Drug and Hormone Sensitivity of Estrogen Receptor-positive and -negative Human Breast Cancer Cells in Vitro

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ABSTRACT

A clonogenic assay of long-term breast cancer cell cultures in vitro has been developed to provide a highly reproducible method with which to quantitate tumor cell killing by hormones and/or cytotoxic chemotherapeutic agents. Monolayer cultures of estrogen receptor-positive MCF-7 human breast cancer cells and of estrogen receptor-negative Evsa T cells are harvested by treatment with 0.01% trypsin:0.02% EDTA in Hank's balanced salt solution. Cell suspensions are treated with drug or hormone in serum-free medium for 1 hr at 37°; treated cells are washed, plated, and cultured for approximately 14 days; and colonies consisting of ≥30 cells are counted. Compared to estrogen receptor-positive cells, estrogen receptor-negative cells were 2-fold more sensitive to melphalan but were conversely 1.9-fold more resistant to Adriamycin; these differences were statistically significant (p < 0.001). Thus, response to cytotoxic chemotherapeutic agents appeared to be independent of estrogen receptor status.

For cells treated with diethylstilbestrol, the dose of drug or hormone reducing the surviving cell fraction to 1/e (D0) for estrogen receptor-positive cells was 2.77 nmol/ml, and that for estrogen receptor-negative cells was 2.80 nmol/ml; this difference was not statistically significant. However, with tamoxifen therapy, the D0 for estrogen receptor-positive cells was 0.611 nmol/ml, and that for estrogen receptor-negative cells was 3.64 nmol/ml; this 6-fold greater degree of resistance to tamoxifen of estrogen receptor-negative cells was highly significant (p < 0.001). Treatment of cells for 24 hr with 17β-estradiol stimulated proliferation not only of estrogen receptor-positive cells but also of estrogen receptor-negative cells. However, estradiol at concentrations up to 200 μM had no apparent cytocidal activity, as measured by the clonogenic assay. Furthermore, treatment of MCF-7 cells simultaneously with estradiol and either diethylstilbestrol or tamoxifen failed to reverse the cytocidal activity of those two agents. These findings suggest that, in the clonogenic assay described herein, diethylstilbestrol and tamoxifen may kill human breast cancer cells by an independent mechanism of action and that the cytocidal activity of diethylstilbestrol and the proliferative effect of 17β-estradiol appear to be independent of estrogen receptor status.

INTRODUCTION

The tumors in approximately 60% of patients with breast cancer contain a cytoplasmic receptor that binds estradiol with a high affinity and in a chemically specific manner. The presence of ER correlates well not only with the biological behavior of the tumor but also with the response of the tumor to endocrine manipulation; approximately 60% of ER-positive tumors are responsive to such therapy compared with only 5 to 10% of ER-negative tumors.

Lippman (9) has argued that the presence of ER may also predict the response of the tumor to cytotoxic chemotherapy, since ER-positive tumors are much less likely to respond than are ER-negative tumors. Others have reported that either no such correlation exists (16, 19), or conversely that ER-positive tumors are more responsive to chemotherapy than are ER-negative tumors (5). In this study, we report the development of a clonogenic assay of long-term breast cancer cell cultures that provides a highly reproducible method with which to quantitate tumor cell kill by hormones and/or cytotoxic chemotherapeutic agents. The assay also provides an opportunity to investigate more directly the mechanism of tumor cell kill by hormones and antiestrogens.

MATERIALS AND METHODS

Drugs and Chemicals. Melphalan (Alkeran) was kindly provided as a gift by Dr. J. R. MacDougal, Burroughs Wellcome and Co., Ltd., Lachine, Quebec, Canada. Adriamycin (doxorubicin hydrochloride) was obtained from Adria Laboratories of Canada, Mississauga, Ontario, Canada. DES was purchased from Sigma Chemical Co., St. Louis, Mo., and tamoxifen citrate [trans-(p-dimethylaminoethoxyphenyl)-1,2-diphenyl-but-1-ene] was kindly provided by Stuart Pharmaceuticals, Division of ICI United States, Inc., Wilmington, Del.

