Growth of a Human Breast Cancer Cell Line in Serum-free Hormone-supplemented Medium

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ABSTRACT

ZR-75-1, a human breast cancer cell line, has been grown in hormone-supplemented medium without serum. The factors required for optimal growth include 17β-estradiol, insulin, transferrin, dexamethasone, and L-triiodothyronine. If estradiol, insulin, or L-triiodothyronine is omitted, cells cease division within 7 days, but viability is retained for at least 14 days. Omission of transferrin leads to cell death within 7 days. The cells have been continuously maintained in this environment without morphological alteration or cessation of growth for more than 5 months. Addition of the anti-estrogen, Tamoxifen (10⁻⁶ M), inhibited cells below the growth rate seen when estradiol was omitted from the medium, even when Tamoxifen was added 4 days and two medium changes after the removal of estradiol from the medium, thus suggesting an action of Tamoxifen which may be independent of competition with estradiol. The availability of a human breast cancer cell line that can be propagated in hormone-supplemented medium without serum should aid in the study of the mechanisms by which hormones effect cell growth.

INTRODUCTION

It has recently been shown that it is possible to grow some cell lines in medium without added serum, provided this culture medium is supplemented with hormones and other factors (9, 11). For example, Hayashi et al. (10) have shown that the GH₄ rat pituitary cell line will grow in Ham’s Nutrient Mixture F-12 supplemented with L-triiodothyronine, thyrotropin-releasing hormone, transferrin, parathyroid hormone, insulin, fibroblast growth factor, and somatomedin C. Even after long-term culture, the growth in this hormone-supplemented medium is equivalent to growth in serum-supplemented medium. Several other cell lines have been adapted to serum-free growth (12, 14, 19).

We felt that the availability of a human breast cancer cell line that could be propagated in serum-free defined medium would aid in the study of the mechanisms of hormone interaction with breast cancer cells. The ZR-75-1 cell line was chosen because we have previously demonstrated receptors for and responses to estrogen, insulin, androgen, and glucocorticoid and because of the unequivocal biochemical, morphological, and chromosomal evidence of its human breast cancer origin (6, 16). We were particularly anxious to develop such a defined growth system in order to be able to study specific hormonal effects.

MATERIALS AND METHODS

Cells. The ZR-75-1 cell line (6) was used for all studies. It is a human breast cancer cell line derived from a malignant ascitic effusion of a patient with infiltrating duct carcinoma of the breast. The cell line has been grown in MEM (3) supplemented with 5 to 10% fetal calf serum for more than 2.5 years and through 68 passages. Its morphological and karyotypic characteristics are similar to the original breast tumor specimen.

Medium and Hormones. IMEM (18) supplemented with L-glutamine (0.6 g/liter), penicillin (62 mg/liter), and streptomycin (135 mg/ml) was the basic culture medium to which hormones and growth factors were added. All media were prepared in the National Institutes of Health Media Unit. Transferrin (Sigma Chemical Co., St. Louis, Mo.) was added at a final concentration of 1 μg/ml (10⁻⁹ M). L-Triiodothyronine (Sigma) (10⁻⁸ M) stock solution was prepared in 0.1 N NaOH and added to medium to yield a final concentration of 10⁻⁸ M. Insulin U-100 (Eli Lilly & Co., Indianapolis, Ind.) was added at a concentration of 5 x 10⁻⁷ M. 17β-Estradiol and dexamethasone (Sigma) in benzene-ethanol were evaporated to dryness, dissolved in ethanol, and stored at -20° until use. Final concentrations in the medium were 10⁻⁸ M 17β-estradiol and 10⁻⁸ M dexamethasone. The final concentration of ethanol is 0.1%, and this concentration has no effect on the growth of the cells. Tamoxifen (ICI 41474) was similarly prepared. Fibroblast growth factor (Collaborative Research, Waltham, Mass.) at a concentration of 0.025 μg/ml was added to the tissue culture flasks when the cells were subcultured, in addition to nucleosides [10⁻⁴ M cytidine, uridine, thymidine, and adenosine (Sigma)] and nonessential amino acids (L-alanine, 18 mg/liter, L-asparagine·H₂O, 30 mg/liter, L-aspartic acid, 27 mg/liter, L-glutamine, 29 mg/liter, glycine, 15 mg/liter; L-proline, 23 mg/liter; L-serine, 21 mg/liter). Other factors tested for growth-promoting activity include epidermal growth factor (Collaborative Research), 5a-dihydrotestosterone (Steraloids Inc., Pawling, N. J.), vasopressin (Calbiochem, Gaithersburg, Md.), oxytocin (Sigma), and human placental lactogen (Sigma).

