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ESTABLISHMENT AND CHARACTERIZATION OF A CAPRINE MAMMARY EPITHELIAL CELL LINE (CMEC)

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SUMMARY

We describe the establishment of a continuous, nontransformed cell line obtained from primary culture of a lactating (114 days postparturition) Anglo-Nubian (*Capra hircus*) goat mammary gland biopsy. These cells (CMEC), have been cultured in the presence of supraphysiologic concentrations of insulin and hydrocortisone for more than 560 population doublings (over 80 passages) without any sign of senescence while maintaining a normal/near-normal diploid chromosome modal number of 2n = 60 and are responsive to contact inhibition of proliferation. Cytoskeletal analysis indicates that CMECs are epithelial, without detectable fibroblastic or myoepithelial cells. When grown at low density on plastic substratum, the cells tend to form island monolayer aggregates with the characteristic cobblestone morphology of epithelial cells. With increasing density, the cells organize into lumen-like structures with various morphology consisting of large and small vacuolized and nonvacuolized cells. Postconfluent cultures form epithelial raised dome-like structures, implying a process of contact-induced differentiation. This is corroborated by positive immunocytochemistry to lactation-specific proteins: β-casein and α-lactalbumin, which were predominantly expressed in dome-forming cells. We also observed an overall modulation of cytokeratin 18/19 expression associated with number of days post subculture and with the expression of lactation-specific proteins. Postconfluent cultures which contain lactation-specific, antibody-reactive, dome-like structures showed a decreased expression of keratin 18 and no (null) expression for keratin 19. Lastly, cells cultured within a collagen matrix show morphological differentiation with the organization of branching duct-like and acini-like structures. This study suggests that CMECs are a useful in vitro model for study of mammary gland development and differentiation, in particular, direct modulation of epithelial cells grown on plastic substratum or extracellular matrix without the influence of stromal elements or the necessity and variability associated with primary cell culture or tissue explants.

Key words: differentiation; dome-like structures; lactogenesis; cytoskeleton.

INTRODUCTION

The mammary gland is a modified cutaneous exocrine organ with diverse physiologic, immunologic, and biochemical functions. The role of this gland is to provide nutritional and developmental support to the suckling neonate. In fulfillment of this function, the gland undergoes characteristic changes necessitating glandular development (mammogenesis), differentiation (lactogenesis), expression of secretory products (galactopoiesis) and lastly, downregulation and cessation of lactation with glandular remodeling (involution). Intrinsically, the complex interaction of systemic and locally produced soluble factors as well as the extracellular matrix (stroma and basement membrane) and mesenchyme-derived cells makes it difficult to identify and dissect the contribution of different components which modulate the development, function, and involution of the mammary gland epithelium. Difficulty in studying the mammary gland is also compounded by species differences and the different stages of the gland's life cycle (i.e., development/differentiation versus lactation or involution) each influenced by different factors or the same factor with a different stage-dependent function. With advances in our understanding of the role of extracellular matrix and tissue-specific local regulation, cell culture systems are more able to answer basic questions of mammary gland modulation both in normal and diseased states. In vitro models of development and function in which primary culture systems are used (reviewed in Ip and Darcy, 1996) range from the complex organ or explant culture to the simpler epithelial cell culture with or without reconstituted extracellular matrix or coculture with stromal cells. The advantage of using newly isolated tissue or cells is that they are more likely to be representative of the in vivo system, maintaining organ-specific function and signal transduction pathways. However, while this approach may work well with rodent models, it is cumbersome to consistently obtain tissue from nonlaboratory species such as humans or ruminants under controlled conditions (i.e., age of the animal, stage of glandular development, or parity) which are known to influence glandular characteristics. Furthermore, due to species differences, rodent models are not necessarily reflective of human or ruminant mammary gland biology. A second approach, the use of established cell lines, has been applied in the study of mammary gland modulation at the epithelial cell level. This method has been

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widely used in studies of normal human mammary cells and breast cancer cells (reviewed in Ethier, 1996).

Similarly, six ruminant mammary epithelial cell lines, five from the bovine and more recently, one cell line from the ovine species have been described. The first described bovine cell line, BMEC+H and subline variance (Schmid et al., 1983a, 1983b) are spontaneously immortalized clones that have an atypical elongated cell morphology and have been used in studies of cytomatrix and desmosome protein expression. A second spontaneously immortalized bovine cell line, HH2A has been shown to produce mammary-derived growth inhibitor (Huynh et al., 1995). The ET-C bovine epithelial cells have been described as having both epithelial and myoepithelial-like characteristics (Zavizion et al., 1995). Yet, none of the three bovine cell lines as described above have been shown to express lactation-specific proteins. Other bovine mammary epithelial cell lines such as BME-UV (Zavizion et al., 1996) have been shown to express low levels, whereas MAC-T has shown variable levels (Huynh et al., 1991) of lactation-specific proteins. The latter three bovine mammary epithelial cell lines (ET-C, BME-UV, and MAC-T) were established by stable integration of the simian virus large T-antigen (SV40LTA) gene to induce immortalization. SV40LTA is known to bind retinoblastoma protein and p53, thereby potentially modulating both the cell cycle and apoptotic pathways leading to an immortal and/or a transformed phenotype (Shay et al., 1991). While this is useful in establishing and maintaining cloned cells, it is not clear how this modification affects the cell's phenotype. Pathways that are altered may include mitogenic responsiveness and certainly, induction of apoptosis. Furthermore, stable integration of foreign genes in the host cell by these techniques is a random event that may affect the function of other genes. This could explain why the two sublines of BME-UV respond differently to growth factors and have distinctive karyotypes. Similar arguments may be used for the MAC-T cell line and could help explain the phenotypic instability associated with protein expression reported by others (Zavizion et al., 1995). The spontaneously derived ovine mammary epithelial cell line NISH (Ilan et al., 1998) has been preliminarily characterized and shown to contain a heterogeneous population of both fibroblastic, epithelial, and myoepithelial-like cells which are α-smooth muscle actin-positive. The cells respond to growth hormone with morphologic differentiation and expression of β-lactoglobulin when cultured with extracellular matrix. To date, no mammary epithelial cell lines of caprine origin are available.

A caprine mammary epithelial cell line would be useful for species-specific studies of protein expression and proliferation, as the transgenic goat has become a useful bioreactor for the biotechnology industry. Furthermore, the physiology of the caprine mammary gland is distinct from the bovine in some key respects: (1) goat and human mammary epithelium secrete proteins by an apocrine mechanism (Schalm et al., 1971), a process by which epithelial cell cytoplasmic projections are released into the lumen of the acini resulting in cytoplasmic particles which become part of the normal composition of the milk, compared to the cow for which the mammary gland is a merocrine organ with relatively few cytoplasmic particles in the normal milk; (2) mammary gland regression and decreased milk production in the goat is associated with a decrease in the number of cells (Knight and Peaker, 1984), rather than a loss of differentiated function and a minimal decrease in cell number as in the bovine mammary gland (Hurley, 1989); (3) the lactating caprine mammary gland retains its potential for proliferation (Knight and Wilde, 1987; Wilde and Knight, 1989) which is distinct from the bovine in which a dry period is essential to allow for glandular redevelopment between successive lactations (Oliver and Sordillo, 1989); and (4) goats are seasonal breeders; therefore, it is easier to study their mammary gland biology without the influence of the estrus cycle or pregnancy during mammary gland involution. Our approach to establishing and using a continuous mammary epithelial cell line was to isolate and propagate cells from a differentiated (lactating) gland and culture them with the well-described mammogenic factors: insulin at supraphysiologic concentration and hydrocortisone. Once the cell line was established, the cells were used for a limited number of passages (30–60) such that they might more closely maintain tissue specific characteristics. The objective of this study was to establish and validate the use of an epithelial cell line for further studies of caprine mammary gland development, differentiation, and involution.

