Culture Conditions and Cell Morphology of Goat Milk-derived Mammary Epithelial Cells in Plate Culture

Susumu TATEYAMA, Akihiro KAWANO, Ryoji YAMAGUCHI, Dai NOSAKA, and Fusao KONDO¹⁾

Department of Veterinary Pathology and ¹⁾Veterinary Public Health, Miyazaki University, Miyazaki 889-21, Japan

(Received 21 April 1988/Accepted 2 August 1988)

ABSTRACT. In order to provide a procedure for studying the mechanism of milk production or carcinogenesis of mammary cells, we attempted to culture mammary epithelial cells from goat milk, and estimated the optimal conditions for the culture system. Cell proliferation was accomplished satisfactorily by using Eagle's minimum essential medium supplemented with 30% fetal calf serum (FCS). However, favorable proliferation was not observed when less than 20% FCS was present. In the course of culture, the cells adhered to plastic dishes within 1 or 2 days after seeding and formed small colonies within 5 days. Such colonies continued to increase in size and made contact with neighboring colonies to form sheets within 10 to 20 days. The sheet-forming cells showed various types of cell morphology, including spindle-like, polygonal and stellate forms. At the 5th or 6th passage, the main proportion of the cell population was comprised of elongated cells and pleomorphic cells. Most of the cells were positive for antikeratin and antiactin antiserum. Electron microscopy of vertical sections of the cell sheet revealed that the cells had flat nuclei and extremely extended cytoplasm. They exhibited an apparent epithelial nature in that they were interconnected by interdigitations and possessed microvilli and desmosomes. Morphological features of differentiation of these cultured cells, such as production of casein granules or lipid droplets, were not observed in the present culture system.—**KEY WORDS:** culture, epithelium, goat, milk.

Jpn. J. Vet. Sci. 50(6): 1192–1199, 1988

There have been many studies on the culture of mammary cells following the first description of the culture of human mammary carcinoma [2]. Most of the cultures in these previous experiments, however, have contained large amounts of fibroblasts, thus making it difficult to determine the inherent role of mammary epithelium in relation to milk production, or to study the carcinogenesis of mammary cells *in vitro*. It has been found that human breast fluids contain epithelial cell clusters, as well as foamy cells, leukocytes and histiocytes, during both pregnancy and lactation [10]. Buehring [1] first observed that mammary cells obtained from human milk could be maintained in culture and formed monolayer colonies without fibroblast contamination. Hallowes *et al.* [9] and Russo *et al.* [15] reported the epithelial nature of colony-

forming cells derived from milk. Gaffney *et al.* [6] reported that foam cells from milk were an active component of colony-forming cells, but Taylor-Papadimitriou *et al.* [18] and Ceriani *et al.* [3] suggested that foam cells resembled macrophages or histiocytes with regard to their adhesiveness, phagocytic ability, ultrastructural features and negative reaction to antiserum against the milk fat globule membrane. In the course of passage of mammary cell cultures derived from human milk, a continuous cell line was established by Gaffney [8].

In order to provide a basic tool for studying the differentiation events and carcinogenesis of mammary epithelial cells, we attempted to passage the culture of pure mammary epithelial cells from goat milk. The present report describes the optimal conditions for cell growth in culture, and the

immunohistochemical and ultrastructural characteristics of the cells.

MATERIALS AND METHODS

Cell Culture: Samples of milk from a late-lactating, 4-year-old, Saanen-breed goat were centrifuged at low speed ($190\times g$). The pellets obtained were resuspended in Eagle's minimum essential medium (E'MEM) (Nissui 1, Nissui Phar. Co., Tokyo). Centrifugation was then repeated 2 or 3 times in the same manner. The collected cells were resuspended in culture medium consisting of E'MEM containing penicillin (100 U/ml), streptomycin (100 μ g/ml) and L-glutamine (29.2 mg/ml) and the numbers of living cells were counted in a hemacytometer. In order to determine the optimal conditions for culture, different concentrations of fetal calf serum, i.e., 0, 5, 10, 20, 30 and 40 V/V%, were added to the culture medium. The cell suspension was diluted so as to contain 2.0×10^5 , 2.7×10^5 and 3.7×10^5 cells, and each dilution was seeded into five dishes (Falcon 3001, Becton Dickinson Co., California) and maintained in a humid 95% air/5% CO₂ atmosphere at 37°C. The medium was changed every 5 days. Observations were carried out with a phase-contrast microscope.

