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INFLUENCE OF CULTURE CONDITIONS ON THE ESTROGENIC CELL GROWTH STIMULATION OF HUMAN BREAST CANCER CELLS

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Summary—17β-Estradiol is a potent mitogen for hormone-dependent cell lines (MCF-7, T47D and ZR 75.1). However, the degree of hormone sensitivity is very much influenced by culture conditions. In order to understand which factors modulate estrogenic effects on cell growth, four different culture conditions were used: (a) medium with dextran-coated charcoal-treated fetal calf serum (DCC-FCS); (b) medium with dextran-coated charcoal-treated growth factor-inactivated serum (DCC-FCSd); (c) serum-free medium, after a 24-h incubation with serum to allow cell attachment; and (d) serum-free medium on collagen IV-treated plates. In all cell lines the highest cell growth stimulation was achieved when estradiol was added in the presence of 5% DCC-FCS, whereas reducing or removing serum from the culture medium resulted in a decrease in cell proliferation stimulation. We postulate that serum contains some still unknown components able to modulate the degree of estrogenic action in endocrine-dependent breast cancer cell lines.

INTRODUCTION

The growth of some human breast cancer cells is under hormonal control. Estrogens have a promoting effect on estrogen receptor (ER)-positive cell lines [1–7] and their presence is required for the formation of tumors from such cells in ovariectomized athymic mice [8]. Cell lines that lack estrogen and progesterone receptors are not affected by estradiol but constitutively produce their own growth factors [9, 10] and grow autonomously. Although the mechanism by which the hormone stimulates growth is not completely understood, it seems clear that stimulation of growth factor expression [11, 12] is an important estradiol-induced process. These peptides, like TGF-α, may act in an autocrine and paracrine manner. It has also been postulated that estradiol plays a role in the neutralization of an inhibitory substance contained in the serum [13]. The mitogenic effect of estrogens has been thoroughly investigated in breast cancer cell lines, but experimental results are still controversial. This can be explained by the different culture conditions used (presence or absence of serum), by the selection of different clones in the various laboratories [14], and by the putative genetic instability of cell lines [15].

The aim of the present study was to understand the mechanism of estrogen action in human breast cancer cell lines using four culture conditions: (a) medium with dextran-coated charcoal-treated fetal calf serum (DCC–FCS); (b) medium with DCC-treated and growth factor-inactivated fetal calf serum (DCC-FCSd); (c) serum-free conditions after an initial 24-h incubation with serum-containing medium to provide cell attachment; and (d) completely serum-free conditions on collagen IV-treated plates.

MATERIAL AND METHODS

Dulbecco's minimal essential medium (DME) and Ham's F_{12} medium (1:1) (DMEM/F_{12}) without Phenol Red, glucose, transferrin, sodium selinite, bovine serum albumin V, 3,3′,5-triiodothyronin, glutathione, 17β-estradiol, ethanolamine, collagen type IV, radioinert 17β-estradiol, Norit A and dextran T 70 were purchased from Sigma Chemical Co. (St Louis, Mo.). Penicillin, streptomycin, amphotericin B were from Whitaker M.A. Bioproducts

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Abbreviations: ER: estrogen receptor; FCS: fetal calf serum; DCC-FCS: dextran-coated charcoal fetal calf serum; DCC-FCSd: dextran-coated charcoal-treated growth factor-inactivated fetal calf serum; PBS: phosphate-buffered saline.

(Walkersville, Md), and fetal calf serum (FCS) was from Biological Industries (Beth Haemek, Israel). Trypsin/EDTA (0.05%, 0.02%) was from Seromed (Biochrom K.G., Berlin F.R.G.), and insulin was from Collaborative Research (Two Oak Park, Bedford, Mass). 16α[125 I]iodo-estradiol (200 Ci/mmol) was from Radiochemical Centre (Amersham, England). All other reagents were analytical grade.

