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PEPSIN CAN BE USED TO SUBCULTURE VIABLE MAMMARY EPITHELIAL CELLS

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SUMMARY

Normal mouse mammary epithelial cells in primary culture can be passaged as viable single cells using 0.5 to 1.0 mg/ml pepsin in Hanks' salt solution. After 5 min the pepsin treatment preferentially removes fibroblasts, leaving a monolayer of purified epithelial cells that can be removed by pipetting and transferred to new culture vessels or injected into animals.

Key words: mammary; epithelial; subcultures; enzyme; pepsin.

INTRODUCTION

A technique to establish mammary epithelial cells in culture from collagenase-dissociated tissue was first described by Lasfargues and Moore (1). From mammary glands of animals early in a first pregnancy it was possible to obtain cultures enriched up to 90% epithelial cells using continued regular collagenase treatment of the cultures and trypsin-versene to subculture. Although this method provides adequate material for a variety of studies (2–4) it does not have universal application when dealing with systems that study membrane and surface interactions (5,6), some mammary tumor cells (7), or larger amounts of tissue using late pregnancy, multiple pregnancy, or estrogen-primed animals (7). In these systems continued use of collagenase is prohibitive, trypsin can be toxic, and purified populations are desired from initiation of the cultures. We have developed a method to transfer mammary cells enzymatically using pepsin. The cells are removed rapidly with high viability. The pepsin also allows separation of fibroblastic and epithelial cells as they are passaged in culture. This technique provides an alternative method for experiments involving normal mouse mammary cells. In the present study we provide details of this transfer method. We also provide evidence of morphology, viability, desmosome formation, and in vivo mammary fat pad growth to demonstrate that pepsin is not harmful to the cells.

MATERIALS AND METHODS

Pregnant BALB/c/Crg1Me females (bred and maintained at Baylor College of Medicine) from 12 to 20 d of gestation were used as the source of mammary tissue. The tissue was dissociated using standard collagenase procedures with final concentrations of 2 mg/ml collagenase (Worthington Biochemical Corp., Freehold, NJ, Type III), 20 mg/ml bovine serum albumin (GIBCO, Grand Island, NY, Fraction V), and a ratio of 24 mg of collagenase to each gram of tissue used. Cells were incubated using one 100 mm petri dish per 0.1 ml packed cell volumes for 4 to 6 h at 37° C to allow preferential fibroblast attachment. The floating and loosely adhering cells were then transferred to 60-mm petri dishes (two 60 mm dishes from each 100 mm dish). The primary cells formed confluent monolayers by 3 to 4 d and were 60% epithelial by morphology.

To subculture the cells, crystalline pepsin (Worthington) was dissolved in Hanks' salt solution (no calcium or magnesium) with 0.2% EDTA. Concentrations of 0.5 and 1.0 mg/ml pepsin were used. With the addition of the pepsin, the pH of the solution was approximately 6.8. The cells were rinsed twice in Hanks' solution (no calcium or magnesium), then 1.0 ml of the pepsin solution was placed in each 60 mm petri dish. The plates were returned to the 37° C, 5% CO_2:95% air incubator for more rapid cell removal, although the procedure could be performed at room

temperature. After 5 to 10 min incubation the cells began detaching from the plates. Gentle rotation of the dishes at this time removed some of the cells that aggregated in suspension. The cells remaining on the dish were an epithelial monolayer, which was removed by gentle pipetting.

Alternative methods of subculturing included trypsin-EDTA (1X-GIBCO), 0.2% EDTA in Hanks' salt solution without calcium and magnesium, scraping with a rubber policeman, 2 mg/ml chymotrypsin, 2 mg/ml collagenase Type III, 10 mg/ml hyaluronidase, 0.1 mg/ml papain, pepsin in complete Hanks' salt solution (all enzymes from Worthington), and colcemid at concentrations of 0.1, 1.0, and 5.0 μg/ml. Viability was tested using trypan blue dye exclusion, and cell growth was assessed by counting cells from replicate plates using a Coulter counter. The ultrastructural morphology of the subcultured cells was examined by electron microscopy. A confluent monolayer of cells grown on plastic petri dishes was fixed in 2% glutaraldehyde, postfixed in 1% osmium made up in 0.1 *M* piperazine-*N*-N¹-bis[2-ethane sulfonic acid] buffer, embedded in situ, sectioned, stained in lead citrate and uranyl citrate, and viewed on a Siemens 101 microscope.