Passage and Stock of Culture Cells: When cultured cells and formed a confluent monolayer, they were washed in 0.2 mM EDTA in phosphate-buffered saline (EDTA-PBS) and digested with 0.02% trypsin in EDTA-PBS by incubation at 37°C. Then E'MEM containing 20% FCS was added to stop the trypsinization. Suspended cells were again centrifuged and the numbers of living cells were counted in the same manner as for the primary culture. After adjustment of the cell number to between 10^4 and 10^5 cells/cm², the cells were subcultured. A 50-mm glass dish containing an 18 $\times$ 18 mm² coverslip for various staining procedures was

subsequently used after the second passage. At the same time, these cells were transferred to a stock medium consisting of E'MEM containing 30% FCS and dimethyl sulfoxide, and then stored at -80°C.

Microscopy: For light microscopy, coverslips with attached cells were fixed in either methacarn fluid (composed of methanol, acetic acid and chloroform) or formalin gas, and then stained with Giemsa or Sudan III, respectively. For examination by electron microscopy, each coverslip on which cells had become confluent at the 15th passage was fixed with 1.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, for 1 hr, and post fixed with 1.5% osmium tetroxide in 0.1 M cacodylate buffer, pH 7.4, for 1 hr as previously described [6]. After dehydration in a graded ethanol series, the coverslip was placed in a glass dish and embedded in Spurr's resin. The dish with the coverslip was immersed in liquid nitrogen to strip the resin block from the dish or the coverslip. Each block was cut into small pieces and reembedded in Spurr's resin in order to locate the specimens in the center of the resin block. Ultrathin sections were sequentially stained with uranyl acetate and lead citrate, and examined with an electron microscope (HITACHI H-800MU, Hitachi).

Immunohistochemical Procedure: Coverslips on which cells had been cultured were washed in PBS, placed in cold acetone (-20°C) and stored in a freezer for 30 min. For immunohistochemical staining using rabbit antihuman keratin antiserum (Ortho Diagnostic Systems Inc., U.S.A.) and rabbit antichickens gizzard actin antiserum (ADVANCE R&D. LSL, Tokyo), each coverslip was processed using a sequence of solutions from a commercial kit (Ortho Diagnostic Systems Inc., New Jersey). The reaction was developed using the peroxidase-antiperoxidase procedure, and hematoxylin was employed as a counterstain.

RESULTS

In order to determine the optimal conditions of culture, the relation between cell proliferation and the concentration of FCS was examined. The number of attached cells increased in proportion to FCS concentration in the range of 0% to 30%, but there was no great difference between the use of 40% FCS and 30% FCS. Cultures containing 30% and 40% FCS formed a monolayer sheet within the shortest period, 18 days (Table 1). However, the cells in E'MEM supplemented with 5% and 10% FCS were unable to reach a confluent monolayer state and most of the cells degenerated after 3 or 4 weeks, before sheet formation. In primary culture from which FCS was omitted, a few cells became attached within 1 or 2 days, but they subsequently exfoliated from the dishes. Even if colonies were sometimes formed, they were few in number and small

in size. Addition of 30% autologous serum to the culture medium instead of FCS produced no satisfactory proliferation like that seen in cultures with 30% FCS. Accordingly, we decided to use E'MEM containing 30% FCS in subsequent experiments.

Table 1. Days from seeding to confluency of mammary cell culture from goat milk

FCS concentration	Density ($10^5/\text{cm}^2$)		
	2.0×10^5	2.9×10^5	3.7×10^5
40% FCS	23 days	20 days	18 days
30% FCS	25 days	20 days	18 days
20% FCS	28 days	25 days	23 days
10% FCS	Attached and grew without reaching confluency		
5% FCS	Same as above		
30% Autologous serum	Same as above		
E'MEM only	Exfoliation after attached and/or formation of small colonies		

Fig. 1. A few single cells attached to a plastic dish, showing various shapes after 2 days in primary culture. Photographed by phase-contrast. $\times 210$.

