Effects of Tamoxifen on Human Breast Cancer Cell Cycle Kinetics: Accumulation of Cells in Early G, Phase

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INTRODUCTION

The antiestrogen tamoxifen is now widely used for the treatment of patients with breast cancer. However, its mechanism of action is not totally defined. Although specific binding sites for tamoxifen have been identified recently in certain tissues (15), tamoxifen, like other antiestrogens, is thought to exert its effects by binding to estrogen receptor and then translocating it to the nucleus, where steps leading to estrogen-regulated events such as tumor growth are inhibited (6). Tamoxifen has been shown to inhibit breast tumor growth in experimental animals (8) and to inhibit breast cancer cells in culture. These studies indicate that tamoxifen inhibits proliferation of MCF-7 human breast cancer cells by invoking a transition delay early in the G1 phase of the cell cycle.

RESULTS

When cells from the well-characterized MCF-7 estrogen recep-

ABSTRACT

We have studied the effects of tamoxifen on the cell cycle kinetics of the endocrine-responsive MCF-7 human breast cancer cells. Tamoxifen inhibits proliferation of MCF-7 cells. The tritiated thymidine labeling index is markedly reduced by tamoxifen, indicating a reduction in the fraction of cells in S phase. Flow cytometry of mithramycin-stained cells reveals that cells accumulate in G, phase, with a concomitant depletion of G_2-M-phase cells with tamoxifen. Mapping of G_1-phase cells by morphology of prematurely condensed chromosomes demonstrated that tamoxifen-treated cells accumulate in early G_1. These studies indicate that tamoxifen inhibits proliferation of MCF-7 human breast cancer cells by invoking a transition delay early in the G1 phase of the cell cycle.

MATERIALS AND METHODS

Cell Proliferation. The tissue culture methodology used for propagation of the MCF-7 human breast cancer cells has been described previously (14). For determination of the effects of tamoxifen on cell proliferation, MCF-7 cells were repopulated into multiwell culture dishes in tissue culture medium (Richter's Improved Eagle's Minimal Essential Medium, Grand Island Biological Co., Grand Island, N. Y.) supplemented with insulin (1.0 nm) and 5% bovine serum. After 24 hr, paring medium was replaced with medium supplemented with 5% dextran-coated charcoal-stripped bovine serum to reduce endogenous estrogen receptor activity. After an additional 24 hr, tamoxifen (1 µM) or ethanol (final concentration, 0.1%) was added to the dishes. At the indicated times, cells were suspended and counted in a hemocytometer.

Cell Kinetic Studies. Cells were plated and cultured as described above. At the indicated times, cells were pulsed with tritiated thymidine (0.3 µCi/ml) and washed twice with PBS, and a cell suspension was prepared. The TLI was determined autoradiographically as described previously (11, 14). The cell cycle distribution of MCF-7 cells was determined by flow cytometry of mithramycin-stained cells (1). Cells were washed once and, then, suspended in 0.02% EDTA in PBS. After a 5- to 10-min incubation, a single-cell suspension was prepared mechanically by passing the cells through progressively smaller gauge needles (22, 25, and 27 gauge). The cells were pelleted by centrifugation (800 rpm for 2 min) and resuspended in 0.4 ml of PBS. The cells were then fixed by adding ethanol (final concentration, 70%) while vortexing and stored at 4°. Fixed cells were pelleted by centrifugation, and the ethanol was discarded. Cells were washed once with PBS and, then, resuspended in staining solution (1.0 mg mithramycin:0.87 g NaCl:0.15 ml 1 M MgCl_2:9.85 ml distilled H_2O) to a concentration of 5 x 10^5 cells/ml. The cells were protected from light at 4° overnight prior to flow cytometry. DNA histograms were obtained by analyzing a total of 1 x 10^6 cells on a Coulter Model TPS-1 cell sorter equipped with a 2-watt argon-ion laser which was tuned to 457.9 nm. Histograms were analyzed by the computer-assisted method of Dean and Jett (2). The coefficient of variation of the G1 peak varied from 4 to 8% using this method.