Materials and Methods

Mammary tissue collection. The caprine mammary epithelial cell line was derived from biopsy tissue obtained from a lactating (112 d postparturition) 16-month-old prima parous Anglo-Nubian goat (*Capra hircus*) registered purebred by the American Dairy Goat Association. The animal was housed at the Spring Hill Isolation Facility, Department of Pathobiology, the University of Connecticut, and tested negative for caprine arthritis encephalitis virus. Weekly monitoring of mammary gland secretions prior to surgery showed no signs of clinical or subclinical mastitis by the California Mastitis Test, somatic cell count, and culture on blood agar plate performed by the Diagnostic Testing Service (DTS) of the University of Connecticut. Mammary tissue was surgically removed according to a procedure modified from previously described methods (Dils and Forsyth, 1981; Knight and Peaker, 1984; Knight et al., 1992). Briefly, 12 h before surgery the udder was shaved and food and water were withheld from the animal. Before surgery, the udder was milked out by hand and the animal was administered 0.4 mg atropine (The Butler Co., Columbus, OH) per kg. After 20 min, xylazine (Miles Inc., Shawnee Mission, KS) was administered intramuscularly at 0.22 mg/kg followed by ketamine hydrochloride (Fort Dodge Laboratories, Inc., Fort Dodge, IA) at 11 mg/kg, 10 min later. Once general anesthesia was induced, a local anesthetic line block of 5 ml 2% lidocaine hydrochloride (J. A. Webster, Inc., North Billerica, MA) injected subcutaneously was administered dorsal to the biopsy site. Skin at the surgical site was aseptically prepared using alternating povodine iodine and alcohol scrubs. Approximately 5 g of secretory tissue was aseptically removed from two sites of each udder half carefully avoiding the gland cistern and major blood vessels. The biopsy site was sutured internally with Dexon "S" (American Cyanamid Co., Danbury, CT). Skin closure was achieved with Dermalon (American Cyanamid Co.). The animal received 40 mg gentamicin sulfate (SoloPak Laboratories, Franklin Park, IL) and 1.5 million units of procaine penicillin (Phoenix Pharmaceuticals, Inc., St. Joseph, MO) immediately after surgery and each day for the following 4 d. Skin sutures were removed 7 d following surgery. The tissue was transported on ice in sterile Hanks' balanced salt solution (Sigma Chemical Co., St. Louis, MO) supplemented with (per ml) 200 U penicillin-G and 200 ug streptomycin sulfate (HBSS 2× P/S).

Dissociation of mammary tissue and culture of cells. Isolation of mammary gland cells was similar to that previously described (Cifrian et al., 1994). The mammary tissue was trimmed of visible fat and connective tissue and washed twice with HBSS 2× P/S. Tissue sections were then minced in a sterile 100-mm petri dish with forceps and scissors. The minced tissue was transferred to a flask with 50 ml HBSS 2× P/S, rocked 5 min at room temperature, and allowed to settle; the supernatant containing lipid and milk protein was decanted. This was repeated until the supernatant was clear. The tissue was further minced with forceps and scalpel, the supernatant with cells was collected and passed through a cell strainer (Falcon, Becton Dickinson & Co., Lincoln Park, NJ) and the resulting single cell–small aggregate suspension was kept and designated as caprine mammary cells-1 (CMC-1). The remaining tissue was transferred to a sterile trypsinization flask with magnetic stirrer and 5 ml of digestion medium per gram of tissue was added. Digestion media consisted of phenol red-free DME/F-12 (Sigma) with (per ml) 300 U

type 1A collagenase (Sigma), 100 Kunitz U deoxyribonuclease 1 (Sigma), 200 U penicillin-G, and 200 μg streptomycin sulfate. The tissue was digested at 37° C with stirring for 1 h, the supernatant was decanted and strained (CMC-2), fresh digestion medium was added, tissue was digested for an additional 2 h, and the cell suspension was strained (CMC-3). After 3 h of digestion it was apparent that the majority of parenchyma was dissociated, since only white stringy aggregates of connective tissue were visible. The cell suspensions (CMC-1, -2, and -3) were separately washed five times with Ca^{++}, Mg^{++}, and phenol red-free HBSS (modified HBSS), (Sigma) at 500 ×g for 5 min. Cell viability and number were determined by trypan blue exclusion. Cells were initially plated in 75-cm^2 tissue culture flasks (Falcon) at 5×10^6 cells/flask/12.5 ml^{-1} in phenol red-free DME/F-12 supplemented with 2.2 mg sodium bicarbonate per ml, 5 m*M* sodium acetate, 5 μg holo-transferrin (Sigma) per ml, 0.5 m*M* ethanolamine (Fisher Scientific Co., Pittsburgh, PA), 10 μg insulin (Sigma) per ml, 5 μg hydrocortisone (Fisher) per ml, 10% fetal bovine serum (FBS), (Rehatuin, Intergen Co., Purchase, NY), 100 U penicillin-G per ml, and 100 μg streptomycin sulfate per ml (growth media with 10% FBS). Cultures were incubated at 37° C with saturated humidity and 5% CO_2. Prior to confluence, media were aseptically siphoned and cells were detached with 0.05% trypsin–0.04% EDTA, washed three times with modified HBSS, and passaged at a threefold dilution. For cryopreservation, cells were prepared as for passage but resuspended at 10^6 cells/ml in 15% glycerol, 30% FBS, and 55% DME/F-12. Aliquots of the cell suspension were then placed in cryotubes (Nunc, Inc., Naperville, IL) at 1 ml per tube, gradually cooled overnight with a biological cell freezer neck plug, type BF-5 (Union Carbide Corp., Linde Division, South Plainfield, NJ), and subsequently stored in liquid nitrogen.

Enrichment of epithelial cells. Whereas 3 h of collagenase digestion resulted in a relatively pure epithelial-like cell population, approximately 10% of the cells showed fibroblastic morphology. To isolate to homogeneity, we enriched the epithelial population by selective detachment and attachment methods. We carried out selective detachment by trypsinization by adding 3 ml trypsin–EDTA (0.05/0.04) to confluent 75-cm^2 flasks. The flasks were incubated at 37° C for 2.5, 5, and 10 min. At each time point, the supernatant was removed and fresh trypsin–EDTA was added. Culture supernatant was washed with HBSS and the cell pellet was resuspended in medium and plated in 25-cm^2 flasks. For selective attachment, trypsinized and washed cell suspensions were plated onto tissue culture flasks, and after 1 h the cell suspensions (cells which had not attached) were removed and plated onto fresh tissue culture flasks. This was done for each hour, up to 4 h. Cell types were assessed morphologically and cell cultures with an epithelioid appearance were selected. On the basis of initial viability, morphology, and rate of proliferation after 30 d in culture (2 passages of CMC-1 and -2, and 5 passages of CMC-3) only CMC-3 was further propagated and maintained in culture. After 182 d in culture (18 passages) the cells were gradually weaned from 10% to 5% FBS in growth media. Further characterization of these cells led to the use of the current nomenclature of Caprine Mammary Epithelial Cells—University of Connecticut (CMEC-UCONN).

Growth characteristics on plastic substratum. Growth curves and doubling time were determined by our seeding 5×10^4 cells/well/2 ml^{-1} in 12-well flat-bottom tissue culture plates (Falcon) in growth media with 10% FBS (Passage 15) or 5% FBS (Passage 37 and 42). Cell number and viability were determined each day for triplicate or quadruplicate wells for 7 to 10 d by trypan blue exclusion. Cells were detached with 0.05% trypsin–0.04% EDTA, washed in modified HBSS and resuspended in medium. Colony-forming ability in semisolid agar was assessed by our overlaying 2×10^4 cells/well/ml^{-1} in 0.5% agar on a basal layer of 3 ml 0.5% agar cultured in growth medium with 5% FBS or growth medium with 5% FBS without insulin and hydrocortisone. Media were changed every other day in 6-well culture plates (Falcon) similar to previously described methods (Macpherson, 1973; Yang, 1978). Growth was assessed daily for 12 d. On Day 12, 1 ml of 0.16% trypan blue in 0.85% NaCl was added to each well and incubated 5 min to allow diffusion into the matrix; excess dye was aspirated, and percent viability was determined microscopically. Morphology of cultured cells was routinely observed with an inverted microscope with phase contrast (Olympus 1M), (Olympus America Inc., Melville, NY), and photomicrographs were made.