Fig. 2. A colony that has formed and increased in size through cell migration after 7 days of culture in E'MEM supplemented with 30% FCS. Photographed by phase-contrast. $\times 210$.

Within 1 or 2 days after inoculation into plastic dishes, phase-contrast observation of cells revealed that most of them floated in the medium, although a few single scattered cells were attached, showing spindle, round, stellate or pleomorphic shapes (Fig. 1). Small colonies were formed after 4 or 5 days in culture, and the numbers of the cells in these colonies increased day by day. Two patterns of colony formation were observed. One was formation by mitosis and the other occurred as a result of cells wandering and making contacts with each other by long projections to form compact colonies (Fig. 2). The colonies were composed of spindle-shaped cells, polygonal cells, foamy cells and multinucleated cells. As culture proceeded, these colonies increased in size by cell proliferation and came into contact with adjacent colonies. Lumen-like structures of various sizes were occasionally observed

within the colonies. Eventually, these cells formed a confluent monolayer with various cell shapes, such as spindle-shaped, stellate and polygonal forms. The spindle-shaped cells were well organized and appeared to have a wavy stream-like arrangement (Fig. 3). The sheet-forming cells were then subcultured as for the 1st passage after about 3 weeks in culture. In the 1st or 2nd passage, degenerated cells which remained unattached to new dishes were removed at the first medium change. At the 1st passage, Giemsa-stained coverslips bore stellate cells and large or small polygonal cells with round to ovoid nuclei, together with multinucleated cells (Fig. 4). Lumen-like structures were also present in small or large colonies. Vacuoles in foamy cells were negative for Sudan III stain. The cells surrounding the growing colonies were de-

Fig. 3. Many spindle-shaped cells arranged in a parallel fashion in a confluent sheet after 3 weeks in primary culture. Photographed by phase-contrast. $\times 210$.

Fig. 4. Cultured cells showing various shapes, such as stellate, polygonal and multinucleated forms, and formation of lumen-like structures (arrows) in a sheet at the 1st passage. Giemsa-stained coverslip. $\times 210$.

Fig. 5. Elongated cells and some pleomorphic cells at the 5th passage, some of which are stained positively (arrows) whereas others are weakly positive for antikeratin antiserum counterstained with hematoxylin. $\times 300$.

generated and detached and they were removed when the medium was changed. After the 5th or 6th passage, two cell types were predominant in the monolayer (Fig. 5). The first type was "elongated" cells showing active multiplication, while the second type was "pleomorphic" cells showing a round to polygonal shape and growing mainly between the elongated cells. The cells were constantly passaged at intervals of 2 or 3 weeks, and the above cell types could still be observed up until the 23rd passage at 11 months, after which no further subculture was carried out.

Upon immunohistochemistry for actin and keratin using the peroxidase-antiperoxidase method, most of the cells were positive for antikeratin and antiactin antisera (Fig. 5). Mitotic cells and some elongated cells were stained strongly with

both antisera. Pleomorphic cells, on the other hand, showed different individual cell reactions to both antibodies, some cells being positive and others weakly positive for antikeratin, while most of them reacted weakly to antiactin.

Ultrastructurally, the cultured cells in the sheets were characterized by flat nuclei and extended cytoplasm with a small number of short blunt microvilli on their apical surface in transverse section (Fig. 6). Most of the flattened cells were interconnected by interdigitations and a few desmosomes, and in some areas, a portion of the cytoplasm overlapped with the surfaces of adjacent cells. The cells possessed numerous polyosomes with a few mitochondria, the endoplasmic reticulum and Golgi apparatus were poorly developed, and no secretory granules were observed in their cytoplasm.

