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CALCIUM REGULATION OF NORMAL HUMAN MAMMARY EPITHELIAL CELL GROWTH IN CULTURE

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SUMMARY

The concentration of Ca^{++} in culture media profoundly affected the growth and differentiation properties of normal human mammary epithelial cells in short-term culture. In media where Ca^{++} was above 0.06 m*M*, longevity was limited to an average of three to four cell divisions. The extended growth fraction (those cells able to divide more than once) was only approximately 50% and diminished to zero quickly with time. Stationary cells inhibited from dividing appeared differentiated in the formation of lipid vacuoles and accumulation of α-lactalbumin. Growth of stationary cultures could be reinstituted in about half the cells, either by disruption and transfer or by a reduction in Ca^{++} to less than 0.08 m*M*.

The reduction of Ca^{++} to levels below 0.08 m*M* extended the longevity of normal cells to eight to nine divisions. The extended growth fraction was 100%. Under these conditions, cells did not differentiate. The effects of Ca^{++} on growth and differentiation were specific (Mg^{++} and Mn^{++} variations were without effect) and reversible and in many respects resembled Ca^{++} effects on epidermal cells. One major difference is that the dual pathways of growth and differentiation in mammary cells were controlled by glucocorticoid and insulin. Based on the kinetics of the reversible Ca^{++}-induced coupling and uncoupling of proliferation and the program of differentiation, we propose that Ca^{++} may be an essential trigger for cell divisions that commit a mammary cell to differentiate progressively in a permissive hormonal milieu.

Key words: calcium; differentiation; cell transformation; cell senescence; cell culture; mammary cells; in vitro.

INTRODUCTION

The prudent use of cell culture systems has provided valuable information on certain mechanisms of neoplastic transformation (6,9,12,27). Most of the existing transformation models have made use of mesenchymal (fibroblast) cell lines or strains that grow well in culture. However, the majority of human cancers are of epithelial origin, and there is sufficient difference in population dynamics between these two kinds of cells (13,14) to raise questions about the applicability of fibroblast models for understanding the origins of carcinomas. This uncertainty has led to an increased emphasis on studies of epithelial cell transformation in culture.

Technology to study transformation of normal epithelial cells in culture is still quite primitive. There are a number of difficulties, but two major ones are a lack of reliably specific markers for *neoplastic* transformation and a very short period of cell division (26) with concomitant differentiation (16). This latter problem abbreviates not only time of carcinogen sensitivity but also expression time for carcinogen effects. Nowhere are these difficulties more manifest than in human epithelial cell culture systems where few examples of transformation exist (19).

In a continuing effort to study the origins and lineages of human mammary carcinomas, we recently established a transformation marker that is based on an alteration in proliferative and differentiative responses of cells to glucocorticoid hormones (16). The functional basis for the marker is an uncoupling of growth from differentiative responses to glucocorticoids, which

occurs in malignant cells. In that qualitative sense the uncoupling of growth from terminal differentiation is similar to early events that occur in epidermal (12,32) and myeloid cell transformation (23).

The coupling of differentiation to proliferation as coordinated responses to glucocorticoids in normal mammary cells results in a longevity of only three or four doublings before terminal differentiation occurs (16). This short proliferative life time and the progressive differentiation that occurs in parallel are not conducive to expression of transformation; we therefore sought to uncouple these two functions of glucocorticoid action. A number of studies have demonstrated that ionic calcium can regulate the longevity of normal epithelium from several tissue sources, including epidermal (10), bronchial (15), and esophageal (28). In each case, restriction of calcium uncoupled growth from end-point differentiation. We sought to determine if calcium was also responsible for coupling growth and differentiation in normal *mammary* epithelial cells in response to glucocorticoid hormones. We found that ionic calcium influenced profoundly several parameters of normal cell growth and differentiation.

Materials and Methods

Tissue source. Breast tissue was obtained locally from subcutaneous mastectomies. All patients were premenopausal, cycling normally, with fewer than three parities. Other patient and tissue characteristics are given in (16).

Cell culture. We have described techniques for cultivating human breast epithelium in detail (16). Briefly, subcutaneous mastectomy tissue was processed for culture on the day of surgery. The tissue was first sliced to 0.5 cm diam with the aid of a Stadie-Riggs microtome to determine the presence of normally dense stroma and parenchyma (with the aid of a dissecting microscope). The slices were then minced to small fragments with scalpels. The fragments were dissociated to "organoids" and cells with the aid of collagenase and hyaluronidase, using the procedure originally recommended by Stampfer et al. (25). Insulin (10 μg/ml) and cortisol (5×10^{-6} *M*) were included routinely (16), with 10% horse serum, in digestion mixtures.

Freshly dissociated epithelium was plated in primary culture at a density of approximately 8×10^4 cells/cm^2. Cell number was calculated roughly from packed cell volumes. Twenty-five and seventy-five-square centimeter flasks (Corning Glass Works, Park Ridge, IL) were used interchangeably for primary culture. Subcultivation of cells was accomplished by means of trypsin (0.05%) and versene (0.025%) in Ca^{++}-Mg^{++}-free balanced salt solution. Growth of cells in primary culture could not be quantitated easily because cell aggregates rather than single cells were plated. Single cells could be prepared readily from primary culture, however, after aggregates had spread on the plastic surface, by the use of trypsin-versene (or 0.05% trypsin alone). Thus, studies on the effects of hormones and calcium on cell growth were conducted on secondary or derivative cultures. Cell counts were made using a hemocytometer.