Cell culture

Human breast cancer cells were kindly provided by K. Horwitz, University of Colorado at Denver (T47D, MCF-7), by G. Daxenbichler, University of Innsbruck (BT-20), by M. J. Siciliano, MD Anderson Hospital and Tumor Institute, Houston (MDAMB 231), and by F. Fox, UCLA, Los Angeles (ZR 75.1). The cell lines were maintained in DMEM/F12 without Phenol Red supplemented with 5% FCS, 4 g/l glucose, streptomycin (50 μg/ml), penicillin (50 U/ml) and amphotericin B (250 μg/ml) under 100% humidity and 5% CO_2/95% air at 37°C. For subculturing in serum-free conditions, basic medium was supplemented with 10 μg/ml transferrin, 10 ng/ml sodium selinite, 20 μg/ml glutathione, 0.3 nM 3,3′,5-triiodothyronine, 1 mg/ml bovine serum albumin V, and 50 μM ethanolamine (MOM-3), and all plastic surfaces of the plates were coated with collagen type IV (4 μg/cm^2) for cell attachment. Estrogens in FCS were removed by stripping with charcoal (0.5% Norit A, 0.05% dextran T70) for 3 h at 37°C as described by Soto and Sonnenchein [7]. DCC–FCSd was prepared essentially according to Van der Burg *et al.* [1]. FCS was incubated with 100 mM dithiothreitol for 2 h under stirring at room temperature. After an ultracentrifugation at 100,000 *g* for 20 min, the supernatant was dialyzed at 4°C against a 50-fold excess of phosphate-buffered saline (PBS) without Ca^{2+} and Mg^{2+}. The following day the solution was treated with iodoacetamide (5 g/l) for 2 h at room temperature while stirring. Another dialysis was performed for two days against PBS and for one day against PBS containing 0.9 mM $CaCl_2$ and 0.5 mM $MgCl_2$. The solution was centrifuged at 100,000 *g*, and the supernatant was sterilized through a Sterivex-GV 0.22 μm filter (Millipore, Bedford, Mass). Such treatment destroys the disulfide bridges of polypeptides, including several growth factors, and neutralizes their biological activity.

All experiments were carried out in the absence of Phenol Red to prevent background estrogenic stimulation [16]. A 100 μM ethanolic 17β-estradiol solution was prepared and diluted in the medium to keep the final concentration of ethanol below 0.01%.

EXPERIMENTAL

Effect of 17β-estradiol on cell proliferation

Cells were harvested using Trypsin/EDTA and plated in 24-well plates at a density of 40,000–100,000 cells/well, depending on the cell line and culture conditions. After a 24-h incubation, medium was removed and substituted by one supplemented with various estradiol concentrations (10^{-7}–10^{-12} M). Culture medium was changed every 3 days. At the end of the experiment (days 7–8) DNA content in each well was determinated by Burton's assay [17]. Cells were grown in four culture experimental media: (a) DMEM/F12 supplemented with 5% DCC-FCS; (b) DMEM/F12 supplemented with 0.2% bovine serum albumin V and 5% DCC-FCSd; (c) MOM-3, after an initial 24-h incubation with 5% DCC-FCS-containing medium; and (d) MOM-3 on collagen type IV-treated plates (4 μg/cm^2) to allow cell attachment.

Interaction between 17β-estradiol and insulin

The experiment was performed as the former one, whereby estradiol (10^{-8} M) was tested alone or in combination with 1 ng/ml insulin for its influence on the growth of MCF-7 cells. Only two culture conditions were tested: (a) in the presence of 5% DCC-FCSd and (b) under completely serum free-conditions (MOM-3), to avoid any growth factor interference.

Cytosolic estrogen receptor assay

Cells were plated in 25 cm^2 flasks at an initial density of 600,000 cells and grown in the presence of 5% FCS for 7 days. The cells were harvested using Trypsin/EDTA and centrifuged at 600 *g*, and the pellet was stored at −20°C. Preparation of the cytosol and DCC-ER assay have been described previously [18].

