Effects of Estrogen and Tamoxifen on the Regulation of Dihydrofolate Reductase Gene Expression in a Human Breast Cancer Cell Line

Richard M. Levine, Elizabeth Rubalcaba, Marc E. Lippman, and Kenneth H. Cowan

ABSTRACT

We have studied the effects of estrogen and the antiestrogen tamoxifen on the regulation of dihydrofolate reductase (DHFR) gene expression in a methotrexate-resistant (MTX\textsuperscript{R}) human breast cancer cell line MCF-7, which contains a 50-fold increase in the cellular level of DHFR enzyme and amplified DHFR gene sequences. Despite their selection for methotrexate resistance, the MTX\textsuperscript{R} cells have retained many characteristics of the parental MCF-7 cell line. Concentrations of estrogen receptors as well as their binding affinity to estradiol are identical in both cell lines. MTX\textsuperscript{R} MCF-7 cells remain sensitive to estrogen and respond to estradiol with an induction of progesterone receptors, as well as increases in the rate of DNA synthesis and cell growth. Incubation of MTX\textsuperscript{R} MCF-7 cells with estradiol results in an additional 1.5- to 3.0-fold increase in their already elevated level of DHFR. The hormone-induced increases in DNA synthesis and DHFR levels are similar both with respect to the time course of inductions, as well as their dose response to estradiol. However, these two estrogen-induced effects are not coupled, since the induction of DHFR occurs even in the absence of concomitant DNA synthesis. Estradiol has no effect on DHFR enzyme stability; thus, the entire effect of estradiol on DHFR levels results from the increased synthesis of this housekeeping enzyme. In contrast, treatment of MTX\textsuperscript{R} MCF-7 cells with the antiestrogen tamoxifen reduces the rate of DHFR enzyme synthesis, resulting in lower levels of DHFR than in MTX\textsuperscript{R} MCF-7 cells. These MTX\textsuperscript{R} MCF-7 cells represent a useful model in which to study the mechanisms involved in the modulation of DHFR gene expression by estrogen and tamoxifen. Since the level of DHFR is a critical determinant of the cytotoxicity of MTX to DHFR (EC 1.5.1.3), an effective antineoplastic agent which is frequently used in chemotherapy regimens with or without concomitant hormonal therapies in the treatment of breast cancer (1). Various factors, including the rate of drug transport (2, 3) and the binding affinity of MTX to DHFR (4, 5), and the intracellular conversion of MTX to polyglutamate derivatives (6–8) have all been shown to influence the sensitivity of cells to this agent. However, studies in drug-sensitive and drug-resistant cell lines have indicated that one of the most important determinants of MTX cytotoxicity is the cellular level of its target enzyme DHFR. Indeed, MTX resistance in animal cell lines has been frequently associated with the overproduction of DHFR enzyme (9, 10). This class of resistant cells is of interest not only because of the frequency of its occurrence in vitro but also because of the findings first noted by Alt et al. (11) in a murine cell line, that the increased levels of DHFR resulted from the amplification of DHFR genes. Subsequent studies have demonstrated the association of DHFR gene amplification with MTX resistance in several other animal cell lines (12–14), as well as in MTX-resistant human cells derived both in vitro (15–18) and in vivo (19–22).

In this paper, we examine the mechanisms involved in the regulation of DHFR in response to hormones using a MTX\textsuperscript{R} human breast cancer cell line (MCF-7) which contains increased levels of DHFR and amplified DHFR gene sequences (15). Although MTX\textsuperscript{R} MCF-7 cells were specifically selected for a high degree of resistance to MTX (>1000-fold), they have retained at least some of the estrogen-sensitive characteristics observed in the parental MCF-7 cell line from which they were derived (35, 36), and incubation of the resistant subline with estradiol results in an increase in their rate of growth. Moreover, treatment of these MTX\textsuperscript{R} cells with estrogen results in an additional 1.5- to 3-fold increase in their already elevated basal level of DHFR, an effect which is similar in magnitude to the regulation observed in animal cell lines (23–29).

