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PRIMARY CULTURE OF MOUSE MAMMARY TUMOR EPITHELIAL CELLS EMBEDDED IN COLLAGEN GELS

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SUMMARY

Mammary tumor epithelial cells from BALB/cfC3H mice were dispersely embedded inside the collagen gels in Ham's F-12 medium containing horse serum. A sustained cell growth leading to a 5- to 10-fold increase in cell number over initial level was observed in less than 2 weeks. The extent of this growth was found to be dependent on serum concentration. However, addition of various protein and steroid hormones, both singly and in combination, to low-serum-containing medium failed to achieve a comparable level of growth to that promoted by higher serum concentration. Mammary tumor cells can now be consistently propagated in primary culture.

Key words: primary epithelial culture; mouse mammary tumor cells; sustained growth; collagen gel; protein and steroid hormones.

INTRODUCTION

We have recently developed the method of embedding mouse mammary tumor epithelial cells inside the collagen gel, resulting in a sustained growth of mammary cells for a prolonged period in primary culture (1). These results are in contrast to studies thus far reported on mammary cell proliferation in primary culture where proliferation has been assessed mostly in terms of [^{3}H]thymidine incorporation in short-term culture (2-6) because of the inability to maintain long term cultures. Mammary tumor epithelial cells in primary culture can now be consistently propagated leading to a significant increase in cell number. In the current report, we extend our earlier findings and show that a level of proliferation much higher than that previously achieved can be attained simply by embedding the cells dispersely throughout the collagen gel and raising the concentration of serum. In addition, the effects of various protein and steroid hormone supplementation have also been tested.

MATERIALS AND METHODS

Primary mammary tumors (spontaneously occurring) from BALB/cfC3H mice were dissociated by a method previously described (1). Stock collagen solution was prepared as originally described (7). Mammary cells obtained from a pool of 3 to 5 tumors were embedded dispersely inside the collagen gel by the following procedure. Four volumes of stock collagen solution (40 ml) were mixed with 1 *V* (10 ml) of 2:1 mixture of 10X-concentrated Waymouth medium and 0.34 *N* NaOH and kept on ice to prevent immediate gelation. One to 2×10^6 cells in 1 to 2 ml of medium was added to 50 ml cold gelation mixture; 0.5 ml, containing 5 to 10×10^4 cells, was overlaid on top of 0.3 ml gelled collagen in each well of a Falcon plastic Multiwell plate and allowed to gel at room temperature. After this second layer had gelled, cultures were fed with 0.5 ml of F-12 (GIBCO, Grand Island, NY) containing various additive together with 100 U/ml penicillin, 100 μg/ml streptomycin, and 2.5 μg/ml amphotericin B. The cultures were fed with fresh medium every 2 days. Horse serum was obtained from both Sterile Systems (Logan, UT) and Flow Laboratories (Rockville, MD) and fetal bovine serum from Sterile Systems.

At the end of the experiment, each gel was transferred into a 10 × 75-mm test tube and 0.1 ml of 1% collagenase (Worthington Biochemical Corp., Freehold, NJ) in Hanks'

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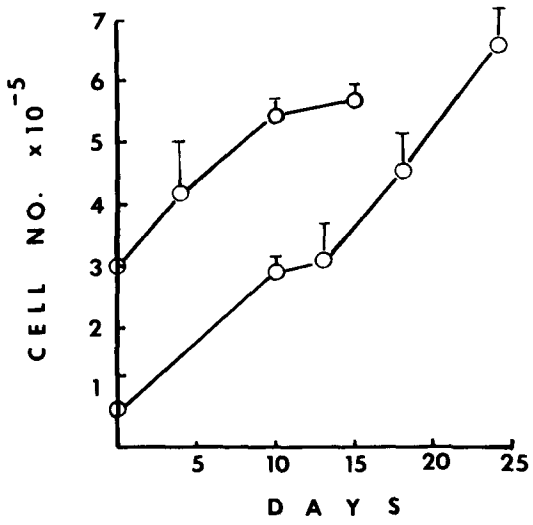

FIG. 1. Growth curves for BALB/cfC3H mammary tumor cells embedded dispersely in collagen gels. Medium was Ham's F-12 with 25% horse serum. Two experiments are shown, each done with cells pooled from three to four tumors. Each point represents the average of three to five gels.

balanced salt solution was added. Cells from the interior of the gel, after digestion on a gyratory water bath shaker for 30 to 60 min at 37° C, were recovered by centrifugation at 100 × *g* for 5 min. The starting cell number was also determined by recovering the cells from the gel 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 (8) utilizing BALB/cfC3H mammary tumor epithelial cells, counted in a hemocytometer, as a standard.

RESULTS AND DISCUSSION

The growth curve for mammary tumor cells embedded locally in collagen gels, as previously reported (1), may have provided us with a rather low estimate of actual cell proliferation that could take place in collagen culture. Radioautography showed most of the DNA synthesis taking place at the periphery of the outgrowth, and histologic analysis showed that many of the cells in the central portion of the locally embedded culture became necrotic, probably due, at least in part, to problems with diffusion. We have now embedded the cells disersely throughout the collagen gel and the results, as shown in Fig. 1, indicate much greater proliferation than that obtained with locally embedded cultures, as previously reported (1). The extent of this growth was found to be dependent on serum concentration, as shown in Fig. 2. Photographs of the resulting three-dimensional outgrowths scattered throughout the gel are shown in Fig. 3.