RESULTS

Proliferation of the two hormone-independent cell lines (MDAMB 231 and BT-20) was not significantly influenced by 17β-estradiol under any of the four culture conditions (Fig. 1). Conversely, estradiol showed a dose-dependent

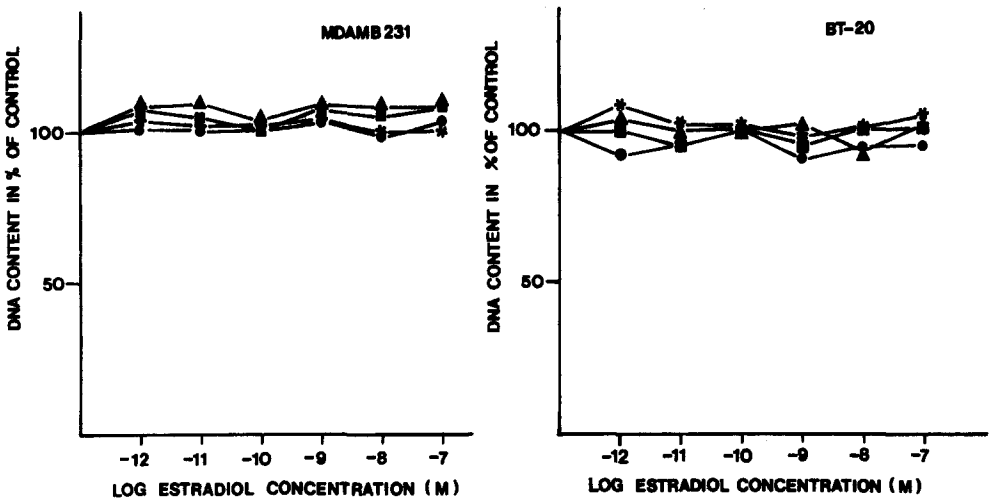

Fig. 1. Effect of different estradiol concentrations (10^{-7}–10^{-12} M) on proliferation of MDAMB 231 and BT-20 cells grown in DMEM/F12 supplement with 5% DCC-FCS (●), with 5% DCC-FCSd (■), in MOM-3 after a 24-h incubation in the presence of 5% DCC-FCS-containing medium (✱), and under completely serum-free conditions (▲). The amount of cellular DNA in each well was determined by Burton's assay after 6 days of estradiol treatment. Each point represents the mean of six wells expressed as the percentage of control. Variation coefficient was always below 10% and is not graphically indicated for clarity.

mitogenic action on cell proliferation of ER-positive cells (MCF-7, T47D and ZR 75.1). Each cell line responded in a different way, and growth stimulation was influenced by culture conditions. In all three cell lines the estradiol action was more pronounced in the presence of 5% DCC-FCS. T47D cells, which are characterized by a low ER content (Table 1), showed 300% growth stimulation in the presence of 1 nM 17β-estradiol in 5% DCC-FCS containing medium, 5% DCC-FCSd reduced the stimulation to 180%, and in serum-free conditions, the proliferative effect was minimal (about 130%) (Fig. 2). The values were obtained after 7 days of culture. The effect of estradiol (0.1 nM) on MCF-7 cell growth was similar (190–210%) under the following culture conditions: 5% DCC-FCS, 5% DCC-FCSd and without serum after an initial 24-h incubation with 5% DCC-FCS-containing medium. Under completely serum-free conditions, estradiol stimulation was weaker (150%) (Fig. 2). Estradiol was more effective on ZR 75.1 cells. In fact, under all four culture conditions, 1 nM 17β-estradiol caused a 3-fold increase in cell yield (Fig. 2).

When the culture medium was supplemented with insulin (1 ng/ml), the estrogenic action was not detectable (106% under serum-free conditions and 102% in the presence of 5% DCC-FCSd) (Fig. 3). Table 1 shows the ER content of each cell line. The two estrogen unresponsive lines (MDAMB 231 and BT-20) did not carry the ER, whereas maximal content was found in the ZR 75.1 cell line (78 fmol/mg protein). MCF-7 and T47D cells contained 52 and 26 fmol/mg protein, respectively.

