations or active growth upon serial subculture, despite the use of various hormones, growth factors, and cell culture substrates.

We describe here our procedures for utilizing surgically derived human breast tissue for growth of HMEC in monolayer culture. An enzymatic dissociation technique, a modification of that originally reported for rat mammary tissue (7), is used in conjunction with crude dissection. This method yields large amounts of pure epithelial tissues from each individual tissue donor. Prolonged growth of these cells in culture has been achieved, first by the use of an enriched undefined medium, MM (12,15), and more recently by growth in a serum-free medium, MCDB170 (8).

II. MATERIALS

A. Equipment

Laminar flow hood, NCB-4, Baker[1]
Magnetic stirrer, Thermolyne nuova 7, S8260-S, American Scientific Products[2]
Centrifuge, table top, HN-SII, with swinging bucket rotor #958, IEC[3]
Incubators, 3331-2, Napco[4]
Microscope, ICM 405, Zeiss[5]
Water bath, B7001-22[2]
pH Meter, 130, Corning[6]
Liquid nitrogen refrigerator, LR-301A, Union Carbide[7]
Ultra-low freezer, 8358, Forma Scientific[8]
Refrigerated, highspeed centrifuge, J2-21, Beckman[9]
Analytic balance, H54AR, Mettler[10]
Tube rotator, #60448, Scientific Equipment Products[11]

B. Culture media and chemicals

MCDB170 and stock L, UCSF cell culture facility[12]
Ham's F12, 320-1765, GIBCO[13]
HEPES, 380-5630[13]
Trypsin, 840-7072[13]
Versene, 890-1266[13]
Fetal bovine serum, 240-6290[13]
Penicillin 10 000 U per ml/streptomycin 1 gm per ml, 600-5145[13]
Fungizone, 43760, Squibb[14]
Dulbecco's Modified Eagles Medium (DME) (H-16) WNEAA 50% and Ham's F12 50% (H/H), 9432, Irvine[15]

Epidermal growth factor (EGF), #40001 Collaborative Research[16]
Insulin, I5500, Sigma[17]
Hydrocortisone, H4001[17]
Ethanolamine, A5629[17]
Phosphoethanolamine, P0503[17]
Transferrin, T2252[17]
3,3′,5-Triiodo-L-thyronine, T2877[17]
Trisbase, T1503[17]
Collagenase Type I, C3010[17]
Hyaluronidase, H3506[17]
Isoproterenol hydrochloride, I5627[17]
β-Estradiol, E8875[17]
Cholera toxin, C3012[17]
L-Glutamine, G3126[17]
Riboflavin, R4550[17]
Phenol red, P4758[17]
Bovine pituitary extract (BPE), University of Colorado[18]
Hs578Bst cells, American Type Culture Collection[19]
Hs767Bl cells[19]
fHs74Int cells[19]
Sodium chloride, 7581, Mallinckrodt[20]
Hydrochloric acid, 2612[20]
Potassium chloride, 6845[20]
Calcium chloride, dihydrate, 4160[20]
Glycerol, 5092[20]
Glucose, 4912[20]
Potassium phosphate, monobasic, 7100[20]
Sodium phosphate, dibasic heptahydrate, 7914[20]
Magnesium sulfate, 6066[20]
Dimethyl sulfoxide (DMSO), 9224-1, J. T. Baker[21]
Ferrous sulfate, 2070[21]
Polymixin B (Aerosporin), Pfizer, Inc.[22]
Ethyl alcohol 95%

C. Small equipment and supplies

Pipet-aid with air filter, 1225-80100, Bellco[23]
Tissue culture dish, 35 × 10 and 60 × 15 mm, 3001 and 3002, Falcon[24]
Tissue culture flask, 25 and 75 cm^2, 3013 and 3024[24]
Disposable pipettes, 1, 5, and 10 ml, 7521, 7543, and 7551[24]
Disposable centrifuge tubes 15 and 50 ml, 2095 and 2070[24]
Petri dish 150 × 15 mm, 1058[24]
Disposable Pasteur pipettes, 14672-380, VWR[25]
Stirring bars 1.5″, 58949-108[25]
Scissors, dissecting 6″, 25608-316[25]
Forceps, dissecting, 25718-100[25]
Scalpel handle, No. 5, 25856-023[25]
Scalpel blades, 25857-048[25]
Media bottles, 500 and 1000 ml, 219759 and 219760, Wheaton[26]
Glass vials with rubber-lined screwcaps, 4, 12, and 16 ml, 224882, 224885, and 224886[26]
Centrifuge tubes, 50 ml Polyallomer, 3119-0050, Nalgene[27]
Disposable filters, sterilization, 0.80, 0.45 and 0.20 μm, 380-0080, 245-0045, and 120-0020[27]
Ampules, cryogenic, 3-63401, Nunc[28]
Gilson pipetteman, p-20, and p-200, Rainin[29]
Pipette tips, RT-20[29]
Hemocytometer, AO 1492, 15170-172[25]
Filter, sterilization, Millex, 0.45 μm, LHA 0250S, Millipore[30]
Sterile specimen containers, C8827-42
Pecap monofilament polyester screen, 150, 95, and 51 μm, HC-7-150, HC-7-95, and HC-7-51, Tetko Inc.[31]
Polyester screen holder, 3″ metal plates with 2″ diameter center opening, machine shop.