Distribution of G, Cells. MCF-7 cells were cultured as described above and then incubated with either ethanol (controls) or tamoxifen (1 µM) for 72 hr. G1 cells were mapped by the method of Hittelman and coworkers (4, 5, 18), which analyzes the morphology of PCC. Briefly, mitotic HeLa cells were obtained following a 24-hr exposure to thymidine (2.5 mM), followed by a 15-hr exposure to colchicine (0.1 µM). This procedure routinely provides a 97% pure population of mitotic cells (as distinguished by light microscopy following acetoorcein staining). Equal numbers of mitotic HeLa cells and MCF-7 cells were mixed in serum-free medium and pelleted (150 x g for 2 min). Cells were resuspended in 0.5 ml of PBS containing 2000 hemagglutinating units of UV-inactivated Sendai virus (a generous gift of Dr. W. Hittelman, Houston, Texas). Cells were then incubated at 4° for 15 min after the addition of 0.05 ml of 20 mM MgCl_2 and Colcemid (5 µg/ml). Samples were warmed to 37° and incubated for an additional 45 min. Cell pellets were obtained by centrifugation (150 x g, 5 min), and 7 ml of 0.075 M KCl (preswapped to 37°) were added for 10 min. Cells were then recentrifuged, the supernatant was removed, and 7 ml of fresh cold fixative (3:1 methanol-glacial acetic acid) were added. Air-dried slides were then prepared and stained with 4% Giemsa (Gurr's R-66). G1 PCC were scored on a scale of 1 through 6, depending on the degree of condensation. PCC given values of 1 to 3 (highly condensed) were considered to be in early G1, whereas those given values of 4 to 6 (less condensed) were judged to be in late G1 (4).

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Tor-positive human breast cancer cell line were incubated with tamoxifen (Chart 1), inhibition of cell proliferation similar to that observed in previous reports (9, 17) was observed. In 5% charcoal-stripped serum, control cultures proliferated rapidly and had reached confluence by Day 6. Tamoxifen (1 μM) slowed the rate of proliferation slightly during the first 48 hr. Thereafter, proliferation was markedly reduced, cell number nearly plateaued, and the monolayers remained at a subconfluent density. These data suggest that more than one passage through the cell cycle is required for expression of maximal effects of tamoxifen at these concentrations. When MCF-7 cells were incubated with tamoxifen for longer durations or were incubated with tamoxifen in serum-free medium, cells would begin to detach from the monolayer and appear as floaters. It is important to emphasize, however, that, under the experimental conditions described in Chart 1, cell number did not decline with tamoxifen. These data suggest that tamoxifen slowed proliferation but did not have an immediate cytocidal effect on the MCF-7 cells.

We next examined the effect of tamoxifen on the distribution of MCF-7 cells in the cell cycle. The TLI was used to estimate the fraction of cells in the S phase of the cell cycle (Chart 2). The TLI of control cells growing in medium supplemented with dextran-coated charcoal-stripped calf serum was 35% at the beginning of the experiment. The TLI decreased slightly over 96 hr in control cultures, probably reflecting depletion of nutrients from the medium and/or density-dependent growth inhibition as the monolayer neared confluence. Tamoxifen resulted in a marked reduction in the TLI, and by 72 to 96 hr, less than 10% of the cell population was in S phase.

To determine the effects of tamoxifen on the distribution of MCF-7 cells in the other cell cycle phases, DNA histograms were obtained by flow cytometry of mithramycin-stained cells (Chart 3). After 72 hr in control medium, 72% of cells were in G1, as revealed by computer analysis of DNA histograms, 19% were in S phase (similar to the TLI result), and 9% were in G2-M phases. Tamoxifen treatment resulted in a marked accumulation of cells in G1 (92%), concomitant with a depletion of cells from the S and G2-M compartments. When experiments were carried out for longer time periods (6 days), cells exposed to tamoxifen remained in G1, indicating that the block in G1 phase was not temporary.

It has been reported that serum-deprived or plateau-phase normal (nontransformed) cells accumulate in early G1, whereas malignant or transformed cell populations accumulate in late G1 (4). Furthermore, certain metabolic inhibitors, such as actinomycin D or cycloheximide, block cells in early G1 in contrast to inhibitors of DNA synthesis, such as thymidine or hydroxyurea, which block cell cycle progression at the G1-S boundary (4). Thus, we were interested in determining where in the G1 phase the tamoxifen block occurred. Utilizing the morphology of PCC, cells can be classified as occurring early or late in the G1 phase based upon the structural criteria of Hittleman and Rao (4). This is possible because the chromosomes from early G1, PCC are highly condensed, whereas those from late G1, PCC are long and extended. Morphological assessment of PCC (Table 1) revealed that 62% of G1, PCC in control cultures were in late G1. In contrast, only 35% of tamoxifen-treated G1, PCC were present in late G1 (p = 0.001). The majority (65%) of tamoxifen-treated cells had accumulated early in G1. Furthermore, exact chromosome counts of G1, PCC revealed that both control and tamoxifen-treated G1, cells had similar chromosome numbers (70), indi-

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Chart 1. Effect of tamoxifen on proliferation of MCF-7 cells. Cells growing in medium supplemented with 5% dextran-coated charcoal-stripped bovine serum were incubated in the absence (O) or presence (C) of tamoxifen (1 μM). Fresh medium and tamoxifen were exchanged for spent medium on Day 4. Points, mean of triplicate determinations; bars, S.D.

