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Cell Biology

Sustained growth in primary culture of normal mammary epithelial cells embedded in collagen gels

(proliferation/growth factors/cholera toxin/passage)

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ABSTRACT **Normal mammary epithelial cells from BALB/cfC3H midpregnant mice were freed from stromal cell types by Percoll density gradient centrifugation after collagenase digestion and were then embedded within collagen gels. Sustained growth leading to an increase in cell number was accomplished in response to cholera toxin and high concentrations of horse serum. The extent of growth was found to be dependent on the horse serum concentration, the maximum growth being attained at 50%. A serum concentration of 12.5% horse serum and 2.5% fetal calf serum, along with cholera toxin at 0.01 μg/ml, allowed maintenance but failed to cause any significant increase in cell number during the experimental period of 2 weeks. This same maintenance medium was used to determine the effects of various exogenously added steroids, protein hormones, and organ extracts on the proliferation of mammary epithelial cells in culture. Hormones failed to elicit any proliferative response, but extracts of kidney, brain, uterus, and spleen produced proliferative responses equal to or greater than the response obtained with 50% horse serum and cholera toxin. Kidney extracts prepared from midpregnant mice, virgin mice, and virgin mice given pituitary isografts all showed comparable activities, suggesting that the concentration of stimulatory factor(s) was not influenced by the hormonal status of the donor. Normal mammary epithelial cells that had undergone a 10- to 15-fold increase in cell number over initial values during 2–3 weeks in culture were passaged to secondary gel cultures. Outgrowths similar to those seen in primary culture were seen again in secondary culture. The present system provides a method for sustaining growth in culture of primary mammary epithelial cells from normal tissues.**

Classical endocrinology involving organ ablation and hormone therapy has developed the concept that pituitary, ovarian, and adrenocortical hormones are involved in mammary gland development (1, 2). Detailed analysis, however, requires an *in vitro* system in which the direct mitogenic effect of hormones can be assessed on appropriate target cells for prolonged periods. Early studies using organ and fragment cultures have shown insulin and growth factors to be the major factors responsible for mammary cell proliferation, but not the *in vivo* mammogenic hormones, an observation presenting a paradox with the *in vivo* situation (1, 2). Even insulin and the growth factors have been shown to cause only one or two rounds of DNA synthesis *in vitro* (3–6) and never a sustained growth.

Recent studies indicate that, while estrogens have a direct mitogenic effect on a human mammary tumor cell line (MCF-7) (7) as assessed by an increase in cell number, estrogen mitogenicity cannot be demonstrated on the MTW9/PL mammary cell line (8). It has been proposed for the latter case that estrogen acts indirectly on the target cells through the production of intermediary growth factors that in turn promote growth of the mammary cell line (18). An inherent limitation of the use of established cell lines to elucidate the role of hormones is that the results may not be indicative of the hormones' physiological role *in vivo*. A better system seems to be primary culture, which is more closely related to the situation *in vivo*. Many investigators have contributed in the development of primary monolayer cultures of mammary cells (6, 9–23). However, the major difficulty with primary mammary culture has been an inability to sustain continuous growth with a multifold increase in cell number characteristic of mammary cells *in vivo* during certain physiological and pathological states. Therefore, the demonstration of mammary cell proliferation in primary monolayer culture has been limited primarily to [^{3}H]thymidine incorporation (9–15). Recent cell culture studies (6, 16–20) have used cell counting to quantitate the effects of hormones and growth factors on the growth of mammary cells in primary monolayer culture. These studies have shown growth-promoting effects of various hormones and growth factors, but the cell number increase has been rather limited.

We have recently developed a system of embedding cells inside a collagen gel and have shown sustained growth of mammary tumor epithelial cells, leading to a multifold increase in cell number, in primary culture (24). The present study describes the use of this culture system to promote sustained growth of normal mammary epithelial cells in primary culture. In addition, preliminary results on growth regulation of normal mammary epithelial cells in culture are presented in an attempt to delineate the role of hormones in mammary cell proliferation.