Preparation of media. A number of media were used in this study, including Eagle's minimal essential medium (MEM, with both Earle's and Hanks' salts), Dulbecco's modified MEM, Ham's F12, and RPMI 1640. These media were all obtained from Grand Island Biological Supply, Grand Island, NY. Antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin) were included routinely in all media. Horse serum (5%; Grand Island Biologicals) was also used routinely for supplementation. This amount of serum maintained viability of normal cells but did not stimulate their growth (16).

Medium RPMI 1640 was used exclusively in studies in which ionic Ca concentration was varied. This medium was prepared in our laboratory completely from constituent compounds, using vitamins and amino acids purchased from Sigma Chemical Company, St. Louis, MO. A basal salt solution of the following composition was prepared: KCl (5.36 m*M*), NaCl (102.7 m*M*), $MgSO_4 \cdot 7H_2O$ (5.6 m*M*), and $NaHCO_3$ (23.8 m*M*) to which $Ca(NO_3) \cdot 4H_2O$ was added to achieve the desired Ca^{++} concentration. The pH of all solutions was adjusted to 7.2 with HCl. Nutrients and salts were combined and sterilized by filtration using 0.22 μm filters.

Divalent cations were removed from serum by Chelex (Chlex 100, sodium form, Bio-Rad Laboratories, Richmond, CA) treatment as recommended by Brennan et al. (2). Serum was sterilized by filtration using 0.45 μm filters and held at −20° C until use. Calcium levels were monitored in all solutions by flame photometry (4).

Cortisol was dissolved in benzene-free ethanol at a concentration of 1.7 mg/ml, diluted to 250 μg/ml in serum-free medium, filtered, and

TABLE 1

IONIC CALCIUM CONCENTRATION IN COMMERCIAL MEDIA

Medium	Calcium Concentration (m*M*)
Ham's F12	0.3
RPMI 1640	0.46
MEM or M199 (Hanks')	1.26
MEM or M199 (Earle's)	1.80
Dulbecco's MEM	1.80
5% Horse serum	0.17 ± 0.006
5% Chelex-treated horse serum	0.003 ± 0.001

stored at 4° C. Insulin was dissolved in 0.005 *N* HCl, diluted to 0.0003 *N* HCl with medium, filtered through a 0.45 μm filter at a concentration of 2500 μg/ml, and held at −20° C. Each solvent was tested at the highest concentration used experimentally and found to have no effect on cell growth.

Growth fraction. Autoradiographs were prepared (29) after labeling cells with [^{3}H]thymidine (6 Ci/mmol; 0.5 μCi/ml). Growth of cells in Costar microwells was under conditions identical to those described above. Cells were stained with Giemsa, and the percentage of labeled nuclei (those with > 5 grains/nucleus) were estimated by counting a minimum of 500 nuclei/well.

RESULTS

Effects of Calcium on Normal Cell Growth

Calcium contents of commercial media. Table 1 shows that ionic calcium varied between 0.3 and 1.8 m*M* in five commercial media commonly used for cultivation of mammary cells. Serum was a considerable additional source of Ca^{++}. Five percent solutions of horse serum contained 0.17 ± 0.006 m*M* Ca^{++} (Table 1). Pretreatment of serum with Chelex resin (2) reduced Ca^{++} to the level of 0.003 m*M* (in 5% solutions) (Table 1). The media shown in Table 1 were all considered "high-Ca^{++}" media. In their effects on growth rate, longevity, growth fraction, and differentiation these media in combination either with untreated horse serum or chelex-treated horse serum were indistinguishable.

Growth rate. Figure 1 *A* shows the growth rate and longevity of human mammary cells in secondary culture, in RPMI 1640, in which Ca^{++} was made 0.46 m*M*. Chelex-treated horse serum (5%) was used as serum supplement in these experiments. The 5% concentration of serum maintained cells in a hormone-responsive state but did not stimulate their growth. Cortisol and insulin acted synergistically to stimulate exponential growth of cells rapidly. The doubling time for cells in exponential growth stimulated by cortisol and insulin was 30 to 36 h. Either hormone alone added to RPMI 1640 stimulated some growth, but cortisol stimulated two to three times more total growth than insulin. Cortisol (5×10^{-6} *M*) and insulin (10 μg/ml) together were as effective in stimulating growth as high (30% or above) concentrations of horse serum [data not shown; *see* (16)]. Growth rate and pattern of response to cortisol and insulin were virtually identical for cells tested from more than 20 different mastectomies.