Rabbit antiserum to goat casein. Casein was isolated by a modification of a previously described method (Koch, 1937). As part of the purification process, skim milk (defatted, acellular soluble component) and casein-free whey (supernatant recovered after casein precipitation) were isolated, dialyzed against dH_2O (MWCO 6-8000) (Spectrum, Houston, TX), and lyophilized. Hyperimmune antiserum was produced by subcutaneous injection of 250 μg/site of purified casein in 0.85% NaCl with 1:1 complete Freund's adjuvant (GIBCO BRL, Grand Island, NY) at two sites on a New Zealand female white rabbit (Millbrook Farms, Amherst, MA). This was followed by 2 booster immunizations at the same antigen concentration, but with Freund's incomplete adjuvant (GIBCO BRL). Whole blood was collected by marginal ear vein bleed; serum was separated and stored at −20° C. To enhance specificity, hyperimmune serum was absorbed against 100 molar excess of casein-free whey and centrifuged at 12500 ×g for 5 min. The antiserum was again absorbed and centrifuged at 10 and 1 molar antigen excess. Specificity of rabbit anti-goat casein was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) and immunoblotting. Briefly, 10 μl/well of 2 mg/ml samples in 0.85% NaCl 1:1 2 × dithiothreitol DTT treatment buffer and for protein standards, low-range molecular weight markers (Bio-Rad Laboratories, Hercules, CA) were applied on a vertical slab gel in a Hoefer SE600 apparatus (Hoefer Scientific Instruments, San Francisco, CA). The samples were run under reduced conditions on a discontinuous SDS-PAGE with 12.5% acrylamide resolving and 4% acrylamide stacking gel. After electrophoresis, the samples were transferred (Towbin et al., 1979) from SDS-PAGE to nitrocellulose (Micron Separations, Inc., Westborough, MA) with a Trans-Blot semi-dry electrophoresis transfer cell (Bio-Rad Laboratories). Nonspecific protein binding sites were blocked with 0.1% gelatin, 0.1% Tween-20 in 100 m*M* Tris-HCl, and 0.9% NaCl buffer (G-TTBS). Rabbit anti-casein or preimmune serum (background control) was applied at 1/250 in G-TTBS. Secondary antibody, horseradish peroxidase conjugated monoclonal anti-rabbit IgG (γ-chain-specific) (Sigma) was applied at 1/10,000 and the reaction was visualized with TMB peroxidase substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD). Total protein was visualized on blotting membranes with alkali pretreated India Ink staining (Hancock and Tsang, 1983).

Immunocytochemistry. We examined cytomatrix and expression of β-casein and α-lactalbumin by seeding 4×10^4 cells on 22 mm × 22 mm nitric acid-washed, heat-sterilized glass coverslips in 6-well plates or on glass 4-well chamber slides (Falcon). Cytostructural protein expression was examined 1, 3, 5, and 10 d after seeding cells (Passage 32 or higher) cultured in growth media with 5% FBS. Before staining, the cells were washed three times with phosphate-buffered saline (PBS) and fixed with ice-cold methanol. Cells were examined for cytomatrix protein expression with anti-cytokeratin 18 ($24A_3$) (Mitchell et al., 1990), anti-cytokeratin 19 (Amersham Laboratories, Arlington Heights, IL), anti-vimentin (Mitchell et al., 1990), and anti-α-smooth muscle actin (Skalli et al., 1986) by previously described methods (Mitchell et al., 1989). Expression of lactation-specific proteins was examined in cells cultured in growth medium with 5% FBS and 5 μg bovine prolactin (bPRL) (USDA-bPRL-B-1, AFP-5300, USDA Animal Hormone Program, Beltsville, MD) per ml for 1, 3, 5, 7, and 10 d, with medium changed every second day. Cells were washed with Tris-buffered saline (TBS) and fixed with Bouin's solution (0.9% picric acid, 9% formaldehyde, and 5% acetic acid) for 20 min. The cells were washed with TBS until clear and equilibrated with TBS 5%–dimethyl sulfoxide (DMSO) for 5 min. The slides were then washed with TBS–0.2% Tween-20, 2 × 5 min and again with TBS–0.05% Tween-20, 2 × 5 min. Nonspecific reactivity was blocked by incubating 0.3% bovine serum albumin (BSA) (Intergen, Bovuminar reagent pure powder clin. reag. grade) with TBS for 1 h at room temperature. Primary antisera, rabbit anti-β casein, or rabbit anti-α lactalbumin (R. M. Akers, Virginia Tech, Blacksburg, VA) were diluted 1/25 in blocking solution and incubated with cells overnight at room temperature. A negative control consisted of rabbit anti-casein preincubated with purified goat casein or preimmune rabbit sera or without primary antibody. The slides were washed 2 × 5 min with TBS–0.05% Tween-20 and once with TBS. Secondary antibody, FITC-conjugated monoclonal anti-rabbit IgG (γ-chain-specific) (Sigma) was diluted 1/200 in blocking solution, added to the slides, and incubated in the dark for 1 h. The slides were washed with TBS and mounted with FA mounting fluid (Difco Laboratories, Detroit, MI); the reaction was visualized microscopically with appropriate excitation and monitoring filters.

Sterility. CMECs were routinely cultured on Thermanox cover slips (Lux Scientific Corp., Newbury Park, CA), every 10 passages and stained with Hoechst stain #33258 (Hoechst LTD, Hounslow, Middlesex, UK) as described (Freshney, 1990) for evidence of contamination. Culture supernatants were tested for evidence of mycoplasma contamination with Randolph Mycoplasma Media (Randolph Biomedical, West Warwick, RI). Reverse transcriptase activity was assessed in cell culture supernatants (Passage 62 and 65) from cells cultured in growth media with 5% FBS or 1 mg BSA per ml. Supernatant samples were obtained at 40%, 80%, and 100% confluence in 75-cm^2 tissue

culture flasks as observed microscopically. The positive control consisted of Moloney murine leukemia virus reverse transcriptase, and media with FBS or BSA served as the negative control. The assay was performed by Mr. Derry Spragion, Duke University Medical Center, Center for AIDS Research, Durham, NC by previously described methods (Goff et al., 1981; Willey et al., 1988).

Chromosomal analysis. CMECs grown in 75-cm^2 tissue culture flasks were treated for 6 h with 10^{-7} *M* colcemide (Sigma), detached with 0.05% trypsin–0.04% EDTA in PBS, washed, treated with hypotonic solution, fixed, dropped onto chilled glass slides, and stained with Giemsa (Worton and Duff, 1979). A combined 123 chromosomal spreads at Passages 42 and 46 were examined and the modal number was determined.

Culture with collagen type 1. Collagen solution and collagen gels were prepared by previously described methods (Michalopoulos and Pitot, 1975) from murine or calf tail. First, 35-mm tissue culture petri dishes or 24-well tissue culture plates were coated with neutral tail (type 1) collagen at 500 or 300 μl, respectively. Cells were then either added directly (2×10^5/ml) to the basal layer and allowed to attach for 6 h, before the media were aspirated and then overlaid with collagen, or cells were suspended in collagen (5×10^5/300 μl) and then layered onto the basal layer. The cultures were incubated in growth media with 5 μg bPRL per ml and the media were changed every other day for up to 10 d. After 5 d in culture, collagen gels were detached and allowed to float.

Data representation. Photomicrographs were prepared by computer scanning (ScanJet 3c/T, Hewlett Packard Co., Hopkins, MN) of processed film negatives or from color prints as sharp black and white photos. Immunoblots were scanned directly. Phase-contrast photomicrographs were prepared by image analysis with a CCD camera (Hitachi Denshi, LTD., Woodbury, NY) and Image-Pro software (Media Cybernetics, L.P., Silver Spring, MD). Scanned image composites and labels were made with Adobe Photoshop 4.0 (Adobe Systems, Inc., San Jose, CA) or Microsoft PowerPoint (Microsoft Corp., Redmond, WA) software.

