A Rat Mammary Carcinoma In Vivo and In Vitro: Establishment of Clonal Lines of the Tumor
(pituitary hormone/fetal-calf serum)

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ABSTRACT Clonal cell lines from a pituitary hormone-dependent rat mammary carcinoma were established. The growth of these cell lines in vitro is markedly stimulated in the presence of prolactin. The cells grow well in a medium supplemented with fetal-calf serum (generation time: 9-10 hr), but do not grow with calf serum. Fetal-calf serum supports little growth when the serum is subjected to antiprolactin-affinity chromatography. Gel filtration of fetal-calf serum indicates that the growth stimulatory activity is in a large molecular weight material (close to 100,000), suggesting that prolactin is associated with another serum component. A variant of the original tumor, which does not require hormone for growth, was obtained, and clonal cell lines of this tumor were also established. These cells, in contrast to the hormone-dependent ones, grow well with either fetal-calf serum (with or without antiprolactin treatment) or calf serum.

An established cell line, retaining the differentiated function of the tissue of origin, provides excellent material for study of the mechanisms of tissue-specific function and differentiation. If, in addition, the cell line requires an agent such as hormone for growth in vitro, it is also valuable for study of the regulatory mechanisms of cell proliferation. In recent years, several cell lines of endocrine tumor origin that show functional response to hormones in vitro have been established (1), and one of them, a rat ovarian cell line, shows that luteinizing hormone (LH) and a glucocorticoid markedly stimulate the cell proliferation in vitro (2).

In this paper, we report the isolation of cell lines that possess a specific growth requirement in vitro from a rat mammary carcinoma that requires pituitary hormone for growth in vivo and, further, we describe some properties of the cell lines.

MATERIALS AND METHODS

Materials. Purified ovine (NIH-P-S-9) and rat prolactin were obtained from the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health. Porcine recrystallized insulin and 6-methyl-8-9-ergolineacet nitride mesylate were the gift of Eli Lilly Co. Estradiol pellets were kindly prepared by Merck, Sharpe and Dohme and the Carter Wallace Laboratory. Hypophysectomy of rats was performed by the Hormone Assay Laboratory, Inc.

Tumor. A transplantable mammary carcinoma was reported by Iglesias et al. (5) in an AxC* rat that had been implanted with an androgen- and estrogen-producing testicular tumor.

Their study indicated that this mammary tumor, when transplanted, grew only in the animals treated with estradiol, irrespective of the animal's sex. We have maintained this tumor since January, 1971, by serially transplanting it subcutaneously in male AxC* rats implanted subcutaneously with an estradiol pellet (25 mg). All have grown to 2-3 cm in diameter within 3-4 weeks and retained the hormone dependency. When, however, the pieces of tumor were implanted without estradiol, tumors arose occasionally in 3-4 months. These tumors grow well, when transplanted, either with estradiol or without estradiol pellets. To date no metastases have been observed.

Establishment and Maintenance of Cell Lines. Sato and his colleagues have developed a systematic procedure for establishing from various tumors permanent cell lines that retain organ-specific functions. The procedure can be summarized briefly: (i) start with a transplantable tumor that possesses organ-specific function, (ii) alternate culture and animal passage of the tumor cells, and finally (iii) use extensive cloning by single-cell plating (3, 4).

The above procedure was applied to establish the mammary carcinoma cell lines. Cells from explants of the tumor have been grown in Dulbecco modified Eagle's medium supplemented usually with 12.5% horse serum and 2.5% fetal-calf serum. All cell lines used in the present study have also been maintained in the above medium. All cultures were grown in disposable 100 × 20 mm and 60 × 15 mm Falcon petri dishes and incubated at 36° in a humidified atmosphere of 10% CO2 and 90% air.

Measurement of Growth Stimulation. For this purpose, cells (about 1 × 10⁶) were plated in 60 × 15 mm petri dishes. After 6-7 days of growth, the cells were released from the plates by treatment with trypsin and counted on a Coulter Counter. At least two duplicate plates were counted for each point in the groups.

RESULTS

Characterization of the Tumor In Vivo. It is important to have a knowledge of the properties of the tumor from which the cell lines are derived. Accordingly, the following studies were done on fast-growing and regressing tumors.

The tumor has been examined both by light and electron microscopy (unpublished results). Light microscopy shows that a fast-growing tumor consists of well-differentiated glandular structures with surrounding connective tissue and a
mass of epithelial tumor cells. This is defined as mixed carcinoma. A microscopic study reveals a glandular structure that closely resembles that of the normal mammary gland of mice (6). The tumor cells appear to possess active secretory function, since an active Golgi apparatus, many lipid droplets, and secretory protein granules are observed within the structure. On the other hand, regressing tumors (10 days after estradiol is removed) show necrosis and degeneration of epithelial cell and an increase in connective tissue and collagen. The number of lipid droplets also drastically decreases. Possibility of the tumor containing virus particles has been carefully examined by electron microscopy. However, no virus-like particles have been observed.