III. PROCEDURE

A. Preparation of media and solutions

1. Stock solutions

a. Phosphate buffered saline (PBS)
To yield a 10X PBS stock, dissolve 80 g NaCl, 2 g KCl, 2 g KH_2PO_4, and 21.6 g Na_2HPO_4 in 1 liter distilled water. Sterilize through a 0.20 μm filter. Check that pH is 7.0. Store at 4° C and dilute 1:10 in sterile distilled water as needed.

b. Solution A
Dissolve 7.149 g HEPES, 1.880 g glucose, 0.2238 g KCl, 7.697 g NaCl, 0.268 g $Na_2HPO_4 \cdot 7\ H_2O$, and 10 mg phenol red in 1 liter distilled water. Sterilize through 0.20 μm filter and adjust pH to 7.6. Store at 4°C.

c. Saline-Trypsin-Versene (STV)
Dissolve trypsin and Versene in solution A to yield a final concentration of 0.05% trypsin and 0.02% Versene. Filter sterilize and store frozen at −20° C.

2. Growth factor stocks

a. Insulin
To yield a 1 mg/ml stock, add 1 g insulin powder to 199 ml distilled water and 1 ml 1 *N* HCl. Stir to dissolve. Add 1 *N* HCl dropwise if powder does not dissolve, not to exceed a final concentration of 0.005 *N* HCl. Bring up to 1 liter and sterilize through a 0.20-μm filter. Store 5, 10, and 20 ml aliquots at −20° C.

b. Hydrocortisone
To yield a 1 mg/ml stock, dissolve 10 mg crystalline hydrocortisone in 10 ml of 95% ethanol. Store at −20° C.

c. EGF
To yield a 100 μg/ml stock, add 1 ml of sterile distilled water or solution A to a vial of 100 μg of EGF. Mix gently. Aliquot 50

and 100-ml portions to nunc ampoules and freeze at −20° C for up to 3 mo.

d. Triiodothyronine
To yield a 2×10^{-4} *M* stock, dissolve 1.3 mg triiodothyronine in 10 ml distilled water with one drop of 0.1 to 0.5 *M* NaOH. Sterilize through 0.20-μm filter and store at 4° C for up to 1 mo.

e. β-Estradiol
To yield a 2×10^{-5} *M* stock, dissolve 0.545 mg estradiol powder in 100 ml 95% ethanol. Store at −20° C.

f. Cholera toxin
To yield a 10 μg/ml stock, add 0.5 ml of sterile distilled water to a 0.5 mg vial of cholera toxin. Dilute from this 1 mg/ml stock 1:100 by removing 0.1 ml of 1 mg/ml stock to 10 ml sterile distilled water. Aliquot 1 ml portions into nunc tubes and store both stocks at 4° C up to 3 mo.

g. Isoproterenol
To yield a 10^{-3} *M* stock, dissolve 99 mg isoproterenol in 40 ml 95% ethanol and store at −20° C.

h. Ethanolamine
To yield a 10^{-1} *M* stock, dissolve 61 mg ethanolamine in 10 ml of solution A. Sterilize through a 0.20 μm filter and store at 4° C.

i. *o*-Phosphoethanolamine
To yield a 10^{-1} *M* stock, dissolve 141 mg phosphoethanolamine in 10 ml of solution A. Sterilize through a 0.20-μm filter and store at 4° C.

j. Transferrin
To yield a 10 mg/ml stock, dissolve 50 mg of transferrin in 5 ml of solution A. Sterilize through a 0.20-μm filter and store at −20° C for up to 6 mo.

k. BPE
Centrifuge BPE at 13, 218 g for 10 min. Sterilize by sequential filtration through 0.8, 0.45, and 0.20-μm Nalgene filters. Filtering proceeds slowly. Aliquot 5 to 10 ml portions into sterile screw-cap vials and store at −20° C for up to 1 yr.

l. L-Glutamine
To yield a 200 *m*M stock, dissolve 2.92 *g* L-glutamine in 100 ml distilled water. Sterilize through a 0.20-μm filter and store in 5-ml aliquots at −20° C.

3. Tissue processing media

a. Tissue mix medium
Add sterile solutions of the following to Ham's F12 medium to yield the indicated final concentrations: insulin (10 μg/ml); penicillin (100 U/ml); streptomycin (100 μg/ml); polymixin B (50 U/ml); and Fungizone (5 μg/ml). Store at 4° C.

b. Enzyme solution

i. Dissolve collagenase (1500 U/ml) in appropriate amount of tissue mix medium at 37° C. Filter sequentially through 0.80, 0.45, and 0.20-μm Nalgene filters to remove impurities.

ii. Dissolve hyaluronidase (1000 U/ml) in appropriate amount of tissue mix medium at 37° C. Filter through 0.20 μm.

iii. Combine equal volumes of the sterile collagenase and hyaluronidase solutions. This is a 5X solution. Aliquot 30-ml vol into 50-ml conical tubes.

iv. Store at −70° C for up to 1 yr.

c. Tissue digestion medium
Add appropriate amounts of the tissue mix medium, enzyme solution, and fetal bovine serum to the dissected breast tissue to yield a final concentration (including the volume of the tissue) of 150 U/ml collagenase, 100 U/ml hyaluronidase, and 10% fetal bovine serum.