We have reported previously the isolation of a MTX\textsuperscript{R} human breast cancer cell line (MCF-7) which contains increased levels of DHFR and amplified DHFR gene sequences (15). Although these MTX\textsuperscript{R} MCF-7 cells were specifically selected for a high degree of resistance to MTX (>1000-fold), they have retained at least some of the estrogen-sensitive characteristics observed in the parental MCF-7 cell line from which they were derived (35, 36), and incubation of the resistant subline with estradiol results in an increase in their rate of growth. Moreover, treatment of these MTX\textsuperscript{R} cells with estrogen results in an additional 1.5- to 3-fold increase in their already elevated basal level of DHFR, an effect which is similar in magnitude to the regulation observed in animal cell lines (23–29).

In this paper, we examine the mechanisms involved in the modulation of DHFR in MTX\textsuperscript{R} MCF-7 cells in response to estrogen as well as to antiestrogens. Because the rate of uptake of MTX into cells is relatively slow and its subsequent binding to DHFR rapid and stoichiometric, even small increases in the cellular level of DHFR can markedly affect drug sensitivity (9–22). Although hormonal therapies as well as chemotherapy are useful treatments for breast cancer, their use in combination has in general been disappointing (37). The studies presented in this paper on the regulation of DHFR in response to hormones may help to provide a rationale for the more effective use of hormonal therapies.
HORMONAL MODULATION OF DHFR GENE EXPRESSION

therapies in combination with chemotherapy for the treatment of this disease.

MATERIALS AND METHODS

Materials. MTX was supplied by Dr. Robert Engle of the Drug Development Branch of the National Cancer Institute. [3H]MTX, [3H]Juridine, [3H]thymidine, and [3H]methionine were all obtained from Amersham-Searle (Arlington Heights, IL). [3H]Leucine was obtained from New England Nuclear (Boston, MA). Methtrexate-Sepharose beads were obtained from Pierce Chemical Co. (Rockford, IL). MTX and [3H]MTX were further purified by ion-exchange chromatography as described previously (15). Tamoxifen [1-(4/3-dimethylaminoethoxyphenyl)-1,2-diphenoxybut-1-ene] was obtained from ICI, Ltd. (Wilmington, DE).

Cells and Tissue Culture. The conditions for the growth of WT MCF-7 cells (36) and the MTX-MCF-7 subline were described previously (15). MTX-MCF-7 cells were grown in drug-free medium for 2 passages prior to their use in growth studies or enzyme assays. Protein determinations were done according to the method of Lowry et al. (38).

Hormonal Stimulation of DHFR. Cells (100,000) were plated in triplicate in 6-well Linbro dishes following 2 passages in 2 ml of IMEM containing 5% CPCS (35). When cells were approximately 50% confluent, the medium was changed to IMEM containing no serum, and the cells were then incubated for 24 h at 37 °C in the presence or absence of hormone. After the incubation period, the cells were washed 3 times with ice-cold PBS containing 0.02% EDTA and harvested by scraping. Following centrifugation at 1500 × g for 20 min, the cell pellets were frozen at −20 °C. The cell pellets were thawed in 1.0 ml of 0.01 M Tris-HCl, pH 6.5; the suspension was sonicated for 10 s, using a microtip of a Branson sonicator (Danbury, CT) at a setting of 1.5; and the cell debris was cleared by centrifugation at 2000 × g for 30 min. DHFR was measured using either a [3H]MTX binding assay (39) or a spectrophotometric assay as described previously (15).

DHFR Synthesis. The rate of DHFR synthesis was measured following incubation of cells in 6-well Linbro dishes in the presence or absence of the antiestrogen tamoxifen (2 μM) for 24 h in serum-free IMEM. [3H]-Methionine (300 μCi; specific activity, 1156 Ci/mmol) in methionine-free IMEM was added to each well, and the cells were incubated for 2 h at 37 °C. At the end of the incubation, the cells were washed and harvested as described above, and the cell pellets were stored at −20 °C. Radio-labeled DHFR was separated from total cell protein using small MTX-Sepharose affinity columns as described previously (15).