Iglesias et al. (5) have shown that the tumor requires a supplement of estrogens for growth in vivo. We transplanted tumor pieces to normal male and hypophysectomized male rats: some normal males and all of the hypophysectomized animals received estradiol. Fig. 1 shows that in the normal animals the tumors grow rapidly with supplemented estradiol, but do not grow without estradiol; on the other hand, in hypophysectomized animals the tumors do not grow even in the presence of supplemental estradiol. The results clearly show that the tumor requires some pituitary hormones for growth. Probably estradiol is acting to raise the level of the pituitary hormones in the plasma. However, the results do not exclude the possibility that estradiol may be directly required in addition.

Although preliminary in nature, one series of experiments indicates that 6-methyl-8β-ergoleneacetanilide mesylate, which inhibits the secretion of prolactin from the pituitary gland and inhibits mammary tumor growth in rats (refs. 7–9, and R. J. Kraay, personal communication), suppresses the growth of tumors to about a half rate in the presence of estradiol.

Establishment of Cell Lines. Several cell lines of the mammary carcinoma were isolated by the method established by Sato and his colleagues (3, 4). The excised tumor was washed, minced with scissors, and plated with medium. After 9 days most of the cells in culture were fibroblasts; the cells were freed from the culture plate by treatment with trypsin and injected into the animal with estradiol. A visible tumor (about 1 cm in diameter) arose in 1 month and was plated again.

Fig. 1. Growth of the mammary tumor in normal and hypophysectomized male rats. Minced tumor pieces (0.3 ml) were injected subcutaneously in each animal, and the size of the tumor was measured every several days. All the animals developed small lumps due to the local inflammation, which disappeared gradually if they did not develop into tumors. A—A, normal male with estradiol pellet; O—O, normal male without estradiol pellet; •—•, hypophysectomized male with estradiol pellet.

Fig. 2. Growth stimulation of 64-24 cells by calf serum and normal and toluene-treated fetal-calf serum. 2.2 x 10^5 cells were plated on each 60 x 15 mm plate in a medium supplemented with different amounts of calf serum and normal and toluene-treated fetal-calf serum. Seven days later the cells were released by treatment with trypsin, and the cell numbers were counted by a Coulter counter. Each point represents the average of two to three plates. O—O, calf serum; •—•, fetal-calf serum; O—O, toluene-treated fetal-calf serum.

The second culture contained rather a large fraction of epithelial cells. Because the epithelial tumor cells attach to the culture plate more firmly than do the fibroblasts, trypsinization of the mixed culture segregated the two cell types. By three successive clonings the several cell lines were thus established.

Characterization of the Cell Lines. Only one of the cell lines isolated (64-24) has been studied extensively. The cells attach firmly to the plate and grow to at least three cell layers thick. However, they cannot grow in suspension. The generation time of the cells is around 8–10 hr and the chromosome number is 46 (normal rat, 2n = 42). The plating efficiency is usually 10–20%.

Studies on growth requirements of the cells revealed that cell growth was supported by fetal-calf serum, but not by calf serum (Fig. 2). Increasing amounts of calf serum do not increase cell growth and division. On the other hand, with 12.5–15% fetal-calf serum the cell number increased about 200-fold in 7 days. Possible presence of a growth inhibitor in calf serum was excluded since the addition of calf serum to medium supplemented with fetal-calf serum did not inhibit cell growth. Similar assays were performed with 3T3 cells, a mouse fibroblast line, and a fibroblast line (F7-1) isolated from the same tumor that was used to isolate the mammary carcinoma cell lines. The results show that the fibroblast lines grow equally well with either calf serum or fetal-calf serum (Figs. 3 and 4). When the mammary tumor cells were grown with steroid-free serum prepared by treating the serum with toluene by the method of Yamamoto and Alberts (personal communication), they grew as well as with normal fetal-calf serum (Fig. 2). Low concentrations of tolenuized serum yield poor growth stimulation. Similarly, tolenuized serum gives lower stimulation for the growth of 3T3 cells (Fig. 3). This result suggests that toluene treatment and extensive dialysis may have resulted in a loss or denaturation of some serum component(s).