4. Cell preservative medium for freezing

a. CPMI: Add 15 ml fetal bovine serum, and 10 ml DMSO to 75 ml of a 1:1 mixture of DME and F12 (H/H). Shake gently to mix and store indefinitely at −20° C. Refrigerate after thawing.

b. CPMII: Add 44 ml fetal bovine serum and 6 ml DMSO to 50 ml of H/H. Mix and store as per CPMI.

c. Glycerol I: Add 10 ml glycerol and 15 ml fetal bovine serum to 75 ml basal MCDB170. Mix and store as per CPMI.

5. Growth media

a. Basal medium (for conditioned media cells and fibroblasts).
To one 470-ml bottle of H/H add 25 ml fetal bovine serum (5% final concentration), 2.5 ml of the insulin stock (5 μg/ml final concentration) and 5 ml of Pen/Strep stock (optional). If medium is over 1 mo. old, add 5 ml of L-glutamine stock. Use with incubator set at 7.5% CO_2 atmosphere.

b. Medium MM

i. Obtain conditioned media from confluent cultures of Hs767Bl and/or fHs74Int cells, and/or Hs578Bst cells growing in T-75 flasks fed with 15 ml of

basal medium per flask; 48 to 72 h spent medium is frozen at −20° C until use.

ii. Mix the following and sterilize through a 0.20-μm filter: 300 ml of Hs767Bl or fHs74Int conditioned medium, or both, 100 ml of Hs578Bst conditioned medium

iii. Add the following to 600 ml of H/H medium in a clean, sterile 1000-ml bottle:

5 ml fetal bovine serum to yield 0.5%
10 ml insulin stock to yield 10 μg/ml
100 μl hydrocortisone stock to yield 0.1 μg/ml
50 μl EGF stock to yield 5 ng/ml
50 μl triiodothyronine stock to yield 10^{-8} *M*
50 μl estradiol stock to yield 10^{-9} *M*
100 μl cholera toxin stock to yield 1 ng/ml
10 ml Pen/Strep stock (optional)

iv. Add the sterile conditioned media to the above to yield 1 liter of MM. Store at 4° C. If H/H medium is over 1 mo. old, add 5 ml of L-glutamine stock. Use with incubator set at 7.5% CO_2 atmosphere.

C. MCDB170 plus serum-free supplements (SFS)

1. Make the following stock solutions:

Stock J. To yield a 200X stock, add 5.88 g $CaCl2 \cdot 2H_2O$ to 100 ml distilled water. Sterilize through 0.20-μm filter and store at room temperature.
Stock K_1. To yield a 200X stock, add 7.39 g $MgSO_4 \cdot 7H_2O$ to 100 ml distilled water. Filter sterilize and store at room temperature.
Stock K_2. To yield a 200X stock, add 27.8 mg $FeSO_4 \cdot 7H_2O$ to 100 ml distilled water with one drop of 1 *N* HCl. Filter sterilize and store at room temperature. Be sure to discard if solution contains a precipitate or becomes orange colored. Best to store under nitrogen gas.
Stock M. To yield a 100X stock, add 1.13 mg riboflavin to 100 ml distilled water. Filter sterilize and store at −20° C in the dark. Place in aliquots so that it is not thawed more than 5X.

2. Just before use, add the following stocks to 967 ml of basal MCDB170:

5 ml each of stocks J, K_1, and K_2
10 ml each of stocks L, and M

Stir vigorously while stocks are slowly being added on magnetic stir plate with sterile stir-bar.

3. Add the following SFS:

5 ml of insulin stock to yield 5 μg/ml
0.5 ml hydrocortisone stock to yield 0.5 μg/ml
100 μl EGF stock to yield 10 ng/ml
1 ml ethanolamine stock to yield 10^{-4} *M*
1 ml phosphoethanolamine stock to yield 10^{-4} *M*
1 ml transferrin stock to yield 10 μg/ml
5 ml BPE to yield 70 μg/ml total protein
10 ml Pen/Strep stock (optional)
10 ml L-glutamine stock if basal medium older than 1 mo.

4. Store at 4° C for up to 2 to 3 wk. Do not freeze.

5. Use with incubator set at approximately 2% CO_2 atmosphere, but adjust CO_2 as necessary to yield dye indicator color around salmon.

B. Tissue processing

1. Obtain human mammary tissue as discard material from surgical procedures, e.g., reduction mammoplasties, mastectomies, biopsies, gynecomastias.

2. Place material in sterile containers containing sterile buffer or tissue mix medium plus 10% fetal bovine serum and transport to laboratory within 5 h of surgery. If not used immediately, tissue may be stored at 4° C for up to 72 h without significant loss of epithelial cell viability.

3. Separate the epithelial areas from the stromal matrix in sterile 150-mm petri dishes using a combination of sterile scalpel, forcep, and scissor.

a. Nontumor specimens: Transfer cut pieces of the tissue into petri dish. Epithelial areas appear as white strands embedded in the stromal matrix. Dissect out these areas, scraping away the grossly fatty material. Lacerate the epithelial tissue using the opposing scalpels. Remove fatty material from dish for disposal in autoclave.
b. Tumor tissue: carefully mince the whole specimen with scalpel and forcep.

4. Transfer the minced epithelial-containing tissue into a conical centrifuge tube (50 or 15 ml) with the tissue comprising no greater than a third of the volume of the tube. Bring the tube up to full volume with tissue digestion medium leaving only a small air space to allow for gentle mixing during rotation. Place tubes on tube rotator and rotate overnight at 37° C.

