Proc. Natl. Acad. Sci. USA
Vol. 79, pp. 4074–4077, July 1982
Cell Biology

Serum-free growth of normal and tumor mouse mammary epithelial cells in primary culture

(collagen gel/growth factors/bovine serum albumin/lithium)

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Communicated by Howard A. Bern, March 29, 1982

ABSTRACT **Freshly isolated normal and tumor mouse mammary epithelial cells embedded within a collagen gel matrix undergo sustained growth when cultured for as long as 3 wk in a serum-free medium composed of a 1:1 (vol/vol) mixture of Hepes-buffered Ham's F12 and Dulbecco's modified Eagle's medium supplemented with insulin, epidermal growth factor (EGF), transferrin, bovine serum albumin fraction V, and cholera toxin. Of these additives, only insulin, EGF, and albumin are required for the growth of most normal cells. Albumin is not always an absolute requirement for growth but greatly enhances it. Lithium has been found to stimulate the growth of normal cells and can replace EGF. The collagen matrix culture system allows sustained growth of primary cultures of both normal and neoplastic mammary epithelium in serum-free conditions. This serum-free system will be useful in identifying and investigating the role of hormones, growth factors, and nutritional factors in regulating the growth of mammary epithelial cells.**

The hormonal control of mammary gland growth and development has long been the subject of intense study. Both *in vivo* and *in vitro* organ culture studies have revealed apparent requirements for hypophyseal polypeptide and steroid hormones for mammary gland growth and development (1, 2). However, the results from *in vivo* studies, with their inherent complexity, and from organ culture studies, which are often characterized by a limited growth response with obligatory hormonal priming of the donor animal, do not permit a clear distinction between direct and indirect effects of hormones upon the growth of the mammary epithelium.

Recently, a collagen gel culture system was developed in which mammary epithelial cells (mouse and human) embedded within the gel undergo sustained (i.e., prolonged) multifold growth in primary culture (3–5). The importance of sustained growth in reducing the impact of endogenous factors with long half-lives, such as steroid hormones carried into culture by the cells (6, 7), should not be overlooked. The collagen gel system has permitted assessment of the ability of hormones such as prolactin, estradiol, and progesterone, known to stimulate growth *in vivo* and in organ culture, to stimulate directly the growth of mammary epithelial cells. In contrast to the *in vivo* findings, prolactin and ovarian steroids did not stimulate the growth of mouse mammary epithelial cells when cultured in this system in medium containing serum (3). These results are compatible with the hypothesis that classical *in vivo* mammogenic hormones may not be direct mitogens for the mammary epithelium (8, 9). However, a limitation of these studies was the use of medium containing serum which may mask, inhibit, or modulate the growth-promoting effects of exogenous hormones. To circumvent this problem, the development of a serum-free culture medium would be of the utmost importance.

Serum-free media have been established for the propagation of cell lines derived from human mammary carcinomas (10, 11) and various primary cell cultures (12–17), including the limited growth of preneoplastic mouse mammary cells (18) and of rat mammary epithelial cells (19). However, attainment of sustained growth of normal or tumor mammary epithelial cells in serum-free primary culture has been an elusive goal.

We now report the development of a serum-free collagen gel culture system in which mouse mammary epithelial cells undergo sustained growth.

MATERIALS AND METHODS

The cell dissociation and culture procedures were essentially the same as described (3) with modifications as indicated. In brief, mammary glands from midpregnant (12–16 days) or virgin BALB/c mice or BALB/cfC3H mammary tumors were minced and dissociated at 37°C in a gyratory water bath by a two-step procedure. Collagenase (0.1%, Worthington CLS III) treatment for 60–90 min at 120–180 rpm was followed by Pronase treatment (0.05–0.1%) for 30 min. This procedure differs from the published procedure by the inclusion of bovine serum albumin, fraction V, at 1 mg/ml in the medium during collagenase and Pronase dissociation and by the use of a lower Pronase concentration (0.05–0.1% instead of 0.5%). At no time during the cell dissociation procedure were the cells exposed to serum.

