Effects of Pharmacological Concentrations of Estrogens on Proliferation and Cell Cycle Kinetics of Human Breast Cancer Cell Lines in Vitro

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ABSTRACT

High dose estrogen therapy has been used effectively in the treatment of human breast cancer. To understand the mechanisms involved, the effects of high concentrations (5-100 μM) of estrogens were studied in estrogen receptor (ER) positive (T-47D and MCF-7) and ER negative (MDA-MB-330) human breast cancer cell lines in vitro. Inhibition of cellular proliferation was seen with the synthetic estrogen diethylstilbestrol (DES) at concentrations >10 μM in each of the three cell lines. In T-47D cells DES was shown by clonogenic survival assays to be cytotoxic. This effect was evident in both plateau phase and exponentially growing cultures, in contrast to the effects of the antiestrogen tamoxifen, which has minimal effects on plateau phase cells. The effects of DES on the proliferation of exponentially growing cultures were accompanied by changes in cell cycle parameters which included an increase in the percentages of S-phase, G2 + M, and polyplody cells and a corresponding decrease in the percentage of G0-G1 cells. These changes, which contrasted with the known effects of tamoxifen, were not seen in the non- or slowly cycling plateau phase T-47D cells. Such results are consistent with two mechanisms of action of high dose estrogens in vitro: a cell cycle phase-specific effect and cell cycle-independent cytotoxicity.

The stereoisomers 17α-estradiol and 17β-estradiol had similar potency to DES in inhibiting cell proliferation and inducing these changes in cell cycle parameters in both MCF-7 and MDA-MB-330 cells. The high-dose estrogen effect was ligand specific in that estrone and estradiol were less potent than DES, 17α-estradiol and 17β-estradiol in inhibiting cell proliferation, and the characteristic cell cycle changes were produced only by concentrations of estradiol >75 μM and not at all by estrone at concentrations up to 100 μM. The androgens testosterone and dihydrotestosterone were similar in effect to estrone.

The cell cycle changes associated with estrogen-induced growth inhibition in vitro are identical to those observed during regression of ER positive but not ER negative human tumor xenografts in nude mice. However, the role of ER in mediating estrogen-induced regression of ER positive tumors in vitro remains undefined.

INTRODUCTION

Although physiological concentrations of estrogens can stimulate breast cancer cell proliferation (1, 2), high-dose estrogens cause regression of some ER+ human breast cancers (3, 4). Little progress has yet been made in elucidating the mechanisms of this paradoxical effect of estrogens on breast cancer cell proliferation. A number of indirect modes of action of high-dose estrogens have been suggested, including changes in circulating hormone levels (5), and induction of an immune response (6). Direct inhibitory effects of pharmacological concentrations of estrogens on cell growth in vitro have been documented for many cell types, both fibroblast (7-11) and epithelial (2, 12-16) in origin. Although a number of these studies have implicated cell membrane effects (8, 10), several lines of evidence point to the interaction of estrogens with the mitotic spindle as a possible mechanism of action (11, 14, 15, 17).

High-dose estrogen therapy of breast cancer has been used less frequently since the introduction of the antiestrogen, tamoxifen, due to the lower toxicity of the latter agent (18). Nevertheless, elucidation of the mechanism of action of high-dose estrogens is important for a deeper understanding of the biology of breast cancer and the mechanisms of hormone-induced tumor regression. In particular, it is unknown whether high-dose estrogens have a mode of action similar to or different from that of antiestrogens. Recent reports from this laboratory have shown that tamoxifen is a cell cycle phase-specific growth inhibitory and cytotoxic agent inducing increases in the proportion of G2-M cells together with decreases in S-phase cells (19-24). The effects of tamoxifen are exerted predominantly during a short interval in mid-G1 (21) and therefore, tamoxifen was more inhibitory to exponentially growing (i.e., cycling) cell populations than to plateau phase cultures which consisted mainly of non- or slowly cycling cells (20, 23).

In this paper, the effects of high concentrations of estrogens on the growth and cell cycle kinetics of ER+ (MCF-7 and T-47D) and ER- (MDA-MB-330) breast cancer cell lines are described and compared with data reported previously for tamoxifen.