DHFR degradation studies were performed by plating cells in 6-well Linbro dishes in IMEM containing 5% CPCS. After 4 days, the cells were incubated with 100 μCi of [35S]-methionine in methionine-free IMEM at 37 °C for 9 h. The medium was then removed and replaced with serum-free IMEM containing unlabeled methionine (15 mg/ml) with or without estradiol (10 nM). The cells were harvested after incubation at 37 °C for various periods of time, and the amount of radioactivity remaining in DHFR was determined as described above. [35S]-Methionine incorporation into total protein was measured by precipitation of aliquots of cell cytosol in 10% trichloroacetic acid on ice, followed by filtration through 0.45-μm HA filters (Millipore, Boston, MA).

RNA, DNA, and protein synthetic rates were measured by incubating cells at 37 °C for 2 h with either [3H]Juridine (2 μCi/ml; 25 Ci/mmol), [3H]-thymidine (2 μCi/ml; 5 Ci/mmol), or [3H]leucine (2 μCi/ml; 45 Ci/mmol). Cells were washed and harvested as described above, following which the cell pellets were resuspended in 1 ml of 0.01 M Tris, pH 7.5, containing 0.001 M EDTA. Following sonication for 10 s, aliquots of cell extracts were precipitated in 10% ice-cold trichloroacetic acid and placed on ice for 30 min. The precipitate was collected by filtration as described above and counted in a Beckman liquid scintillation counter following the addition of 10 ml of Hydrofluor.

Receptor Analyses. Estrogen receptors were determined using a whole-cell assay. Cells were passaged in IMEM containing 5% CPCS for 2 weeks and then plated in the same medium in 6-well Linbro dishes (10,000 cells/well). After 4 days, the cells were washed with serum-free IMEM and then incubated in 2 ml of serum-free IMEM containing various concentrations of [3H]estradiol (0.2 to 10 nM; 130 Ci/mmol). Following incubation at 37 °C for 2 h, the cells were washed 3 times in ice-cold PBS and then lysed by the addition of 2 ml of NaOH. After incubation of the cell lysate at 60 °C for 1 h, 0.20 ml of 10 N HCl were added to each sample, and aliquots were counted in a liquid scintillation counter as described previously. Nonspecific binding was determined by simultaneous incubation of a duplicate set of cells in the presence of unlabeled diethylstilbestrol (200 nm).

Progestrone receptor assays were done using cell cytosol as described previously (40). Radiolabeled synthetic progestin, [3H]Promegestone (R5020; 70 Ci/mmol), was used as the ligand. Specific binding to both the estrogen and progesterone receptors was analyzed by Scatchard plots (41).

RESULTS

Estrogen Sensitivity of WT and MTX-MCF-7 Cells. Cells containing a 50-fold excess of DHFR enzyme and amplified DHFR genes represent a useful model system in which to study the regulation of expression of this housekeeping gene. Before studying the effects of hormones on the regulation of DHFR in the MTX-MCF-7 cells, it was first necessary to determine whether or not, during their subsequent selection for a high degree (>1000-fold) of resistance to MTX, these cells retain the same sensitivity to hormones as the parental WT MCF-7 cells (35). Since the presence of high-affinity receptors is a prerequisite for steroid hormone action, we first compared the concentration of estrogen receptors as well as their relative binding affinities for estradiol in the MTX-MCF-7 and the parental WT MCF-7 cells. As shown in Table 1, both the concentration of estrogen receptors (181 versus 177 fmol/mg) as well as their apparent affinity constants for estradiol (2.7 versus 1.5 nM) were essentially identical in both cell lines. Thus, although the MTX-MCF-7 cells were selected for a relatively high degree of resistance to MTX (>1000-fold), these cells have retained the same concentration of high-affinity estrogen receptors which are present in the parental estrogen-sensitive MCF-7 cell line.

Although the presence of steroid receptors is necessary for estrogen action, the hormone-binding results presented in Table 1 do not, by themselves, indicate whether these receptors are actually functional. Thus, further studies were required in order to evaluate the response and relative sensitivity of the MTX-MCF-7 cells to estrogen. Since one of the specific effects of estrogen on hormone-sensitive cells is the induction of progesterone receptors (42), the concentrations of these receptors in WT and MTX-MCF-7 cells were compared following their incubation in the presence or absence of 10 nm estradiol. As shown in Table 2, although the basal level of progesterone receptors is somewhat lower in the MTX-MCF-7 cells (363 versus 885 fmol/mg)...