Cell Growth Experiments. Cells growing exponentially in MEM + 5% fetal calf serum were suspended with trypsin-EDTA (trypsin, 0.05%; EDTA, 0.02%) and were plated in replicate in MEM supplemented with 5% charcoal-treated calf serum (2). The cells were plated into sterile 6-well (35-mm) plastic tissue culture dishes (Linbro Scientific Inc., Hamden, Conn.) After sufficient time for the cells to become adherent had elapsed (usually 12 to 16 hr), the
medium was changed to improved minimal essential medium supplemented with estradiol, L-triiodothyronine, insulin, dexamethasone, and transferrin (IMEM-HS), or to IMEM-HS minus a specific hormone. After 24 hr, the medium was replenished with fresh medium of identical composition. At various times, cells were collected by suspension in trypsin-EDTA and counted in a hemocytometer.

Cell Transfer. The serum-free cells are transferred by treating with 0.02% EDTA in saline A (NaHCO₃, 0.35 g/liter; NaCl, 8.0 g/liter; KCl, 0.4 g/liter; dextrose, 1.0 g/liter; and phenol red, 0.02 g/liter). The cell layer was rinsed with this solution and the excess removed. When the cells began to detach, the action of the EDTA was stopped by simple dilution in IMEM-HS. Cells were passed at a 1:2 dilution.

Plating efficiency experiments were performed by plating 25,000 cells in a 75-sq cm tissue culture flask. After 7 days, the cell colonies were counted. Plating efficiency is defined as the number of colonies formed × 100 divided by the number of cells added to the tissue culture flask.

Precursor Incorporation. To assess the effect of Tamoxifen on thymidine incorporation in the ZR-75-1 breast cancer cells growing in IMEM-HS minus 17β-estradiol, radiolabeled thymidine (Amersham-Searle, Arlington Heights, Ill.) diluted in Dulbecco's phosphate-buffered saline (KCl, 0.20 g/liter; KH₂PO₄, 0.20 g/liter; NaCl, 8.0 g/liter; Na₂HPO₄·7 H₂O, 2.16 g/liter; pH 7.4) was added to each dish 1 to 2 hr before the cells were harvested. Each dish usually contained 1 μCi of tritium. Cells were harvested by washing the dishes once with Dulbecco's phosphate-buffered saline, suspending in EDTA (0.02%), and collecting cell pellets by centrifugation. Cell pellets were suspended in water and sonically dispersed for 3 sec in a Branson sonic extractor (Branson Sonic Power Co., Danbury, Conn.) at the lowest setting. Aliquots were then used for the determination of protein by the method of Lowry (15) or thymidine incorporation by precipitation in 10% trichloroacetic acid. Acid-insoluble counts were collected and washed on a 0.45-μM Millipore filter. After drying, the filters were solubilized in Aquasol (New England Nuclear, Boston, Mass.) and counted in a Packard scintillation counter (Packard Instrument Co., Downers Grove, Ill.) (efficiency for tritium, ∼35%).

RESULTS

Growth Experiments. ZR-75-1 cells grow rapidly in IMEM-HS. In Chart 1, the growth of the ZR-75-1 cells in IMEM-HS is compared to their growth in IMEM supplemented with an optimal concentration of fetal calf serum (5%). In this experiment the cells in IMEM-HS are growing at a more rapid rate than those in fetal calf serum. In other experiments not shown, their growth rates are usually equal. As will be shown later, if hormones and serum are omitted, the cells die. The ZR-75-1 human breast cancer cell line grows rapidly in improved MEM supplemented with hormones and the iron transport protein transferrin. The optimal concentration of 17β-estradiol in IMEM-HS has previously been shown to be 10⁻⁸ M (6). As described later, 5 × 10⁻⁷ M insulin, 10⁻⁸ M L-triiodothyronine, 10⁻⁸ M dexamethasone, and 10⁻¹¹ M transferrin (1 μg/ml) are optimal for cell growth.