DISCUSSION

We have investigated the effect of 17β-estradiol on *in vitro* growth of several human breast cancer cell lines. Different culture conditions were used in order to understand the mode of action on proliferation of hormone-dependent cell lines (MCF-7, T47D and ZR 75.1): such cells are characterized by expression of estrogen and progesterone receptors and by hormonal cell growth regulation. In contrast, the growth of hormone receptor-negative cell lines (MDAMB 231 and BT-20) were not influenced by the tested estrogen concentrations and were regulated by a completely different mechanism, probably because the cells produced their own growth factors constitutively. In fact, high amounts of TGF-α, IGF-II and PDGF-like molecules have been found in the conditioned medium of hormone-independent breast cancer cell lines [11, 12].

Although some laboratories failed to show the estrogenic stimulation on breast cancer growth [2–7], we observed in all of our culture systems, both in the presence and in the absence of serum, and estradiol-induced cell growth of

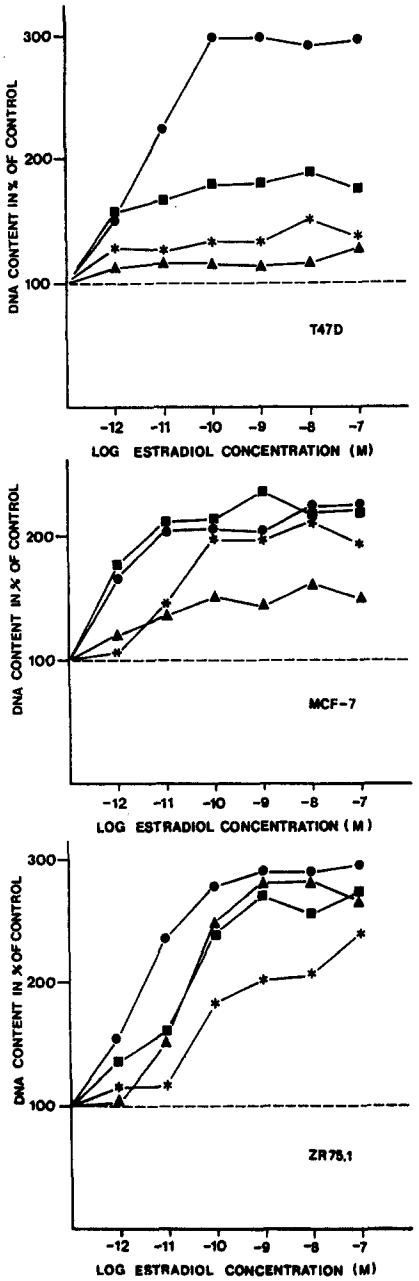

Fig. 2. Stimulatory effect of different estradiol concentrations (10^{-7}–10^{-12} M) on proliferation of T47D, MCF-7 and ZR 75.1 cells. The cells were grown in DMEM/F12 supplemented with 5% DCC-FCS (●), with 5% DCC-FCSd (■), in MOM-3 after a 24-h incubation in the presence of 5% DCC-FCS (✱), and under completely serum-free conditions (▲). The experiments were performed as described for Fig. 1. Each point represents the mean of six wells expressed as percentage of control. Variation coefficient was always below 10% and is not graphically indicated for clarity.

