Growth Control of Human Mammary Cancer Cells (MCF-7 Cells) in Culture: Effect of Estradiol and Growth Factors in Serum-containing Medium

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ABSTRACT

The growth control of estrogen-dependent mammary cancer is very complex and only partly understood. The present study was undertaken in order to establish conditions for growth control of MCF-7 cells in monolayer culture with focus on the effect of estradiol-17β, fetal calf serum, and growth factors. The effect of charcoal-stripped fetal calf serum (CSFCS) on cell growth was dependent upon the presence of hormones or growth factors in the medium. In the presence of insulin (or insulin-like growth factor 1) and in the absence of estradiol-17β, increasing concentrations of CSFCS, 0.625–20%, produced a bell-shaped growth response curve. Serum concentrations >2.5% inhibited cell growth in the absence of estradiol-17β, whereas CSFCS in a dose-dependent way up to 10% stimulated growth in the presence of estradiol-17β (5 x 10⁻⁸ mol/liter). The growth inhibitory effect of CSFCS could not be demonstrated in the absence of insulin (or insulin-like growth factor 1) and estradiol-17β. CSFCS stimulated growth in a dose-dependent way in the presence of estradiol-17β and also in the absence of insulin. Both the putative growth inhibitor and stimulator were found to be heat stable and not dialyzable. Epidermal growth factor stimulated growth but was unable to eliminate the growth inhibitory effect of 5–10% CSFCS. Interleukin-1α inhibited MCF-7 cell growth in a dose-dependent way and produced a 75% reduction in cell number at a concentration of 5 x 10⁻⁸ mol/liter. This inhibition was almost totally overcome by estradiol-17β. It is concluded that serum appears to contain factors with both stimulatory and inhibitory effects on the growth of MCF-7 cells. The inhibitory effect can be eliminated by estradiol (5 x 10⁻⁸ mol/liter). In the presence of estradiol cell growth is stimulated by CSFCS in a dose-related way up to 5–10%. Taken together these data seem to indicate that estradiol stimulates cell growth in two principal ways: partly by eliminating the effect of an inhibitor, in support of a “negative hypothesis,” and partly by an effect whereby estradiol permits a growth stimulator in CSFCS to be expressed, in support of the “indirect positive hypothesis.”

INTRODUCTION

During recent years the study of growth control has shifted from almost exclusively being concerned with the effect of growth stimulators to include also the effect of growth inhibitors (1). The concept of growth control including both growth stimulators and inhibitors obviously provides a more effective way to understand the mechanism(s) by which one may forecast the effect of such therapy in a large group of patients. Although many different treatment modalities have been used and are still in use, most of them aim to remove estrogens from the blood (and tissue) of the patients or their effect at the level of the cancer cell.

There are two principal concepts or models to explain the estrogen-stimulated growth: (a) the positive hypothesis according to which estrogens exert a growth stimulation, either directly or indirectly, and (b) the negative hypothesis according to which estrogens stimulate growth by removing the effect of a growth inhibitor. This second mode of operation may also be either direct or indirect. One may add that a combination of these, with both negative and positive effects, may possibly be operating.

MCF-7 cells have been used extensively as a model system for such studies, since they have been shown to be estrogen responsive and contain estrogen receptor (4). Soto and Sonnenschein (5) have strongly promoted the negative hypothesis and shown that MCF-7 cells grown in monolayer culture are growth inhibited by fetal calf serum, and the growth inhibition is overcome by estrogens. Dell’Aquila et al. (6) have characterized a factor from human serum that inhibits cell growth of the MCF-7 cells, and Markaverich et al. (7, 8) have, in a number of publications, presented evidence for the presence of a ligand in fetal calf serum which binds to the nuclear type II estrogen receptor and inhibits estrogen-dependent growth. Lippman et al. (9, 10) have strongly supported the indirect positive hypothesis and presented evidence that TGFα or EGF may be produced by estrogen-dependent cells and acts as a growth stimulator in an autocrine manner. The present work was undertaken in order to further establish the conditions for growth control of MCF-7 cells in monolayer culture by estrogens and serum.