5. Centrifuge the tubes at 600 *g* for 5 min. Discard the supernatant fat and medium. Dilute a small aliquot of the pellet in medium to check in the microscope for degree of digestion. Digestion is complete when microscopic examination shows clumps of cells (organoids) with ductal, alveolar, or ductal-alveolar structures free from attached stroma (*see* Fig. 1). Tumor tissue may only show unstructured clumps of epithelial cells.

6. If the tissue is not fully digested, resuspend the pellet in fresh tissue digestion medium at ap-

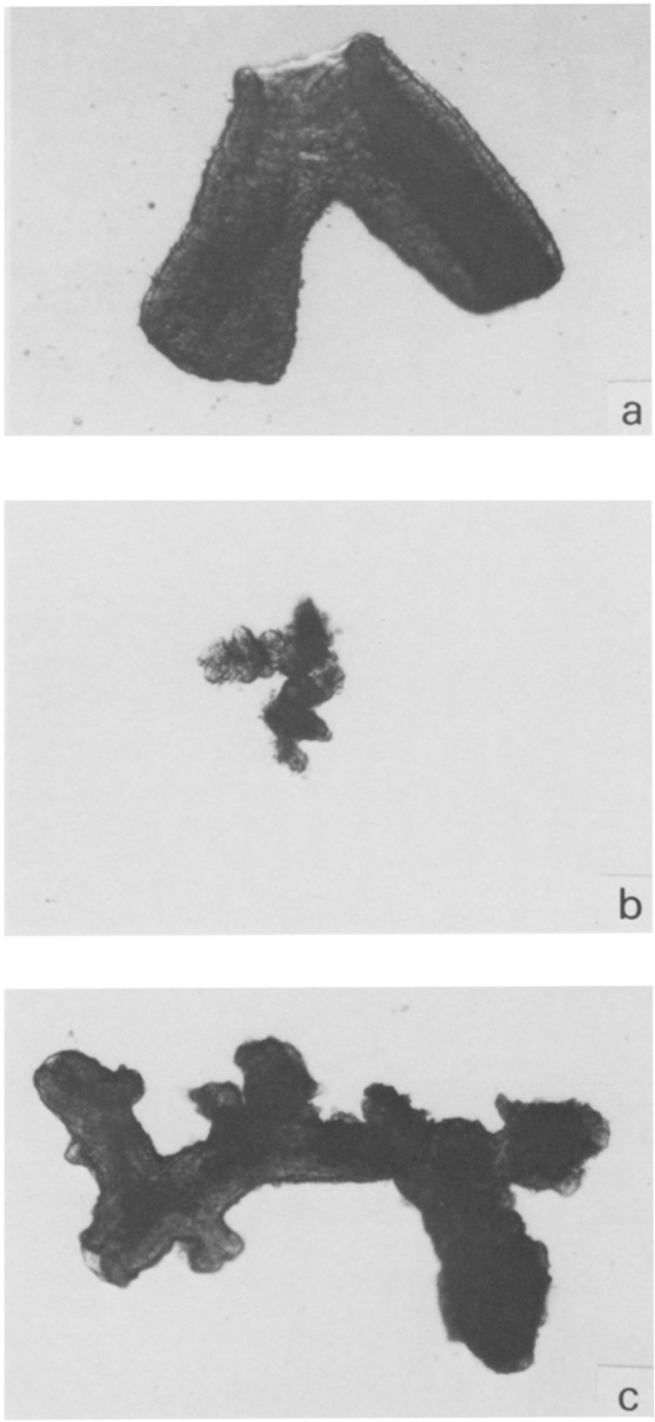


FIG. 1. Organoids derived from enzymatic digestion of reduction mammoplasty tissue. The organoid population contains ductal (*a*), alveolar (*b*), and ductular-alveolarlike (*c*) structures. ×42.

proximately the same ratio of pellet to medium. Reincubate with rotation at 37° C for another 4 to 12 h. Recentrifuge tubes and recheck pellet. If digestion is still not complete add fresh digestion medium and incubate again overnight. The concentration of enzymes in the digestion medium can be varied according to how much more digestion is required.

7. When digestion is complete, centrifuge tubes at 600 *g* for 5 min, carefully aspirate supernatant, and resuspend pellet in tissue mix medium (approximately 15 ml/50 ml tube, 5 ml/15 ml tube).
8. Place a sterile 150-μm filter holder (filter cloth cut and secured by clips between two metal plate holders) on top of a sterile disposable container. Wet one side of the filter cloth with about 1 ml of tissue mix medium, and turn the filter over to the other side. Transfer the resuspended organoids to the top of the filter cloth a few milliliter at a time, letting the medium drain into the beaker. Wash the organoids on the filter a few times with 2 to 3 ml of medium. If there are too many organoids on the filter cloth, the medium will no longer drain through the filter. In that case, either additional filters should be used or the collected organoids removed (*see below*) and the filter reused.
9. Carefully flip the filter cloth with the collected organoids on top of another clean container with the organoids facing down. Wash the organoids off the filter with medium. This is the 150-μm organoid pool, which contains mostly ductal structures.
10. Take the filtered medium from the 150-μm cloth and repeat the filtering procedure with a 95-μm filter holder. The organoids collected from this filter contain mostly smaller ductal and alveolar structures. The filtered medium from this second container contains mostly small epithelial clumps and stromal cells. This is the filtrate. *Note:* For tumor tissue that contains only smaller epithelial structures, the size of the filters used may vary, e.g., use first a 95-μm and second a 51-μm filter.
11. Transfer the 150-μm and 95-μm organoids pools, and filtrate, to 50-ml tubes and centrifuge at 600 *g* for 5 min.
12. Aspirate the supernatant. Reconstitute the pellets in each tube in CPMI by adding 1 ml of CPMI/0.1 ml of packed pellet.
13. Seed a test dish for each tube by placing 0.1 ml of resuspended material into 35-mm dishes drop by drop to fill in different areas of the dish. Disperse the organoids on dish by gently knocking the dish sideways to spread out the medium. Let sit approximately 1 min, then add 1 ml of growth medium to dish. Incubate at 37° C and check for attachment and sterility the following day.
14. Aliquot the remainder of the resuspended material into nunc ampules (1 ml/tube). Freeze slowly to −70° C and then transfer to storage in liquid nitrogen refrigerator.

