Effect of Estrogen and Antiestrogen on the Human Breast Cancer Cell Line MCF-7 Adapted to Growth at Low Serum Concentration

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ABSTRACT

The human breast cancer cell line MCF-7 has been adapted to long-term growth at 0.5 and 0.05% fetal bovine serum. Free cytoplasmic and filled nuclear estrogen receptors were found in cells grown at 5, 0.5, and 0.05% fetal bovine serum. Cells grown in medium with 0.05% dextran-charcoal-treated serum contained cytoplasmic receptors but no filled nuclear receptors, indicating that this medium did not contain biologically active concentrations of estrogen. Addition of estradiol to the medium translocated the cytoplasmic receptor to the nucleus and stimulated estrogen receptor synthesis but did not increase the growth rate. The antiestrogen tamoxifen (TAM) inhibited cell growth at all serum concentrations investigated, at least in part by a reduction of the growth fraction. The sensitivity to TAM was highest at low serum concentrations. The effect of TAM could be reversed by estradiol at TAM concentrations of $10^{-8}$ M or lower.

It is concluded that estradiol does not have a direct growth-stimulatory effect on our MCF-7 cells in monolayer cultures although the cells contain functional estrogen receptors and growth of the cells in athymic mice requires estrogens. TAM has an estrogen-competitive, inhibitory effect on the growth of MCF-7 cells at concentrations lower than $10^{-8}$ M. At higher concentrations, the growth inhibition is unspecific and noncompetitive by estradiol.

INTRODUCTION

The presence of hormones in the serum required in tissue culture media complicates the demonstration of specific hormone effects on growth of cells in culture. Attempts to overcome this problem have included the use of charcoal-treated serum or serum-free medium. Charcoal-treated serum has been used in many studies on steroid hormone action since steroid hormones can be adsorbed to the charcoal and easily removed from the serum by centrifugation (13). However, it is well known that the nonsterrogenic compound, estrone sulfate, is not completely removed by the charcoal treatment and that some cells, e.g., MCF-7 cells, convert estrone sulfate to estrone (31). Therefore, medium with charcoal-treated serum may not be a sufficiently estrogen-depleted medium for MCF-7 cells, unless the serum concentration is very low. Serum-free medium has been used in 1-week growth experiments with MCF-7 cells propagated in 10% FBS (15). However, we have found that a shift from high to low serum concentrations influences the growth of cells so dramatically that we consider this approach unsatisfactory for the study of hormone action. In addition to using medium with FBS, we have therefore adapted MCF-7 cells to growth at low serum concentrations in order to study the effect of estradiol and TAM in cultures under stable growth and at low estrogen concentrations. We have used the presence of filled nuclear estrogen receptors in the cells as a marker of biologically active concentrations of estrogens in the medium and the stimulation of progesterone receptor synthesis as an indicator for an intact estrogen receptor mechanism.

MATERIALS AND METHODS

Cell Culture. MCF-7 is a cell line originating from a pleural effusion containing tumor cells from a mammary adenocarcinoma (26). The Human Cell Culture Bank, Mason Research Institute, Rockville, MD, has kindly supplied us with MCF-7 cells. The cells were propagated in plastic T-flasks (Falcon, Oxnard, CA; Nunc, Roskilde, Denmark) in growth medium composed of Eagle's minimum essential medium supplemented with 2 mA glutamine, 0.1 mA nonessential amino acids, insulin (6 ng/ml); either porcine, from Nordisk Insulin, Copenhagen, Denmark, or bovine, from Collaborative Research, Waltham, MA), and 5% heat-inactivated FBS. When serum concentrations of 0.5% or lower were used, the growth medium contained Dulbecco's minimal essential medium: Ham's F-12 medium (1:1) supplemented with 2 mA glutamine and insulin (6 ng/ml). In the experiments, growth medium was replaced by experimental medium from Day 2. The experimental medium consisted of growth medium supplemented with $10^{-8}$ M hydrocortisone and prolactin (1 ng/ml) (12). When indicated, FBS was used in the experimental medium in order to diminish the content of estrogens. No change in the growth rate was observed after shifting from FBS to the same concentration of FBS. The concentrations of estrogens in treated and untreated serum are shown in Table 1.

At each subcultivation, 0.25% trypsin (Difco Laboratories, Detroit, MI) plus 1 mA EDTA was used to detach the cells. At 0.5% serum concentrations or lower, 0.1% trypsin plus 0.4 mA EDTA was used; at 0.05% FBS, soybean trypsin inhibitor (Sigma Chemical Co., St. Louis, MO; 50 $\mu$g/ml) was added to the cell suspension before centrifugation. The cells were subcultured once per week with inocula of 5 to $10^3$ cells/sq cm.