The ability of the cells to grow in vivo was tested using transplantation back into empty fat pads of syngeneic mice (8). Cells harvested for injection into mammary fat pads were collected in the same manner as the subculturing method. The cells were centrifuged from the pepsin solution, washed in medium with serum, washed twice in medium without serum, and concentrated to 10^7 cells/ml in medium without serum. 10^5 Cells in 0.01 ml were injected into the cleared fat pads of 3 wk old syngeneic mice (8). The mammary glands were examined as stained whole mount preparations 3 to 10 months later (9).

RESULTS

Confluent dishes of primary mammary cell cultures were available for transfer 3 to 4 d after they had been established. After pepsin subculturing techniques, 70 to 80% cell viability was found routinely by trypan blue dye exclusion. Mammary cells subcultured with the alternate enzymes or scraping did not produce viable cultures. The

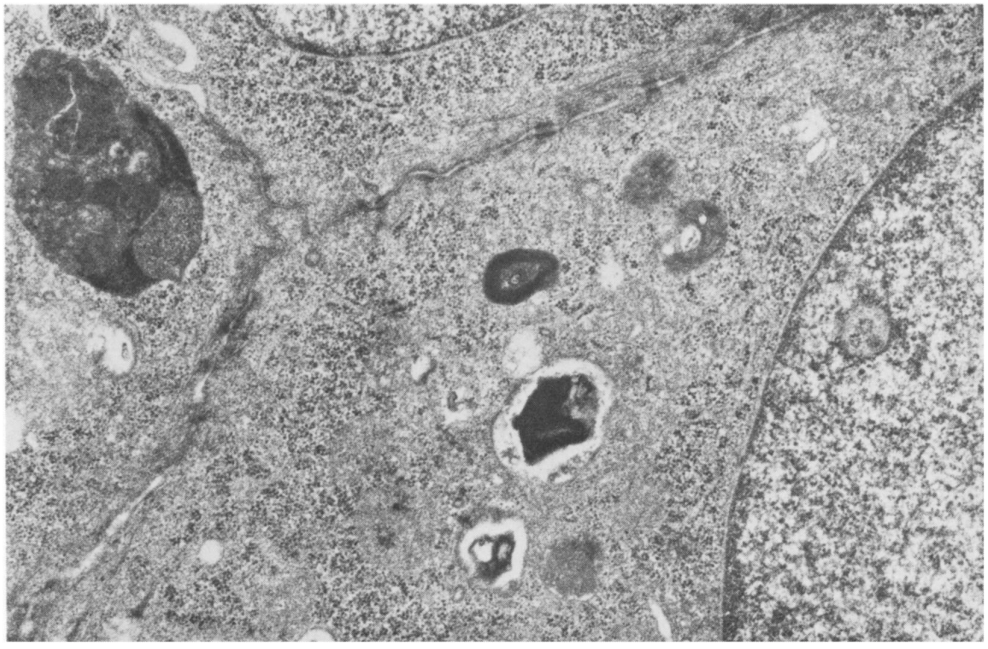

FIG. 1. Electron micrograph of three interfacing cells in a normal mammary epithelial monolayer culture. The cells had been subcultured using pepsin. Note the desmosomes along the cell membranes. ×24,000.

majority of the cells removed by pepsin were in a single cell suspension. The growth potential of cells exposed to pepsin was studied by counting triplicate plates harvested as single cells with pepsin. The mammary cells seeded at 10^5 cells/cm^2 had 80% plating efficiency and were capable of growth, reaching a saturation density of 1.77×10^5 cells/cm^2 in 7 d. As in previous reports (10,11), the saturation density was proportional to seeding density. At passage 1, cells seeded at densities of 0.4×10^5 cells/cm^2 also became confluent in 7 to 8 d at a saturation density of 1.6 to 1.8×10^5 cells/cm^2. If the plates were seeded at densities greater than those attained at confluency through growth, more cells attached initially but began to detach even when fed daily. At an initial density of 2×10^5 cells/cm^2 the cultures were confluent within 1 d, and at 4×10^5 cells/cm^2 the cells filled the dish in a crowded condition the next day with an additional floating population of cells.

One method to demonstrate that morphologically identifiable cells are epithelial is the reformation of desmosomes. The ability of the epithelial cells to reform desmosomes in culture was confirmed by transmission electron microscopy. Figure 1 shows an interfacing of three cells in culture with desmosomes formed along the contiguous cellular membranes.