Figure 2 shows growth rate of normal cells in secondary culture in RPMI 1640 medium in

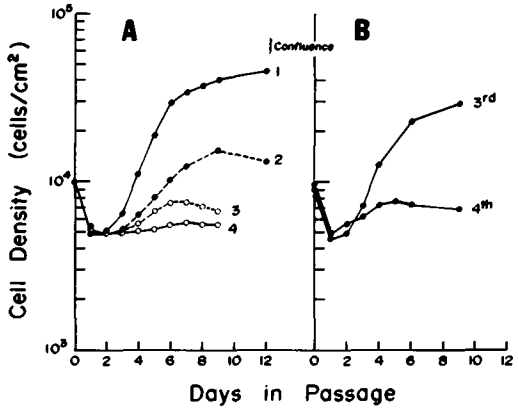

FIG. 1. Growth rate and longevity of normal cells in high Ca^{++} medium. Normal cells were plated as single cells in secondary culture in RPMI 1640, containing 0.46 m*M* Ca^{++} supplemented with 5% horse serum. Primary cultures had been grown under identical conditions in Corning 25 cm² flasks for 5 d before transfer. Cells were plated in Costar microwells at a density of 1×10^4 cells/cm². *A*, Cells were grown for 1 d and medium was then changed to include insulin (10 μg/ml) or cortisol (5×10^{-6} *M*), or both. *Curve* 1, insulin and cortisol; *curve* 2, cortisol only; *curve* 3, insulin only; *curve* 4, no hormones added (5% horse serum only). *B*, Cells grown for 12 d in secondary culture in medium containing insulin (10 μg/ml), cortisol (5×10^{-6} *M*), and horse serum (5%) were removed from Costar wells with trypsin (0.05%) and replated as 3rd passage cells. After 9 additional d of growth, cells were again trypsin-transferred (4th). In all cases, media were replenished on Days 3, 5, 7, and (where applicable) 10 after plating. Data are given as the average of duplicate samples for a given condition of time and additive, for each of three different mastectomy specimens tested separately. Maximum variation in cell counts at the time of maximum variation between experiments groups (Day 9) was ± 9%. The density at which cells would form a confluent monolayer is indicated in *A*.

which Ca^{++} was reduced from 0.46 to 0.046 m*M*, and the cell inoculum decreased from 1×10^4 cells/cm² to 2×10^3 cells/cm². The population doubling time (24 to 28 h) was 6 to 12 h faster in this reduced-Ca^{++} medium than in high (0.46 m*M*) Ca^{++} media. [The reduction in cell inoculum size from 1×10^4 cells/cm² did not contribute to the enhanced growth rate (unpublished observation of the authors)]. Insulin and cortisol were still required for growth of normal cells in this medium, and data of Fig. 2 show that the relative effects of the two hormones, including a synergistic effect when added together, were the same in 0.046 m*M* Ca^{++} as in 0.46 m*M* Ca^{++}.

Attached cells grew on the plastic surface during exponential growth in low-Ca^{++} (0.046 m*M*) media until they approached confluence. As cultures approached confluence, cells continued to divide, but they did not increase their density on the substrate. Instead, cells detached and free floated into culture supernatant fluids (Fig. 2 *A*). The free-floating cells did not divide readily in suspension ($< 10^{-3}$ frequency of mitotic figures) but did remain viable for a period of at least 2 to 3 d. The free-floating cells attached readily to fresh culture dishes; and, whereas plating efficiency of cells trypsinized from substrates was ca. 50% (Fig. 1 *A*) plating efficiency of free-floating cells was routinely greater than 75% (Fig. 2 *B*).

Longevity. Even with a rapid (30 to 36 h) doubling time, the cell population only divided three to four times in high-Ca^{++} (0.46 m*M*) medium in secondary culture before growth ceased (Fig. 1 *A*). Growth cessation was not due either to depletion of nutrients (because the addition of fresh media to staionary culture did not restimulate growth) or to the attainment of confluence (because growth stopped when cultures were only 60 to 70% confluent; Fig. 1 *A*).

Stationary secondary cultures could be subcultured readily. Upon passage, growth was renewed and the same pattern of growth occurred in tertiary as in secondary culture: exponential growth followed a 1 to 2 d lag period, which in turn was followed, after 7 to 8 d, by a slower rate until growth ceased (Fig. 1 *B*). Although we have noted exceptions (16), the longevity of normal cells was characteristically only 2 to 3 passages (Fig. 1 *B*) corresponding to a total of 8 to 10 population doublings.

The longevity of normal cells was extended from 3 to 4 to as many as 8 to 9 doublings within a single (secondary) passage in low Ca^{++} (0.046 m*M*) medium. Free-floating cells were additionally capable of dividing when transferred to tertiary culture, but the longevity of free-floating cells was dependent on the history of parent cultures. In general, the number of doublings the parent population had undergone in secondary culture was inversely related to the divisional capability of replated free-floating cells (Fig. 2 *B*). Free-floating cells taken from primary cultures that had undergone negligible multiplication before approaching monolayer density exhibited nearly the same growth potential in