Cell Lines and Cultures. MCF-7, a cloned ER-positive cell line originally established at the Michigan Cancer Foundation, Detroit, Mich., from a malignant pleural effusion in a female patient with metastatic breast cancer (17), was generously provided by Dr. Robert Shiu, Department of Physiology, University of Manitoba. Evsa T, an ER-negative cell line which was established by Dr. Marc Lippman, National Cancer Institute, Bethesda, Md., from a malignant ascitic effusion from a female patient with metastatic breast carcinoma (10), was kindly provided by Dr. Peter Lam, Manitoba Institute of Cell Biology. Both cell lines were grown in monolayer cultures in Corning tissue culture flasks in Eagle’s MEM [Grand Island Biological Co., Grand Island, N. Y.] supplemented with glutamine (0.6 g/liter), penicillin (100 units/ml), streptomycin (100 μg/ml), and 10% FBS (Grand Island Biological Co.). Cells were grown at 37° in a humidified atmosphere containing 6% CO2 in air.

Clonogenic Assay. Stock cultures of MCF-7 or Evsa T cells in exponential growth phase were harvested by treatment with 0.01% crystalline trypsin (Grand Island Biological Co.):0.02% EDTA in calcium- and magnesium-free Hank's balanced salt solution (Grand Island Biological Co.) at 37° for 3 to 5 min. The cell suspension was centri...
fuged at 1000 rpm for 5 min in a Sorvall GLC-2 centrifuge, and the cell pellets were resuspended in serum-free MEM at an approximate concentration of $2 \times 10^5$ cells/ml. Cell suspensions were treated with either drug or hormone, which was added to the suspension in a 1:100 dilution, and was incubated in a shaking bath at 37°C for 1 hr. The drug or hormone treatment was terminated by addition of an equal volume of ice-cold MEM, and the cells were promptly centrifuged at 1000 rpm for 5 min. The cells were washed twice with cold MEM to remove residual drug or hormone and were resuspended in MEM containing 10% FBS stripped of endogenous hormone by a 45-min incubation at 50°C twice with DCC as described previously (14). The cells were counted with an electronic particle counter (Model ZBi, Coulter Counter; Coulter Electronics, Inc., Hialeah, Fla.) and were diluted in MEM containing DCC-stripped 10% FBS. Treated cell suspensions were seeded at a density ranging from 100 to 100,000 cells/well in sextuplicate in 9.6-sq cm Linbro multiwell tissue culture plates (Flow Laboratories, Inc., Mississauga, Ontario, Canada) and were incubated for approximately 14 days at 37°C in a humidified atmosphere containing 6% CO$_2$ in air. The colonies, which consist of $\geq$30 cells, were fixed with absolute methanol, stained with 2% Giemsa stain (Fisher Scientific Co., Fair Lawn, N. J.), and counted over a background grid using an inverted microscope (Fig. 1).

The plating efficiency for untreated MCF-7 cells ranged from 75 to 95%, and that for Evsa T cells ranged from 25 to 50%. The cloning efficiency of treated cells was determined at each drug concentration and the surviving cell fraction was calculated. Linear regression analysis of each dose-survival curve was obtained, and the $D_0$ was derived from the negative reciprocal of the regression slope as described previously (1).

**ER Assay.** The ER content of MCF-7 and Evsa T cells was monitored periodically using minor modifications of the DCC assay (7, 8). Cells prepared for binding studies were grown as noted above but were incubated in serum-free medium overnight before harvesting in order to deplete estrogen receptors of endogenously bound steroid. These assays were performed in the laboratories of Dr. Lorne J. Brandes and Dr. Peter Lam, Manitoba Institute of Cell Biology. Over a period of 1 year considerable variability in ER content was noted, particularly in MCF-7 cells; however, the distinction between "ER-positive" and "ER-negative" status was maintained. ER levels for MCF-7 cells ranged from 35 to 173 fmol/mg of cytosol protein, and that for Evsa T cells varied from 2 to 8 fmol/mg of cytosol protein.

**RESULTS**

**Dose-Survival Curves of ER-positive and -negative Human Breast Cancer Cells Treated with Melphalan.** Dose-survival curves of ER-positive and -negative cells treated with melphalan are shown in Chart 1. The $D_0$ for Evsa T cells treated with melphalan was 2.02 nmol/ml, and that for MCF-7 cells was 4.16 nmol/ml. ER-negative Evsa T cells were 2-fold more sensitive to the cytocidal action of melphalan than were ER-positive MCF-7 cells.

**Dose-Survival Curves of ER-positive and -negative Human Breast Cancer Cells Treated with Adriamycin.** Dose-survival curves of cells treated with Adriamycin are presented in Chart 2. The $D_0$ for Evsa T cells treated with Adriamycin was 0.365 nmol/ml, and that for MCF-7 cells was 0.194 nmol/ml. Contrary to the finding with melphalan, Evsa T cells were 1.9-fold more resistant to Adriamycin than were MCF-7 cells.