C. Culture of human mammary epithelial cells

1. Initiation and maintenance of primary cultures

   a. Freshly digested epithelial tissue is placed directly into a tissue culture vessel (*see* section B.13) in either medium MCDB170 plus SFS or medium MM.

   b. Epithelial tissue frozen in ampules is quickly thawed in a 37° C water bath. Ampules containing approximately 0.1 ml of the pelleted organoids are seeded into approximately six T-25 flasks. The thawed organoids are placed, drip by drip, onto the surface of the flasks with a Pasteur pipette to yield an even distribution. Two milliliter of growth medium is then added to the other side of the flask, stood on end. Slowly tip the flask over to have the medium cover the organoids without dislodging them.

   c. After 1 d of incubation at 37° C check that the organoids are attached. Add an additional 2 ml of fresh medium. Cell outgrowth should be visible by 24 to 48 h after seeding (*see* Fig. 2 *A*).

   d. Cultures should be fed every 48 h or three times a week.

   e. If fibroblastic cell growth is observed (particularly from 51 or 95-μm organoid pools), remove by differential trypsinization.

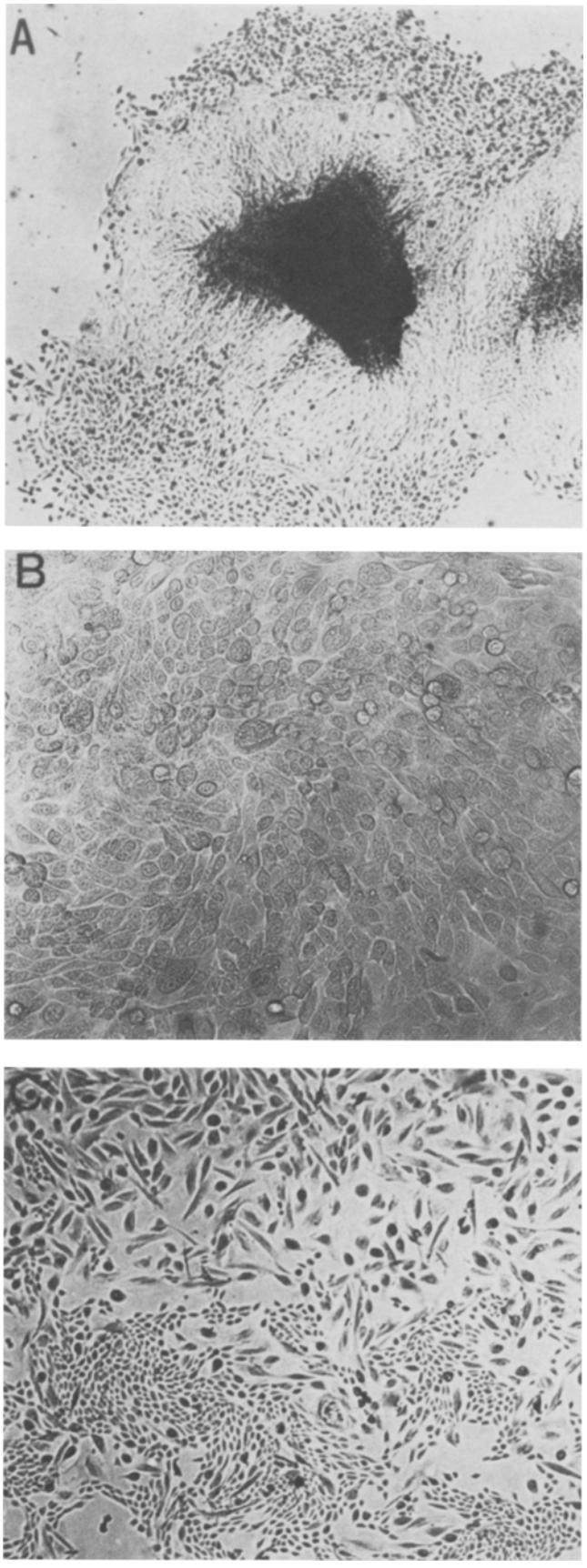
      i. When epithelial patches are large, aspirate medium, wash 1X with STV, and add 0.5 ml STV/T-25 flask. Leave STV on cells at room temperature for about 1 to 2 min, with continued observation in the microscope. Knock flask gently. When the fibroblasts are observed to detach, while the epithelial cells remain adherent, remove the STV.

      ii. Wash cells growing in MCDB170 3X with sterile PBS and refeed flask with fresh growth medium. Cells growing in MM need only one wash.

   f. Primary cultures should not be permitted to remain at confluence, or growth potential upon subculture will be diminished.