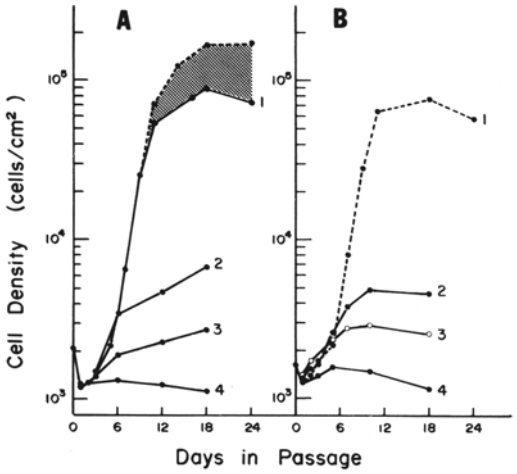

FIG. 2. Growth rate and longevity of normal cells in low Ca^{++}. Normal cells from the same mastectomy specimens described in Fig. 1 were plated as single cells in secondary culture in RPMI 1640 containing 0.046 m*M* Ca^{++}. Primary cultures had been grown in high-Ca^{++} medium including cortisol (5×10^{-6} *M*), insulin (10 μg/ml), and serum (5%) for 5 d before passage. Cells were plated in Costar microwells at 2×10^3 cells/cm². Media were supplemented with 5% horse serum only for 24 h after plating. *A*, Medium was changed and supplemented with insulin (10 μg/ml) or cortisol (5×10^{-6} *M*), or both 1 d after plating. *Curve* 1, insulin and cortisol added; *Curve* 2, cortisol only added; *Curve* 3, insulin only added; and *Curve* 4, no hormones added (5% horse serum only). Media were replenished every 3 d. In *Curves* 1-4, only attached cells were counted. The shaded area of *Curve* 1 corresponds to the full range in number of free-floating cells observed in addition to attached cells. *B*, Free-floating cells were taken from either primary (*Curve* 1) or secondary (*Curves* 2-4) cultures grown in medium 0.046 m*M* in Ca^{++} supplemented with cortisol (5×10^{-6} *M*), insulin (10 μg/ml), and serum (5%) and replated in tertiary culture in the same medium. *Curve* 2, cells taken from an 11-d secondary culture; *Curve* 3, cells taken from an 18-d culture; *Curve* 4, cells taken from a 24-d culture. Data are given as the average of duplicate samples for a given condition of time and hormone additive. Maximum variation in cell counts was ±9%.

secondary culture as did attached cells from primary cultures (Fig. 2 *B*).

Growth fraction. In high-Ca^{++} (0.46 m*M*) medium only a subpopulation of cells in secondary culture had the prolonged capability of incorporating thymidine into DNA. This result is shown in Fig. 3 where the [^{3}H]thymidine labeling index during an 8 d continuous labeling, 6 to 14 d after plating, was only approximately 50%. Also shown in Fig. 3 is the cumulative labeling index during 2-d labeling intervals. Soon after plating (2 to 4 d), labeling index was approximately 90%. During the 6 to 8-d interval after plating, only approximately 50% of cells were labeled; and during the 8 to 10-d interval, only about 25% of cells were labeled. Thus, a minimum of 90% of cells were initially capable of DNA synthesis, but this capability was not maintained for longer than approximately one generation time.

The increased longevity of cells in reduced Ca^{++} in secondary culture correlated with an increase in extended growth fraction. When grown in 0.046 m*M* Ca^{++}, nearly 100% of the cells incorporated thymidine into DNA during a continuous 8-d label between 6 and 14 d of culture and during each of three 2-d labeling intervals during the life time of the culture (Fig. 3).

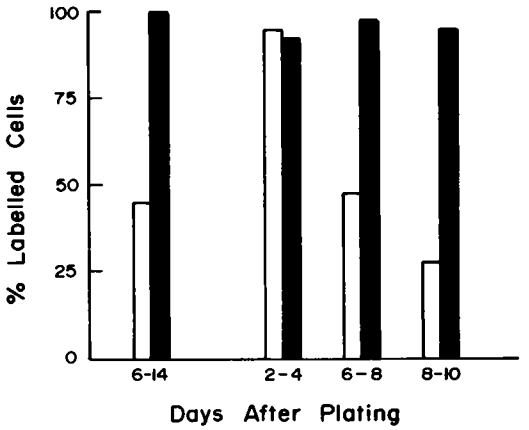

FIG. 3. Growth fraction of cells in 0.46 and 0.046 m*M* Ca^{++}. The growth fraction was estimated by determining the labeling index (percent of labeled cells) in autoradiographs after labeling with [^{3}H]thymidine (6 Ci/mmol; 0.5 μCi/ml) for the intervals indicated. Cells were in secondary culture and plated and grown in cortisol/insulin/serum-containing media as described in the legends to Figs. 1 and 2, except that the plating densities were 1×10^4 cells/cm^2 in both cases. Cells were fed with fresh medium every 2 d. Cells in 0.46 m*M* Ca^{++} (*open bars*); cells in 0.046 m*M* Ca^{++} (*solid bars*). Labeling percents represent the average from triplicate samples. There was a maximum of 8% variation between triplicates.

TABLE 2

REVERSAL OF GROWTH INHIBITION BY REDUCTION IN Ca^{++}[a]

Condition	Labeling Index (%)
0.46 m*M* Ca^{++}, 18 d culture + 48 h — 0.46 m*M* Ca^{++}	0.2 ± 0.008
0.46 m*M* Ca^{++}, 18 d culture + 48 h — 0.046 m*M* Ca^{++}	55 ± 9

[a] Cells were cultured and labeling indexes measured as described in the legend to Fig. 3. After 18 d in culture, no growth (labeled cells) was evident; addition of fresh media for 48 h (containing either 0.46 m*M* or 0.046 m*M* Ca^{++}) had the effect shown. Variation between triplicate samples is also shown.

Inhibition of growth that occurred in high-Ca^{++} (0.46 m*M*) medium could be reversed by switching to low-Ca^{++} (0.046 m*M*) medium. By 48 h after the switch, the labeling index had increased from 0.2% to approximately 55% (Table 2). The maximum increase in labeling index observed in switching stationary cells to low-Ca^{++} conditions has been from 0 to 62% (data not shown).