DISCUSSION

Mammary epithelial cells derived from goat milk were cultured and passaged using E'MEM supplemented with 20% or 30% FCS. The most satisfactory result was obtained when 30% FCS was applied. Although E'MEM containing 30% FCS was recommended [1], in the first report of cultured cells from breast milk, autologous serum was substituted for FCS in order to obtain confluent sheets of epithelial cells in a subsequent study [4] using human mammary epithelial cells. We also tried the same method, but it was found that autologous serum was not as effective as FCS for the growth and differentiation of goat mammary cells. In fact, the rate of cell attachment increased in proportion to FCS concentration. With a low FCS content, only a few cells became adherent to the plastic dishes, and therefore the numbers of such cells did not appear to be sufficient for growth and formation of a confluent monolayer. On the other hand, several reports

Fig. 6. The ultrastructural features of cultured cells in sheets, characterized by a flat nucleus (N) and poorly developed cell organelles in extended cytoplasm with a small number of short blunt microvilli on the apical surface. No secretory granules are apparent. $\times 6,300$.

have described that prolactin induces morphological changes [5], that hydrocortisone in combination with FCS leads to an increase in the attachment of cell clusters and colony formation [7], and that insulin, hydrocortisone, epidermal growth factor and steroid hormones stimulate mammary epithelial growth [17]. In addition, it has been suggested that mammary fibroblasts are transformed into preadipocytes, which stimulate the multiplication of mammary epithelial cells under the influence of growth hormone [11]. In the present study, however, no hormones were added to the culture medium and the cells produced were considered to be fibroblast-free mammary epithelial cells on the basis of their immunohistochemical and electron microscopic features. Therefore, a high content of FCS may be required for the growth of these cells. This might also be the reason why final differentiation, indicated by features such as the production of casein granules, was not obtained in these cultures.

The morphological cell types observed in the present study corresponded to cultured human mammary gland cells. Epithelial cells have been distinguished into two types,

one of which, E cells, are slightly elongated and appear only in cultures from normal human breast [9]. Taylor-Papadimitriou *et al.* [18] have classified cultured cells into three types, including D-type cells which are elongated like E cells. Because both E cells and D-type cells have an elongated shape and the outlines of their colonies are smooth with an unbroken edge, we consider that the elongated cells in our cultures were identical to E cells and D-type cells. Morphological differences among cultured cells are said to be related to their derivation from different parts of the alveolar duct system at various stages of differentiation in the mammary gland [18]. It also seems highly likely that pleomorphic cells are stem cells, which are able to differentiate to some extent or change into elongated cells, finally undergoing degeneration. Therefore, the properties of this cell type should be examined in detail.

Most of the cultured cells were positive for staining with antikeratin antiserum. It has been suggested that these cells originate from an epithelial element, since cytokeratin, which is one of the constituent proteins of cytoskeletal filaments, is specific for

epithelial cells [13]. It is well known that actin is widely present in other cells as well as in myocytes. However, the distribution of actin filament bundles is closely related to cell morphology. It has been reported that during growth in culture dishes, bundles of actin filaments changed their distribution from the whole cytoplasm to the periphery of pigmented epithelial cells from chick embryo [12]. The different kinds of staining properties of the cells positive for antiactin antiserum in the present study seemed to reflect the different stages of the cell cycle, and cell condition.

The results of ultrastructural observations did not agree with those obtained by Russo and Frumanski [14]. Russo *et al.* [15, 16] and Gaffney *et al.* [6] established cell lines from samples of human milk and postweaning breast fluid. The greatest difference lay in the development of cell organelles and the presence of lipid droplets. In the former reports, these organelles, such as the Golgi complex, were well developed and showed polarity resembling that of glandular epithelium, whereas the cultured cells in the present study did not show cell organelle development. It was thus concluded that the cause of the morphological difference between these cells could be attributed to the composition of the culture medium. From the present study, it is concluded that this culture method is useful for studying the mechanism of milk production or the carcinogenesis of mammary cells. A pure population of mammary epithelial cells was obtained easily from goat milk without any contamination by fibroblasts, in comparison with cells derived from dissociated mammary tissue.