Table 2

<table>
<thead>
<tr>
<th>Cells</th>
<th>Total sites (fmol/mg protein)</th>
<th>Kd (nM)</th>
<th>t</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT MCF-7</td>
<td>181</td>
<td>2.7</td>
<td>−0.92</td>
</tr>
<tr>
<td>MTX-MCF-7</td>
<td>177</td>
<td>1.5</td>
<td>−0.97</td>
</tr>
</tbody>
</table>

*Note: Cells were grown for 2 weeks in IMEM containing 5% CPCS. Estrogen receptors were determined using a whole-cell assay as described in "Materials and Methods."
mg), there is a similar (approximately 3-fold) induction in the concentration of these receptors in both cell lines 24 h after the addition of estradiol. Thus, MTXR MCF-7 cells not only contain high levels of estrogen receptors, but they have retained at least some of the hormonal responsiveness observed in the WT MCF-7 cells.

Since estrogen also induces a mitogenic response in hormonestensitive cells, we next examined the effects of estradiol on the rate of thymidine incorporation into MTXR MCF-7 cells at various times following the addition of hormone. As shown in Chart 1, beginning between 15 and 20 h after the addition of 10 nM estradiol to the MTXR MCF-7 cells, there is an increase in the rate of incorporation of thymidine into the acid precipitate, with the maximal effect occurring at approximately 27 h.

As mentioned earlier, studies in animal cell lines have indicated that the expression of the DHFR gene is cell cycle dependent (33, 34). Therefore, the level of this enzyme in the MTXR MCF-7 cells was examined at various times following the addition of estradiol. As shown in Chart 1, the time course of the induction of DHFR in these cells nearly parallels the effect of estradiol on the rate of thymidine with the maximal increase in enzyme occurring simultaneously with the maximal increase in the rate of precursor incorporation into DNA (approximately 27 h). Although the data in Chart 1 are expressed relative to the enzyme level present in the cells at time zero, it should be noted that estradiol induces an increase both in the total amount of DHFR as well as a specific increase in the intracellular level of DHFR relative to other cell proteins (see also Table 3). These data are consistent with results in animal cell lines which demonstrate that the maximal synthesis of DHFR enzyme occurs during S phase of the cell cycle (33, 34).

In order to examine the relative sensitivity of the MTXR MCF-7 cells to the stimulation of cell growth by estrogen, these cells were treated with varying concentrations of estradiol for 24 h, and the rate of DNA synthesis was measured by incubation with [3H]thymidine. As shown in Chart 2, incubation with 10 nM estradiol results in approximately a 2-fold increase in the rate of DNA synthesis. Moreover, this stimulation of the rate of thymidine incorporation in the MTXR cells is observed over a wide range of hormone concentrations, beginning as low as 10⁻¹⁰ M.

In a similar fashion, the effects of varying doses of estradiol on the induction of DHFR in the MTXR cells were also examined. As shown in Chart 3, the maximal hormonal effect is observed following incubation of these cells with 10 nM estradiol. In addition, the hormonal induction of DHFR, like that observed for the increase in thymidine incorporation, occurs over a broad range of estradiol concentrations (1 nM to 1 μM estradiol).

Although the effect of estrogen on DNA synthesis and the induction of DHFR levels are similar both with respect to their dose response to estradiol as well as the time course of their inductions, these 2 effects are not tightly coupled. Shown in Table 3 are the results obtained during the incubation of MTXR MCF-7 cells with estradiol in the presence or absence of 1-β-o-arabinofuranosylcytosine. Although the incorporations of thymidine and the level of DHFR obtained at time zero is set equal to 100%.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>DHFR (pmol/mg protein)</th>
<th>Fold increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>5.2</td>
<td>1.0</td>
</tr>
<tr>
<td>+ 1-β-o-Arabinofuranosylcytosine</td>
<td>9.1</td>
<td>1.8</td>
</tr>
<tr>
<td>+ Cycloheximide</td>
<td>3.1</td>
<td>1.0</td>
</tr>
<tr>
<td>+ Actinomycin D</td>
<td>5.2</td>
<td>3.8</td>
</tr>
</tbody>
</table>