Differentiation and senescence. Results of other studies showed that growth of normal cells in response to cortisol and insulin in high-Ca^{++} media occurred in small colonies of cells called "growth units" (16). Borders circumscribing individual cells were much less distinct than the borders circumscribing colonies, giving the colony rather than the cell a "unit" appearance. A typical growth unit is shown in Fig. 4 *A*, 5 d after plating. Growth units developed on the surface of the culture vessel within 1 to 3 d after plating (corresponding to the lag phase of growth in Fig. 1 *A*) from the migration together of plated single cells (16).

The progressive decline in cell growth that occurred in growth units was accompanied by accumulation of lipid and small amounts of α-lactalbumin (αLA) (16). Lipid-containing droplets were detected by staining cells with Oil Red O. These droplets increased in size with time, progressing from single droplets to coalescent droplets or vacuoles (Fig. 4 *B*, *C*). The fraction of cells with lipid vacuoles also increased progressively with time to a maximum of approximately 50% (Fig. 5). Lipid vacuoles were not routinely detected until 8 to 9 d in culture (after the exponential growth phase); and the maximum number of vacuolated cells was attained by 14 to 15 d in culture. The accumulation of αLA to

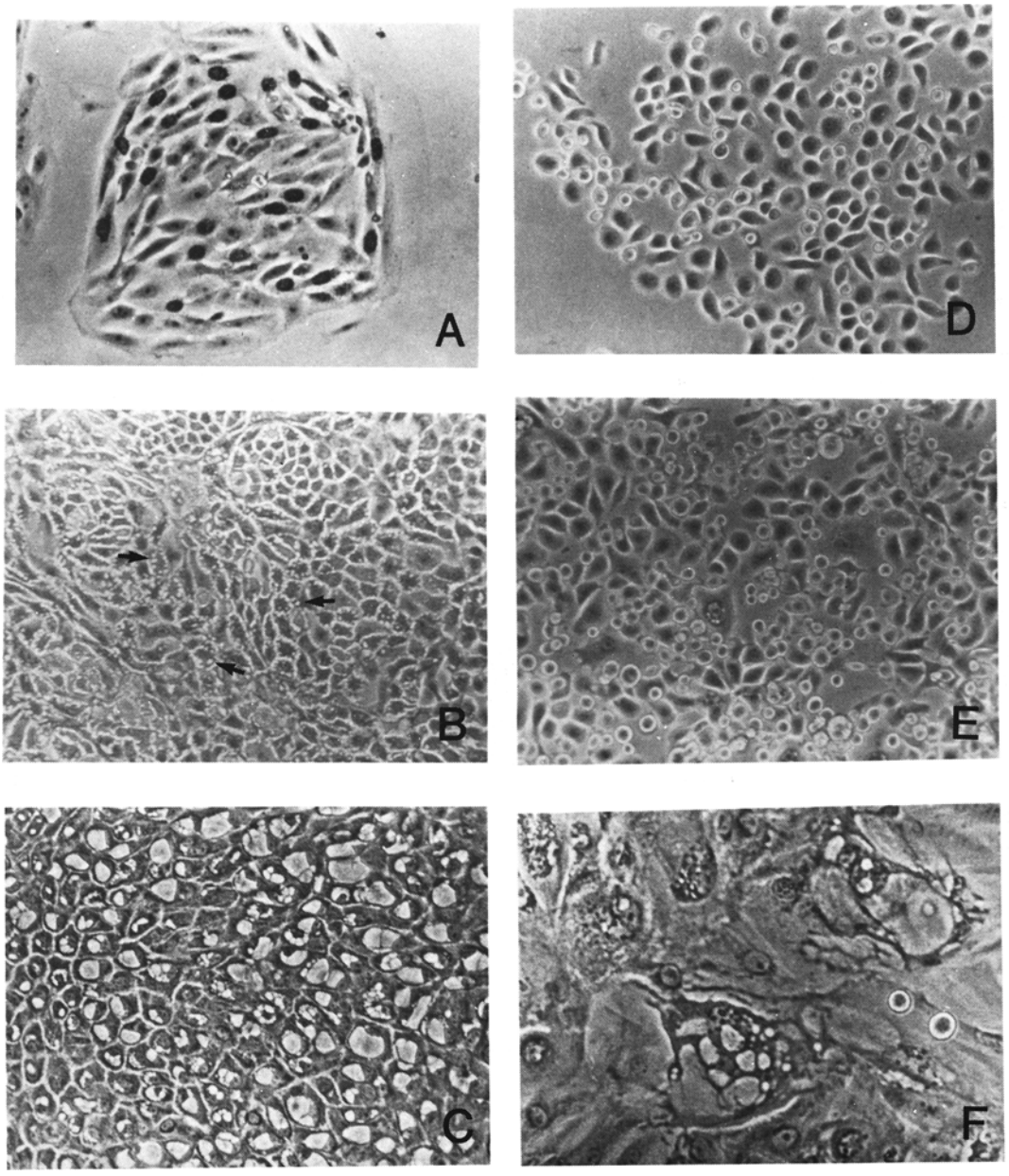

FIG. 4. Cell morphology in 0.46 and 0.046 m*M* Ca^{++} containing medium. *A-C*, Cells grown in 0.46 m*M* Ca^{++}; *D-F*, cells grown in 0.046 m*M* Ca^{++}. *A*, A grown unit in a 3-d secondary culture of [^{3}H]thymidine labeled cells. Note, relative to *D*, the well-circumscribed and cohesive nature of the colony. DNA synthetic cells (dark nuclei from exposed silver grains) are also shown. Toluidine blue stained autoradiography. ×300. *B*, An area of a growth unit containing cells with simple and coalesced lipid droplets (*arrows*). The growth fraction in this 8-d culture would be between 50 and 25%; unstained. ×220. *C*, An area of a growth unit with heavily vacuolated cells. These cells, 12 d in culture, are not DNA synthetic; the entire growth unit is stationary. Compare these cells with stationary (senescent) cells in *F*. Toluidine blue stained autoradiography. ×220. *D*, A colony of cells in a 3-d secondary culture in 0.046 m*M* Ca^{++}. Note, relative to *A*, the unit appearance of the cell rather than the colony. ×300. *E*, A semiconfluent culture of cells grown 12 d in 0.046 m*M* Ca^{++}. Note the free-floating in addition to the attached cells, and the absence of cells with lipid droplets. The growth fraction of attached cells would be > 80%. ×300. *F*, Senescent cells in a stationary 22-d culture. Note the large irregular shaped cells with fibrillar or foamy cytoplasm. The vacuoles in these cells do not stain with Oil Red O. ×300.

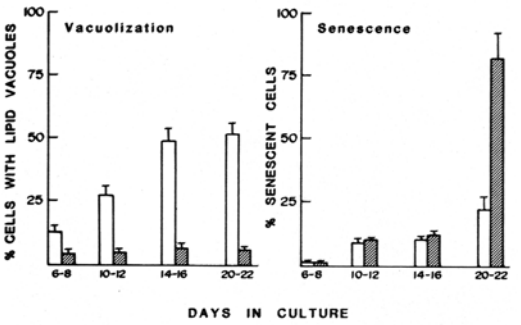