MATERIALS AND METHODS

Cell Culture. Human breast cancer cell lines were obtained as follows: T-47D (25) and MDA-MB-330 (26) from E. G. and G. Mason Research Institute, Worcester, MA, for the National Cancer Institute Breast Cancer Program Cell Bank, and MCF-7 (27) from Dr. C. McGrath, Meyer L. Prentis Cancer Center, Detroit, MI. Stock cultures were maintained in Roswell Park Memorial Institute 1640 medium supplemented with 5 mM glutamine, 14 mM sodium bicarbonate, 20 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid buffer, 10 μg/ml porcine insulin, 20 μg/ml gentamicin, 6 μg/ml phenol red, and 10% FCS. The stock cultures were maintained in continuous exponential growth by weekly passage of the appropriate numbers of cells following trypsinization with 0.05% trypsin-0.02% EDTA in phosphate-buffered saline (1.5 mM KH2PO4,8.1 mM Na2HPO4, 2.7 mM KCl, 140 mM NaCl). Cells were routinely monitored for, and shown to be free of, Mycoplasma contamination. Experiments were done with medium which differed from that used for stock cultures only in that it was supplemented with 5% instead of 10% FCS.

Plateau phase T-47D cultures were generated by inoculating 1 × 10⁶ cells into 150-cm² flasks in 50 ml medium and replacing the medium daily from day 7 as described previously (23).

Drug Treatment. Estrone, 17α- and 17β-estradiol, estradiol, DES, testosterone, and DHT were obtained from Sigma Chemical Co., St. Louis, MO, and stock solutions were prepared in ethanol and stored at −20°C. After appropriate dilution in ethanol, these drugs were added to culture medium such that the final ethanol concentration was 0.1% (v/v). Except for the experiments using plateau phase cultures, cells (2.5 × 10⁶) were inoculated into 25-cm² flasks with medium containing steroid hormone or ethanol vehicle. Flasks were harvested after 48 and 72 h...
and viable cells were counted using a hemocytometer and phase contrast microscopy, or stained for DNA flow cytometry. In one experiment, MCF-7 cells were harvested for flow cytometry daily or every second day for a week. The effect of DES on plateau phase T-47D cells was examined by replacing the medium of day 21 cultures with medium containing DES, repeating the medium change after 24 h, and followed after another 24 h by cell enumeration and DNA flow cytometry.

To test the effect of DES on the viability of T-47D cells, exponentially growing cells were harvested by trypsinization, counted, diluted, pipetted repeatedly to obtain single cell suspensions, and inoculated into 50-mm diameter Petri dishes in 6 ml medium, then incubated at 37°C in sealed plastic boxes containing a humidified atmosphere of 5% CO₂ in air. After the cells had attached to the plastic substratum, the medium was replaced with medium containing DES and 5% FCS. After 48 h this latter medium was removed, and the dishes were washed twice with fresh medium and then incubated for a further 14 days with medium containing 10% FCS. The colonies were then rinsed in normotonic saline, fixed in 95% ethanol, stained with 0.5% aqueous crystal violet, rinsed with water, air dried, and counted. Only colonies containing more than 50 cells were scored.

DNA Flow Cytometry. Cells were stained with ethidium bromide/mithramycin (28) with chicken RBC as an internal standard. Flow cytometry was carried out on an ICP 22 flow cytometer (Ortho Instruments, Westwood, MA). The percentages of cells in G₀-G₁, S, and G₂ + M of the cell cycle, and the coefficient of variation of the G₂-M peak were computed using a curve-fitting method of analysis (29). The number of cells to the right of the G₂ + M peak, i.e., polyploid cells, was also quantitated (20).

RESULTS

Effects of High Concentrations of Estrogens on ER+ Breast Cancer Cell Proliferation. High-dose DES inhibited the proliferation of exponentially growing T-47D cells; 10 μM DES caused a 17% reduction in proliferation rate, 25 and 50 μM DES were completely cytostatic, and 100 μM DES caused a rapid decline in cell numbers indicative of cytotoxicity (Fig. 1A). The occurrence of DES-induced cytotoxicity was confirmed by T-47D colony forming assays: DES at concentrations of 10, 25 and 50 μM caused 0, 35, and 73% decreases, respectively, in the number of colonies formed, and cells treated with 100 μM DES were mostly nonviable (Fig. 1B).

Cytotoxicity was also observed in DES-treated plateau phase T-47D cultures: 25 μM DES caused nearly 60% decline in cell numbers under conditions in which it had previously been established that only about 15% of the cell population would by cycling rapidly (23). Higher concentrations of DES caused larger decreases in cell numbers (Fig. 2).