We next wondered whether fetal calf serum could increase the growth of cells already growing rapidly in IMEM-HS. Chart 2 compares the growth of the cells in IMEM-HS and IMEM-HS supplemented with 5% fetal calf serum. Again, over this 9-day period no differences in growth were observed, and thus we conclude that the addition of fetal calf serum to IMEM-HS does not result in more rapid growth of these breast cancer cells.

The effects of each of the hormones and transferrin on growth are assessed in Chart 3. As can be seen in this chart, the cells in IMEM-HS grow rapidly over the 14-day period. Control cells in IMEM alone remain viable for 4 to 7 days as judged by attachment to the plastic tissue culture dishes, and then detach and die, as do the cells in IMEM-HS that lack transferrin. Cells in IMEM-HS minus estradiol or insulin or L-triiodothyronine appear to grow for approximately 7 days and then become static, although viability is maintained for the 14-day period. The addition or subtraction of 10⁻⁸ M dexamethasone had no apparent affect on growth but appeared to increase plating efficiency and thus was included in IMEM-HS (see below).

The dose-response curve for transferrin is illustrated in Chart 4. As low a concentration as 0.25 μg/ml (2.5 × 10⁻¹² M) decreases the growth of cells already growing rapidly in IMEM-HS.
Cell Growth in Hormone-supplemented Medium

M) causes an increase in cell number after 4 days, compared to control cells. The curve has a slow increase and is relatively flat between the concentration range of 1 to 7 µg/ml (10^{-11} to 7 × 10^{-11} M). Although this experiment demonstrates that addition of transferrin leads to an increase in cell number, the absolute dependence of the cells for transferrin is better demonstrated in the growth curve shown in Chart 3.

Chart 3. Requirements for serum-free growth of ZR-75-1 human breast cancer cells. Cells were plated at a density of 50,000 cells/dish in MEM supplemented with 5% charcoal-treated calf serum. Over the next 4 days, the medium was exchanged twice with fresh MEM plus 5% charcoal-treated calf serum. On Day 0, the medium was changed to IMEM-HS, IMEM-HS minus 17β-estradiol, IMEM-HS minus insulin, IMEM-HS minus T3, IMEM-HS minus transferrin, or IMEM. Arrows, days on which the cells were refed with fresh medium. Standard deviations of triplicate cell counts are generally less than 10%.

Chart 4. Effect of transferrin on growth of ZR-75-1 cells. Cells were plated at a density of 50,000 cells/dish in MEM supplemented with 5% charcoal-treated calf serum. After sufficient time for the cells to become adherent had elapsed, the medium was changed to IMEM-HS with varying concentrations of transferrin. After 24 hr, the medium was exchanged for fresh medium, and the experiment was allowed to continue for 4 days, at which time the cells were harvested and counted. Standard deviations of triplicate cell counts are generally less than 10%.

Chart 5. Effect of L-triiodothyronine on growth of ZR-75-1 cells. Cells were plated at a density of 50,000 cells/dish in MEM supplemented with 5% charcoal-treated calf serum. After sufficient time for the cells to become adherent had elapsed, the medium was changed to IMEM-HS with varying concentrations of L-triiodothyronine. After 24 hr, the medium was exchanged for fresh medium, and the experiment was allowed to continue for 4 days, at which time the cells were harvested and counted. Standard deviations of triplicate cell counts are generally less than 10%.

Chart 5 shows the dose-response curve for L-triiodothyronine in IMEM supplemented with all of the other required growth factors. As can be seen in this chart, an effect on growth of L-triiodothyronine is observed at concentrations as low as 10^{-10} M with maximal stimulatory effects on growth at 10^{-8} M. The effect on growth is diminished at higher concentrations, and 10^{-6} and 10^{-5} M exhibit no growth-promoting activity.