Chart 1. Time course of estradiol effects. MTXR MCF-7 cells were plated (100,000 cells/well) as described in "Materials and Methods." When the cells were 50% confluent, the medium was removed and replaced with serum-free IMEM containing 10 nM estradiol. At the times indicated, one-half of the cells were incubated with [3H]thymidine (4 μCi/well) for 1 h, and the incorporation of radiolabel into DNA was determined as described in "Materials and Methods." The other half of the cells were harvested, and DHFR levels were determined using a [3H]MTX binding assay as described in "Materials and Methods." The results represent the amount of [3H]thymidine incorporated into the trichloroacetic acid precipitate (dpm/mg protein) (□) or the intracellular level of DHFR (pmol of [3H]MTX bound/mg of protein) (O) at each time point. Points, mean of triplicate samples plotted as the mean percentage of the ratio of the value obtained at each time point (hours after the addition of estradiol) divided by the results obtained at time zero (before the addition of estradiol); bars, SD. The value for both the incorporation of thymidine and the level of DHFR obtained at time zero is set equal to 100%.

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<th>Fold increase</th>
</tr>
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<tbody>
<tr>
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<td>1.0</td>
</tr>
<tr>
<td>+ 1-β-o-Arabinofuranosylcytosine</td>
<td>9.1</td>
<td>1.8</td>
</tr>
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<td>1.0</td>
</tr>
<tr>
<td>+ Actinomycin D</td>
<td>5.2</td>
<td>3.8</td>
</tr>
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</table>

Table 2

<table>
<thead>
<tr>
<th>Progesterone receptor induction by estradiol</th>
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<tbody>
<tr>
<td>Cells were grown in IMEM containing 5% CSCS for 2 weeks. The medium was changed to serum-free IMEM for 24 h containing 10 nM 17β-estradiol. Progesterone receptors in cell cytosol were determined as described in &quot;Materials and Methods.&quot;</td>
</tr>
<tr>
<td>17β-Estradiol (fmol/mg protein)</td>
</tr>
<tr>
<td>WT MCF-7</td>
</tr>
<tr>
<td>MTXR MCF-7</td>
</tr>
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</table>

Table 3

Effect of inhibitors on the hormonal induction of DHFR

MTXR MCF-7 cells were grown in IMEM containing 5% CSCS in 6-well Linbro dishes. When the cells were 50% confluent, the medium was changed to serum-free IMEM in the presence or absence of 10 nM estradiol and containing either 1-β-o-arabinofuranosylcytosine (10 μg/ml), cycloheximide (10 μg/ml), or actinomycin D (1 μg/ml), or no additional drugs. Following 24 h of incubation at 37 °C, the cells were harvested, and DHFR levels were determined using a [3H]MTX binding assay as described in "Materials and Methods." Results are expressed as the mean of triplicate samples.
Effects of Estradiol on DHFR Stability. We have demonstrated previously that the estrogen-induced increase in DHFR levels in these MTX/MCF-7 cells is associated with an increase in the rate of synthesis of this enzyme (15). Since steroid hormones can also regulate the level of a protein by altering its stability, we have measured the rate of degradation of DHFR in these MTX/MCF-7 cells during incubation at 37 °C in the presence or absence of estradiol. In the experiments shown in Chart 4, MTX/MCF-7 cells were preincubated with [35S]methionine in the absence of hormone for 12 h. Following the removal of the radiolabel from the medium, the cells were then incubated in the presence of excess unlabeled methionine with or without estradiol for 24 h, and the amount of radioactivity remaining in DHFR was determined at various times during the incubation as described in "Materials and Methods."