The cultures were passaged four times using a seeding density of 10^5 cells/cm^2 before they would not grow to confluency. The cells enlarged but did not divide. After a 5th passage few cells attached. Those that attached also enlarged but did not divide. Additional cultures were initiated with 50 ng/ml progesterone, 10 μg/ml insulin, 10^{-8} *M* estradiol, 50 μg/ml hydrocortisone and 100 ng/ml prolactin (all hormones from Sigma

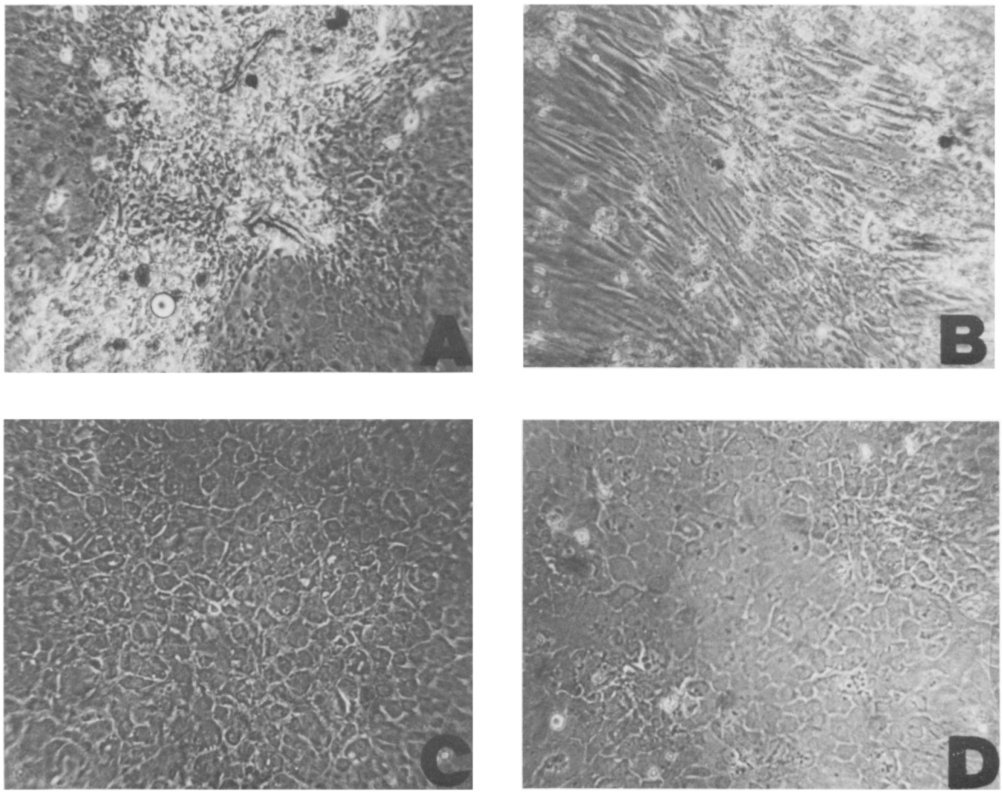

FIG. 2. Light micrographs of live mammary cultures. *A*, Mixed culture of epithelial and fibroblast cells from primary collagenase digestion. *B*, Replating of cells first removed as an aggregate by pepsin and termed "fibroblasts." *C* and *D*, "Epithelial" components of cultures from two independent initial primary cultures. The cells have been passaged and purified twice using 1 mg/ml pepsin.

Chemical Co., St. Louis, MO) supplemented to the medium. The cells were grown continuously in this medium but still were unable to grow past the 4th passage. The cells were transplanted from culture into mice to determine if lack of growth was due to the pepsin treatment or to lack of proper culture conditions. Cells from Passages 1, 2, and 3 were transplanted back into the mammary fat pads of 3 wk old female BALB/c mice. The mammary fat pads were cleared of any host tissue immediately before the injections. There were 24 of 28 successful transplants from Passage 1 cultures and 29 of 34 successful outgrowths from cells passaged three times with pepsin, or 85% overall. The mean of the fat pad filled by growth of these cells was 70% at 19 wk (range of 20 to 100%), 98% (90 to 100%) at 34 wk for Passage 1 cells, and 86% (50 to 100%) at 34 wk for Passage 3. This is greater growth compared to scraping of cells before transplantation (12). Thus, pepsin did not compromise cell viability as judged by the ability of the cells to grow and differentiate into ductal outgrowths in vivo.

The pepsin subculture can be used to transfer all cell types that grow from the primary tissue or it can be used as a selective enzyme treatment. After 5 min of pepsin treatment and rotation of the culture dishes, some of the cells aggregated into a floating population. The cells remaining constituted an epithelial monolayer. These remaining epithelial cells could be removed into a single cell suspension by pipetting with 1 ml of additional pepsin solution. Both populations were 70 to 80% viable as judged by trypan blue dye exclusion. The two populations of cells were replated separately at 1×10^5 cells/cm^2. The cells first removed formed a fibroblast monolayer; the cells that required pipetting for removal formed epithelial cultures with few fibroblasts as judged by morphology. Figure 2 shows mixed and purified populations of cells separated by pepsin. When "epithelial" cultures were harvested selectively and counted every other day for 7 d, the "fibroblast" component never constituted more than 9% of the total population as judged by this technique. The fibroblast cultures could be passaged using standard trypsin techniques.