MATERIALS AND METHODS

MCF-7 cells were obtained from the American Type Culture Collection (Rockville, MD) and grown in monolayer in Nunc culture dishes (six wells of 9.6 cm²/plate). Cells were maintained in Dulbecco’s minimum essential medium (Gibco) supplemented with 5% FCS (Gibco Bio-Cult, Paisley, Scotland), glutamine (2 mmol/liter), streptomycin (100 μg/ml) and penicillin (100 IU/ml), and phenol red as pH indicator (standard medium). During growth experiments cells were grown in the absence of the pH indicator phenol red (11), and CSFCS replaced the normal FCS (experimental medium). The charcoal stripping was performed by shaking 200 ml of FCS with 1.0 g of Norit A charcoal and 0.1 g of dextran (Sigma Chemical Co., St. Louis, MO) at 37°C for 3 h. The serum was then centrifuged at 9000 x g for 15 min. Growth factors and inhibitors added to the medium were: insulin, EGF, and estradiol-17β (obtained from Sigma), interleukin-1α and IGF-1 (from Boehringer-Mannheim, Mannheim, Germany), and TGFβ (gift from Sangvino, Bergen, Norway). Cells were seeded at a concentration of about 150,000 cells/ml of standard medium/well. After 24 h this medium was changed to experimental medium (day 0) and the growth
experiment continued for 5 days. Cell number was determined in a Coulter Counter after detachment of the cells by trypsination. The cell number presented represents the mean of six replicate wells ± SD. Statistical analysis was done with Student's t-test.

[^3H]Thymidine incorporation was assayed by pulse labeling for 2 h at 37°C prior to harvest, with 2.3 μCi [^3H]thymidine (Amersham, Amersham, United Kingdom) of specific activity 1.2 Ci/mmol. The thymidine-containing medium was removed and cells were washed twice with ice cold medium and treated three times with 1 ml of ice cold 10% trichloroacetic acid and twice with 1 ml of ice cold methanol. One ml of 1 N NaOH was added; the contents of the wells were transferred to tubes and wells were washed with 1 ml of water which was added to the tubes. Aliquots were taken for determination of ^3H in a liquid scintillation spectrometer, and samples were also analyzed for protein content by the method of Lowry et al. (12).

**RESULTS**

The effect of increasing concentrations of CSFCS on cell growth is presented in Fig. 1. Cell growth was stimulated by increasing serum concentration up to 1.25-2.5% in the absence of estradiol and in the presence of 50 nM insulin, and cell doubling time was reduced from 63-54 h. Further increase in CSFCS concentration attenuated cell growth, and cell doubling time increased to 69 h at 10% CSFCS. Estradiol at a concentration of 5 × 10^{-10} M stimulated cell growth at all serum concentrations, and furthermore, the presence of estradiol abolished the growth inhibitory effect of increasing serum concentrations. The cell-doubling-time in the presence of estradiol was reduced by increasing CSFCS from 52 h at 0.625% to 36 h at 10%. The growth inhibitory effect of serum was not attenuated by heating CSFCS to 56°C for 60 min, nor did this treatment modify the response to estradiol (Fig. 2). Dialysis of fetal calf serum did not remove the growth inhibitory action of the serum (data not shown).

The effect of increasing concentrations of CSFCS was tested at different levels of estradiol in the medium up to 1 × 10^{-9} mol/liter (Fig. 3). In this experiment a different batch of fetal calf serum was used. It can be seen that growth inhibition was augmented up to 20% CSFCS. Growth stimulation by CSFCS in the presence of the highest level of estradiol reached a plateau at 10%.

The growth stimulatory effect of estradiol could be demonstrated within about 8 h of treatment when a significant increase in thymidine incorporation could be observed (Fig. 4). The greatest thymidine uptake was observed after about 17-22 h, when the incorporation reached about 245% of control levels. The hypothesis of an indirect stimulation by estradiol on cell growth involves a paracrine or autocrine secretion of some growth factor induced by the estradiol treatment, and there is some evidence of such an effect by estradiol (13). One candidate growth factor is TGFα, and the effect of EGF was therefore
tested on the growth inhibition induced by increasing serum concentrations. It can be seen in Fig. 5 that EGF (25 ng/ml) was able to stimulate growth of MCF-7 cells at all serum concentrations tested. Increasing CSFCS from 2.5–5.0 and 10.0% increased the cell-doubling time from 61–74 and to 101 h, respectively. The corresponding cell-doubling times in the presence of 25 ng/ml EGF were 53, 58, and 70 h. EGF was, however, unable to eliminate the pattern of serum-induced growth inhibition and mimic the estradiol effect on serum-induced growth stimulation. In addition to the experiments presented in Fig. 5 larger doses of EGF were tried, up to 100 ng/ml, and also additions of 5 to 25 ng/ml were repeated every 12 h, but the growth inhibition by increasing serum concentration could not be prevented by EGF (data not shown).