2. Subculture of primary cultures

   a. Primary cultures are subcultured when large epithelial patches are present, but before they reach confluence. The density of organoid seeding and attachment will influence the time required (approximately 7 to 14 d) to reach subconfluence. To retain the primary culture and to generate multiple secondary cultures, spaced over time, remove only about 50% of the cells (called partial trypsinization).



b. Cells are washed 1X with STV and exposed to 0.5 ml STV/T-25 flask at room temperature. After a few minutes, knock flask gently and observe under the microscope. Continue exposure, with gentle knocking, until the desired number of cells have detached. Flasks may also be placed at 37° C in the incubator with careful microscope monitoring at short time intervals.

c. Trypsinization is stopped for cells grown in MM by addition of 2 ml MM. Repipette liquid in flask, washing the cell monolayer. Transfer wash to 15-ml tube and repeat with additional 2 ml of MM. Refeed primary flask and return to incubator. Cells in tube are ready to be seeded.

d. Trypsinization is stopped for cells grown in MCDB170 by two additions of 2 ml sterile PBS to flask, as above. However, the primary flask is washed twice more with PBS (discard washes) before refeeding to eliminate all traces of trypsin.

e. Count an aliquot of the cells transferred to the 15-ml tubes above in a hemocytometer. Note exact volume in tubes.

f. For cells grown in MCDB170, now bring volume of PBS in tube up to 10 ml and centrifuge at 600 *g* for 5 min. Aspirate supernatant and resuspend cells in fresh MCDB170 and SFS.

g. Seed cells in second passage at a density of 3 to 5 × $10^3/cm^2$.

h. Partial trypsinization can be repeated, producing good growing secondary cultures, approximately three to six times.

i. Cells removed from primary flasks may be stored frozen instead of seeding. *See* section 5 below.

3. Propagation of HMEC in MM

a. Most reduction mammoplasty-derived specimens will display active growth (18 to 24 h doubling times) for 2 to 4 passages. Tumor-derived specimens are more heterogeneous in growth potential. Procedures for subculture and seeding densities are as described in the previous section. It is again important to subculture the cells as soon as they reach confluence.

b. By 4th or 5th passage (reduction mammoplasties), or 2nd to 5th passage (tumor tissues), the cell population becomes morphologically heterogeneous. Pockets of small, actively growing cells are mixed with larger, vacuolated, nondividing populations. When these indications of senescence appear, cell cultures are no longer used for general experimental purposes.

c. Confluent populations of cells grown in MM can produce large quantities of lactic acid (13). If they become acidic (pH dye yellow-orange to yellow), it is important to remove the cultures to an incubator with a reduced CO_2 atmosphere.

d. Cells grown in MM have a tendency to grow in multilayers when confluent, producing domes and ridges (15). Addition of cAMP stimulators enhances this tendency. Cholera toxin is therefore routinely omitted from MM of primary reduction mammoplasty cultures to reduce multilayering in the organoid outgrowths. MM including the cholera toxin is used after subculture to 2nd passage. For some tumor specimens, addition of cholera toxin to primary cultures significantly increases overall growth.

4. Propagation of HMEC in MCDB170 plus SFS

a. HMEC will grow actively, with a typical epithelial cobblestone appearance (*see* Fig. 2 *B*) for about 2 passages. Procedures for subculture and seeding densities are as described previously.

b. At Passage 2 to 3, most of the cell population gradually changes morphology, becoming larger, flatter, striated, and with irregular edges (*see* Fig. 2 *C*). When assayed for presence of cell-associated fibronectin by indirect immunofluorescence (16), these cells now contain large amounts of fibrillar-appearing fibronectin.

c. When this process of "selection" first appears, maintain the cell cultures with regular feeding, but without subculture. Within a few days to 2 wk, small pockets of actively growing cells with the typical epithelial morphology will appear (Fig. 2 *C*). These cells retain the epithelial pattern of powdery, cell-associated fibronectin (16).

d. When the epithelial-appearing cells have grown to large patches, but before the patches become heavily confluent, subculture these cells.

FIG. 2. Morphology of normal human mammary epithelial cells grown in MCDB170 plus SFS. *A*, primary culture 5 d after seeding. The remnant of the organoid is in the center, with actively dividing cells on the outside of the patch. ×38. *B*, confluent 9th passage cells, with typical epithelial polygonal morphology and continuing mitoses. ×152. *C*, 3rd passage cells. Note the two distinctly different cell morphologies. The smaller cells seem similar to those in (*A*) and (*B*). The larger, elongated cells do not maintain mitotic activity.

e. Within 1 to 2 passages the cell population will consist uniformly of the morphologically epithelial-appearing cells, with 18 to 24 h doubling times.

f. Depending on the individual specimen donor, growth will slow at the 10th to 20th passage. Cells will become larger and more vacuolated, but retain the epithelial cobblestone, smooth-edged appearance and powdery, cell-associated fibronectin pattern. When these indications of senescence appear, cell cultures are no longer used for general experimental purposes.

g. Some specimen donors seem to require the presence of a cAMP stimulator to maintain the actively growing epithelial-appearing population during "selection." Other donors show greater numbers of growing patches. MCDB170 plus SFS containing 10^{-6} *M* isoproterenol is therefore routinely added to half of all secondary cultures for each new specimen donor. If growth is enhanced in the presence of isoproterenol, it is maintained in the culture medium thereafter.

h. MCDB170 plus SFS will support clonal growth of HMEC with cloning efficiencies of 10 to 35%.