REFERENCES

1. Buehring, G. C. 1972. Culture of human mammary epithelial cells: Keeping abreast with a new method. *J. Natl. Cancer Inst.* 49: 1433-1434.
2. Cameron, G., and Chambers, R. 1937. Neoplasm study III. Organization of cells of human tumors in tissue culture. *Am. J. Cancer* 30: 115-129.
3. Ceriani, R. L., Taylor-Papadimitriou, J., Peterson, J. A., and Brown, P. 1979. Characterization of cells cultured from lactation milks. *In vitro* 15: 356-362.
4. Frumanski, P., Longley, C., Fouche, D., Rich, R., and Rich, M. A. 1974. Normal human mammary cells in culture: Evidence for oncornavirus-like particles. *J. Natl. Cancer Inst.* 52: 975-977.
5. Gaffney, E. V., Polanowski, F. P., Blackburn, S. E., Lambiase, J. T., and Burke, R. E. 1976. Cultures of normal human mammary cells. *Cell Differ.* 5: 69-81.
6. Gaffney, E. V., Polanowski, F. P., Blackburn, S. E., and Lambiase, J. P. 1976. Origin, concentration and structural features. *Cell Tiss. Res.* 172: 269-279.
7. Gaffney, E. V., and Pigott, D. 1978. Hydrocortisone stimulation of human mammary epithelial cells. *In vitro* 14: 621-624.
8. Gaffney, E. V. 1982. A cell line (HBL-100) established from human breast milk. *Cell Tiss. Res.* 227: 563-568.
9. Hallows, R. C., Millis, R., Pigott, D., Shearer, M., Stoker, M. G. P., and Taylor-Papadimitriou, J. 1977. Results on a pilot study of cultures of human lacteal secretions and benign and malignant breast tumours. *Clin. Oncol.* 3: 81-90.
10. Holmquist, D. G., and Papanicolaou, G. N. 1956. The exfoliative cytology of the mammary gland during pregnancy and lactation. *Clin. Oncol.* 3: 81-90.
11. Houdebine, L. M. 1985. Regulation hormonale de la grande mammaire normale. *Bull. Cancer (Paris)* 72: 121-141.
12. Owaribe, K. 1985. Epithelium formation and cytoskeleton. *The Cell* 17: 9-13 (in Japanese).
13. Ramaekers, F., Broers, J., and Vooijs, G. P. 1986. pp. 390-400 *In: Immunocytochemistry*, 2nd ed. (ed. by Polak, J. M., Nooren, S. V.) Wright, Bristol.
14. Russo, J., and Frumanski, P. 1974. Differentiation of human breast epithelial cells in culture. *J. Cell Biol.* 63: 292.
15. Russo, J., Frumanski, P., and Rich, M. A. 1975. An ultrastructural study of normal human mammary epithelial cells in culture. *Am. J. Anat.* 142: 221-232.
16. Russo, J., Frumanski, P., Bradley, R., Wells, P., and Rich, M. A. 1976. Differentiation of normal human mammary epithelial cells in culture: An ultrastructural study. *Am. J. Anat.* 145: 57-78.
17. Stemper, M., Hallows, R. C., and Hackett, A. J.

1980. Growth of normal human mammary cell in culture. *In vitro* 16: 415-425.
18. Taylor-Papadimitriou, J., Shearer, M., and Tilly, R. 1977. Some properties of cells cultured from early-lactation human milk. *J. Natl. Cancer Inst.* 58: 1563-1571.

要 約

平板培養における山羊乳汁由来乳腺上皮細胞の培養条件と細胞形態：立山 晉・河野明広・山口良二・野坂大・近藤房生¹⁾（宮崎大学農学部家畜病理学教室，¹⁾獣医公衆衛生学教室）——泌乳や乳癌の研究の手段の一つを提供する目的で，山羊乳汁由来の乳腺上皮細胞の培養を試み，その最適培養条件を検討した．細胞は30%の割合で牛胎児血清（FCS）を含むイーグル MEM 培地で良好な発育が得られたがそれ以下の FCS 濃度では不可能であった．細胞は培養開始後 1 ないし 2 日で培養器に接着し，5 日程度で小さなコロニーを形成した．この様なコロニーは次第にその大きさを増して他のものと融合しシートを形成した．シートを形成する細胞は種々の形態を示したが，5 ないし 6 代継代すると伸長した細胞と多型性を示す細胞が主成分となった．これらの細胞は抗ケラチン及び抗アクチン抗体に陽性で電子顕微鏡的にもデスモゾーム，細胞陥合あるいは微絨毛等を認め，上皮細胞の形態を示した．しかしながらカゼイン粒子や脂肪滴等の泌乳に関する細胞分化は本培養法では認められなかった．