MATERIALS AND METHODS

Cell Dissociation. Normal mammary glands from midpregnant (8–12 days) BALB/cfC3H mice were dissociated according to the procedure described (25), with the following modifications. Finely minced mammary tissues, containing both parenchymal (ducts, alveoli, and myoepithelia) and stromal elements, were placed in 250-ml erlenmeyer flasks containing 0.1% collagenase (Worthington) in Hanks' balanced salt solution (HBSS) (10 ml per g of tissue) and swirled on a gyratory water bath shaker at 120–150 rpm at 37°C for approximately 90 min or until the suspensions were uniform without macroscopic lumps. The suspension was passed through Nitex cloth (mesh size, 150 μm), and the cells were collected by centrifugation at 80 × *g* for 5 min. The resulting preparation consisting of large clumps of cells was further dissociated in 0.5% Pronase (Calbiochem) in HBSS until the preparation consisted mainly of small clumps and single cells. This was usually accomplished by gently swirling the flask on a shaker for approximately 30 min and, if necessary, pipetting the suspension a few times. Cells were collected by centrifugation after addition (about 1/10 vol) of fetal calf serum directly into the suspension. Over 90% of the cells were found to be viable, as determined by the trypan blue

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Abbreviations: HBSS, Hanks' balanced salt solution; EGF, epidermal growth factor.

exclusion test. Cell number was estimated by mixing 1 vol of cell suspension with 9 vol of 0.02% crystal violet in 0.1 M citric acid and counting stained nuclei in a hemocytometer.

Cell Separation. Collagenase/Pronase-dispersed cells were separated through density gradients of Percoll (Pharmacia) as described (26). Approximately 3×10^7 cells were mixed with 30 ml of 42% Percoll, (12. 6 ml of Percoll, 1.4 ml of 10× Waymouth medium, and 16 ml of saline A) and centrifuged at $10,000 \times g$ for 1 hr. Epithelial cells, which band preferentially at a higher density (1.07–1.08 g/ml) compared to stromal cell types (fibroblast and others), were collected, pelleted, and counted. In some cases, cells were layered on top of preformed gradients generated by prior centrifugation at $10,000 \times g$ for 30 min and centrifuged at $800 \times g$ for 15 min.

Culture Procedure. Collagen solution and gel were prepared as described (27). Briefly, 1 g of rat tail collagen fibers was sterilized in alcohol overnight and dissolved in 300 ml of 1:1000 acetic acid in sterile distilled water; the supernatant after centrifugation at $10,000 \times g$ for 30 min was the stock collagen solution. Eight volumes of stock solution was mixed with 2 vol of a 2:1 mixture of 10× Waymouth medium and 0.34 M NaOH and kept on ice to prevent immediate gelation. An appropriate number of Percoll-separated cells was added to cold gelation mixture: 0.5 ml, containing $5–10 \times 10^4$ cells, was overlaid on 0.3 ml of gelled collagen in each well of Falcon multiwell plates and allowed to gel at room temperature. After this layer had gelled, cultures were fed with basal medium—0.5 ml of F12 medium (GIBCO) containing 12.5% horse serum (Sterile Systems, Logan, UT, and Flow Laboratories, McLean, VA), 2.5% fetal calf serum (Sterile Systems), and, per ml, 100 units of penicillin, 0.01 μg of cholera toxin, 100 μg of streptomycin, and 2.5 μg of amphotericin B. The cultures were fed with basal medium every 2 days.

Cell Number Estimates. At the end of the experiment, each gel was transferred into a 10 × 75 mm test tube and 0.1 ml of 1% collagenase made up in HBSS was added. Cells inside the gel, after digestion on a gyratory water bath shaker for 30–60 min at 37°C, were recovered by centrifugation at $100 \times g$ for 5 min. Estimates of initial cell number were determined by digesting several gels with collagenase 24 hr after embedment. Recovered cell samples were stored frozen until the time of DNA assay. The DNA content was determined by a fluorometric assay (28) utilizing BALB/cfC3H mammary tumor epithelial cells, counted in a hemocytometer, as a standard. Standard curves using these tumor cells and normal mammary cells from midpregnant BALB/cfC3H mice were virtually identical. Therefore, tumor cells were routinely used because of the ease of obtaining these cells.