5. Freezing of HMEC

a. Monolayer populations of HMEC in primary culture or higher passages are removed from the culture flasks as previously described (C.2, b-e) for cells grown in MM or MCDB170, and an aliquot counted in the hemocytometer.

b. Centrifuge the removed cells at 600 × *g* for 5 min in MM or PBS.

c. Aspirate the supernatant fraction and add cell-preservative medium (CPMII for MM grown cells, GLYI for MCDB170 grown) to yield a cell concentration of 10^6 cells/ml. Keep the preservative medium and resuspended cells at 4° C in an ice bucket.

d. Label nunc ampoules appropriately and add 0.5 to 1.0 ml of resuspended cells to each ampoule.

e. Slowly freeze ampoules to −70° C and transfer to storage in a liquid nitrogen freezer. Do not store ampoules for long times at −70° C.

f. Growth is renewed by quick thawing of the frozen ampoule in a 37° C water bath. The contents of the ampoule are added to a 15-ml tube containing 9 to 10 ml of the appropriate medium for seeding. Repipette gently to break up cell clumps and seed. Cells to be seeded in MCDB170 may be first pelleted at 600 *g* for 5 min in PBS to remove the serum present in the preservative medium.

IV. DISCUSSION

The combined use of these digestion procedures and media can yield actively growing populations of pure HMEC capable of serial subculture. The identity of the HMEC can be ascertained by several criteria. Use of indirect immunofluorescence or immunoperoxidase can demonstrate that the populations are 100% positive for epithelial specific keratin (14), have powdery patterns of cell-associated fibronectin (except during "selection" in MCDB170) (16), are 100% positive for the mammary specific enzyme thioesterase II (11), and have variable degrees of expression of the human milk fat globule antigens (2). Keratin and fibronectin antibodies are readily available from several commercial sources and provide simple methods for monitoring the epithelial purity of the cultures. For example, even a single fibroblast cell can be easily visualized with the fibronectin antibodies. However, markers to unambiguously identify these cells on the basis of ductal, ductular, or alveolar origin are not presently available.

The prolonged serial subculture provided by the MCDB170 medium makes available virtually unlimited quantities of HMEC for experimental purposes. However, these cells have undergone some form of "selection" in vitro. We do not yet understand the nature of this process. Possibly, the cells that maintain growth represent some form of stem cells. Further studies using a variety of different antibody probes are currently underway to elucidate this phenomenon. The cells' postselection do not seem to represent any abnormal subpopulation inasmuch as they maintain a normal diploid karyotype and similar expression of mammary epithelial properties as found in second passage cells grown in MCDB170.

Cells grown in MM vs. MCDB170 plus SFS do display some differences in expression of some mammary epithelial properties (13). For example, cells in MM display more cells positive for human milk fat globule antigens, synthesize greatly increased amounts of glycogen and lactic acid from glucose [similar to cells in culture from pregnant mouse mammary glands (3)], and secrete newly synthesized proteins that are recognized by antibodies to human whey protein. Nevertheless, cells grown for long periods in MCDB170 and then switched to MM were capable of expressing these latter two properties within 24 h of the medium switch. Thus, although by these criteria the MCDB170 grown cells may seem to be in a less differentiated state, they are still capable of modulation by changes in the culture environment.

Although the procedures described here have also been used to grow HMEC from tumor tissues, we cannot say for sure which cells from the very heterogeneous tumor populations are those that grow well under these culture conditions. Although some prop-

erties have been found that distinguish between tumor-derived and normal reduction mammoplasty-derived cells (14), most of the tumor-derived cells display a normal karyotype (19). Possibly, growth of the more abnormal or malignant tumor cell population is not favored by these culture conditions.

Many aspects of the procedures described here have not been carefully tested to determine optimal conditions. This is particularly true of the media and methods used to preserve the frozen cell populations and the solutions and conditions used to subculture the cells. Also, the exact filter pore sizes, enzyme concentrations, and times and temperature used for the tissue digestion procedures are suggested as general guidelines rather than optimized techniques. It is important to maintain gentle methods of rotation during tissue digestion to avoid degradation of the organoids. For example, we found the use of stir-bars on magnetic stirers to yield organoids with decreased capacity for attachment. Continued monitoring of cultures to avoid overly acidic pH is also important.

Further studies varying medium composition, growth factor additions, and cell culture substrates may improve the capacity of HMEC in vitro to express differentiated cell properties. The procedures described here provide large quantities of pure HMEC as a starting point for experimentation on human cellular physiology and carcinogenesis.