Results

Morphology and growth characteristics demonstrate a nontransformed "normal" mammary epithelial cell line. Selective digestion of caprine mammary tissue resulted in the isolation of three primary cultures with distinct cellular morphology and proliferation rates. Primary cultures obtained from mincing (CMC-1) or after 1 h (CMC-2) of collagenase digestion contained a heterogeneous population of cells which included "fibroblast-like" spindle-shaped (fusiform) cells as well as stellate, polygonal, and cuboidal cells. These isolated populations also had a lower percent viability and lower recovered cell number (CMC-1: 60% viable, 1×10^7 viable cells recovered; CMC-2: 56% viable, 2×10^7 viable cells recovered) when compared to primary cultures obtained after 3 h of digestion (CMC-3: 89% viable, 7×10^7 viable cells recovered) from an original 20 g of tissue. Since our objective was to establish a continuous culture of mammary gland cells, CMC-3 were maintained and further propagated due to their 2.5-fold greater proliferation rate compared to CMC-1 and CMC-2. Morphology of CMC-3 in primary culture (Passages 1–10) was consistent with a mixed population containing polygonal and cuboidal epithelial cells and stellate myoepithelial cells. Continued propagation (greater than 10 passages) and selective attachment and differential trypsinization of these cells on plastic substratum resulted in the selection of a homogenous population of polygonal and cuboidal cells which formed monolayer island aggregates with no evidence of contaminating stromal or myoepithelial cells (Fig. 1*a*). Confluent (850 cells/mm^2) or near-confluent cultures consisted of large and small cells which were often vacuolated and organized into lumen-like structures with definition of boundaries where extended islands have converged (Fig. 1*b*). Postconfluent cultures (approximately 3–4 d after confluency) formed dome structures initially where lumen-like structures were observed (Fig. 1*c*) and later sporadically throughout the monolayer. We observed that dome-forming cell morphology was consistent with detachment from the plastic substratum. However, the cells maintained a monolayer configuration through desmosomal junctions and the dome-like structures were not a simple aggregation or piling of cells.

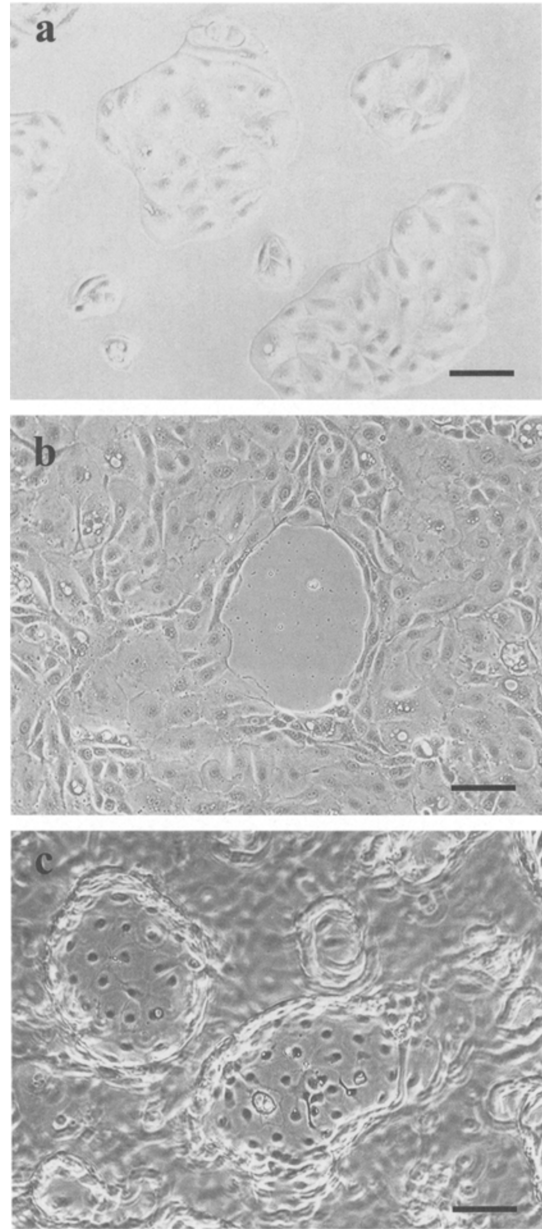

FIG. 1. Phase-contrast photomicromicrographs of CMECs cultured on plastic substratum. *a*, One day after plating, cells formed monolayer island aggregates with the typical epithelial cobblestone morphology. *b*, After 5 days, preconfluent and confluent cultures formed lumen-like structures with elongated cells within the lumen margins and between areas where islands have converged. *c*, Two to 3-day postconfluent cultures formed raised epithelial dome-like structures. Bar, 100 μm.

Growth kinetic assessment of the cell line indicated a stable population with a doubling time of 28 h with little variation between early (Passage 15) versus later (Passage 42) continuous subcultures.

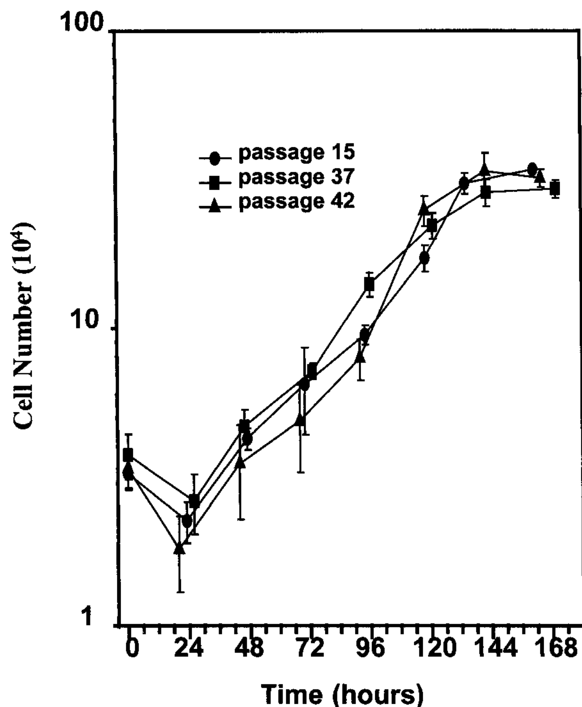

FIG. 2. Growth curves for CMECs indicated a doubling time of 28 h with little variation between early passage (15) versus later continuous passage (42) or after storage in liquid nitrogen (Passage 37). Results are means ± SD of a minimum of three independent experiments.

The cell line also maintained its growth characteristics after being stored in liquid nitrogen for 1 yr, thawed, and cultured for two passages prior to a growth curve experiment (Passage 37) (Fig. 2). CMECs have been maintained in continuous culture for more than 560 population doublings (over 80 passages) without evidence of senescence. CMECs do not express a transformed phenotype since no foci or overgrowths have been observed during routine culture, and a doubling time of 28 h is consistent with that of a normal anchorage-dependent cell culture. Proliferation is modulated by density limitation as observed by growth cessation, contact inhibition, and morphology in confluent cultures in which there is strict monolayer formation and membrane ruffling (Fig. 1*b*) even with fresh media replacement. Further evidence of a nonmalignant phenotype is indicated by culture in soft agar. CMECs (Passage 65) are not able to form colonies in soft agar either in the presence or absence of supplemented insulin and hydrocortisone after 12 d in culture while maintaining a greater than 90% viability (not shown). These observations are consistent with serum/growth factor and anchorage dependence.