Extract Preparation. Tissues and organs were excised from mice and homogenized with a Polytron apparatus (Brinkmann) in 3 vol of distilled water per g of tissue. The homogenates were centrifuged at $10,000 \times g$ for 30 min and the supernatant was placed in dialysis tubing with a molecular cutoff of 3500 daltons. Urine was placed directly in dialysis tubing. After dialysis against water for 2–3 days at 4°C, the volume was concentrated by absorption with polyethylene glycol 6000 (J.T. Baker Chemical, Phillipsburg, NJ). The concentrated extract was centrifuged at $105,000 \times g$ for 1 hr and the supernatant was sterilized by Millipore filtration. The protein content was determined by the Bio-Rad protein assay.

RESULTS

Serum Dependence of Growth. Normal mammary epithelial cells produce duct-like outgrowths extending into the collagen gel matrix within a few days after cultivation in the presence of 0.01 μg of cholera toxin per ml and 50% horse serum. Cholera toxin was included in most cultures because of its ability to inhibit the growth of fibroblastic cells (29) while also stimulating epithelial cell proliferation (30). Growth was apparent by light microscopy and was confirmed by measuring the total DNA content of the outgrowth. The extent of growth was dependent on the concentration of horse serum, as shown in Fig. 1. A higher serum concentration was found to affect the size as well as the number of outgrowths distributed throughout the collagen gels.

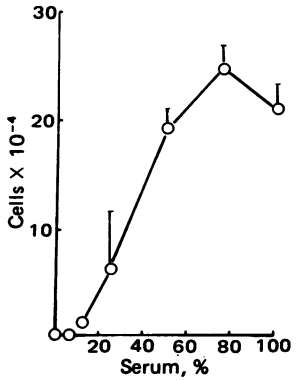

FIG. 1. Horse serum dose response. Normal mammary epithelial cells (5×10^4 cells) were cultured for 13 days in Ham's F12 medium containing 0.01 μg of cholera toxin per ml with various horse serum concentrations. Bars indicate positive standard deviation.

Morphology and Functional Capability of Mammary Cells That Have Undergone Proliferation *in Vitro*. In 2–3 weeks, primary mammary epithelial cells, having undergone proliferation to approximately 10–15 times the initial cell number, were recovered by collagenase digestion, dissociated, and plated at high density ($5–10 \times 10^5$ cells per cm^2) in conventional monolayer culture. The resulting continuous sheets of polygonal cells were indistinguishable from primary monolayer cultures of mouse mammary epithelial cells. Dome formation (31) was also noted when cortisol at 5 μg/ml was added to the basal medium. In a preliminary experiment, mammary epithelial cells from C57BL/Crgl mice and derived from collagen gel outgrowths similar to those obtained here from BALB/cfC3H mice were transplanted into parenchyma-free fat pads of syngeneic mice. These transplanted cells gave rise to mammary ductal structures with normal morphology.

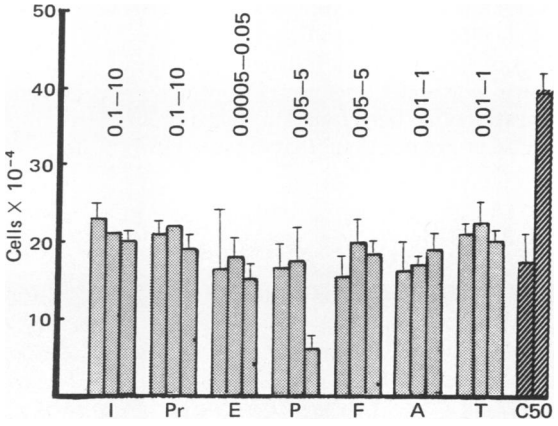

FIG. 2. Dose response of various protein and steroid hormones added singly. Normal mammary epithelial cells (10^5 cells) were cultured for 8 days in the presence of various additives used to supplement Ham's F12 medium containing 12.5% horse serum, 2.5% fetal calf serum, and 0.01 μg of cholera toxin per ml (the control): I, insulin; Pr, prolactin; E, estradiol, P, progesterone; F, cortisol; A, *d*-aldosterone; T, testosterone; C, control; 50, 50% horse serum. Concentration ranges, lowest for the bars on the left to highest for bars on the right, are in μg/ml as shown, and the increment is by a factor of 10. Error bars indicate positive standard deviation.