Since substantial growth resulting in 5- to 10-fold increase in cell number over initial value can be achieved within a relatively short period in culture (10 to 14 days), the effect of various additives can now be assessed directly in terms of increase in cell number. This has not been possible in primary culture of mammary tumor epithelial cells, since cells cultured at low density in the conventional monolayer system become flattened and multinucleate and rarely attain confluence (9). A concentration of 12.5% horse serum and 2.5% fetal bovine serum in the medium elicited only a modest increase, if any, in cell number compared to initial value within a period of 2 weeks, as shown in Figs. 4 and 5. Various protein and steroid hormones were supplemented to this "maintenance" medium to determine whether proliferation comparable to that achieved with higher serum concentration can be obtained. Protein hormones (insulin, prolactin) and steroids (estradiol, progesterone, cortisol, *d*-aldosterone,

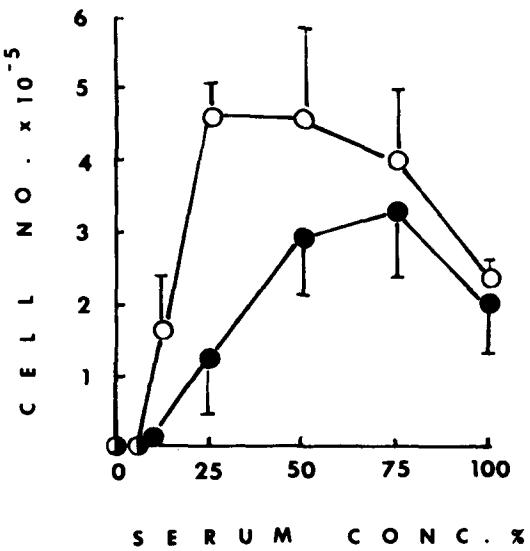

FIG. 2. Horse serum dose response. BALB/cfC3H mammary tumor cells (5 × 10^4 cells) were embedded dispersely in collagen gels. The medium was Ham's F-12 with varying concentrations of horse serum. Cultures were terminated after 13 days (●) and after 22 days (○).

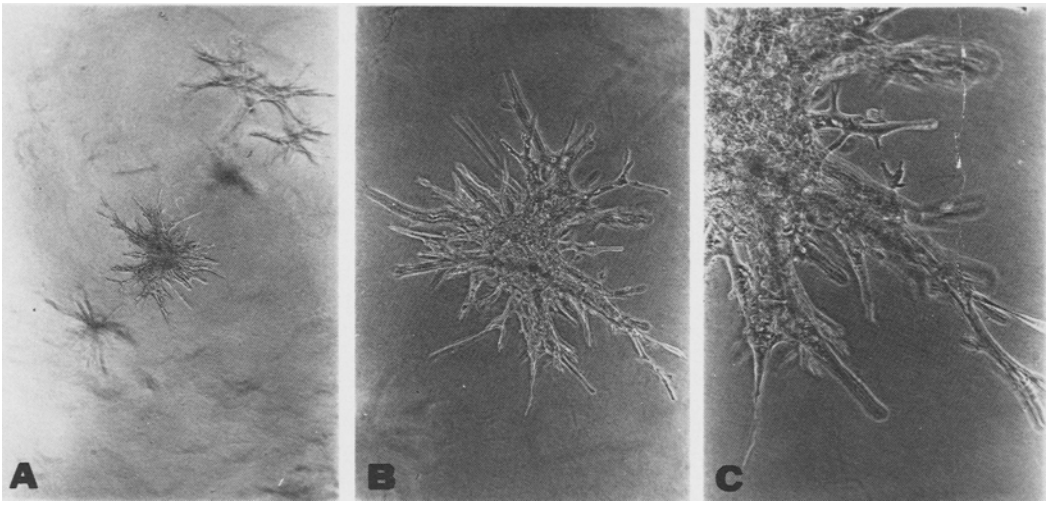

FIG. 3. Photomicrographs of mammary tumor epithelial cells cultured for 13 days. The medium was Ham's F-12 containing 50% horse serum. *A*, ×19; *B*, ×48; *C*, ×95.

and testosterone), singly at various dosage levels, showed little, if any, proliferative effect on mammary tumor cells as shown in Fig. 4. Similarly, various combinations of steroids and protein hormones (IPrl, IF, IEP, IPrlEP, IPrlA, IPrAF) also failed to show any significant proliferative effect as shown in Fig. 5. However, addition of corticoids, such as cortisol and aldosterone, either singly or in combination with other hormones, has consistently shown a slight increase over the control culture. Nevertheless, all the additives thus far tested failed to elicit a response as great as the level achieved with higher serum concentration.