HBL-100 originated from cells in normal human milk (21). The cells were a gift from the Breast Cancer Task Force Cell Culture Bank (Worcester, MA). The cells were propagated in McCoy's Medium 5A supplemented with 0.5% FBS. They do not contain estrogen receptors. Both cell lines were regularly tested and found to be free of Mycoplasma.

Preparation of Flasks Coated with Polysine. Plastic T-flasks were coated with polysine according to the method described by McKeehan and Ham (18).

Preparation of Flasks Coated with Collagen. Collagen IV (human placental collagen; Sigma) was sterilized by irradiation and dissolved in 0.5 M acetic acid. The collagen solution was dialyzed against distilled water for 48 hr. The coating procedure was carried out according to the method of Schor and Court (22).

Growth Curves. All growth studies with adapted cells were carried...
One receptor followed the standards for the assessment of hormone receptors in human breast cancer (8). Tritiated ORG-2058 (Radiochemical Centre; specific activity, 48 Ci/mmol) was used as ligand.

**Protein**. Protein content was measured by the Bio-Rad method using Kabi human serum albumin (Kabi Diagnostica, Stockholm, Sweden) as standard (3). The protein concentration for receptor determinations by the hydroxylapatite assay was 0.2 mg/ml or higher, whereas the protein concentration for progesterone receptor determination was 1 to 3 mg/ml.

**RESULTS**

MCF-7 cells routinely propagated in 5% FBS were successfully adapted to growth at 0.5 and 0.05% serum. The growth rate, as roughly evaluated from the N/F ratio (Chart 1), decreased 2 weeks after the cells were shifted from 5% to 0.5%, and growth continued at a lower rate. After six months, the growth rate at 0.5% was equal to that at 5% serum. When the serum content of the medium was decreased from 0.5 to 0.05%, cell growth almost stopped and thereafter gradually increased. The introduction of polylysine-coated flasks further improved the growth by increasing the plating efficiency of the cells. A higher cell plating was also obtained by diluting the trypsin-EDTA solution to a concentration of 0.1% trypsin and 0.4 mM EDTA and by adding soybean trypsin inhibitor (50 µg/ml) at each subcultivation.

In order to investigate whether adaptation to low serum concentrations selectively permitted propagation of cells devoid of estrogen receptor, receptor determinations were carried out on cells maintained at 5, 0.5, and 0.05% FBS and FBS-DC. Cytoplasmic receptors were found in cells at all serum concentrations. Filled nuclear receptors were found in cells at 5, 0.5, and 0.05% FBS and at 5% FBS-DC. The amount of filled nuclear receptors at 0.5% FBS-DC was close to the detection limit (10 fmol/mg protein) and undetectable at 0.05% FBS-DC (Table 2). Cells grown at 0.05% FBS-DC with 10^(-6) M estradiol for 6 days had no free receptors in the cytosol, and 114 fmol filled receptors/mg protein in the nucleus. The progesterone receptor level in these cells increased 13-fold after growth in the presence of estradiol for 6 days (65 fmol/mg protein in control culture and 875 fmol/mg protein after estradiol treatment).

No stimulatory effect on cell growth could be demonstrated at any of the serum concentrations investigated when estradiol was added to the medium at concentrations ranging from 10^(-11) to 10^(-7) M (Chart 2). The slightly higher growth rate at 5% FBS-DC with estradiol was not reproducible in ensuing experiments. Cells
Table 2

<table>
<thead>
<tr>
<th>Serum concentration (%)</th>
<th>FBS (fmol/mg protein)</th>
<th>FBSocc (fmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RC</td>
<td>RNE</td>
</tr>
<tr>
<td>0.05</td>
<td>61 ± 17 (6)</td>
<td>91 ± 25 (7)</td>
</tr>
<tr>
<td>0.5</td>
<td>88 ± 45 (5)</td>
<td>79 ± 5 (5)</td>
</tr>
<tr>
<td>0.05 + 10^{-6} M estradiol</td>
<td>152 ± 58 (5)</td>
<td>206 ± 18 (3)</td>
</tr>
</tbody>
</table>

*RC, free cytoplasmic estrogen receptor; RNE, filled nuclear estrogen receptor; ND, not detectable (less than 10 fmol/mg protein). writes mean ± S.D. Numbers in parentheses, number of experiments.
Estradiol was added to the medium 6 days before the cells were harvested for receptor determination.