EGF exerts a growth stimulatory action by interacting with the EGF receptor on the plasma membrane whereby the receptor undergoes autophosphorylation (14). This autophosphorylation may be inhibited by EGF receptor kinase inhibitors which will block the EGF-dependent cell proliferation (15). A number of such EGF receptor kinase inhibitors have been synthesized, some of which are very specific for the EGF receptor with hardly any effect of the IGF-1 receptor (15).

Such an inhibitor (RG 50863, Rorer Biotechnology) was tested on MCF-7 cells stimulated with EGF and estradiol (Fig. 6). It can be seen that the modest growth stimulatory effect of EGF was totally abolished with 40 μmol/liter RG 50863, and a moderate inhibition of estradiol-stimulated growth was observed.

The growth inhibitory effect of increasing serum concentrations was tested at various levels of insulin and/or IGF-1. Insulin stimulated cell growth in the presence of serum (Fig. 7), and IGF-1 was more potent than insulin on a molar basis (data not shown). In the absence of insulin cell growth was very modest, and no inhibitory or stimulatory effect was observed with increasing serum concentrations. In the presence of increasing insulin concentrations increasing serum concentrations exerted attenuated growth (Fig. 7). Growth stimulation by 50 nmol/liter of insulin was almost totally abolished in the presence of 10% CSFCS. This serum effect could be reproduced in the presence of IGF-1, although at lower IGF-1 concentrations (data not shown). Whereas no effect of CSFCS on cell growth was observed in the absence of insulin and estradiol (Fig. 7), increasing concentrations of CSFCS stimulated growth in the presence of estradiol at a concentration of 5 × 10⁻¹⁰ mol/liter even in the absence of any insulin (Fig. 7).

The nature of the putative growth inhibitor in serum is not known. Various types of lymphokines, peptides produced by the immune system, have been shown to interfere with cell growth, and IL-1α is one of these (16). In Fig. 8 it can be seen that IL-1α in a medium of 2.5% CSFCS, containing 50 nmol/liter of insulin, inhibited significantly the growth of MCF-7 cells at a concentration of 0.1 pmol/liter, and at 500 pmol/liter the cell number was reduced to about 25% of control level. In the presence of estradiol (5 × 10⁻¹⁰ mol/liter) the growth inhibitory effect of the IL-1α was greatly reduced but not totally abolished (Fig. 8). At the highest concentration of IL-1α tested, 5 × 10⁻¹⁰ mol/liter, the cell number in the presence of estradiol was still 25% higher than control cultures without IL-1α and estradiol.

The effect of another potential growth regulator, TGFβ, was
tested in both the absence and presence of estradiol at a concentration of $5 \times 10^{-10}$ mol/liter. Concentrations of TGF/3 between 0.3 and 30 ng/ml were used, but no effect of TGF/3 could be observed (data not shown).

**DISCUSSION**

The growth response of MCF-7 cells to increasing concentrations of CSFCS in estradiol-free medium revealed a bell-shaped curve (Fig. 1). Concentrations of CSFCS up to about 2.5% exhibited increased growth, whereas higher concentrations induced growth inhibition. This growth inhibition of higher serum concentrations is in agreement with Soto and Sonnenschein (5). Also in agreement with their observations is the finding that estradiol was able to abolish the growth inhibitory effect of increasing concentrations of CSFCS. This finding fits well within the negative hypothesis; estradiol would remove or counteract a growth inhibitor present in CSFCS (3).

In order to substantiate the presence of a growth inhibitor in serum one would prefer to be able to define a serum dose that is able to saturate the effect of estradiol. This is obviously very difficult since the serum effect is as complex as the data show, being both inhibitory and stimulatory. The results in Fig. 3 do, however, demonstrate that the putative growth inhibitor in serum saturates the estradiol stimulation. These results also indicate that the level of inhibitor may vary from batch to batch of FCS. It is obvious from the data presented in Fig. 7 that the growth inhibition of CSFCS was dependent upon the concentration of insulin or IGF-1 in the medium. In the absence of insulin (or IGF-1) increasing concentrations of CSFCS had no effect on cell growth even in the absence of estradiol; in the presence of estradiol CSFCS stimulated cell growth. Therefore, CSFCS may function as a growth stimulator or a growth inhibitor or have no effect on the growth of MCF-7 cells, depending entirely on the conditions under which the cells are grown. It should be pointed out that estradiol stimulated growth under all the conditions tested, also when CSFCS did not exert any growth inhibitory effect (Fig. 7). This observation calls for other explanations in addition to the negative hypothesis to explain the growth stimulatory effect of estradiol.