Cytoskeleton expression. Cytoskeleton expression is consistent with an epithelial cell line and associated with an increase in the cytokeratin 18/19 ratio as the cells form dome-like structures. Although all of the established CMEC cultures appeared to have epithelial morphology, we further investigated cell line homogeneity by examining cytoskeletal protein expression. CMECs were stained with antibodies which recognize cytokeratins, which are generally specific for epithelial cells, anti-vimentin, which is usually expressed in various nonepithelial cells (i.e., cells of mesenchymal origin such as fibroblasts) and anti-smooth muscle α-actin, which is expressed in mammary myoepithelial cells but not in nonmuscle cells (e.g., fibroblasts and luminal epithelial cells). The criteria for a positive staining reaction relied on structure as well as intensity (i.e., wavy filaments for keratin and vimentin and straight fibers for smooth muscle actin). The caprine mammary cell line was identified as more than 99% epithelial by a positive staining pattern with cytokeratin antibodies and revealed tonofilament junctions between cells which are important for intercellular communication and cellular polarity (Fig. 3*a*,*b*). While up to 40% of the cells stained lightly with antibody to the mesenchymal intermediate filament protein vimentin at 1 d postsubculture, the staining pattern was predominantly perinuclear with some evidence of filament degradation

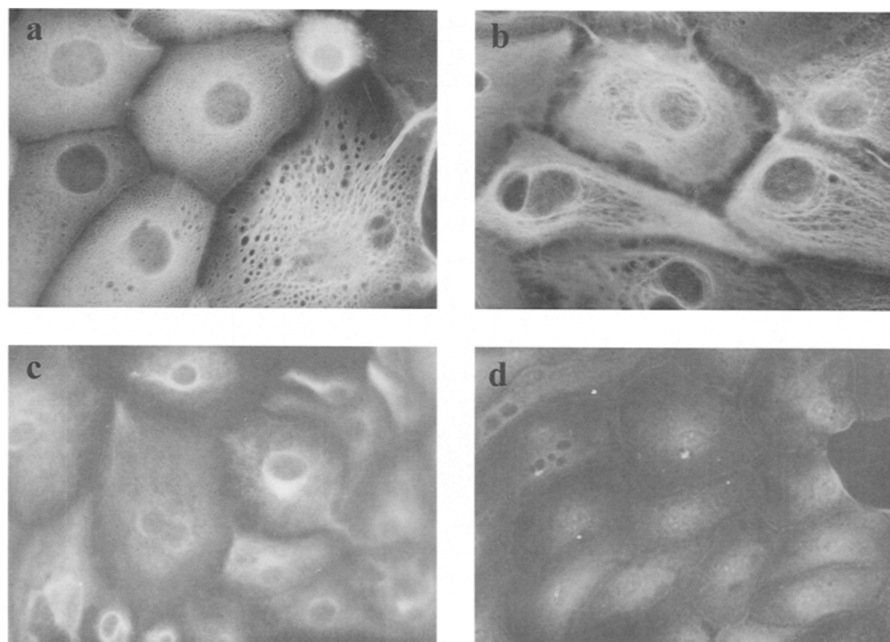

FIG. 3. Immunofluorescence of CMEC cytoskeleal protein expression 24 h postsubculture. *a*, Cytokeratin 18; *b*, cytokeratin 19; *c*, vimentin atypical perinuclear expression with evidence of tonofilament degradation; *d*, α smooth muscle actin-negative. Magnification, ×400.

TABLE 1

MODULATION OF CYTOMATRIX PROTEINS POSTSUBCULTURE ON PLASTIC SUBSTRATUM[a]

Protein	Response of cells postsubculture on: Day 1	Day 3	Day 5	Day 10
Cytokeratin 18	All cells positive	All cells positive	All cells positive	Most cells (60–80%) positive
Cytokeratin 19	Most cells positive	General decreased intensity and number	General decreased intensity and number	No positive cells observed
Vimentin	30–40% Perinuclear staining	<3% Perinuclear staining	<10% Perinuclear staining	40% Positive tonifiliment staining
α Smooth muscle actin	All cells negative	All cells negative	All cells negative	All cells negative

[a] Addition of prolactin to the cultures had no obvious affect on cytoskeleton expression.

(Fig. 3*c*) which is not consistent with what has been observed for mesenchyme-derived cells. We do not consider the vimentin-staining cells to be contaminating fibroblasts since 1) the morphology is consistently epithelial; 2) CMEC vimentin staining varies with culture conditions (i.e., number of days postsubculture); 3) the staining pattern is not consistent with mesenchymal cells; 4) CMECs that stain with anti-vimentin also react with anti-cytokeratin; 5) vimentin expression by epithelial cells has been shown to be associated with culture adaptation (Dairkee et al., 1985) and influenced by media and growth conditions (Schmid et al., 1983a); and 6) cell cultures on Days 1–7 were negative for collagen expression by hydroxyproline assay (Woessner, 1961) with a linear lower detection limit of 500 ng/ml (data not shown). No cells reacted with anti-α smooth muscle actin, discounting contamination by myoepithelial cells (Fig. 3*d*).

We further examined cytostructural protein expression as a potential marker for cell differentiation with this in vitro system. The cells were plated and grown in the presence or absence of prolactin and examined for cytomatrix expression on Days 1, 3, 5, and 10. These time points represent different stages of the cell growth curve: Day 1, cell attachment/lag phase; Day 3, exponential growth; Day 5, confluency; and Day 10, postconfluent culture with multiple dome-like structures. In particular, we were interested in cytomatrix protein expression, comparing proliferating cells to nonproliferating confluent and postconfluent (containing dome-like structures) cultures for evidence of contact-mediated differentiation associated with cytoskeleton expression. Overall we found the addition of prolactin to have no obvious effect on cytostructural protein expression compared to that of cells grown in media alone. Upon extensive examination, we observed some differential staining patterns for cytomatrix proteins associated with number of days postsubculture and a subjective association between staining intensity and cell morphology. Cytokeratin 18, normally associated with simple epithelium and all luminal, but not basal cells of the human mammary gland (Taylor-Papadimitriou et al., 1989) was expressed in all CMECs on Days 1, 3, and 5 and decreased in staining intensity and numbers of cells on Day 10 to as few as approximately 60% (Table 1). The intensity varied with cell morphology; larger flat cells and vacuolated cells stained with lesser intensity than smaller nonvacuolated cells. However, we found it difficult to discern dome structures when staining for cytomatrix proteins which suggests that cells which formed the dome-like structures were not any different in their expression of cytokeratin 18 than cells which form the surrounding monolayer. These experiments demonstrate that the expression of cytokeratin 18 is a consistent feature of this cell line which overall is not altered by culture condition, except in postconfluent dome-forming cultures. Cytokeratin 19, which can be expressed by both simple and stratified epithelia and is associated with luminal cells in terminal ductal lobular units and some basal cells in the larger ducts (Taylor-Papadimitriou et al., 1989) was expressed by most CMECs at Day 1. Interestingly, both the number of cells and intensity of staining decreased dramatically over time to Day 10, where no positive cells were observed. Morphologically, cytokeratin 19 was most prominently expressed in larger vacuolated cells after 1 d postsubculture and noticeably decreased in intensity primarily in the larger flat cells at Day 3. Therefore, cytokeratin 19 seems to be primarily expressed in attaching and proliferating cells whereas null expression is observed in confluent and postconfluent cultures. Vimentin expression was variable with respect to number of cells and staining pattern related to number of days postsubculture. At Day 1, vimentin was atypically expressed in a predominant perinuclear pattern with some evidence of filament degradation. By Days 3 and 5 the number of cells staining with vimentin decreased to less than 10% but again, predominantly as a perinuclear pattern. At Day 10, we observed an increase to nearly 40% of the cells expressing vimentin with filament staining, and to a lesser degree many cells retained the perinuclear staining pattern which was observed at Day 1. In summary, expression of vimentin is not reflective or characteristic of the in vivo cell of origin, but is a cellular response to culture adaptation and/or culture conditions. Furthermore, we observed no relationship between the dome-like structures and vimentin expression since vimentin-expressing cells were individually scattered throughout the culture with no evidence of grouping or structure association. However, vimentin expression may be related to cells expressing extracellular matrix proteins. Lastly, there was null expression of α-smooth muscle actin for all the cells and time points tested. Repeat experiments with CMECs from various passages between 32 and 60 show a similar cytomatrix profile indicating differential matrix expression associated with number of days postsubculture and growth status of the cells. The most remarkable and consistent feature of the cell line's cytomatrix expression is the decrease and eventual loss of cytokeratin 19 expression and a modest decrease in cytokeratin 18 during subculture. To further investigate the relationship between cytomatrix expression and functional differentiation, we examined expression of lactation-specific proteins.