As shown in Chart 4, there is little loss of prelabeled DHFR enzyme during the 18-h period of incubation (6 to 24 h) under control conditions (no hormone). Although there was insufficient enzyme degradation to calculate a half-life for DHFR enzyme in these MTX/MCF-7 cells, it is apparent from these data that it is in excess of 18 h. This result is similar to that observed in animal cell lines (9, 10), in which the half-life of DHFR was estimated to be in excess of 60 h. Furthermore, incubation with estradiol has no effect on the stability of DHFR enzyme in these MTX/MCF-7 cells (Chart 4). Thus, the entire effect of the hormone on the modulation of DHFR levels in these breast cancer cells is apparently mediated through an estrogen-induced increase in the rate of DHFR enzyme synthesis (15).

Effects of Antiestrogen on DHFR. Whereas incubation with estrogen increases the rate of growth of hormone-sensitive breast cancer cells, treatment with antiestrogens, such as tamoxifen, inhibits their growth. In order to determine what effects antiestrogens might have on the regulation of DHFR, MTX/MCF-7 cells were incubated in the presence or absence of 2 µM tamoxifen for 24 h, and the rate of DHFR enzyme synthesis and the overall level of enzyme were measured. As shown in Table

Chart 2. Effect of estradiol on thymidine incorporation. MTX/MCF-7 cells (100,000/well) were plated in 6-well Linbro dishes in IMEM containing 5% CSCS. When cells were 50% confluent, the medium was replaced with serum-free IMEM and various concentrations of estradiol. Following 24-h incubation at 37 °C, [3H]-thymidine (2 µCi/ml) was added to each well, and the cells were incubated for an additional h. After the incubation period, the cells were harvested, and the amount of incorporation of radiolabel into DNA was determined as described. Columns, mean of triplicate samples; bars, SD.

Chart 3. Effect of estradiol on DHFR levels. MTX/MCF-7 cells were treated as described in the legend of Chart 1. Following incubation in the presence of varying concentrations of estradiol for 24 h, the cells were harvested, and the level of DHFR was measured as described in "Materials and Methods." Columns, mean of triplicate samples; bars, SD.

dione in the MTX/MCF-7 cells are inhibited >99% under these conditions, there is no inhibition of the induction of DHFR by estradiol. Thus, the modulation of DHFR in these cells by estrogen does not require concomitant DNA synthesis.

In other experiments, the requirement of protein or RNA synthesis in the hormonal induction of DHFR was examined by incubating cells with estradiol in the presence or absence of either cycloheximide or actinomycin D. As shown in Table 3, simultaneous incubation of MTX/MCF-7 cells with cycloheximide (10 µg/ml), which reduces the rate of [3H]-leucine incorporation into cell protein by >95% of control values (in both hormone-treated and untreated cells), eliminates the induction of DHFR by estradiol. Likewise, incubation of these cells with estradiol in the presence of actinomycin D (1 µg/ml) to inhibit RNA synthesis (>95% inhibition of [3H]-uridine incorporation) also abolishes the hormone-induced increase in DHFR. Thus, the hormonal induction of DHFR enzyme apparently requires both RNA and protein synthesis. These results will be discussed further below.

Chart 4. Effect of estradiol on DHFR degradation. MTX/MCF-7 cells were plated as described in the legend of Chart 1. When the cells were 50% confluent, the medium was changed to methionine-free IMEM containing 5% CSCS. [35S]-Methionine (200 µCi/well) was added, and the cells were incubated at 37 °C for 12 h. Following removal of the medium, the cells were washed and then incubated at 37 °C in the presence or absence of 10 nM estradiol in IMEM containing 5% CSCS and unlabeled methionine (15 mg/ml). After the appropriate periods of time, cells were harvested, and the amount of radioactivity remaining in DHFR was determined by MTX affinity chromatography as described in "Materials and Methods." Points, mean of triplicate samples; bars, SD.
Effects of tamoxifen on DHFR in MTX<sup>®</sup> MCF-7 cells

MTX<sup>®</sup> MCF-7 cells were grown in IMEM containing 5% CSCS as described in the legend of Chart 1. Cells were treated for 24 h in serum-free IMEM in the presence or absence of 2 μM tamoxifen. DHFR enzyme activity and the rate of DHFR enzyme synthesis were determined as described in "Materials and Methods."