The indirect positive hypothesis of estradiol-stimulated growth includes paracrine or autocrine secretion of growth factors in response to estradiol treatment (2). Both IGF-1 and EGF or TGF/3 are candidates for such a role. Although EGF did stimulate the growth of MCF-7 cells, this growth factor could not replace estradiol in order to overcome the growth inhibition exerted by CSFCS (Fig. 5). This observation seems to exclude EGF or TGF/3 from playing the role of the only estradiol-induced auto- or paracrine-secreted growth factor. Other experimental systems have also suggested that EGF alone (or TGF/3) is an unlikely candidate for such a role (17). The inhibitory effect of the EGF receptor kinase inhibitor on estradiol-stimulated growth (Fig. 6) does suggest that EGF (or TGF/3) is partly involved in the estradiol stimulation.

Huff et al. (18) presented strong evidence that estradiol was able to induce secretion of IGF-1 in MCF-7 cells in culture, and it is evident that insulin or IGF-1 are potent mitogens for MCF-7 cells at all concentrations of CSFCS tested (Fig. 7). If, however, estradiol-induced growth was mediated via autocrine secretion of IGF-1, one would expect no additional effect of estradiol at a concentration of insulin which had reached a plateau. The fact that additional growth stimulation was observed with estradiol at a concentration of insulin at which no further insulin stimulation could be observed indicates that neither of these two peptide growth factors alone or in combination seem to mediate the overall estradiol-induced growth stimulation.

The present study confirms the presence of an activity in fetal calf serum that inhibits growth of MCF-7 cells in culture in the absence of estradiol (5), and estradiol is able to abolish this growth-inhibiting activity. Also, human sera from patients with breast cancer contain a growth inhibitor, and estradiol is able to abrogate this inhibitor (19). Whether this activity in serum is of significance under physiological conditions remains to be demonstrated.

In this context one might mention the multihormonal regulation of the progesterone receptor in MCF-7 cells (20). It was observed that progesterone receptor in MCF-7 cells was inhibited by increasing concentrations of charcoal-stripped serum in the absence of estrogen or phenol red, whereas serum stimulated progesterone receptor in the presence of estradiol. The striking similarity between this effect on progesterone receptor and the present effect on cell growth may suggest that these effects involve similar mechanisms.

It is clearly demonstrated in the present study that, when the inhibitory effect of CSFCS is abolished by estradiol, serum exerts growth stimulation. Whereas growth inhibition by serum requires insulin (or IGF-1) to be expressed, growth stimulation by serum in the presence of estradiol was observed also in the absence of insulin or IGF-1 in the medium (Fig. 7). Cell growth of MCF-7 cells in culture is thus under complex control, and the effect of a single factor, stimulatory or inhibitory, is dependent upon the presence or absence of other growth factors or inhibitors, and one may assume that the growth control under in vivo situations is not less complex. The hypothesis that the growth stimulation of estradiol is exerted solely via autocrine or paracrine secretion of TGF/3 (or EGF) is not supported by the present study, since EGF could not replace estradiol to overcome the growth inhibition of CSFCS. It should, however, be noted that the EGF receptor kinase inhibitor partly inhibited estradiol-stimulated growth (Fig. 6).

It has been postulated that TGF/3 and TGF/5 play important roles as para- or autocrine growth regulators of estrogen-dependent mammary cancer cells, with TGF/3 as the growth stimulator and TGF/5 as the growth inhibitor (21). We were unable to demonstrate that TGF/5 had any inhibitory effect on growth of MCF-7 cells in culture at TGF/5 concentrations between 0.3 and 30 ng/ml, which is in agreement with other investigators (22). This lack of effect was observed both in the presence and in the absence of estradiol ($5 \times 10^{-10}$ mol/liter). Furthermore, the effect of EGF as a growth stimulator was modest which seems to exclude this substance as the estradiol-mediated growth stimulator.

On the basis of the present results one may conclude that estradiol-stimulated growth of MCF-7 cells seems to involve both the negative hypothesis and the positive hypothesis discussed in the "Introduction." Of particular interest is the growth-promoting effect of higher concentrations of charcoal-stripped serum which seems to require the presence of estradiol to be expressed. There are many possible explanations for this phenomenon and it is considered futile to speculate on alternative mechanisms on the basis of the present data.

Serum is a complex mixture containing a large number of unknown substances. From the present study it appears that the complexity of the experimental situation is so great that it must be simplified before one can make any definite conclusions.
as to the mechanisms by which defined substances, such as growth factors and inhibitors, exert their functions on the cells in culture. Obviously, the effect of a single substance is dependent upon the presence of other factors in the system. One approach is, therefore, to study growth under defined conditions in totally serum-free media. Such studies are now in progress in various laboratories.

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