Chart 2. Effect of estradiol on the growth of MCF-7 cells in vitro. A, 5% FBSocc; B, 0.5% FBS; C, 0.05% FBS; D, 0.05% FBSocc, collagen-coated flasks. O, control; □, 10^{-11} M estradiol; △, 10^{-9} M estradiol; △, 10^{-7} M estradiol. Lines are drawn between median cell number of 3 flasks (in C, only 2 flasks). When the results are too close to each other, only the median value of the experimental cultures is shown.

Cells propagated at different serum concentrations responded differently to TAM treatment (Chart 3). At 5% FBSocc, the cell number did not increase after 4 days of treatment with 5 × 10^{-6} M TAM, whereas a similar effect was obtained only at 10^{-6} M TAM with cells propagated in 0.5% FBS and at 5 × 10^{-7} M TAM in 0.05% FBS. Cells propagated at 5% FBS and shifted to and investigated at 0.05% FBS did not grow continuously during the experimental period; the effect of TAM was therefore difficult to evaluate (data not shown).

By adding estradiol together with TAM, it was possible to counteract the effect of TAM at concentrations lower than 10^{-6} M (Table 3). At 0.05% FBS, the effect of estradiol could not be evaluated since estradiol increased cell detachment.

In agreement with the growth studies, estradiol in concentrations ranging from 10^{-10} to 10^{-6} M did not influence the growth fraction of MCF-7 cells grown in 5% FBSocc (Chart 4). TAM decreased the growth fraction at 10^{-7} and 10^{-6} M. At 10^{-5} M TAM, the growth fraction could not be determined due to cell detachment. When estradiol was added together with TAM, the decrease in growth fraction could be prevented at concentrations less than or equal to 10^{-6} M.

The HBL-100 cell line that does not contain estrogen receptors is less sensitive to TAM than MCF-7 cells (Chart 5). At 10^{-6} M TAM, growth of HBL-100 cells is unaffected, whereas growth of MCF-7 cells is inhibited 50%. At 2 × 10^{-6} M TAM, the HBL-100 cells detach.

DISCUSSION

Ideally, a serum-free, defined medium should be used when the effect of hormones on cell growth in vitro is to be studied. Rapid adaptation of MCF-7 cells from medium with 10% FBS to serum-free conditions has been obtained by others by supplementing the medium with insulin, transferrin, epidermal growth factor, prostaglandin F2, fibronectin, and serum-spreading factor (Holmes' α1-protein) (1). However, in our hands, this medium did not permit continuous growth of MCF-7 cells directly transferred from medium containing 5% FBS mainly due to detachment of the cells. However, by decreasing the serum content of the medium in steps, MCF-7 cells have now been cultivated for more than 1 year at 0.05% FBS, and the cells contain estrogen receptors. An important improvement of the growth conditions was obtained by coating the flasks with poly-o-lysine. Recently, we have succeeded in transferring these cells from 0.05% FBS to serum-free medium and obtained continuous growth.

It has been discussed whether estradiol has a direct growth-stimulatory effect on MCF-7 cells (2). No effect of estradiol on the in vitro growth of MCF-7 cells has been found by several authors (4, 6, 11, 12, 14, 23), while others (5, 15, 17, 19) have reported a small stimulation when the estrogenic effect was investigated after 7 days in medium containing charcoal-treated serum or no serum. However, these experiments were performed on cells transferred to low-serum or serum-free medium immediately prior to the experiment. In our hands, such experimental conditions are unacceptable, since a dramatic decrease in the growth rate of the control culture occurs within 2 weeks. Furthermore, estrogens can be demonstrated in appreciable amounts in the cells more than 7 days after they have been transferred to estrogen-free medium (17, 27). In some experiments, we have shifted the cells from FBS to the same concentration of FBSocc without any influence on the growth rate for more than 2 weeks.
Estrogen Effect on Human Breast Cancer Cell Line

Table 3
Effect of TAM and TAM plus estradiol on the growth of MCF-7 cells cultivated at 5 and 0.5% FBS

<table>
<thead>
<tr>
<th>Serum concentration</th>
<th>TAM concentration (M)</th>
<th>Estradiol concentration (M)</th>
<th>Cell no. x 10^4 after 7 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>10^{-7}</td>
<td>4 x 10^{-5}</td>
<td>2.0 ± 0.4*</td>
</tr>
<tr>
<td></td>
<td>10^{-6}</td>
<td>4 x 10^{-5}</td>
<td>1.0 ± 0.3</td>
</tr>
<tr>
<td>0.5</td>
<td>10^{-7}</td>
<td>10^{-6}</td>
<td>1.6 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>10^{-6}</td>
<td>10^{-7}</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>5 x 10^{-7}</td>
<td>4 x 10^{-5}</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>5 x 10^{-7}</td>
<td>5 x 10^{-9}</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.8 ± 0.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1.5 ± 0.2</td>
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<td></td>
<td></td>
<td></td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.9 ± 0.1</td>
</tr>
</tbody>
</table>

* Mean ± S.D.