ER-positive cells. Each cell line was characterized by a different degree of sensitivity to estrogens, but all tested hormone concentrations stimulated cell proliferation. There was a trend towards a decrease in cell growth stimulation when serum was partially (only 24-h preincubation with 5% DCC-FCS) or completely removed from culture medium (in serum free conditions). In fact, estradiol was able to maximally increase proliferation only in the presence of DCC-treated serum. Under completely serum-free conditions only ZR 75.1 cells achieved the maximal growth rate even with lower estradiol doses (10^{-10}–10^{-9} M), which instead could not be reached with any of the tested estrogen concentrations (10^{-7}–10^{-12} M) in MCF-7 and T47D cells. It therefore seems possible that a serum component may modulate the estrogen action. Soto and Sonnenschein [13] postulated the presence of an inhibitory substance in the serum, called "estrocolyone", which was able to control cell growth. Its neutralization through binding with estradiol would allow the cell replication. This implies that no estrogen-induced stimulation should be seen in the absence of serum [7]. The hypothesis is not supported by our experimental results, since we observed an increase in cell number even under completely serum-free conditions. However, it should be noted that our serum-free medium did not contain any insulin. As our experiments showed (Fig. 3), insulin, which is usually added to serum-free medium as a supplement substance [19], can mask the action of 17β-estradiol. This may explain why some authors did not observe estradiol-induced proliferation in the absence of serum. At physiological concentrations, insulin causes an increased macromolecular synthesis and growth in human breast cancer cells [20]. Van der Burg *et al.* [1] reported a synergistic action for insulin and estrogen on two human breast cancer cell lines (MCF-7 and CG5) and demonstrated also that insulin, at high concentrations (10 μg/ml), bypasses the estrogen requirement. Butler *et al.* [21] demonstrated the ability of insulin to lower the ER levels in MCF-7 cells and concluded that such a decrease could be correlated with a reduced responsiveness to estradiol and antiestrogens. Other growth factors, for example EGF, bFGF and IGF-I, did not mask such stimulation. In fact, a synergistic interaction between these peptides and 17β-estradiol was observed [22].

Our data point out that the responsiveness of human breast cell lines to estrogens depends much on the culture condition used. Thus extrapolation of results obtained under a single culture condition should be done cautiously. Our data also support the existence of an unknown serum component that is able to modulate the

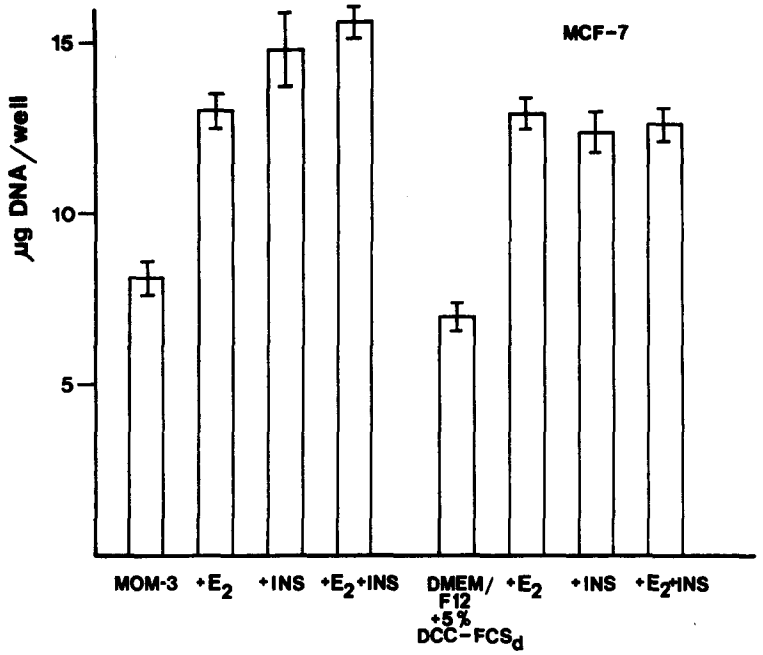

Fig. 3. Interaction between estradiol (10^{-8} M) and insulin (1 ng/ml) on proliferation on MCF-7 cells in completely serum-free conditions (MOM-3) and in DMEM/F12 supplemented with 5% DCC-FCSd. The experiment was carried out as described for Fig. 1. Each bar represents the mean of six wells expressed as μg DNA/well $\pm$SD.

Table 1. Cytoplasmic estrogen receptor concentrations in different human breast cancer cell lines

CELL type	ER content (fmol/mg protein)
MCF-7	52
ZR 75.1	78
T47D	26
MDAMB231	0
BT-20	0

action of estratiol on hormone-dependent cell lines. It can therefore be concluded that defined media may be useful for insight into the action and role of single substances, but that different culture conditions, at least in the presence of serum, should also be applied to include important and still unknown mechanisms of potentiation or quenching exerted by serum components.

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