Since spontaneous tumors in BALB/cfC3H mice are generally regarded as hormone independent (10), it is possible that cells derived from these mammary tumors may not respond to any

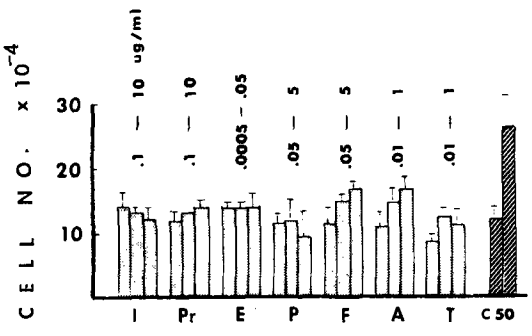

FIG. 4. Dose response of various protein and steroid hormones added singly. Mammary tumor cells (10×10^4 cells) were embedded dispersely inside the collagen gels and cultured in the presence of various additives for 8 days: I, insulin; Pr, prolactin; E, estradiol; P, progesterone; F, cortisol; A, aldosterone; T, testosterone; C, control; 50, 50% horse serum. Concentration range, lowest on the left to highest on the right, are as shown and the increment is by a factor of 10.

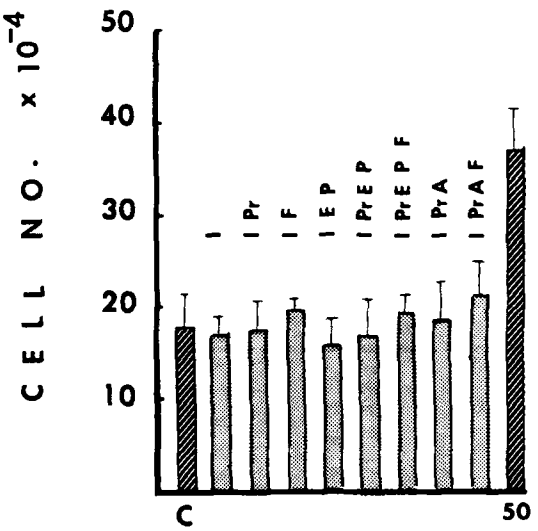

FIG. 5. Effect of various protein and steroid hormone combinations. Mammary tumor cells (10×10^4) were embedded dispersely inside the collagen gels and cultured in the presence of various additives for 13 days. Abbreviations are the same as in Fig. 4. Concentrations of various hormones were as follows: 10 μg/ml insulin, 5 μg/ml prolactin, 0.5 μg/ml cortisol, 0.005 μg/ml estradiol, 0.5 μg/ml progesterone, 1 μg/ml aldosterone.

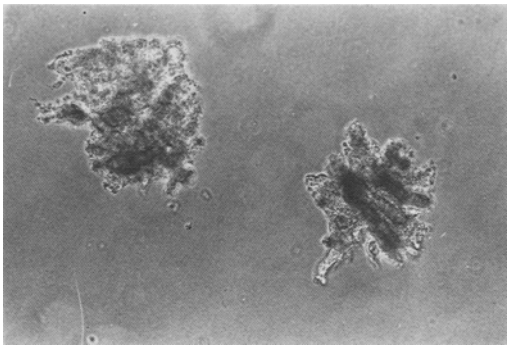

FIG. 6. Photomicrograph of outgrowths from primary culture recovered by collagenase digestion. Mammary tumor epithelial cells were embedded inside the collagen gels and cultured for 2 weeks in Ham's F-12 medium containing 50% horse serum. Outgrowths from the gels were recovered by digestion with 0.1% collagenase in Hank's balanced salt solution. Note that the projections have retracted after being freed from the collagen matrix. ×49.

hormones in vitro. These results also suggest that the mitogen(s) present in higher serum concentration may not be pituitary and ovarian hormones. Current efforts are directed at identifying various additives that will promote growth comparable to the level achieved with higher serum concentration. In this regard, it has been reported recently (11) that certain organ extracts, but not estrogen, are mitogenic for the rat mammary epithelial cell line derived from estrogen-dependent tumors in vivo.

Mammary tumor epithelial cells that have undergone a 5- to 10-fold increase in cell number over the initial value during 2 weeks in culture have been passaged to secondary culture. This was done by initially recovering the outgrowths from the gels by collagenase digestion, as shown in Fig. 6. Note that the projections that extended into the collagen matrix have retracted when the outgrowths were freed from the matrix, suggesting that these projections may have been under tension. These outgrowths were then broken into smaller clumps and single cells by pronase, using the procedure described elsewhere (12). These cells were then re-embedded inside the collagen gel. Outgrowths similar to that shown in Fig. 3 were seen scattered throughout the gels after 2 weeks in culture in Ham's F-12 medium containing 50% horse serum.

The results reported herein show that rapid growth of mammary tumor cells in primary cultures can be attained by dispersing the cells in collagen gels and culturing them in F-12 medium containing 50% horse serum. The optimization of the culture condition now enables propagation of mammary tumor cells in primary culture within a relatively short period. Hormone and drug sensitivity analyses, heretofore performed mostly in established cell lines, can now be performed in primary culture, thus avoiding the criticism that cell lines may not be representative of the original cell population. In addition, from a practical point of view, sustained growth also provides a means of propagating enough cells in primary culture for further analyses.

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