β-casein and α-lactalbumin expression in CMECs. CMECs when grown in the absence of exogenous stromal matrix express β-casein

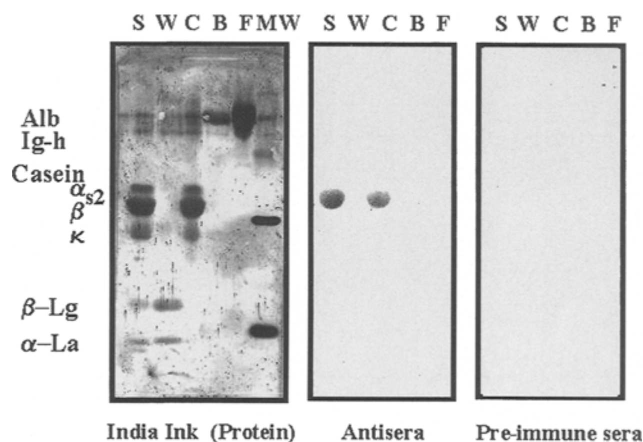

FIG. 4. Specificity of rabbit anti-goat β-casein assayed by immunoblot. *Lanes:* skim milk (S), whey (W), casein (C), bovine serum albumin (B), fetal bovine serum (F), and molecular weight markers (MW). *Abbreviations:* serum albumin (Alb), immunoglobulin-heavy chain (Ig-h), beta-lactoglobulin (β-Lg), alpha-lactalbumin (α-La).

and α-lactalbumin (lactogenesis) most prominently in cells forming dome-like structures. For a mammary epithelial cell line to have practical application to the study of mammary gland biology the cell line must reflect the in vivo process of lactogenesis. CMECs were cultured with insulin, hydrocortisone, and prolactin, which have been described as the minimal requirements for induction of caprine explant culture functional differentiation (Forsyth and Turvey, 1984). Although plastic and glass culture wares are not considered good substrata for the support of differentiation, we cultured cells on tissue culture treated glass slides and examined production of β-casein and α-lactalbumin by immunocytochemistry for cells grown for 1, 3, 5, 7, and 10 d postsubculture. These experiments were designed to determine whether our continuous culture of mammary epithelial cells could undergo "contact-mediated functional differentiation" when cultured without the addition of exogenous stromal matrix. To evaluate the expression of casein, a specific polyclonal rabbit anti-goat β-casein antiserum which does not cross-react with other milk proteins or BSA or any components found in fetal bovine serum by immunoblot assay was produced and used (Fig. 4).

CMECs cultured with prolactin showed less than 5% reactivity with antisera to β-casein and α-lactalbumin when cultured for 1 or 3 d. By Day 5, confluent or near confluent cultures contained 8–10% positive specific-staining cells which were as single cells within the boundaries of lumen-like structures (Fig. 5*a*) or more often as cell aggregates or groups of 3–7 cells (Fig. 5*b*). We have observed during routine culture that dome-like structures arise from lumen-like areas (Fig. 1*b*) and seem to be initiated from single cells which protrude from the lumen margin. These single cells are often vacuolated and as can be seen in Fig. 5*a*, stain in a pattern reminiscent of secretory vacuoles. As the number of days of cells in culture increased, so did the number of cells reacting with antibody to lactation-specific proteins. Day 7 postconfluent cultures, which are morphologically characterized by the formation of raised dome-like structures, showed an increase to nearly 20% of all cells reacting with both anti-casein and anti-lactalbumin. The positive staining was consistently associated with the dome-like structures (Fig. 5*c*); however, scattered single cells were also positive. Day 10, with an increased number of dome-like structures and increased number of cells making up the dome-like structures, we observed a positive staining reaction for approximately 30% of the cells, again predominantly associated with dome structures (Fig. 5*e,f*). The number of positive cells is a conservative estimate, since despite using various fixation methods and despite various modification of the staining protocol, the final product of the immunocytochemistry experiments always resulted in the loss/detachment of many dome structures. In addition, since almost all of the positive cells were associated with dome structures, the actual number of cells expressing β-casein and α-lactalbumin was most likely higher than reported, possibly by a factor of 1.5 to 2 (i.e., at Day 10, 45–60% of the cells might actually be positive). Cytocentrifuge preparations of detached cells obtained from the washing stages during the staining procedure showed more than 80% antibody reactivity, suggesting that many of the detached cells expressed lactation-specific proteins (data not shown). Negative controls of normal rabbit serum, omission of the primary antibody, or absorption of the antisera with either goat casein (Fig. 5*d*) or lyophilized whey protein for anti-α lactalbumin assay were similar and showed no reactivity.

Comparison of our results described above for expression of cytoskeletal proteins and expression of lactation-specific proteins revealed no direct association between cytokeratin, vimentin, and actin expression and the number of cells positive for casein and lactalbumin. The cytomatrix profile was not distinguishable between dome-forming and non-dome-forming cells, except as the number of dome-like structures increased, the overall expression of cytokeratin decreased and the expression of vimentin increased. Since dome-forming cells were consistently positive for reactivity to anti-β casein and anti-α-lactalbumin, there is a general association between the decrease in cytokeratin 18/19 expression and increased vimentin expression and synthesis of lactation-specific protein. However, since we have not yet demonstrated that null cytokeratin expression is directly related to the cells expressing lactation proteins, this relationship needs to be further defined with this in vitro model.

Sterility. There is no evidence of microbial contamination of the cell line or evidence that immortalization of the cell line is due to retrovirus-mediated transformation. Cultures of CMECs were routinely (every 10 passages) grown in the absence of penicillin and streptomycin and evaluated for evidence of contamination by bacteria, yeast, mold, and mycoplasma. None of the cultures showed signs of contamination. Culture supernatants submitted to the University of Connecticut, Department of Pathobiology DTS were consistently negative for growth on culture agar or mycoplasma enrichment media. CMECs grown on coverslips were also negative for contamination by Hoechst #33258 stain. Retroviruses or retroviral products can potentially induce a transformed cell phenotype by insertional mutagenesis. As such, the establishment of the CMEC line could be due to a naturally acquired or laboratory acquired retroviral infection. To examine immortalization of the cell line, we assayed for reverse transcriptase activity in culture supernatant from cells grown in FBS or BSA throughout the cell growth curve. Relative to a Moloney murine leukemia virus reverse transcriptase-positive control, there was no detectable reverse transcriptase activity in any of the samples tested (data not shown). These results demonstrate that immortalization is not associated with retroviral infection.