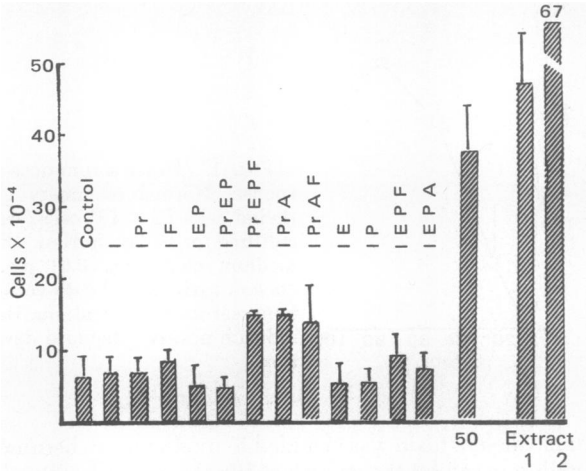

FIG. 3. Effect of various protein and steroid hormone combinations. Normal mammary epithelial cells (5×10^4 cells) were cultured for 13 days in the presence of various additives used to supplement Ham's F12 medium containing 12.5% horse serum, 2.5% fetal calf serum, and 0.01 μg of cholera toxin per ml. Abbreviations are the same as in Fig. 2. Concentrations of various hormones were as follows: insulin, 10 μg/ml; prolactin, 5 μg/ml; cortisol, 0.5 μg/ml; estradiol, 0.005 μg/ml; progesterone, 0.5 μg/ml; aldosterone, 1 μg/ml. Extract 1 was prepared from brains of female BALB/c mice (3–5 months). Extract 2 was prepared from kidneys of female BALB/c mice having pituitary isografts for 1–2 months.

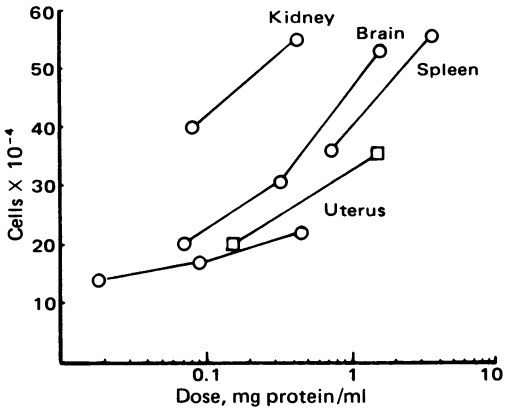

FIG. 5. Effect of various tissue extracts. Results are from two experiments (○, □) each using normal mammary epithelial cells (10^5 cells) and cultured for 12 days in Ham's F12 medium containing 12.5% horse serum, 2.5% fetal calf serum, cholera toxin at 0.01 μg/ml, and various concentrations of extracts from different organs as shown. Organs were from 3- to 5-month virgin BALB/cfC3H mice.

Effect of Steroids and Protein Hormones. A concentration of 12.5% horse serum and 2.5% fetal calf serum, with cholera toxin at 0.01 μg/ml, in the medium maintained cell number but failed to cause any significant increase compared to the initial value within a period of 2 weeks. This basal medium was then used in subsequent studies as a "maintenance" medium to determine the proliferative potential of steroid and protein hormones and to compare their effects with the effect obtained with the optimal combination of 50% horse serum and cholera toxin. Protein hormones (insulin and prolactin) and steroids (estradiol, progesterone, cortisol, *d*-aldosterone, and testosterone), singly at various dosages, showed little, if any, proliferative effect on mammary epithelial cells, as shown in Fig. 2. Similarly, various combinations of these steroids and protein hormones also failed to show any proliferative effect on mammary epithelial cells, as shown in Fig. 3.