After separation from stromal cells by Percoll density gradient centrifugation, epithelial cells were collected and embedded within neutralized collagen gels (rat tail collagen dissolved in acetic acid) at an initial density of $0.5–1 \times 10^5$ cells per well on a Falcon multiwell plate.

The cells were cultured in a medium consisting of a 1:1 (vol/vol) mixture of Ham's F12 and Dulbecco's modified Eagle's medium (DME medium) (both from GIBCO) containing $NaHCO_3$ at 1.2 g/liter and buffered with Hepes at 20 mM. This medium was supplemented with insulin, transferrin, cholera toxin, fraction V or crystalline bovine serum albumin (Sigma), and epidermal growth factor (EGF; Collaborative Research, Waltham, MA).

For some experiments, fraction V bovine serum albumin was dialyzed against deionized, double-distilled water in dialysis tubing (M_r 3,000 pore size; Specpor) or delipidized by extraction of an aqueous solution with butanol/diisopropyl ether, 2:3 (vol/vol) followed by dialysis and lyophilization (20). Albumin was added directly to the medium, and the pH was adjusted to 7.4 if necessary. The dialyzed and delipidized/dialyzed albumin were taken from the same lot of untreated fraction V used in these experiments.

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Abbreviations: EGF, epidermal growth factor; DME medium, Dulbecco's modified Eagle's medium.

Cell number was determined as described (3) by a DNA fluorometric assay (21) with BALB/cfC3H tumor or normal cells for standards. Because the cells in primary culture are overwhelmingly diploid, the DNA determination reflects cell number.

RESULTS

Mammary epithelial cells from midpregnant and virgin BALB/c mice underwent sustained growth when cultured for 10–12 days in the 1:1 mixture of Hepes-buffered (20 mM) Ham's F12 and DME medium supplemented with insulin (10 μg/ml), transferrin (10 μg/ml), EGF (0.1 μg/ml), cholera toxin (0.01 μg/ml), and bovine serum albumin fraction V (5 mg/ml). The mammary epithelial nature of these cultured cells has been demonstrated by the observed induction of casein synthesis in serum-free culture (J. Yang, personal communication) and by the *in vivo* transplantation of cultured cells (after 3 weeks in culture) which produced mammary outgrowths in cleared fat pads of syngeneic hosts (R. Guzman, personal communication). The results of dose–response studies in which the concentration of each supplement was varied in turn while the concentrations of the others were maintained at the above values are summarized in Table 1. Insulin and EGF were required for the growth of cells from midpregnant and virgin animals in serum-free medium. Growth was decreased by about 50% when cholera toxin was omitted. Transferrin was not required for growth; in some experiments, it enhanced growth slightly. Other factors, including biotin (0.1–10 μg/ml), ascorbate (1–50 μg/ml), α-tocopherol (1–50 μg/ml), selenium (1–100 nM), and triiodothyronine (1–100 pM), did not significantly enhance growth when added to the serum-free medium. Putrescine (10–50 μg/ml) enhanced growth but not consistently.

Table 1. Summary of dose–response studies

Additive	% of growth at optimal concentration: Midpregnant	Virgin
Insulin (μg/ml):		
0	3.2 ± 1.3	3.7 + 5.1
0.01	4.8 ± 1.4	4.0 ± 5.7
0.1	15.4 ± 6.6	4.0 ± 5.7
1.0	53.2 ± 13.9	32.3 ± 15.4
10.0	100	100
20.0	96.6 ± 1.6	115.7 ± 4.9
Bovine serum albumin fraction V (mg/ml):		
0	69 ± 31.3	33.7 ± 31.7
1	58.6 ± 10	41.7 ± 25.2
5	100	100
10	104.6 ± 4.3	176 ± 8.5
15	100.7 ± 13.3	236 ± 62.6
20	89.2 ± 17.8	275 ± 162
EGF (μg/ml):		
0	9.0 ± 2	19 ± 6.5
0.005	72.4 ± 9.1	77.3 ± 21.7
0.01	100	100
0.05	94.6 ± 7.4	103.3 ± 24.9
0.1	91.3 ± 8.5	90.3 ± 15.2
Transferrin (μg/ml):		
0	80.2 ± 8	96.7 ± 19.9
0.1	72 ± 13.1	111 ± 19.8
1.0	80 ± 22.4	114.7 ± 16.8
10	100	100
Cholera toxin (μg/ml):		
0	56.1 ± 12.3	42.7 ± 6.2
0.001	74.4 ± 17.6	66.7 ± 14.3
0.01	100	100
0.1	98.3 ± 3.4	82.7 ± 4.2