Effects of High Concentrations of Estrogens on ER+ Breast Cancer Cell Cycle Kinetics. The most marked changes in the cell cycle parameters of exponentially growing T-47D cells were seen with 25 μM DES (Fig. 3). The predominant effect was a marked depletion of G₀ cells and an accumulation of G₂ + M cells. This was accompanied by an increase in the proportion of S phase cells, an increase in polyplloid cells (from 1.8% in control cultures to 5.3% with 25 μM DES; n = 3; P < 0.001), and a large increase in the nuclear debris to the left of the G₀ peak. At other concentrations there were less marked changes in the DNA profiles: a 2-fold increase in the percentage of G₂ + M cells was seen with 50 μM and only minor changes with the lowest (10 μM) and highest (100 μM) concentrations of DES tested (Fig. 3).

In plateau phase T-47D cultures (Fig. 4), DES concentrations >25 μM caused significant increases in the percentage of S phase cells (all P < 0.005), and at concentrations >50 μM a small decrease in the percentage of G₂ + M cells (P < 0.025) was apparent.

Further studies were undertaken with another ER+ cell line, MCF-7. Exponentially growing cells were treated with 40 and 80 μM 17β-estradiol and cell numbers and cell cycle kinetic parameters were measured over a 7-day period. These concentrations of drug resulted initially in a concentration-dependent decrease in proliferation rate followed by a decline in cell numbers (Fig. 5A). Changes in cell cycle kinetic parameters were maximal at 3 to 4 days and consisted of a large increase in the percentage of G₂ + M and polyploid cells (Fig. 5B).

Steroid Specificity of High-Dose Estrogen Effect in ER+ Cells. When exponentially growing MCF-7 cells were treated with a number of different sex steroids, considerable differences in potency were observed (Fig. 6). The stereoisomers 17α- and 17β-estradiol and the synthetic estrogen, DES, had similar potencies as judged by decreases in cell numbers and induction
of changes in cell cycle kinetic parameters. The estrogens estrone and estriol were much less potent and similar in effect to the androgens, testosterone, and DHT. The DNA histograms following treatment with estrone, estriol, or androgens showed either no change from control or a small decrease in the percentage of S phase cells with a corresponding increase in the percentage of G1 cells, i.e., an effect similar to that of a weak antiestrogen (Fig. 7). The exception to this was 75 and 100 \( \mu \)M estriol which caused changes characteristic of lower doses of 17\( \beta \)-estradiol (e.g., 31 and 40% G2 + M cells for 75 and 100 \( \mu \)M estriol, respectively).

Effects on ER- Cells. The MDA-MB-330 cell line was also sensitive to the growth-inhibitory and cytotoxic effects of high-dose estrogens (Fig. 8). However, the sensitivity of this cell line was markedly reduced especially at the lower concentrations of 17\( \beta \)-estradiol and DES studied. While cell numbers were reduced to about 50% of control by 5 \( \mu \)M 17\( \beta \)-estradiol and DES in MCF-7 cells (Fig. 6), 2 to 3 times that concentration was required to cause a similar decrease in cell number in MDA-MB-330 cells (Fig. 8).

The changes in cell cycle kinetic parameters associated with growth inhibition and cytotoxicity in MDA-MB-330 were qualitatively similar to those seen in the ER+ cell lines, i.e., increased in the percentage of S and G2 + M cells and in fragmented nuclei, accompanied by decreases in the percentage of G1 cells (Fig. 9). There was also a markedly greater increase in polyploid cells, including cells with more than four times the DNA content of the original G1 cells, in this cell line (compare data in Figs. 3, 5, and 7 with those in Fig. 9).

At the concentration for which the histograms are shown in Fig. 9, i.e., 50 \( \mu \)M, DES caused a large increase in the percentage of G2 + M cells without the increase in polyploidy seen with 17\( \beta \)-estradiol, but at lower concentrations of DES a large increase in polyploidy was seen similar to that illustrated for 50 \( \mu \)M 17\( \alpha \)- and 17\( \beta \)-estradiol in Fig. 9. DHT and testosterone caused minimal changes in the DNA histograms of MDA-MB-330 cells at concentrations up to 50 \( \mu \)M, but at higher concentrations there was a slight increase in the percentage of S phase cells. Estrone at all concentrations and estriol at concentrations <50 \( \mu \)M had no effect on the DNA histograms, but 75 and 100 \( \mu \)M estriol produced changes similar to those seen with the "potent" estrogens (data not shown).