The dose-response curve for insulin is shown in Chart 6. Insulin in varying concentrations was added to IMEM supplemented with all of the other required growth factors. An effect by insulin on growth is observed at concentrations as low as 10^{-11} M. The growth-promoting effect is maximal at 10^{-10} M, and this maximal effect persists at a concentration range between 10^{-10} and 10^{-9} M.

The effect of other factors on growth of the ZR-75-1 cells was also investigated. No further growth-promoting activity was observed when the nucleosides, adenosine (10^{-8} M), uridine (10^{-8} M), thymidine (10^{-8} M), or cytidine (10^{-8} M), nonessential amino acids, human placental lactogen (2 µg/ml), fibroblast growth factor (0.025 µg/ml), epidermal growth factor (0.01 µg/ml), vasopressin (1 milliunit/ml), oxytocin (1 milliunit/ml), or 5α-dihydrotestosterone (10^{-8} M) was added to IMEM-HS.

The effect of the antiestrogen, Tamoxifen, on thymidine incorporation and growth of the ZR-75-1 cells is examined in Chart 7. In this experiment, the cells were plated in MEM plus 5% charcoal calf serum. Charcoal-treated calf serum was used because it is steroid depleted and contains very low concentrations of estrogen. This medium was exchanged twice over a 4-day period, and the medium was then changed to either IMEM-HS minus 17β-estradiol or IMEM-HS minus 17β-estradiol plus 10^{-6} M Tamoxifen. Over the 10-day period there was a difference in cell number, suggesting that Tamoxifen has an action on the breast...
Insulin Concentration (mM) vs. Cell Number

Chart 6. Effect of insulin on growth of ZR-75-1 cells. Cells were plated at a density of 200,000 cells/dish in MEM supplemented with 5% charcoal-treated calf serum. After sufficient time for the cells to become adherent had elapsed, the medium was changed to IMEM-HS with varying concentrations of insulin. After 24 hr, the medium was exchanged for fresh identical medium, and the experiment was allowed to continue for 3 days, at which time the cells were harvested and counted. Standard deviations of triplicate cell counts are generally less than 10%.

Cell Transfer and Long-Term Culture. The transfer of the ZR-75-1 cell line in IMEM-HS is difficult. Cells are passed at a dilution ratio of 1:2 because of a low plating efficiency. Table 1 lists the results of the plating efficiency experiments. The plating efficiency of the cells in IMEM-HS is approximately 1%. This can be improved to approximately 3 to 4% by the addition of nonessential amino acids, nucleosides (10^{-8} M adenosine, cytidine, thymidine, uridine), and fibroblast growth factor, 0.025 µg/ml. We routinely add these factors at the time of subculture and, since none of these factors appear to have any growth-promoting activity on the ZR-75-1 cells, the flasks are refed with fresh IMEM-HS after plating has occurred. The plating efficiency of the cells in IMEM alone is less than 0.1% and is not significantly improved by the addition of bovine serum albumin (100 µg/ml). The plating efficiency of the ZR-75-1 cells in IMEM plus 5% fetal calf serum is greater than 50%. If one omits dexamethasone from IMEM-HS, the plating efficiency decreases to 0.1%.

We also attempted to improve plating efficiency by the addition of conditioned medium. We have preliminary data that the ZR-75-1 cells condition their medium and that this conditioned medium, when added to the cells, leads to an increase in thymidine incorporation at 24 hr and an increase in cell number at 48 hr (Allegra and Lippman, unpublished data). As can be seen in the table, 50% (by volume) conditioned medium did not increase plating efficiency when added to IMEM-HS or IMEM-HS supplemented with nucleosides, nonessential amino acids, or fibroblast growth factor.

Thus far, the cells have been carried in IMEM-HS for 5 months and through 13 passages. No morphological changes have been observed during this period, and there has been no alteration in growth rate. Figures 1 and 2 compare control cells that have been growing continuously in MEM + 5% fetal calf serum with cells that have been growing in IMEM-HS for 3 months. No significant differences can be seen.