Effect of Crude Organ Extracts. In contrast to the effects of classical hormones, inclusion of optimal concentrations of various extracts has been found to elicit a proliferative response as great as, or greater than, that obtained by 50% horse serum and cholera toxin. Fig. 3 shows that under conditions in which various combinations of steroid and protein hormones elicited virtually no response, crude extracts prepared from brain and kidney stimulated growth over that achieved with 50% horse serum. Growth-stimulatory activity was found to be present in a number of different organ extracts, as shown in Figs. 4 and 5. We have also observed a stimulatory effect of urine extracts on mammary cell proliferation, but the response was found to be lower than that achieved with organ extracts. Because kidney was found to be a rich source for stimulatory activity, further analyses were performed on kidney extract. Extract alone elicited proliferative response, and the inclusion of cholera toxin resulted in synergism. This synergism between kidney extract and cholera toxin is shown in Fig. 6. Kidney extracts were then prepared from mice of different physiological states. As shown in Fig. 7, the level of stimulatory activity did not seem to be affected by the hormonal status of the donor. In addition, kidney extracts prepared in dialysis tubings with two different molecular weight cutoffs (3500 and 13,000) revealed about the same activities.

Passage to Secondary Culture. Normal mammary epithelial cells that had undergone a 10- to 15-fold increase in cell number over the initial value during 2–3 weeks in culture have been passaged (1-to-8 split) to secondary culture. This was accomplished by recovering the outgrowths from the gels by collagenase digestion and further dissociating them into smaller clumps by Pronase as is done in primary dissociation. These cells

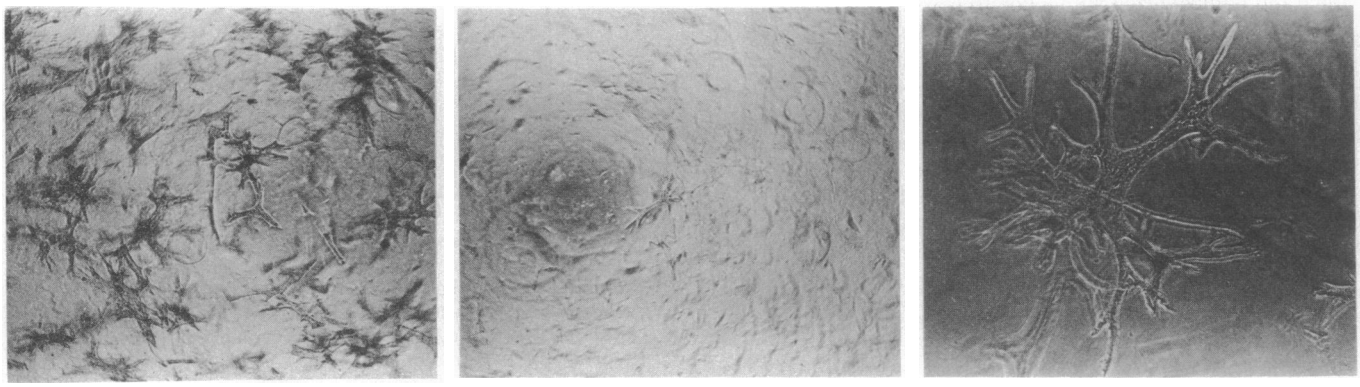

FIG. 4. Phase-contrast photomicrographs of normal mammary epithelial cells (10^5 cells) cultured for 6 days in Ham's F12 medium containing 12.5% horse serum, 2.5% fetal calf serum, and 0.01 μg of cholera toxin per ml in the presence (*Left*) and absence (*Center*) of kidney extract. (×13.) (*Right*) One of these outgrowths at higher magnification. (×52.)