Chromosomal analysis. CMECs display a normal/near normal

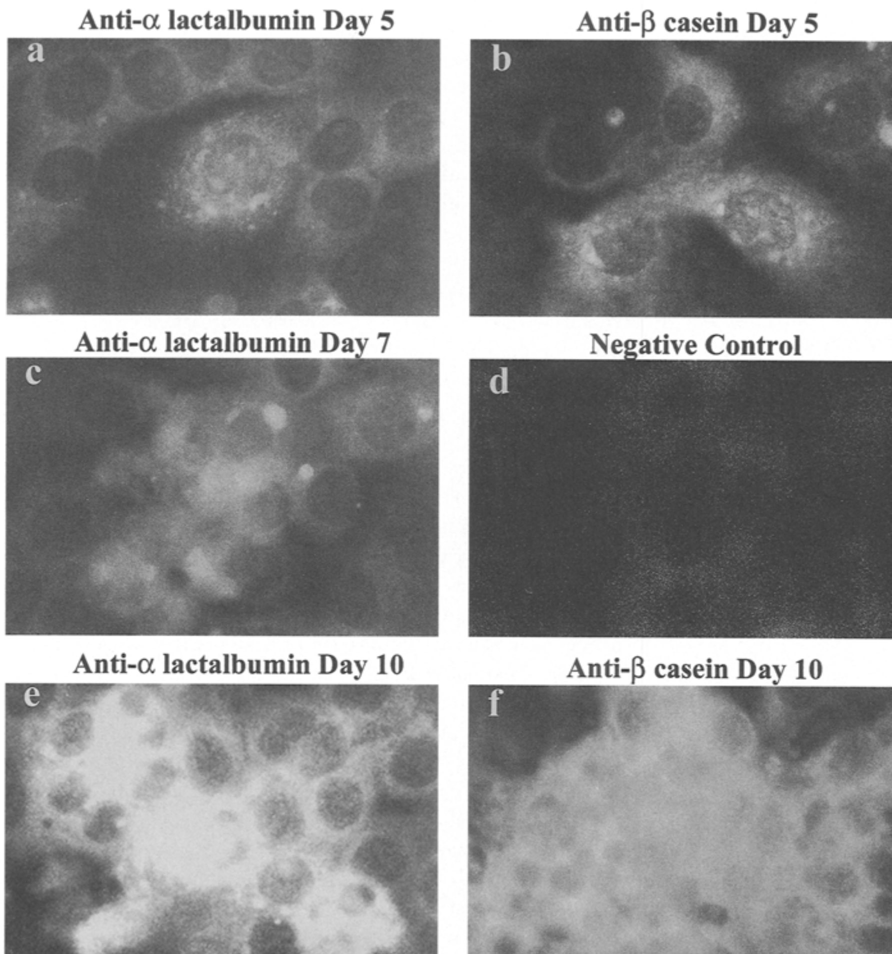

FIG. 5. Immunofluorescence assay of CMEC lactation-specific protein expression on different days postsubculture on plastic substratum. *a*, Positive vacuole pattern-staining cell within a lumen-like structure as observed in a near confluent culture; *b*, groups of 3–5 cells forming an early dome-like structure; *c*, more complex dome-like structure with strong staining of centrally located cells and nonreacting surrounding cells; *d*, negative control of rabbit anti-goat β-casein preincubated with purified goat casein; *e*, large dome-like structure; and *f*, area where two large dome-like structures have converged. Magnification, ×330.

chromosome number consistent with that of cells of caprine origin. Chromosomal analysis of CMECs after more than 290 population doublings showed a modal number of 60 which is consistent with the normal diploid chromosome number for caprine species (Mensher et al., 1989). However, there is evidence of chromosomal drift (pseudodiploidy) which is expected in long-term cell cultures (Fig. 6).

Evidence of morphologic differentiation when cultured with collagen type I. The influence of tissue microenvironment on cellular function and development is well recognized. To complement the induction of relative functional differentiation as indicated by expression of lactation-specific proteins, we examined morphological differentiation of CMECs for organ-like structures when cultured with reconstituted stromal proteins. At Day 0, well-dispersed single cells were observed embedded within the collagen type I matrix (Fig. 7*a*). Within 24 h, cells developed an elongated morphology and formed simple three-dimensional branching structures by migration and congregation (Fig. 7*b*). Continued incubation of attached embedded cultures resulted in the formation of complex branching aggregates with crude luminal structures formed by extensions of fusiform-fibroblastic cells (Fig. 7*c*). After 5 d in culture, the collagen matrix was physically detached from the plastic substratum and allowed to float. Within 2 d, the gels were noticeably contracted and upon examination, revealed morphologic structures reminiscent of branching duct-like structures as well as terminal bud/acini-like formations.

DISCUSSION

We describe the establishment of a caprine mammary epithelial cell line and our initial characterization focused on growth morphology, cytosketetal expression, and evidence of differentiation. Our objective was to develop and validate an in vitro system for the further study of caprine mammary gland growth, differentiation, and involution at the epithelial cell level. The important distinctions in the biology of the caprine mammary gland from that of the bovine and a consideration for establishment of this cell line is that the goat lactating mammary gland retains its potential for proliferation (Wilde and Knight, 1989) and secondly, the reduction in post-peak milk production is association with cell loss (Knight and Peaker, 1984), similar to human, rather than the loss of cell function as in the bovine (Hurley, 1989). As such, our strategy for establishing a continuous mammary cell line took advantage of these characteristics. Since the lactating caprine mammary gland retains its proliferative capacity, stimulation of tissue-derived differentiated cells would likely retain their biological function and may be representative of a semidifferentiated proliferating cell type found within the lactating gland. Furthermore, those cells which survive tissue cul-

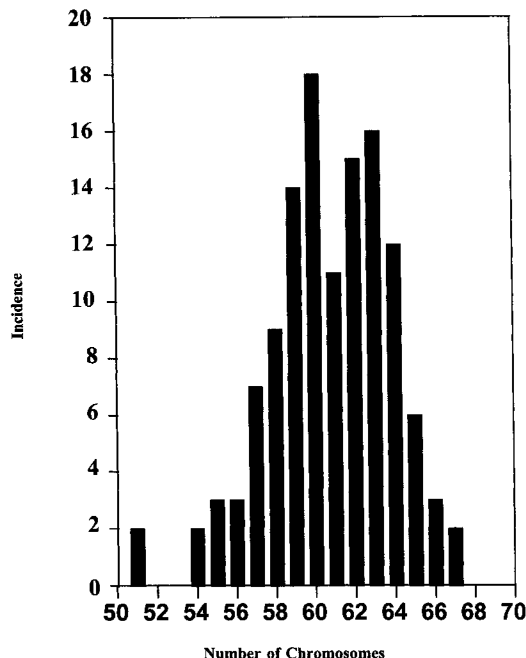

FIG. 6. Chromosomal analysis of CMECs after more than 290 population doublings showing a modal number of 60, consistent with cells of caprine origin and evidence of pseudodiploidy which is expected in long-term cell cultures.

ture adaptation may retain functional capacity and may represent a semidifferentiated epithelial stem cell population, because in the caprine mammary gland, cell death is associated with loss of lactation function.

Isolation of mammary cells by selective digestion with collagenase resulted in three distinct populations with differing morphology, viability, and growth capacity. Cells obtained by mincing of the tissue or after 1 h of digestion were morphologically mixed and difficult to maintain in culture with an obvious increasing doubling time between successive passages and eventually entered crisis within 15–20 passages after isolation. On the contrary, cells which were separately obtained after an additional 2 h of digestion showed few fibroblast-like cells but contained a morphological mixture of epithelial and myoepithelial-like cells. During early passages we enriched the proportion of cells with epithelial morphology to purity based on their relative resistance to detachment by trypsinization and their ability to more rapidly attach to plastic substratum than cells with a stellate myoepithelial-like appearance. Most importantly, these cells, which are the progenitors of the mammary epithelial cell line, maintained a consistent proliferative capacity in media with the mammogenic factors insulin and hydrocortisone and never showed any evidence of senescence during successive passages. This demonstrated that we cultured a population of epithelial cells which retain their proliferative capacity and suggested that since these cells were derived from a lactating rather than a developing caprine mammary gland, they are more likely responsive to the induction of lactation-specific protein expression.