V. REFERENCES

1. Buehring, G. C. Culture of human mammary epithelial cells: keeping abreast with a new method. J. Natl. Cancer Inst. 49:1433–1434; 1972.
2. Ceriani, R. L.; Blank, E. W.; Peterson, J. A. Variability in surface antigen expression of human breast epithelial cells cultured from normal breast, normal tissue peripheral to breast carcinomas, and breast carcinomas. Cancer Res. 44:3033–3039; 1984.
3. Emerman, J. T.; Bartley, J. C.; Bissell, M. J. Glucose metabolite patterns as markers of functional differentiation in freshley isolated and cultured mouse mammary epithelial cells. Exp. Cell Res. 134:214–250; 1981.
4. Flaxman, B. A. In vitro studies of the normal human mammary gland. J. Invest. Dermatol. 63:48–57; 1974.
5. Gaffney, E. V.; Pigott, D. Hydrocortisone stimulation of human mammary epithelial cells. In Vitro 14:621–624; 1978.
6. Gaffney, E. V.; Polanowski, F. P.; Blackburn, S. E. et al. Origin, concentration and structural features of human mammary gland cells cultured from breast secretions. Cell Tissue Res. 172:269–279; 1976.
7. Hallowes, R. C.; Rudland, P. S.; Hawkins, R. A. et al. Comparison of the effects of hormones on DNA synthesis in cell cultures of nonneoplastic and neoplastic mammary epithelium from rats. Cancer Res. 37:2492–2504; 1977.
8. Hammond, S. L.; Ham, R. G.; Stampfer, M. R. Serum-free growth of human mammary epithelial cells: Rapid clonal growth in defined medium and extended serial passage with pituitary extract. Proc. Natl. Acad. Sci. USA 81:5435–5439; 1984.
9. Janss, D. H.; Hillman, E. A.; Malan-Shibley, L. B. et al. Methods for the isolation and culture of normal human breast epithelial cells. In: Methods in cell biology, vol. 21B. Academic Press; 1980:107–123.
10. Russo, J.; Furmanski, P.; Bradley, R. et al. Differentiation of normal human mammary epithelial cells in culture: An ultrastructural study. Am. J. Anat. 145:57–77; 1976.
11. Smith, S.; Pasco, D.; Thompson, B. J. et al. Thioesterase II, a new marker enzyme for human cells of breast epithelial origin. J. Natl. Cancer Inst. 73:323–329; 1984.
12. Stampfer, M. R. Cholera toxin stimulation of human mammary epithelial cells in culture. In Vitro 18:531–537; 1982.
13. Stampfer, M. R.; Bartley, J. C. Development of human mammary epithelial cell culture systems and their use for studies of carcinogenesis and differentiation. In: Weber, M.; Sekely, L. eds. In vitro models for cancer research vol. 3. Boca Raton, Fl: CRC Press; 1985, in press.
14. Stampfer, M. R.; Hackett, A. J.; Smith, H. S. et al. Growth of human mammary epithelium in culture and expression of tumor specific properties. Cold Spring Harbor Symp. Cell Prolif. 9:819–830; 1982.
15. Stampfer, M.; Hallowes, R. C.; Hackett, A. J. Growth of normal human mammary cells in culture. In Vitro 16:415–425; 1980.
16. Stampfer, M. R.; Vlodavsky, I.; Smith, H. S. et al. Fibronectin production by human mammary cells. J. Natl. Cancer Inst. 67:253–261; 1981.
17. Stoker, M. G. P.; Pigott, D.; Taylor-Papadimitriou, J. Response to epidermal growth factors of cultured human mammary epithelial cells from benign tumors. Nature 264:764–767; 1977.
18. Taylor-Papadimitriou, J.; Shearer, M.; Tilley, R. Some properties of cells cultured from early lactation human milk. J. Natl. Cancer Inst. 58:1563–1571; 1977.
19. Wolman, S. R.; Smith, H. S.; Stampfer, M. et al. Growth of diploid cells from breast cancers. Cancer Genet. Cytogen. 16:49–64; 1985.

[1] Baker Co., Inc., Sanford, ME 04073.
[2] American Scientific Products, McGaw Park, IL 60085.
[3] International Equipment Company, Needham, MA 02191.
[4] National Appliance Co., Portland, OR 97223.
[5] Carl Zeiss, D-7082 Oberkochen, West Germany.
[6] Corning, Corning, NY 14831.
[7] Union Carbide, Indianapolis, IN 46224.
[8] Forma Scientific, Marietta, OH 45740.
[9] Beckman Instruments, Palo Alto, CA 94304.
[10] Mettler Instruments, Hightstown, NJ 08520.
[11] Scientific Equipment Products, Baltimore, MD 21218.
[12] UCSF Cell Culture Facility, San Francisco, CA 94143.
[13] GIBCO, Grand Island, NY 14072.
[14] Squibb, Princeton, NJ 08540.
[15] Irvine Scientific, Santa Ana, CA 92705.
[16] Collaborative Research, Waltham, MA 02154.
[17] Sigma, St. Louis, MO 63178.
[18] Susan Hammond, Dept. of Molecular and Cellular Developmental Biology, P.O. Box 347, University of Colorado, Boulder, CO 80309.
[19] American Type Culture Collection, Rockville, MD 20852.
[20] Mallinckrodt, Paris, KY 40361.
[21] J. T. Baker Chemicals, Phillipsburg, NJ 08865.
[22] Pfizer, Inc. New York, NY 10017.
[23] Bellco, Vineland, NJ 08360.
[24] Falcon, Coekeysville, MD 21030.
[25] VWR, San Francisco, CA 94120.
[26] Wheaton Scientific, Millville, NJ 08332.
[27] Nalgene, Rochester, NY 14602.
[28] Nunc, R15, DK-4000 Roskilde, Denmark.
[29] Rainin, Emeryville, CA 94608.
[30] Millipore, Bedford, MA 07130.
[31] Tetko, Inc., Monterey Park, CA 91754.