FIG. 5. Differentiation and senescence of cells in 0.46 and 0.046 mM Ca^{++}-containing medium. Cells were plated in secondary culture as described in legends to Figs. 1 and 2. Regularly shaped cells with coalesced vacuoles that stained with the lipid-specific dye Oil Red O (Fig. 4 *B* and *C*) were counted as lipid-vacuolated cells. Senescent cells were large, irregular shaped cells with either fibrillar or foamy cytoplasm. *Open bars*, 0.46 mM Ca^{++}; *cross-hatched bars*, 0.046 mM Ca^{++}. Standard errors are shown for replicates of two different mastectomy specimens.

levels sufficient for detection by immunofluorescence techniques coincided in time with maximum accumulation of lipid. At 8 and 10 d in culture (Fig. 4) no αLA was detected; at Days 15 and 22, 11 and 18% of cells, respectively, reacted with anti-αLA serum.

Figure 4 illustrates morphologic differences between cells in 0.046 and 0.46 mM Ca^{++}. In low Ca^{++} individual cells were less spread and more polygonal than in high Ca^{++}. There were distinct intercellular spaces giving cell sheets a paving stone appearance (Fig. 4 *D*). Although cells still grew as colonies (Fig. 4 *D*), the cellular borders were more distinct than the borders of colonies, giving the cell rather than the colony a "unit" appearance.

Although a fraction of cells in low-Ca^{++} medium demonstrated the formation of lipid droplets, that fraction was considerably smaller than in high-Ca^{++} medium, and progressive coalescence of droplets to vacuoles was not observed (Fig. 5). α-Lactalbumin was not detected in cells in low-Ca^{++} medium at any interval shown in Fig. 5. After growth ceased in low- (0.0046 mM) Ca^{++} medium, cells became abnormally large with an extensive fibrillar or foamy cytoplasmic mass (Fig. 4 *F*). These "foamy" cells never stained with anti-αLA antibodies. They were confined almost exclusively to the periphery of growth units in high-Ca^{++} conditions of growth, but in low-Ca^{++} conditions were distributed throughout the surface culture. Because of morphologic qualities classically attributed to aging in culture, and the lack of αLA production, we tentatively consider the large, vacuolated foamy cells, cells that predominated in stationary low-Ca^{++} cultures, to be senescent and not differentiated.

Titrations of calcium effects on growth. Figure 6 shows the results of an experiment in which Ca^{++} added to culture medium was titrated for its effect on growth, formation of growth units, and formation of lipid-containing vacuoles. The optimum Ca^{++} concentration for growth rate and longevity (total number of population doublings) in 1 passage was between 0.02 and 0.06 mM). Below 0.02 mM, cells attached too tenuously and free floated too readily to achieve any extended growth.

The optimum Ca^{++} concentration for growth unit and lipid vacuole formation was a broad range between 0.24 and 20 mM. Figure 6 also shows in a quantitative way the concordance between growth unit formation and lipid vacuole