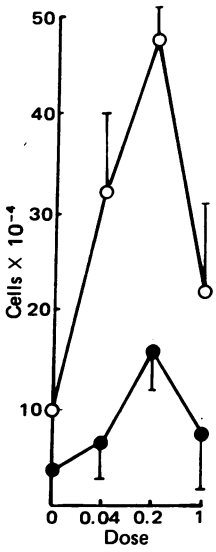

FIG. 6. Kidney extract dose response in the presence (○) and absence (●) of cholera toxin at 0.01 μg/ml. Normal mammary epithelial cells (10^5 cells) were cultured for 12 days in Ham's F12 medium containing 12.5% horse serum, 2.5% fetal calf serum, and various amounts of kidney extract. A dose of 1 refers to a protein concentration of 2 mg/ml.

were then reembedded in the collagen gels. After 2–3 weeks in culture in Ham's F12 medium containing 50% horse serum and cholera toxin, outgrowths similar to those seen in primary cultures were consistently observed.

DISCUSSION

Normal mammary epithelial cells in primary culture on plastic have a limited growth capacity. Because the cells undergo only a few rounds of division, mammary epithelial cells cannot be propagated for prolonged periods in a manner similar to fibroblasts in culture (6, 32, 33). In addition, fibroblasts eventually take over in longterm culture. Yet, an *in vitro* system in which normal mammary epithelial cells can undergo sustained DNA synthesis and mitosis for prolonged periods, leading to a continuous increase in cell number, is necessary to delineate the role of hormones and growth factors in mammary proliferation. Our previous success in growing mammary tumor epithelial cells embedded in collagen gels (24) has prompted us to apply the system to achieve sustained growth of normal cells. By incorporating the step of isopycnic centrifugation for cell separation and the inclusion of cholera toxin in the medium, we have

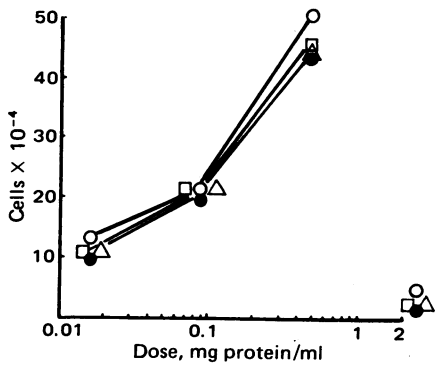

FIG. 7. Effect of kidney extracts prepared from mice in different physiological states. Normal mammary epithelial cells (9×10^4 cells) were cultured for 13 days in Ham's F12 medium containing 12.5% horse serum, 2.5% fetal calf serum, cholera toxin at 0.01 μg/ml, and various amounts of kidney extract prepared from BALB/c mice of different physiological status as follows: ○, midpregnant mice (dialysis tubing with a molecular weight cutoff of 3500); ●, midpregnant mice (dialysis tubing with a molecular weight cutoff of 13,000); □, adult virgin mice (dialysis tubing with a molecular weight cutoff of 3500); and △, adult virgin mice given pituitary isografts for 1–2 months (dialysis tubing with a molecular weight cutoff of 3500).

now accomplished this goal. Percoll gradient centrifugation removes the stromal cell types sufficiently so that fibroblast overgrowth does not take place. In addition, cholera toxin, known to cause an irreversible increase in intracellular adenylate cyclase activity, is also known to inhibit proliferation of fibroblasts (29). Cholera toxin is also a positive stimulus for normal mammary epithelial cell proliferation, because its deletion results in a significantly lower increase in epithelial cell numbers. Interestingly, the tissue content of cyclic AMP in rat mammary gland rises continuously to the end of pregnancy, suggesting that the growth and development of the gland are related to the tissue content of cyclic AMP (34). That the cholera toxin effect on mammary epithelial cells might be mediated by cyclic AMP is also implied by our finding that replacement of cholera toxin by agents (prostaglandin E_1 and E_2, isoproterenol, theophylline, dibutyryl cyclic AMP) known to increase the level of cellular cyclic AMP also favors proliferation of these cells (35).