Results are shown as mean ± SEM (n = 3). The medium was supplemented with insulin (10 μg/ml), transferrin (10 μg/ml), EGF (0.1 μg/ml), cholera toxin (0.01 μg/ml), and bovine serum albumin fraction V (5 mg/ml). The concentration of each supplement was varied in turn while the concentrations of the others were maintained at the above values.

Bovine serum albumin was not absolutely required in all experiments, but its omission could drastically limit growth. Fraction V may contain both low and high molecular weight contaminants such as serum globulins, lipoproteins, trace elements, hormones, and lipids which might be used by the cells. Dialyzed, dialyzed and delipidized, and crystalline [free of globulin but not lipoprotein contamination (22)] bovine serum albumin fraction V were tested for their growth-supporting capacities in comparison to untreated albumin. The medium for these experiments also contained insulin (10 μg/ml), EGF (10 ng/ml), transferrin (10 μg/ml), and cholera toxin (0.01 μg/ml) and is designated SFM. The growth response to the different albumin preparations was variable. Table 2 gives a summary of the growth responses from three separate experiments. These data indicate that the growth response in general was decreased with crystalline or dialyzed/delipidized albumin but not with dialyzed albumin.

It was noticed that some colonies seemed to disintegrate with concurrent cell death during the culture period. This degeneration was rarely seen in cells from virgin animals but was more prevalent in cultures of cells derived from midpregnant animals. To assess the effect of this degeneration upon the long-term growth of the cultures, cells were maintained for 3 weeks in

Table 2. Growth with different types of bovine serum albumin: A summary of three experiments

Albumin	Conc., mg/ml	% growth relative to absence of albumin: A	B	C
Untreated	1	164 ± 26	172 ± 3	100 ± 4
	5	361 ± 100	186 ± 3	218 ± 25
	10	443 ± 26	158 ± 7	>246
	15	284 ± 71	142 ± 4	>246
	20	324 ± 87	130 ± 9	>246
Dialyzed	1	289 ± 24	152 ± 5	124 ± 12
	5	311 ± 82	152 ± 6	220 ± 17
	10	364 ± 221	156 ± 6	228 ± 13
	15	377 ± 86	128 ± 20	>246
	20	389 ± 117	120 ± 14	>246
Crystalline	1	13 ± 10	152 ± 4	106 ± 4
	5	32 ± 24	142 ± 15	44 ± 8
	10	10 ± 19	131 ± 2	64 ± 16
	15	16 ± 11	69 ± 19	66 ± 27
	20	(0)	22 ± 3	32 ± 17
Dialyzed/ delipidized	1	94 ± 9	138 ± 14	102 ± 4
	5	152 ± 43	136 ± 17	92 ± 16
	10	156 ± 42	119 ± 12	99 ± 1
	15	190 ± 20	104 ± 18	90 ± 3
	20	269	65 ± 7	43 ± 5

Results are shown as mean ± SEM for three separate experiments.

SFM lacking cholera toxin. There was no observed decrease in cell number, indicating that, although some colonies degenerated, this degeneration was minor in comparison to the total growth of the cultures (Fig. 1).

Tumor cells were cultured 12–14 days in SFM in an effort to detect differences in the growth requirements of normal and tumor cells which might permit their selective growth *in vitro*. The concentrations of EGF, albumin, and insulin were varied (as in Table 1) in turn while the concentrations of the other components were maintained at standard. In contrast to normal cells, the growth of some tumors was independent of EGF (four of six) and more responsive to insulin (three of three)—that is, growth did not decrease at an insulin concentration of 1 μg/ml as seen in the normal cells. All the tumors were dependent upon albumin for growth; maximal growth occurred at 5 mg/ml in five of six tumor cultures. Fig. 2 shows growth responses of an EGF-independent and an EGF-dependent tumor.