Discussion

The use of pepsin as a method to transfer mammary cells has several advantages over trypsin which can be toxic, over scraping which is destructive of cells, and over the buffer of White et al. (12). The present pepsin method removed all of the cells in 10 min with 70 to 80% minimum viability when tested immediately for trypan blue dye exclusion. The cells had no less than 80% plating efficiency in new culture dishes and were capable of growth, as demonstrated by an increase in the cell number. The epithelial cells also formed desmosomes in culture after pepsin subculturing. Thus, pepsin does not destroy the capability of the cells to regenerate epithelial specializations. Whereas Lasfargues and Moore (1) were able to propagate cells using medium additions of hydrocortisone and prolactin these components did not promote growth in the purified epithelial cells. It is possible that fibroblast cells aid the continued growth of the epithelial cells by providing extracellular matrices and other stromal support elements. The proliferative potential of the cells subcultured by pepsin was intact after pepsin treatment as shown by the ability to grow and differentiate into mammary tissues in vivo. The epithelial cells apparently are lacking certain factors to sustain growth in culture.

The pepsinization has also been successful to subculture viable normal human breast epithelial cells (Dr. Gertrude Buehring, personal communication), and has been used to transfer dimethylbenz(*a*)anthracene-induced rat mammary tumor cells (Dr. Mark Johnson, personal communication). With new developments in medium factors to support growth of epithelial cells, the ability to subculture viable cells becomes important. Pepsin represents a viable alternative for transferring epithelial cells when standard techniques have not been workable.

References

1. Lasfargues, E. Y.; Moore, D. H. A method for the continuous cultivation of mammary epithelium. In Vitro 7: 21-25; 1971.
2. Marcus, S. L.; Smith, S. W.; Sarkar, N. H. Quantitation of murine mammary tumor virus-related RNA in mammary tissues of low- and high-mammary tumor-incidence mouse strains. J. Virol. 40: 87-95; 1978.
3. Vaidya, A. B.; Lasfargues, E. Y.; Sheffield, J. B.; Coutinho, W. G. Murine mammary tumor virus (MuMTV) infection of an epithelial cell line established from C57B1/6 mouse mammary glands. Virology 90: 12-22; 1978.
4. Lasfargues, E. Y.; Lasfargues, J. C. Possible role of genetic cell variants in the viral induction of mouse mammary tumors. In Vitro 17: 805-810; 1981.
5. Asch, B. B.; Medina, D. Concanavalin A-induced agglutination of normal, preneoplastic, and neoplastic mouse mammary cells. J. Natl. Cancer Inst. 61: 1423-1430; 1978.

6. Ceriani, R. L.; Peterson, J.; Abraham, S. Removal of cell surface material by enzymes used to dissociate mammary gland cells. In Vitro 14: 887-894; 1978.
7. Hosick, H. L.; DeOme, K. B. Plating and maintenance of epithelial tumor cells in primary culture: Interacting roles of serum and insulin. Exp. Cell Res. 84: 419-425; 1974.
8. Daniel, C. W.; DeOme, K. B. Growth of mouse mammary glands after monolayer culture. Science 149: 634-636; 1965.
9. Medina, D. Preneoplastic lesions in mouse mammary tumorigenesis. Methods Cancer Res. 7: 353-414; 1973.
10. Hosick, H. L. A note on growth of epithelial tumor cells in primary culture. Cancer Res. 34: 259-261; 1974.
11. Voyles, B. A.; McGrath, C. M. Markers to distinguish normal and neoplastic mammary epithelial cells *in vivo:* Comparison of saturation density, morphology and concanavalin A reactivity. Int. J. Cancer 18: 498-509; 1976.
12. White, M. T.; Hu, A. S.; Hamamoto, S. T.; Nandi, S. In vitro analysis of proliferating epithelial cell populations from the mouse mammary glands: Fibroblast-free growth and serial passage. In Vitro 14: 271-281; 1978.

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ERRATUM

Air Bladder Cells: A New Continuous Cell Line From Goldfish

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Volume 19, No. 3, Part II, p. 255, abstract no. 83. The following statement should have been placed after the last line: The Air Bladder III cell line was originally initiated by Dr. Janis Gabliks in the Department of Biology, Northeastern University, and the cells used in this study were courteously supplied by Dr. Gabliks.