**Dose-Survival Curves of ER-positive and -negative Human Breast Cancer Cells Treated with DES.** The survival of MCF-7 cells and of Evsa T cells exposed to tamoxifen for 1 hr is shown in Chart 3. The $D_0$ for ER-positive cells was 2.27 nmol/ml, and that for ER-negative cells was 2.80 nmol/ml. The difference was not statistically significant.

**Dose-Survival Curves of ER-positive and -negative Human Breast Cancer Cells Treated with Tamoxifen.** Dose-survival curves of MCF-7 cells and of Evsa T cells exposed to tamoxifen for 1 hr are illustrated in Chart 4. The $D_0$ for MCF-7 cells was

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**Fig. 1.** A photograph of 1 of the 6 wells in a Linbro multiwell tissue culture plate illustrating macroscopic colonies of MCF-7 human breast cancer cells after 12 days in culture. The colonies consisting of 30 or more cells were fixed with absolute methanol, stained with 2% Giemsa, and counted as described above. The actual area of the well is 9.6 sq cm.
Sensitivity of Human Breast Cancer Cells in Vitro

1.0

< 0.01

0.001

0.0001

0.601 nmol/ml, and that for ER-negative cells was 3.64 nmol/ml. Unlike the response to DES, ER-negative cells were 6-fold more resistant to tamoxifen than were ER-positive cells, and the difference was highly significant (p < 0.001).

Effect of Estradiol on MCF-7 Cells. Treatment of MCF-7 cells for 1 to 24 hr with 17β-estradiol at concentrations of ≤200 μM had no apparent cytocidal effect as measured by the clonogenic assay. An increase in the number of MCF-7 cells was noted 24 hr after treatment at various concentrations of estradiol (Table 1); this apparent stimulation of cell proliferation by estradiol has been reported previously for ER-positive cell lines (10). However, apparent stimulation of proliferation of the ER-negative Evsa T cells was also observed 24 hr after exposure to estradiol (Table 1).

Using estradiol, attempts were undertaken to rescue MCF-7 cells from the clonogenic-inhibitory effects of DES and tamoxifen. Cells were treated simultaneously either with DES and estradiol or with tamoxifen and estradiol, and dose-survival curves were compared to those obtained for cells treated with DES or tamoxifen alone. The survival curves of MCF-7 cells treated with DES alone or DES and estradiol were identical (Chart 5A); similarly, no difference was noted in the survival of cells treated with tamoxifen alone compared to those treated with tamoxifen and estradiol (Chart 5B).

DISCUSSION

This study describes clonal growth of long-standing cultures of human breast cancer cell lines using a single-layer cell culture technique. A high cloning efficiency varying from 75 to 95% for MCF-7 cells and from 25 to 50% for Evsa T cells has been attained. Using this procedure, a quantitative assay has been developed to investigate the mechanism of cytocidal activity of drugs and hormones on ER-positive and -negative human breast cancer cell lines.

In a retrospective study, Lippman et al. (9) evaluated the
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Table 1
Stimulatory effect of 17-ß-estradiol on the growth of MCF-7 and Evsa T human breast cancer cells in vitro

<table>
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<th>Estradiol concentration (μM)</th>
<th>MCF-7</th>
<th>Evsa T</th>
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Chart 5. A, dose-survival curves of MCF-7 cells treated with DES alone (○) or with DES and 40 μM 17-ß-estradiol (●) for 1 hr at 37°. Each point represents the mean of 6 determinations; confidence intervals were too small to be illustrated. The linear regression equation for cells treated with DES alone was

\[ \log_2 y = -0.262x + 2.31 \]

with a correlation coefficient of -0.942, and that for cells treated with DES + estradiol was

\[ \log_2 y = -0.245x + 1.61 \]

with a correlation coefficient of -0.986. A t test comparing the significance of the difference of the slopes of regression lines was not statistically significant. B, dose-survival curves of MCF-7 cells treated with tamoxifen alone (○) or with tamoxifen and 10 μM 17-ß-estradiol (●), for 1 hr at 37°. Each point represents the mean of 6 determinations; confidence intervals were too small to be illustrated. The linear regression equation for cells treated with tamoxifen alone was

\[ \log_2 y = -2.38x + 12.49 \]

with a correlation coefficient of -0.998, and that for cells treated with tamoxifen + estradiol was

\[ \log_2 y = -2.37x + 12.26 \]

with a correlation coefficient of -0.946. A t test comparing the significance of the difference of the slopes of the regression lines was not statistically significant.