To determine whether the characteristic growth responses of the cells to fetal-calf serum and calf serum shown in vitro
reflect the true hormonal requirement of growth of the tumor in vivo, we did the following experiments. Several clonal cell lines were established from a variant tumor not requiring hormonal supplements that was derived from the original hormone-dependent tumor; their growth stimulation in fetal-calf serum and calf serum media was tested (e.g., Fig. 5). Calf serum supported the growth of the cells quite well in all cell lines tested, although the cell still preferred fetal-calf serum (see Fig. 5).

Sera from adult female rats, adult male rats with and without estrogen treatment, and hypophysectomized males were isolated and tested for the ability to stimulate cell division in 64-24 cells (Table 1). Sera from all sources markedly stimulated growth when the cells were grown in 2.5% serum concentration. However, a difference in growth stimulation among sera from the different animal types was consistently detected when the serum concentration was reduced to 0.25% (Table 1). Sera from normal females and males treated with estrogen stimulated growth equally well. Normal male serum gave an intermediate response, whereas serum from hypophysectomized animals gave very poor stimulation.

The results obtained from in vivo studies suggest that the cells require prolactin for growth. Accordingly, we prepared medium devoid of prolactin. The immunoglobulin fraction of rabbit serum containing antibodies against ovine prolactin was coupled to Sepharose 4B by the method of Cuatrecasas et al. (10), and fetal-calf serum was chromatographed on this Sepharose column. The results of the growth response of different cell lines using the treated serum are shown in Table 2. The growth of 64-24 cells (hormone-dependent line) was markedly reduced when the "prolactin-free" serum was used, while mouse 3T3 cells and 22-1 cells (a hormone-independent line) grew equally well (considering the dilution factor—see Table 2) whether the serum was treated or not.

Purified prolactin and various hormones thought to be necessary for development of the mammary gland were tested for growth stimulation in vitro. Purified rat prolactin, ovine prolactin, insulin, estradiol, and hydrocortisone did not give any consistent stimulation when added singly or in all possible combinations to the medium with calf serum. Insulin stimulated cell growth considerably when the medium supplemented with fetal-calf serum was used. Other pituitary hormones and steroids have not been tested.

Fetal-calf serum was fractionated to isolate the active component. We used a procedure similar to that used by Holley and his colleagues to fractionate serum factors for mouse 3T3

### Table 1. Growth stimulation by rat sera

<table>
<thead>
<tr>
<th>Serum source</th>
<th>Average no. of cells per plate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female without estrogen</td>
<td>$2.5 \times 10^6$</td>
</tr>
<tr>
<td>Male without estrogen</td>
<td>$1.4 \times 10^6$</td>
</tr>
<tr>
<td>Male with estrogen*</td>
<td>$2.4 \times 10^6$</td>
</tr>
<tr>
<td>Male hypophysectomized</td>
<td>$3.2 \times 10^6$</td>
</tr>
<tr>
<td>No serum</td>
<td>$1.3 \times 10^6$</td>
</tr>
</tbody>
</table>

$2 \times 10^6$ cells were plated with a medium supplemented with 5% calf serum, and each plate received 0.01 ml of one of the sera to be tested in addition. Cell number was determined 6 days after plating.

* Blood was withdrawn 14 days after estradiol was implanted in the animals.
cells and their virus-transformed cell lines using Sephadex G-100 columns (11–13). Fig. 6 shows the results of chromatography of fetal-calf serum on Sephadex G-100 columns at neutral pH (pH 7.2). Growth-stimulating activity is eluted with the bulk protein behind the void volume.

Gel filtration at a lower pH (pH 3.0) was then tried. Maintenance of fetal-calf serum at pH 3.0 for 2 days, the duration required to chromatogram and neutralize the fractions, inactivates the growth-stimulatory activity considerably, although treatment at low pH for 1 or 2 hr does not affect the activity appreciably. Fig. 7 shows the results of chromatography of fetal-calf serum at pH 3.0. The growth-stimulatory activity of the fractions was reduced as expected, but the activity again eluted behind the void volume with the leading edge of the bulk protein as at pH 7.2. Synergistic effects of the fractions were also tested by combining fractions, but in no instance did this result in more than an additive effect.

**DISCUSSION**

Prolactin, hydrocortisone, and insulin are required for proliferation of epithelial cells and lactation of adult mammary glands (14). If the tumor was induced during development of the mammary gland under hormonal influence, it is possible that one or all of these hormones stimulate growth of the tumor in vivo, and in extreme cases, growth of the tumor may be dependent on these hormones. Our results show that the rat mammary carcinoma used to establish the cell lines requires pituitary hormone(s) (most likely prolactin) and possibly other hormones in addition.