DISCUSSION

Comparison of the inhibitory effects of high concentrations of estrogens with the previously described effects of tamoxifen on human breast cancer cells in vitro revealed several differences. Estrogens appeared to be considerably less potent than tamoxifen in causing inhibition of T-47D cell growth, e.g., 10 \( \mu \)M DES caused a 17% reduction in T-47D cell proliferation rate in the experiment depicted in Fig. 1, whereas similar inhibition under the same cell culture conditions was seen at concentrations of <0.5 \( \mu \)M tamoxifen (23, 24), a difference in potency of >20-fold. In contrast MCF-7 cells were relatively more sensitive to estrogens, 50% inhibitory dose values for both 17\( \beta \)-estradiol and DES were approximately 5 \( \mu \)M (Fig. 6) while under similar assay conditions tamoxifen had 50% inhibitory dose of 5 to 7.5 \( \mu \)M (24). Thus in MCF-7 cells there was little difference in sensitivity to estrogens and antiestrogens in this concentration range.

The potential involvement of estrogen metabolism in differ-
ESTROGEN INHIBITION OF BREAST CANCER CELL GROWTH

CONTRIBUTED RESEARCH

Fig. 4. Effect of diethylstilbesterol on the cell cycle kinetics of plateau phase T-47D cells. The experimental design is described in the legend to Figure 2 and the presentation of the DNA histograms is as described in Figure 3.

Fig. 5. Effect of estradiol on the growth and cell cycle kinetics of MCF-7 breast cancer cells. Cells (2.5 × 10^6) were inoculated into 25-cm^2 flasks in 5 ml medium. After 24 h the medium was replaced with 8 ml fresh medium containing 40 or 80 μM 17β-estradiol or ethanol vehicle. At the times indicated cells from duplicate flasks were harvested, counted, subjected to DNA flow cytometry, and the percentage of G2 + M and polyploid cells calculated from the resulting DNA histograms as described in “Materials and Methods.” A, cell numbers for control cells (•) and cells treated with 40 (■) or 80 (▲) μM 17β-estradiol. Bars, SE. B, mean ± range (bars) of the percentage of G2 + M cells following treatment with 40 (■) and 80 (▲) μM 17β-estradiol. The proportion of cells to the right of the G2 + M peak (polyploid cells) was also calculated (△); the results for 40 and 80 μM 17β-estradiol were pooled since they were not significantly different (n = 4).

 conquestions of high-dose estrogens and tamoxifen on plateau phase cultures. The potency of DES in reducing cell numbers below control concentration was similar in exponentially growing and plateau phase T-47D cultures (compare Figs. 1A and 2), whereas plateau phase cultures are resistant to the action of tamoxifen (20, 23). Since few cells in such cultures are cycling rapidly (20, 23), DES may be concluded to be active against both cycling and noncycling cells.

The effects of high concentrations of estrogens on breast cancer cell cycle kinetic parameters were also distinctly different from those of antiestrogens. Estrogens (DES, 17α- and 17β-estradiol, and to a lesser extent estriol) caused an accumulation of S, G2 + M, and polyploid cells, with depletion of G1 cells (Figs. 3, 5, 7, and 9), whereas tamoxifen and its metabolites caused a decrease in the percentages of S and G2 + M cells with a concomitant increase in the percentage of G1 cells in ER+ cell lines (19–24). DNA histograms of estrogen-treated cells showed substantial accumulation of nuclear material to the left of the G1 peak; it was also observed that many cells remaining on the monolayer were nonviable, as judged by phase-contrast microscopy, in contrast to tamoxifen-treated cultures where very few nonviable cells were seen on the monolayer even under conditions where the rate of decrease in cell numbers indicated rapid cell death. It seems likely, therefore, that cells treated with high concentrations of estrogens undergo nuclear fragmentation before disintegrating or detaching from the plastic substratum.

These differences in effects of high-dose estrogens and antiestrogens suggest different mechanisms of action in agreement with the finding that high-dose tamoxifen and 17β-estradiol are synergistic in inhibiting the growth of several human breast cancer cell lines (24). Perhaps this synergism could be exploited therapeutically, although in the sole published study comparing tamoxifen with tamoxifen plus DES in the treatment of breast cancer no additive effect was seen (31). A cross-over trial of tamoxifen and stilbestrol, however, showed significant responses to either agent after failure with the other, leading to the conclusion that the two agents act independently (32).