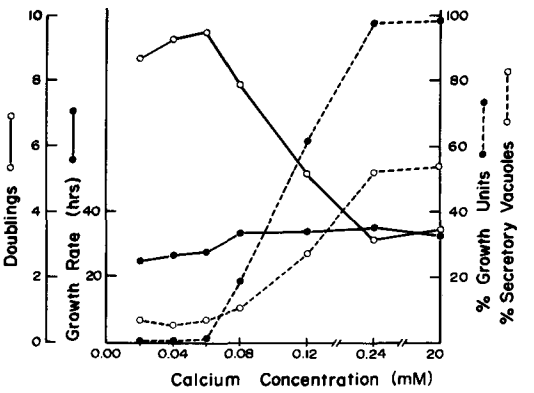

FIG. 6. Titration of biological effects of Ca^{++}. Normal cells from one mastectomy specimen were plated in secondary culture in RPMI 1640 0.046 mM in Ca^{++} and containing cortisol (5×10^{-6} M), insulin (10 μg/ml), and horse serum (5%). After 1 d (to normalize plating efficiency) media were switched to Ca^{++} concentration shown on the abscissa. Ca^{++} at 20 mM corresponds to 50% horse serum-containing RPMI 1640. The number of cell doublings (O—O) corresponds to the total increment (including free-floating cells) in cell number in one (secondary) passage. Growth rate (h) (●—●) was determined as the slope of the growth curve during maximum cell incrementation. Percent growth units (●---●) is the percent of total cell colonies appearing as units rather than islands (compare Fig. 4 *A* with *D*), counted 5 d after plating. Percent vacuolated cells (O---O) corresponds to the fraction of regularly shaped cells determined after cultures became stationary, which contained coalescent lipid-containing droplets.

formation and the inverse relationship between those two Ca^{++}-sensitive functions and population longevity.

Inhibition of growth by an increase in Ca^{++}. After growth for 5 d (corresponding to approximately 1.5 population doublings) (Fig. 2 *A*) in 0.046 m*M* Ca^{++}, elevating the Ca^{++} concentration to 0.42 m*M* Ca^{++} induced a rapid change in cell appearance. Distinct spaces between cells became much less apparent within 4 to 6 h. As individual cell borders became less distinct, cell colonies took on the appearance of growth units. Within 24 to 36 h, all cells were organized as growth units and growth began to slow until after a total of 3 to 4 doublings, when growth ceased (Fig. 7). Large lipid-containing vacuoles formed as growth ceased. If, instead of 0.046 m*M* Ca^{++}, 0.046 m*M* was added back, cells continued to proliferate for 8 to 9 doublings and no growth units or lipid vacuoles formed (Fig. 7).

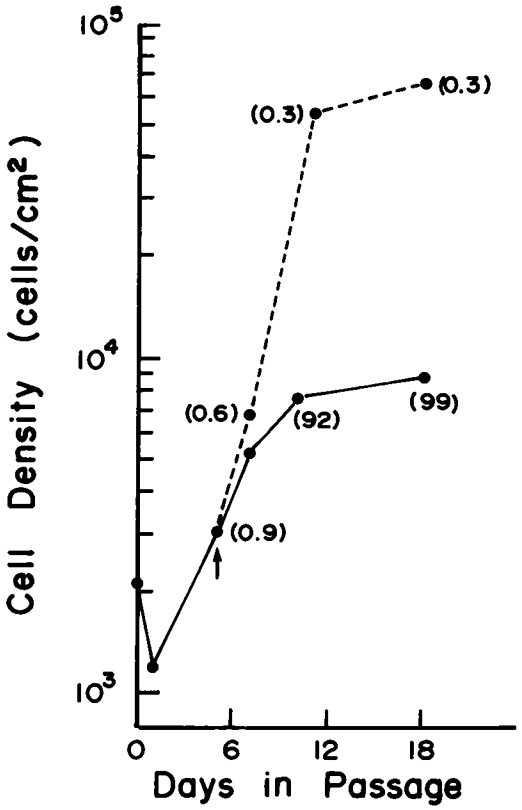

FIG. 7. Reversibility of low Ca^{++} effects on cell growth. Cells were plated in secondary culture as described in Fig. 2. After 5 d growth in 0.046 m*M* Ca^{++} (RPMI 1640), Ca^{++} was increased to 0.46 m*M* in half the cultures. ●---●, Growth in low-Ca^{++} (0.046 m*M*) medium; ●—●, growth in high-Ca^{++} (0.046 m*M*) medium. Numbers in parentheses represent the percentage growth units in cultures at each time point cells were counted.

DISCUSSION

The Ca^{++} concentration in commercial media commonly used for culture of mammary cells was not conducive to extended growth. The Ca^{++} concentration in minimal essential medium (Earle's salts), for example, was 20 to 30 times higher than the maximum allowable for extended growth. The use of horse serum at or about 50% levels (30) created an excess of Ca^{++} nearly 40 times the maximum allowable for extended growth. In view of the inverse relationship between differentiation and transformability of cells (17,22), the difficulty in transforming mammary cells in vitro (8,21) may be related to excessive Ca^{++} contents of media used for growing cells.

Other limitations to the extended cultivation of mammary cells can be overcome by low Ca^{++} growth. Low Ca^{++} decreases the cell density needed to initiate growth. How that is achieved is purely speculative but it should facilitate cloning of normal mammary epithelium. The fact that cells free float in reduced Ca^{++} when the population approaches monolayer density and the free-floating cells exhibit equivalent growth and even better plating efficiencies than trypsin-dissociated cells suggests a way, using reduced Ca^{++}, to end the difficulties associated with transfer of enzymatically treated epithelial cells (31).