Chart 2. Effect of tamoxifen on the TLI of MCF-7 cells.

Control

G1 = 71.7%  
S = 19.1%  
G2 M = 9.2%

TAM 1 μM

G1 = 91.9%  
S = 8.1%  
G2 M = 0.0%

Chart 3. Effect of tamoxifen (TAM) on the cell cycle distribution of MCF-7 cells. A, control cells; B, tamoxifen for 72 hr. Hatched area, computer estimate of the S- phase fraction of cells.
We have demonstrated that the antiestrogen, tamoxifen, inhibits proliferation of estrogen receptor-positive human breast cancer cells. These results are similar to those reported by Lippman et al. (9) and Sutherland et al. (17) who found that, at concentrations of tamoxifen observed in women treated for breast cancer (≤1.0 μM), the antiestrogen slowed proliferation but was not lethal to MCF-7 cells in short-term culture. At suprapharmacological concentrations (10 μM), a cytotoxic effect was observed (17), although the relevance of this effect to in vivo drug mechanism of action is speculative. The slight differences observed in the effects of tamoxifen in these studies may be related to differences in the type (charcoal stripped or not) and concentration of serum used.

Our data suggest that tamoxifen inhibits cell proliferation by invoking a transition delay or block in early to mid-G1 phase of the cell cycle. This delay may require more than one complete passage of some cells through the cell cycle, since cell number more than doubled before plateau growth was observed, and since 72 to 96 hr (2 to 3 generation times) were required for maximal accumulation of cells in G1. A small percentage of cells was refractory to tamoxifen and remained in the proliferative pool (5 to 10% in S phase). The mechanism of resistance of these cells to tamoxifen will require further study. We have shown recently that the tamoxifen effect can be reversed by the addition of 17β-estradiol and that tamoxifen has no effect on the cell cycle kinetics of the receptor-negative MDA-MB-231 cells, suggesting that the antiestrogen effect is mediated through the estrogen receptor (13). Furthermore, these inhibitory effects are not restricted to tamoxifen. In our preliminary studies, the antiestrogens nafoxidine and trioxifene have identical effects on breast cancer cell kinetics.

These data are consistent with the hypothesis that antiestrogens, and perhaps other forms of endocrine therapy, induce regression of breast cancer in vivo by simply blocking progression of estrogen-dependent cells through the cell cycle rather than by a direct cytotoxic effect. With cell replication inhibited, tumors might then regress because of ongoing cell shedding or death or because of interaction with normal host defenses. This hypothesis would account for the very slow rate of tumor regression observed in many patients undergoing endocrine therapy.

These results may have important clinical implications for the treatment of breast cancer. (a) If antiestrogens are simply putting endocrine-responsive breast cancer cells into a "resting" phase of the cell cycle (G0-G1), then treatment of patients after surgery for primary breast cancer with adjuvant antiestrogens may have to be continued indefinitely or at least long enough for host defense or normal cell attrition to eradicate residual tumor cells. Premature discontinuation of therapy would lead to tumor regrowth. (b) The combined use of antiestrogens with cytotoxic chemotherapy in an attempt to obtain an additive or synergistic effect will require careful evaluation. Tamoxifen might enhance the killing effect of drugs most active in G1 cells, whereas the cytotoxic effect of drugs acting specifically during DNA synthesis (S phase) might be diminished. This hypothesis is currently being investigated.

**ACKNOWLEDGMENTS**

We would like to acknowledge the technical assistance of Philip Estrada, Sharon Olson, and Kathy Mosty.

**REFERENCES**


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**Table 1**

<table>
<thead>
<tr>
<th>G1 distribution of control or tamoxifen-treated MCF-7 cells</th>
<th>Early G1 (Stages 1–3)</th>
<th>Late G1 (Stages 4–6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>31 (38)*</td>
<td>51 (62)</td>
</tr>
<tr>
<td>Tamoxifen</td>
<td>49 (65)</td>
<td>26 (35)</td>
</tr>
</tbody>
</table>

*p = 0.001

*Numbers in parentheses, percentage of cells.
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