The relationship between ER status and the response rate to cytotoxic chemotherapy in 70 patients with metastatic breast cancer. The study concluded that ER status was an important predictor of chemotherapeutic response since 34 of 45 ER-negative patients responded to chemotherapy, whereas only 3 of 25 ER-positive patients had objective remissions. However, in a study of 143 patients with advanced breast cancer at the University of Minnesota, the response rate to chemotherapy was 86% in ER-rich tumors, which was significantly higher than the response rate of 36% in ER-poor tumors. In this study, the ER-negative Evsa T cell line was significantly more sensitive to melphalan than was the ER-positive MCF-7 cell line (Chart 1), but conversely, Evsa T cells were more resistant to Adriamycin than was their ER-positive counterpart (Chart 2). This finding suggested that response to cytotoxic chemotherapy as determined by the clonogenic assay appeared to be independent of ER status. Furthermore, a review of several clinical reports in the literature indicated that 53% of ER-positive and 54% of ER-negative subjects responded favorably to chemotherapy (Table 2).

The sensitivity of ER-positive and -negative breast cancer cells to DES was identical (Chart 3). This observation together with the inability of equimolar concentrations of estradiol to reverse or protect cells from the growth-inhibitory effect of DES suggest at least 2 possible interpretations: (a) that the mechanism of cell kill of DES as measured by the clonogenic assay in this study is ER independent; and (b) that the observed cytotoxicity is "nonspecific" and of questionable biological relevance.

Although the data do not permit a clear choice between these 2 options, we favor the first interpretation. Quantitatively, the cytotoxic effect of DES appears to be pharmacologically relevant. With 1 hr of DES treatment, the \( D_0 \) was 2.27 nmol/ml for MCF-7 cells and 2.80 nmol/ml for Evsa T cells. Plasma levels of DES, as determined by radioimmunoassay, in patients with prostatic cancer receiving 1 mg DES 3 times/day, ranged from 0.15 to 6 ng/ml or 0.56 to 22 nm (4). Patients with breast cancer have been treated with as much as 15 to 20 mg DES daily (2), so that plasma DES levels of 3.7 to 149 nm would be a realistic expectation. In evaluating the pharmacological significance of these dose-survival studies, the product of drug concentration × treatment time is particularly pertinent. If one extrapolates and theoretically extends the treatment time from 1 to 24 hr, then the "corrected" \( D_0 \) for DES treatment of MCF-7 and Evsa T cells would be 0.095 and 0.117 nmol/ml, respectively (or 95 and 117 nm). Thus, it would be reasonable to assume that such plasma levels would be clinically attainable in patients receiving 15 to 20 mg DES daily.

In this study, it is unclear why the cytotoxic action of DES on breast cancer cells should be ER independent, particularly since the correlation between ER positivity and response to endocrine manipulation is clinically well established (12, 13, 15).

Unlike the response to DES, the ER-positive MCF-7 cell line was 6-fold more sensitive to tamoxifen than were Evsa T cells (Chart 4). Although this finding suggests a possible correlation
between ER positivity and tamoxifen sensitivity in human breast cancer cells, the inability of estradiol to block tamoxifen-related cytotoxicity suggests an independent mechanism of action for tamoxifen and estrogen analogs. The identical response of ER-positive and ER-negative cell lines to DES, compared to the 6-fold difference in response to tamoxifen, also suggests separate mechanisms of action. Evidence for separate receptors for estrogen and tamoxifen in MCF-7 breast cancer cells has been presented recently by comparing properties of a tamoxifen-resistant variant, R27, with those of the parent cell line (14). The concentration range of tamoxifen used in the dose-survival curves was similar to that used by others in studies of hormone effect on cell proliferation and thymidine incorporation (14). However, the treatment time in our study was restricted to 1 hr, whereas others have exposed cells to tamoxifen for time intervals as long as 15 days (10, 14). Finally, estradiol-induced stimulation of cell proliferation of both ER-positive and ER-negative breast cancer cells was observed 24 hr after treatment (Table 1). Although this phenomenon has been described for MCF-7 cells (10, 11, 14), it has not been observed previously with ER-negative cells such as MDA-231 (18). Of interest was the observation that estradiol, at concentrations up to 200 μM with 24 hr of treatment, had no apparent antitumor activity on either cell line as measured by the clonogenic assay. This contrasts with the ‘nonspecific’ inhibition of thymidine incorporation into DNA of breast cancer cells reported by others with μM concentrations of estradiol (10).

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REFERENCES

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