Several observations strongly indicate that the collagen gel outgrowths are normal mammary epithelial in origin: (*i*) when embedded cells were recovered and transferred to monolayer culture, they formed a continuous sheet of polygonal cells that were indistinguishable from primary culture of mouse mammary epithelial cells. In addition, dome formation in response to cortisol is indicative of transporting epithelia (31). (*ii*) Electron microscopy observation of growing cells has demonstrated polarized cells containing tight junctions (D. R. Pitelka, personal communication). These are well-established characteristics of mammary epithelial cells (36). (*iii*) Transplantation of cells from collagen gel-derived outgrowths from C57BL mice similar to those reported here from BALB/cfC3H, to parenchyma-free fat pads *in vivo*, gave rise to mammary structures with normal morphology (ductal outgrowths).

Studies on growth regulation of normal mammary epithelial cells showed that steroids and protein hormones, both singly and in combination, failed to stimulate growth of these cells over the control level under conditions in which increased horse serum concentration (50%) elicited a proliferative response. This suggests that the component(s) present in horse serum that was responsible for the proliferative response may not be the steroid and protein hormones tested. In contrast, organ (kidney, brain, uterus, spleen) and urine extracts at optimal concentrations were found to promote growth. Recent studies have shown that organ extracts are mitogenic in primary cultures of rat Schwann cells (37) and bovine epithelial lens cells (38). Moreover, synergism of cholera toxin with extracts (37) and with epidermal growth factor (EGF) (30) has also been reported recently. The nature of the growth factor(s) in various tissue and urine extracts has not been determined, but it has been reported that urine is a relatively rich source of EGF (39) and that EGF has a stimulatory effect on mammary epithelial cells (18, 40, 41).

The lack of a proliferative response to steroids and peptide hormones by normal mammary epithelial cells reinforces recent findings that seem to challenge the classical concept that trophic hormones directly stimulate their target cells to proliferate. For example, corticotropin, thyrotropin, and gonadotropins, thought to be responsible for both the growth and functioning of the adrenal cortex, thyroid, and ovary, respectively, have been found to elicit no effect or actually inhibit the growth of their target cells *in vitro* (42–44). Similarly, three established cell lines requiring estrogens for optimal tumor formation in host animals have been found not to be responsive to estrogen for growth *in vitro* (8). It has been shown that certain tissues contain growth factor activity for these three cell lines and that estrogen influences the tissue levels of these activities. On the basis of this observation, it has been proposed that the role of

estrogen *in vivo* is to induce an intermediary growth factor (8). In contrast, in our studies using normal mammary epithelial cells in primary culture, the stimulatory activity found in the kidney extract was found not to be affected by the physiological status of the donors. A possible alternative to the hypothesis of hormonal involvement in inducing an intermediary growth factor(s) is that conventional hormones may have an indirect effect by somehow making the mammary cells responsive to a rather ubiquitous growth factor(s). Experiments using kidney extracts prepared by dialysis in tubings of two different molecular weight cutoffs seem to suggest that the factor may be larger than 13,000 daltons. However, it is possible that a higher molecular weight form of smaller growth factor may exist, as has been reported for EGF (45). The ultimate role of classical hormones in mammogenesis must await development of serum-free conditions for sustained growth of primary mammary cells, as well as identification of the mitogen(s) present in various extracts. Additionally, it will be necessary to demonstrate that the outgrowths derived in serum-free conditions, inside a collagen gel matrix, contain all the epithelial components of the mammary tree and are not a selected population. That mammary glands may contain two epithelial cell populations responding to different growth factors has been previously suggested (5, 46).

Finally, our system will greatly facilitate other studies that may be dependent on the proliferation of normal mammary epithelial cells. Mammary epithelial transformation is one such area in which growth may increase the period when cells are vulnerable to transformation; once transformed, the cells would increase their number, allowing further analysis. This system may increase the incidence of transformation compared to that achieved in the monolayer system (47). The system is also amenable to studies requiring growth of representative cell populations from normal tissues and isolation of individual cell types from these populations for further growth studies and cell type analysis.

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