Medina and Oborn (18) reported that the optimum Ca^{++} concentration for growth of mouse mammary cells was approximately 0.8 m*M*. This concentration is in vast excess of the Ca^{++} optimum we observed for extended growth of human cells. The reason for the discrepancy is not clear; it may be due to a difference in Ca^{++} tolerance between mouse and human cells or to differences in the way the Ca^{++} effect was measured. In the Medina-Oborn study, only short-term growth *rate* was measured; in our study short-term rate was the least responsive parameter of growth to fluctuations in Ca^{++}. Indeed, in view of the 40 to 50% difference in extended growth fraction we observed under low- and high-Ca^{++} conditions, it is not clear whether the 15 to 20% maximum difference we observed in growth rate in low and high Ca^{++} was not completely due to the more substantial effect of Ca^{++} on growth fraction.

Other investigators (3,25,26) have reported extended growth of human mammary cells in high-Ca^{++} media with various other supplements. Such growth was accomplished by subcultivation or trypsin lavage, however, treatments that disrupt the Ca^{++}-sensitive inhibition signals demonstrated in our studies (16). Thus, growth in those cases was probably still coupled to differentiating effects of hormones in culture media.

The effect of Ca^{++} on growth of mammary cells was similar in several respects to its reported effects on epidermal cells (10). The optimum Ca^{++} concentration for extended growth was essentially identical for mammary and epidermal cells and the low-Ca^{++} effects were reversible in both systems. Serving to illustrate fundamental differences in growth control transformation of mesenchymal and epithelial cells, both normal mammary and epidermal cells grow extensively in Ca^{++} concentrations that support growth of only malignant fibroblasts (1); and they differentiate under maximum growth conditions for fibroblasts (1).

One difference in the action of Ca^{++} in the mammary and epidermal systems is especially noteworthy. We were able to identify a major hormone pathway (glucocorticoid) through which Ca^{++} acted in growth and differentiation of mammary cells. We also have obtained preliminary data (unpublished) from experiments with cholera toxin and cyclic nucleotides that Ca^{++} intersects the glucocorticoid pathway via a direct effect of the cation on cyclic nucleotide metabolism. Thus, the preliminary data suggest a direct, calmodulin-mediated effect of Ca^{++} in glucocorticoid-induced growth. The cyclic nucleotide pathway does not seem to be involved in the Ca^{++} effects on epidermal cells (S. Yuspa and H. Hennings, personal communication). The idea that Ca^{++} is acting directly on cells through a specific pathway is consistent with the observation that neither Mg^{++} nor Mn^{++} replaced or competed with Ca^{++} in its effects on mammary cells (data not shown). We must emphasize, however, that Ca^{++} probably acts through multiple direct pathways, and indirect effects of the cation have not been ruled out.

Proliferation in normal mammary cells in vitro is regulated at two key control points. The first control is one that requires glucocorticoid and insulin to produce more cells that can then differentiate. The second control is the arrest of cell multiplication. The first control point has wide representation among cell types and is certainly not specific for mammary epithelium (5,7). Whether these hormones stimulate DNA synthesis and subsequent cell division directly or indirectly (11,24) has not been determined. However, reducing Ca^{++} to levels that maximally extended cell longevity did not mitigate the requirement for insulin and cortisol, suggesting that Ca^{++} was not acting to regulate growth at this "positive" control point.

Ca^{++} exerts its effect at the second, "negative" point of growth control, the arrest of proliferation. This arrest seems to occur, as it does in epidermal cells (10,32), within the cell-specific program of differentiation. The accumulation of lipids into droplets and vacuoles has been used extensively as a marker of secretory differentiation for mammary cells in vitro (22). Cells with lipid vacuoles are clearly distinguishable from foamy, fibrillar, "senescent" cells, not only morphologically but by parallel accumulation of αLA (16). However, α-LA accumulation is less sensitive than lipid accumulation, which leaves, in high Ca^{++}, many cells with only a lipid marker that is not a sufficiently specific marker for mammary differentiation.

In attempting to determine how Ca^{++} regulates the program of mammary cell growth and differentiation, two observations seem especially noteworthy: First, the reduction of extracellular Ca^{++} to levels below 0.08 mM, did uncouple proliferation from the differentiation program, but it did not increase significantly the final number of doublings the population underwent. The population simply expressed its full proliferative potential without the need of trypsin lavage or subcultivation. Second, the inhibition of growth unit formation and the uncoupling of the differentiation program from proliferation titrated extracellular Ca^{++} identically.

This latter observation suggests that the formation of growth units is involved in the program of differentiation and that increased longevity in low Ca^{++} is related to the inability of cells to form growth units. Previously (16) we have shown that within growth units there is a complex balance between an irreversible termination of proliferation (marked by inability of certain nondividing cells to reattach and grow when transferred) and reversible inhibition of proliferation (marked by the ability of other nondividing cells to reattach and grow when transferred). The reversible inhibition conserves a fraction of cells for entry into the differentiation program, which occurs after disruption of the growth unit and subcultivation of the cells (16). Ca^{++} seems to affect both the

reversible (inhibition) and irreversible (termination) arms of this glucocorticoid-determined program of differentiation; cells were neither inhibited nor differentiated in low Ca^{++} media and at the end of 8 to 9 doublings cells were uniformly senescent. It is tempting to speculate that these pleiomorphic effects of Ca^{++} on cell behavior all have their origin in its effect on the integrity of intercellular junctional complexes (20), especially in as much as growth units are largely defined morphologically in terms of intercellular borders and the establishment of junctional complexes precedes the reversal of the low Ca^{++} effects on longevity and differentiation of cells.

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