**DISCUSSION**

Growth of cells in a defined medium without serum supplementation has been a goal in tissue culture for many years. The challenge lies in creating a medium that is both biologically and chemically defined, allowing for the growth of a wide range of cell types. The ZR-75-1 cell line, which we have been working with, presents a unique challenge due to its dependency on estradiol for growth.

**Table 1**

| Effect of factors on plating efficiency of ZR-75-1 human breast cancer cells |
|-----------------|-----------------|
| **Medium**      | **Plating efficiency (%)** |
| IMEM            | 0.1             |
| IMEM plus bovine serum albumin | 0.1 |
| IMEM-HS         | 1.2             |
| IMEM-HS minus dexamethasone | 0.1 |
| IMEM-HS plus bovine serum albumin | 0.8 |
| IMEM-HS plus nucleosides | 1.1 |
| IMEM-HS plus nonessential amino acids | 1.0 |
| IMEM-HS plus fibroblast growth factor | 1.8 |
| IMEM-HS plus nucleosides, nonessential amino acids and fibroblast growth factor | 3.4 |
| IMEM-HS plus nucleosides, nonessential amino acids, fibroblast growth factor, and conditioned medium | 3.2 |
The establishment of the cell line in defined medium without the unknown effects of serum factors would better enable investigators to study the mechanisms of hormone interaction with breast cancer. Our preliminary data on the effect of the antiestrogen, Tamoxifen, are encouraging. In an environment in which extensive incubation in media devoid of estrogen (IMEM-HS minus 17β-estradiol) has occurred, we are able to show a significant inhibition of cell growth and precursor incorporation which suggests a Tamoxifen effect that may be independent of competition with estradiol. This system also allows the investigation of hormone stimulation in an environment in which the specific hormone studied can be eliminated for a fixed period of time. With this system, we are able to see large stimulatory effects of 17β-estradiol and insulin as measured by thymidine incorporation in cells which are grown for 7 to 10 days in IMEM-HS minus estradiol and insulin. The conversion of cells from a stable nondividing state to exponential growth is a much closer approximation of hormone-dependent tissue than the relatively paltry stimulation usually observed in culture. The attainment of these effects of hormones may be important in allowing investigators to isolate specific cellular products induced by hormone.

Interestingly, none of these hormonal components, either alone or in any combination attempted by us, permits growth of another human breast cancer cell line (MCF-7).

Finally, the ZR-75-1 cell line growing in IMEM-HS may become a useful system to study the effects of single drugs, drug combinations, and hormone and drug interactions in human breast cancer. It provides a growing cell system, free of serum contamination, for the study of drug interaction. It allows for the study of mechanisms of drug action without the presence of nucleosides which are present in serum (17). The system may be easily modified by the addition of purines, pyrimidines, amino acids, and rescue agents such as folinic acid in order to evaluate the mechanisms of drug action and drug toxicity. Furthermore, the growth rate of the system can be altered by the omission of a single hormone, allowing one to study the relationship of drug action and growth rate.

The major problem with the system, thus far, lies in the low plating efficiency. At present, the production of large quantities of cells is not practical; however, in studying hormone or drug interactions, this is not a problem, since it is possible to plate the cells in charcoal-treated calf serum and change to IMEM-HS as soon as the cells become adherent. This low plating efficiency has also been noted in many of the other cell lines that have been adapted to

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defined medium. The HTC cells growing in IMEM-ZO reported by Thompson et al. (19) have a plating efficiency of 0.1 to 1%. The HTC cells were fastidious in their requirements for gentle handling and quality of medium. Burks and Peck (4) also report a low plating efficiency for their primary bone cell cultures in serum-free medium. They found their plating efficiency to be density dependent, with the cells exhibiting a low plating efficiency and a failure to proliferate at a low density.

In summary, the ability to grow the ZR-75-1 human breast cancer cell line in a defined serum-free medium supplemented with hormones and transferrin will be advantageous to the study of hormone interaction with human breast cancer.

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REFERENCES


Fig. 1. ZR-75-1 human breast cancer cells. Cells growing continuously in MEM plus 5% fetal calf serum. × 220.

Fig. 2. ZR-75-1 human breast cancer cells. Cells growing continuously for 3 months in IMEM-HS. × 220.
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