Chart 3. Effect of TAM on the growth of MCF-7 cells in vitro. A, 5% FBS; B, 0.5% FBS; C, 0.05% FBS. O, control; Δ, 2 x 10^{-9} M TAM; □, 10^{-7} M TAM; ⊗, 5 x 10^{-7} M TAM; ⊠, 10^{-6} M TAM; A, 3 x 10^{-6} M TAM; ⊠, 5 x 10^{-6} M TAM; •, 8 x 10^{-6} M TAM. Lines are drawn between median cell number of 3 flask.

chart4. Effect of estradiol (E) and/or TAM on the growth fraction in MCF-7 cultures grown in 5% FBS. For experimental procedure, see "Materials and Methods.'

Chart 4. Effect of TAM on MCF-7 cells (O) and HBL-100 cells (•). Cells were grown at 0.5% FBS for 7 days with and without the addition of TAM. Median cell number of 3 flasks are shown. Conc., concentration.

The cell detachment observed at high estradiol concentrations (10^{-5} M) is probably due to a nonspecific effect as suggested by others (16).

In previous studies using charcoal-treated serum or low serum, it cannot be excluded that the difficulties in demonstrating an effect of estrogen on growth of MCF-7 cells is due to the presence of small amounts of estrogens in the medium. Charcoal treatment does not completely remove estrone sulfate which...
may be converted to estrone by the cells (31). The concentration of estrone sulfate in the medium containing 0.05% FBS or 5% FBS_diss is 2 to 4 × 10^{-11} M which is sufficient to translocate estrogen receptor to the nucleus as shown by the presence of filled nuclear receptors (Table 2). However, we have found that no filled nuclear receptors could be measured in cells grown in 0.05% FBS_diss corresponding to 2 × 10^{-13} M estrone sulfate. However, no growth stimulation by estradiol was observed at 0.05% FBS_diss even though estradiol treatment results in translocation of the receptor to the nucleus and stimulation of progesterone receptor synthesis. This demonstrates that under our culture conditions estradiol has no direct growth-stimulatory effect on these estrogen receptor-containing MCF-7 cells, although estrogens are required for the growth of the MCF-7 cells transplanted s.c. into athymic mice (results not shown).

The apparent controversy between the estrogen dependence of MCF-7 cells in vivo (25) and the difficulties in demonstrating a growth response in vitro may be explained by the in vivo liberation of an estrogen-induced growth factor from cells outside the mammary gland. A factor stimulating the growth of a rat mammary tumor cell line in vitro has been found in extracts of uterus and kidney of estrogen-treated rats and not in extracts of ovariec-tomized rats (24). Alternatively, estrogens may increase the effect of another growth factor present in vivo and eliminated in the low-serum medium, since Page et al. (20) have found that growth of MCF-7 cells with 15% calf or human serum (and not with 0.5% serum) could be stimulated by estradiol. Finally, growth in vivo may be subject to inhibition that can be overcome by estrogens (23).

The growth inhibition produced by TAM in concentrations lower than 10^{-6} M was due to a decrease in the growth fraction and could be counteracted by the addition of estradiol, indicating that the effect is mediated by the estrogen receptor although the existence of specific TAM receptors has also been proposed (28, 29). In contrast to previous reports, our experiments have been carried out on cells during stable growth. Under such conditions, the sensitivity to TAM was found to be related to the serum concentration. This has been described by others previously in cultures shifted to low serum concentrations immediately before the experiment (4). Some authors have used TAM concentrations above 10^{-6} M. We found that TAM at concentrations higher than 10^{-6} M had an unspecific cytotoxic effect since the effects of TAM at these high concentrations could not be reversed by estradiol. Furthermore, concentrations of TAM above 10^{-6} M also inhibited growth of HBL-100 cells that lack the estrogen receptor. This result supports other reports on the unspecific cytotoxic effect of TAM at high concentrations (30).

We conclude that estradiol under our culture conditions does not stimulate growth of MCF-7 cells in vitro, although the cells contain a functional estrogen receptor mechanism and depend on estrogen for in vivo growth. Low concentrations of TAM have an estrogen-competitive, inhibitory effect on the growth of MCF-7 cells. At concentrations above 10^{-6} M, TAM has an unspecific, noncompetitive cytotoxic effect. The lower sensitivity to TAM at high serum concentrations may be due either to the higher content of estrogen in the serum or to binding of TAM to serum components.

ACKNOWLEDGMENTS

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