The estrogen-induced changes in cell cycle parameters appear
Fig. 6. Effect of high concentrations of estrogens and androgens on the growth of MCF-7 cells. Cells (2.5 × 10^5) were inoculated into 25-cm² flasks in 5 ml medium. After 24 h the medium was replaced with 8 ml fresh medium containing 5 to 100 μM steroid hormone. Triplicate flasks were harvested and counted after 3 days. Data are mean ± SE (bars) of cell numbers of triplicate flasks for estrone (Δ), 17α-estradiol (■), 17β-estradiol (▲), estradiol (×), DES (•), testosterone (O), and DHT (□).

...to indicate a disturbance of mitosis, with accumulation of cells in G2 + M and the appearance of tetraploid cells, i.e., cells which have failed to divide after DNA synthesis. The hypodiploid nuclei seen in DNA histograms may represent the products of unequal mitoses, which would also account for an increase in cells with DNA content intermediate between G1 and G2, i.e., an apparent increase in the percentage of S phase cells. As reviewed previously (33), a number of earlier studies have pointed to an effect of high-dose estrogens on mitosis. These included the observation of colchicine-like effects (10, 15, 17), and the induction of chromosomal nondisjunction (11).

Not all of the cell cycle effects of high-dose estrogens have been localized to mitosis, however. A recent study of DES- or 17β-estradiol-treated human lymphocytes demonstrated prolongation of G2 phase but not mitosis (34). 17β-Estradiol induced prolongation of G2 and early prophase in HeLa cells and since this could be reversed by calcium chloride or putrescine, the author speculated that the anionic substituents of 17β-estradiol interfered with the condensation of chromatin, thus delaying the onset of mitosis (14).

The activity of high-dose estrogens against breast cancer cells is not a non-specific effect of the steroid nucleus since the androgens, testosterone and DHT, and the estrogens, estrone and estriol, had little effect on cell proliferation and, with the exception of estriol at relatively high concentrations (75 and 100 μM), did not cause an accumulation of G2 + M or polyploid cells (Figs. 7 and 9). In addition the relative potency of estrogens in inhibiting MCF-7 proliferation (Fig. 8) appeared to be broadly correlated with their relative binding affinities for ER (i.e., DES = 17β-estradiol > 17α-estradiol > estriol = estrone) (34, 35). These results are in agreement with a study using suspension cultures of HeLa cells in which 17β-estradiol, estriol, ethinyl estradiol, DES, and Colcemid induced mitotic chromatid nondisjunction, but estrone, testosterone, hydrocortisone, fluoxymesterone, and progesterone had no visible effects on mitosis (15). The observed specificity was also in general agreement with a study of human lymphocytes which found that DES and 17β-estradiol strongly inhibited proliferation but...
that estriol was only a weak inhibitor. However, only DES and not 17β-estradiol or estriol induced an increased level of polyploidy (36).

Because cytotoxicity occurs in both ER+ and ER− cell lines (Figs. 6 and 8 and Refs. 2 and 14) and since tumor regression in response to additive estrogen therapy is restricted mainly to ER+ tumors (37), the relevance of these phenomena in vitro to tumor regression in vivo must be questioned. However, when detailed dose-response data for the MCF-7 and MDA-MB-330 cell lines were compared significant differences in sensitivity were observed. Whether this difference between ER+ and ER− cell lines will hold when a wider range of breast cancer cell lines are studied must await further experimentation. The most compelling evidence for a relationship between the in vitro effects of high-dose estrogens and tumor regression in vivo comes from a study in which athymic nude mice carrying xenografted human breast carcinomas were treated with 17β-estradiol and sequential fine needle aspirates of the tumors were analyzed by DNA flow cytometry (38). In an ER+ carcinoma, 17β-estradiol induced complete tumor regression and DNA histograms which were indistinguishable from those reported here for estrogen-treated cells in vitro. An ER− tumor did not regress and no changes in DNA histograms were observed (38). Perhaps the crucial difference between ER+ and ER− tumors in vivo is the extent to which intratumor accumulation of hormone occurs, a difference which may be less marked in culture.

Although the cell cycle kinetic changes accompanying growth inhibition of breast cancer cells by high-dose estrogens are markedly different from those observed following treatment with antiestrogens (19–24) and progestins* they are not unique. In a recent collaborative study we have shown that the previously reported inhibition of T-47D cell proliferation by 1,25-dihydroxyvitamin D3 (39) is associated with accumulation of G0 + M and perhaps polyploid cells while the percentage of G2-M cells decreased.7 These effects were qualitatively similar but quantitatively less marked than the changes observed here with high-dose estrogens. Further studies are required to establish whether or not these two hormonal agents share common molecular mechanisms of action as antiproliferative agents in human breast cancer cells.

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REFERENCES


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