Clonal cell lines that exhibit specific growth requirement in vitro have been established from the mammary tumor. Calf serum does not support the growth, although fetal-calf serum does. This characteristic indeed reflects the true hormonal requirement in vivo, as shown by results with cell lines isolated from a variant tumor that grows independently of estradiol.

When prolactin is removed from fetal-calf serum, growth of the cells decreases markedly. However, addition of purified rat or ovine prolactin alone or in combination with insulin, estrogen, and/or hydrocortisone to the medium does not clearly stimulate cell growth. Gel filtration of fetal-calf serum indicates that the active component has a large molecular weight (close to 100,000); the molecular weight of prolactin is 22,000–24,000. Our present working hypothesis is that: (i) prolactin is the major hormone required by the cells and calf serum does not contain it in sufficient amounts, and (ii) prolactin is bound to some large plasma protein and this complex is biologically active, or the activity of prolactin is protected by binding to the protein.

**Table 2. Growth stimulation by “prolactin-free” serum**

<table>
<thead>
<tr>
<th>Serum source</th>
<th>3T3</th>
<th>22-1</th>
<th>64-24</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calf serum</td>
<td>$1.07 \times 10^6$</td>
<td>$4.57 \times 10^5$</td>
<td>$6.30 \times 10^4$</td>
</tr>
<tr>
<td>Fetal-calf serum</td>
<td>$1.15 \times 10^4$</td>
<td>$7.98 \times 10^3$</td>
<td>$2.12 \times 10^2$</td>
</tr>
<tr>
<td>Prolactin-free fetal-calf serum</td>
<td>$7.49 \times 10^5$</td>
<td>$6.91 \times 10^4$</td>
<td>$2.55 \times 10^3$</td>
</tr>
</tbody>
</table>

6 $\times 10^4$ cells of 3T3 cells, 4 $\times 10^4$ cells of 22-1 cells, and 1.6 $\times 10^4$ cells of 64-24 cells were plated with a medium supplemented with 5% calf serum. Each plate received 0.2 ml of one of the sera to be tested in addition. Cell number was determined 6 days after plating. Protein content of the "prolactin-free" serum was 66% of the untreated serum.

**Fig. 6.** Sephadex G-100 filtration of fetal-calf serum at pH 7.2. 4 ml of fetal-calf serum was eluted on a column of 1 $\times$ 115 cm at 5 ml/hr with saline–phosphate buffer (10 mM phosphate buffer–15 mM NaCl [pH 7.2]) at 4°. 5-ml Fractions were collected, $A_{280}$ of each fraction was determined, and selected fractions were sterilized by Millipore filter. A fraction (0.3 ml) was added per plate to examine the growth stimulatory activity. The stimulatory activity was expressed as the ratio of the cell number attained with 5% calf serum plus a given sample to the cell number attained with 5% calf serum only. $\bullet--\bullet$, $A_{280}$; $O--O$, stimulatory activity.

**Fig. 7.** Sephadex G-100 filtration of fetal-calf serum at pH 3.0. 4 ml of fetal-calf serum, pH of which had been adjusted to 3.0, was filtered through a G-100 column of 1 $\times$ 110 cm as in Fig. 6. The elution buffer was 0.05 M glycine–HCl at pH 3.0 in 15 mM NaCl. After fractionation, the pH of each fraction was adjusted to neutral, sterilized, and tested for growth-stimulatory activity as in Fig. 6. $\bullet--\bullet$, $A_{280}$; $O--O$, stimulatory activity.
Three of the tumor cell lines have been carried in culture since January 1972, and we find that their growth characteristics have been extremely stable. These cell lines should be profitable material for study of biochemical mechanisms of hormone action on normal compared to abnormal cell growth and division. Study of biochemical events taking place during the cell cycle would also profit from the use of a cell line requiring hormone for growth. Synchronous culture provides obvious advantages for such a study. Deprivation of the required hormone may be better than blocking some biosynthetic process, such as DNA or protein synthesis, to obtain synchrony since the latter process is more likely to bring the cells to an unbalanced physiological condition.

The initial work toward establishing the tumor cell lines was done in Dr. G. Sato’s laboratory at the University of California at San Diego. We deeply appreciate his helpful advice and that of Dr. D. Sirbasku in his laboratory. We thank Drs. N. Sueoka, A. Levine, and M. Steinberg at Princeton University for the use of their laboratories and valuable discussions with them. This work was supported by Grants NP 78E from the American Cancer Society, GM10923 from the National Institutes of Health, and GB19560 from the National Science Foundation. Mr. Hsieh’s work on this project in part satisfied his junior level undergraduate independent research project at Princeton University.