Recent studies (23, 24) have demonstrated that lithium ions stimulate DNA synthesis in mouse mammary cells in culture. The effect of lithium upon the proliferation of mouse mammary epithelial cells was tested in a serum-free medium composed of a 1:1 mixture of Ham's F12 and DME medium buffered with bicarbonate only and supplemented with insulin (10 μg/ml), transferrin (10 μg/ml), albumin (1 or 5 mg/ml), and cholera toxin (0.01 μg/ml) with or without EGF (10 ng/ml). These studies reveal that lithium (added as lithium chloride) at a concentration of 5–10 mM can stimulate the growth of mammary epithelial cells from virgin and midpregnant mice cultured in this serum-free medium. In dose–response studies similar to those in Table 1, lithium potentiated the growth response to all the components of this serum-free medium but its potentiating effect required the presence of insulin and albumin. Although lithium could not substitute for insulin or albumin in the promotion or maintenance of growth, it could replace EGF. Cells cultured in the absence of EGF could be stimulated to grow by inclusion of lithium in the medium. However, preliminary results indicate that lithium does not stimulate the growth of tumor cells cultured in the absence of EGF.

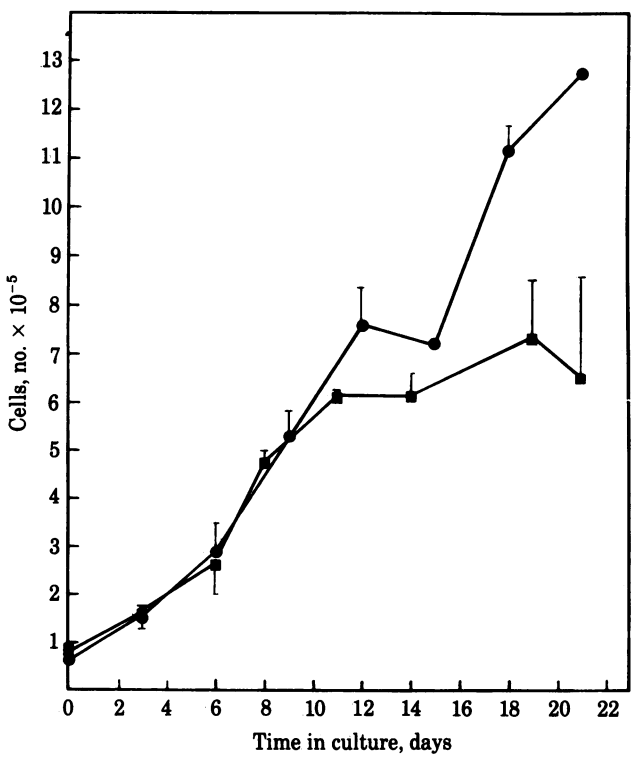

FIG. 1. Growth curves of cells from midpregnant mice cultured in SFM lacking cholera toxin for 3 weeks. Two separate experiments (●, ■) are shown as mean ± SD.