Morphologically, CMECs grown on plastic substratum have an epithelioid morphology and, at low density, form cobblestone monolayer aggregates (Fig. 1*a*). Interestingly, despite thorough single cell suspension preparation prior to passage, the cells when plated, predominantly form island aggregates of 3–4 cells rather than attach in a random single cell pattern. Cell attachment to plastic substratum is dependent on protein synthesis. A dose-dependent inhibition of attachment was observed with the addition of cyclohexamide up to 1 μg/ml where no cells attached even in the presence of fibronectin (data not shown). We have also observed that early postsubcultured isolated cells and cell islands form pseudopodial extensions between neighboring cells. These observations suggest that the cells may produce an autocrine chemotactic factor(s) which facilitates the formation of cell groups. With increasing density, the cells form lumen-like structures with elongated cells within the luminal area and elongated cells forming the boundaries where island groups have converged (Fig. 1*b*). We have observed that the majority of cells contained large and small vacuoles. Immunocytochemical staining of cells from at-

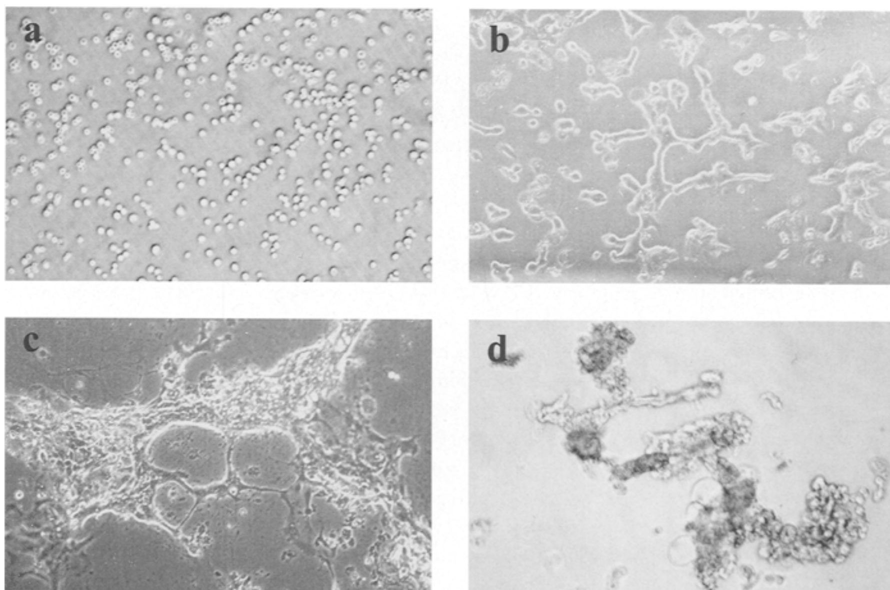

FIG. 7. Phase-contrast photomicromicrographs showing morphologic differentiation of CMECs cultured within a type I collagen matrix. *a*, Day 0 showed a well-dispersed single cell suspension. *b*, Within 1 d of culture, cells developed an elongated morphology and formed three-dimensional branching structures. *c*, Day 5 cultures contained complex branching structures with lumen-like formations. *d*, Detached gels contained duct-like and terminal bud/acini-like structures. *a*, Magnification, ×25; *b–d*, ×33.

tachment to the near-confluency growth phase suggested that some of the vacuoles contained aggregates of carbohydrate/protein as shown by staining for periodic acid Schiff (PAS) reaction and hematoxylin and eosin. However, preconfluent cell cultures grown on plastic were negative for lipid oil red-O staining, and the majority (approximately 75%) of the vacuoles contained water as indicated by negative staining (data not shown). Maintaining the cells during postconfluent culture results in the formation of raised dome-like structures (Fig. 1*c*) which are often initiated from lumen-like areas and later appear sporadically throughout the monolayer. The dome-like structures or hemicysts have been associated with the accumulation of fluid under the epithelial pavement for cells grown on plastic or glass substratum (Pickett et al., 1975); however, dome structures formed in cultures with exogenous basement matrix occur by cells drawing up the flexible matrix around them, leading to the formation of lumina and rudimentary acini-like structures (Barcellos-Hoff et al., 1989). These in vitro formations reflect the induction of functional/morphological differentiation by augmented expression of lactation-specific proteins and cell polarization relative to non-dome-forming cells. Many cell lines, including many mammary epithelial cell lines, have been described as forming dome-like structures depending on the culture conditions. Cells grown on plastic substratum for which dome formation has been described include primary human mammary epithelial cells (Stampfer et al., 1980), the mouse cell line, COMMA-D (Danielson et al., 1984), the rat mammary adenocarcinoma cell line Rama 25 cultured in the presence of DMSO (Warburton et al., 1981) and the ovine NISH cell line. Bovine MAC-T cells forming dome structures on plastic substratum have not been described; however, dome-like structures have been described when cultured on collagen (Huynh et al., 1991) or when cocultured with bovine myoepithelial cells (Zavizion et al., 1992). Others have observed a strong positive anti-β casein antibody reaction in sections of mammary cell monolayers which have become detached from glass coverslips (Parry et al., 1987). Therefore, our working model of this in vitro caprine mammary epithelial system suggests that confluent CMEC cultures are induced to undergo a contact-mediated differentiation pathway which results in the secretion of basement membrane components which facilitate the formation of dome/acini-like structures and the expression of lactation-specific proteins in the absence of exogenous stromal matrix.

Mammary epithelial cells have been shown to synthesize and secrete basement matrix components when cultured on plastic substratum including laminin (Blum and Wicha, 1988; Streuli and Bissell, 1990), collagen type IV, fibronectin (Streuli and Bissell, 1990), and sulfated glycosaminoglycans (Parry et al., 1985). However, optimal expression of lactation-specific proteins requires not only basement membrane but also stromal matrix which supports differentiation by organizing the basement membrane. This facilitates the interaction between extracellular matrix and the cell's integrin receptors (Howlett and Bissell, 1990) and allows for changes in cell shape for enhanced cell-to-cell interaction as with the in vitro floating collagen gel model, which we are currently optimizing for use with CMECs. While we have described expression of lactation-specific proteins by immunofluorescence of cells cultured on plastic substratum as representative of lactogenesis, we have been unable to show that these proteins are secreted into the culture media. Experiments with cells cultured on plastic and treated with ^{35}S methionine indicated casein and α-lactalbumin are expressed in the cell fraction, consistent with the observations by immunofluorescence, but are not present in the culture supernatant (data not shown). This suggests that CMEC dome-like structures support the induction of lactation-specific protein expression, presumably by basement membrane deposition and detachment from the plastic substratum as with dome formation, but does not provide an adequate microenvironment for the secretion of these products. Further studies are needed to confirm basement membrane expression. We have observed ^{35}S methionine protein bands of approximately 400 and 200 kDa consistent with the comigrating laminin and type IV collagen products (Kleinman et al., 1986) or 220-kDa fibronectin (Warburton et al., 1981) from supernatants of confluent and postconfluent CMEC cultures by gradient SDS-PAGE. We also are able to consistently visualize matrix-like deposition on postconfluent tissue culture flasks. As such, CMEC represent a model to study the induction of protein synthesis and basement membrane deposition.

To further characterize this model system we examined the relationship between dome formation, lactation protein expression, and modulation of the cytoskeleton. Previous studies have described a relationship between the cytoskeleton and mammary gland lactation gene expression (Blum and Wicha, 1988). Milk protein gene expression was associated with the expression of laminin and dependent upon the integrity of actin and microtubule cytoskeleton. In vivo studies of the lactating murine mammary gland demonstrated that luminal cells positive for β-casein expression were negative for cytokeratin markers AE1 (k10,14,15,16,19) and AE3 (Fil8) (Sapino et al., 1990). In the human breast, cytokeratin 19 expression is characteristic of the fully differentiated luminal cell in the terminal ductal lobular unit; all luminal cells express cytokeratins 7, 8, and 18 and primary culture of milk-derived shed luminal epithelial cells retain this expression (Taylor-Papadimitriou et al., 1989). In the goat, a preliminary study suggests that cytokeratin modulation is distinct from that of the human, since the number of cytokeratin 18-positive cells is decreased in acini cells of the pregnant goat mammary gland and null expressed in acini cells of the lactating gland, "although cytokeratin 18 was found in cells, distinct from the myoepithelial phenotype, in alveoli during lactation," suggesting loss of expression is associated with the differentiation pathway (Li et al., 1996). Similarly, we have been unable to observe a positive cytokeratin reaction by immunohistochemistry of caprine lactating mammary tissue fixed in Histochoice (Amresco, Solon, OH) or buffered formalin and paraffin embedded. These data support our observations that CMECs are a valid model by which to study in vitro mammary gland development and differentiation. The characteristics of cell growth on plastic are evidence of modulation of cytokeratin expression such that cytokeratin 18 and 19 are expressed during cell growth and decreased or lost as cells form dome-like structures and express lactation-specific proteins.

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