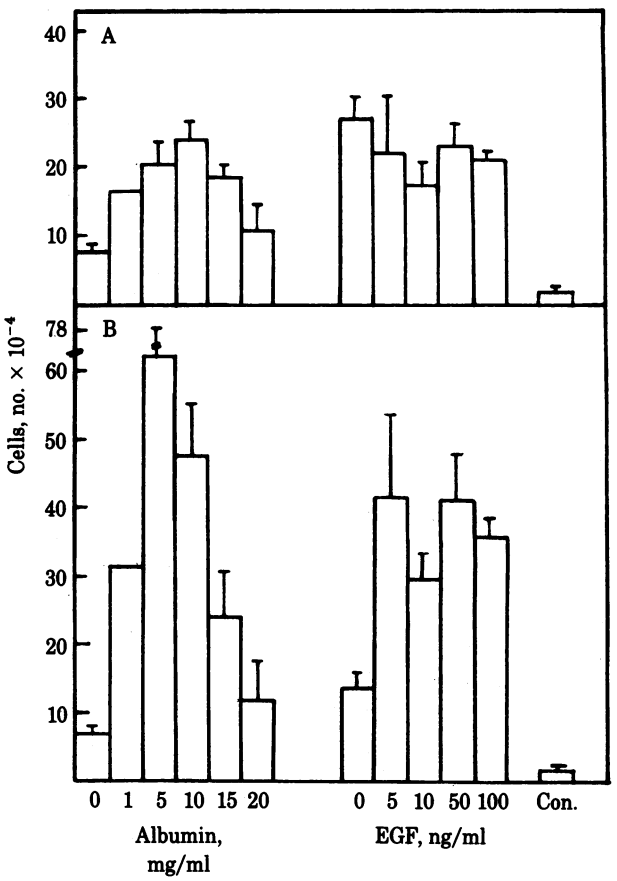

FIG. 2. Growth of BALB/cfC3H mammary tumor cells cultured for 12–14 days in SFM (mean ± SD) in two experiments showing albumin-dependent but EGF-independent (*A*) and EGF-dependent (*B*) growth. Con., time 0 value.

DISCUSSION

When cultured for 10–12 days in serum-free medium, mouse mammary epithelial cells can sustain as much as a 10-fold increase in cell number. EGF and insulin are absolutely required for the growth of normal cells. In contrast, some tumors are EGF-independent.

A supraphysiological concentration, 10 μg/ml, of insulin is required for optimal growth, although appreciable growth will occur at 1 μg/ml. This requirement for a high insulin concentration may reflect binding to the collagen and degradation in culture (10) or, alternatively, low-affinity insulin binding to receptors for as yet undefined mammotropic insulin-like growth factors.

It is known that EGF can stimulate the growth of cultured human (5, 25, 26) and mouse mammary epithelial cells (4). The results reported here demonstrate that EGF not only promotes growth but also is required for the growth of cells from both midpregnant and virgin mice. EGF is required for the second round of growth of mouse mammary glands in organ culture (27) and is present in mouse plasma and milk in nanogram quantities (28), findings compatible with the hypothesis that EGF may be important *in vivo*, possibly in the regulation of basement membrane synthesis (29).

Preliminary studies examining the effect of lithium upon growth indicate that lithium can replace EGF for the growth

of mammary epithelial cells from normal mice. This effect may suggest a possible relationship between EGF and ion transport or the ionic composition of the cell relevant to the mode of action of EGF upon cellular growth. The EGF-independent growth of some tumors may indicate that they are producing an endogenous growth factor capable of stimulating growth or have become less dependent upon growth factors for growth (30–32).

The nature of the substances that bovine serum albumin may provide to the cells is unknown but may be lipids or lipid soluble as suggested by the decrease in growth observed with crystalline or solvent-extracted albumin preparations. Crystalline albumin can inhibit growth. This inhibition may result from decanol contamination of the preparation (33). Lipids have been shown to be required for or to enhance the growth of cultured cells, including mammary epithelial cells (17, 29, 33–35). It must be remembered that, although crystalline and solvent-extracted albumin may contain relatively less fatty acid than fraction V albumin, they are not completely lipid-free and are contaminated with lipoproteins (22, 33). Thus, growth in the presence of either of these albumin preparations does not necessarily indicate an absolute independence of growth from exogenous lipid. Our lot of albumin fraction V was assayed for steroids by radioimmunoassay (kindly provided by Paul Licht) and was found to contain picogram amounts of estradiol and progesterone per gram of albumin. Therefore, the albumin is not a significant source of ovarian steroids. In addition, the addition of ovarian steroids and prolactin, at various concentrations, to this serum-free mammary cell culture system does not stimulate cell growth (unpublished data).

Serum-free collagen gel primary culture of mouse mammary epithelium allows prolonged multifold growth in a system to which steroids can be added to test for their direct mitogenicity with no or minimal endogenous steroid present. It is our hope that the development of this serum-free culture system will greatly facilitate the identification of the hormones and growth factors that regulate the growth and development of the mammary gland. Only then can questions concerning the interplay and mechanism of action of hormones and growth factors in normal and abnormal growth regulation be meaningfully approached. Serum-free culture may also lead to the design of selective media for the growth of transformed cells which will be useful in studies of carcinogenesis in the mammary gland.

This investigation was supported by U.S. Public Health Service Grants CA05388 and CA09041.

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