<table>
<thead>
<tr>
<th>Treatment</th>
<th>DHFR (μmol/mg/h)</th>
<th>DHFR synthesis (dpm × 10&lt;sup&gt;-3&lt;/sup&gt;/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.3 ± 0.14&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.8 ± 0.9</td>
</tr>
<tr>
<td>+ Tamoxifen</td>
<td>2.7 ± 0.25</td>
<td>2.8 ± 0.1</td>
</tr>
<tr>
<td>% of decrease</td>
<td>18</td>
<td>39</td>
</tr>
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</table>

<sup>a</sup> Mean ± SD of triplicate samples.

4, there is approximately a 40% decrease in the rate of DHFR enzyme synthesis in the tamoxifen-treated cells. This is also associated with approximately a 20% decrease in DHFR enzyme level. Thus, in contrast to the results obtained during incubations with estradiol, treatment with tamoxifen results in an inhibition in the rate of DHFR enzyme synthesis and lower intracellular levels of this enzyme.

**DISCUSSION**

The studies presented in this paper demonstrate that, during their selection for resistance to MTX (>1000-fold), these MTX<sup>®</sup> MCF-7 cells have retained not only high concentrations of estrogen receptors but have also maintained a sensitivity to hormones that has been noted previously in the parental MCF-7 cell line (35). Thus, incubation of these drug-resistant cells with estradiol results in a 3-fold induction in the level of progesterone receptors. Furthermore, following incubation of these MTX<sup>®</sup> MCF-7 cells with physiological concentrations of estrogen, there is an increased rate of DNA synthesis as well as an additional 1.5- to 3-fold increase in the level of DHFR enzyme in these cells.

As alluded to earlier, the similarity in the time course of the increased rates of thymidine incorporation and the increased levels of DHFR following the addition of estradiol (see Chart 1) are consistent with observations in animal cell lines which indicate that the synthesis of DHFR enzyme is cell cycle regulated and is maximal during S phase (23, 34). However, since the induction of DHFR by estrogen occurs during simultaneous incubations with an inhibitor of DNA synthesis, these 2 hormonally regulated events are not tightly coupled. A similar dissociation of the regulation of DHFR levels and the synthesis of DNA has been reported in murine cells stimulated to grow following the addition of serum to quiescent cells (33).

The results on the turnover of DHFR enzyme in these MTX<sup>®</sup> MCF-7 cells indicate that the half-life of this enzyme in these breast cancer cells is relatively long (see Chart 4). These data are consistent with observations of the half-life of DHFR enzyme in murine and hamster cell lines (9, 10) but are somewhat longer than the 20-h half-life found in human KB cells (31). The reasons for these differences in DHFR enzyme half-life among different cell lines are not known. Furthermore, the results presented in Chart 4 demonstrate that estradiol has no apparent effect on the stability of this enzyme. Thus, in these MTX<sup>®</sup> MCF-7 cells, the estrogen-induced increase in DHFR apparently results entirely from an increase in the rate of DHFR enzyme synthesis (15). In contrast, treatment of these cells with the antiestrogen tamoxifen results in a decrease in the rate of DHFR enzyme synthesis and lower levels of DHFR.

Incubations performed in the presence of actinomycin D or cycloheximide suggest that both RNA and protein synthesis are required for the hormonal induction of DHFR. While results based on the use of such inhibitors are difficult to interpret because of the multiple metabolic effects that occur under these conditions, other studies in our laboratory using RNA-DNA hybridizations have shown that treatment of the MTX<sup>®</sup> MCF-7 cells with estradiol results in an increased concentration of DHFR mRNA in these cells (Ref. 43; Footnote 2). Since the regulation of DHFR gene expression in animal cells has been observed to involve transcriptional (28, 44) as well as posttranscriptional mechanisms (27, 29, 45), it will be of interest to determine the level(s) by which the modulation of DHFR gene expression by estrogen as well as antiestrogens is mediated in these breast cancer cells.

It must be emphasized that the studies presented in this paper do not address the question of whether the hormonal induction of DHFR in these breast cancer cells represents a primary (direct) or secondary (indirect) effect of estrogen on the regulation of DHFR gene expression. Indeed, since the synthesis of DHFR in murine and hamster cells appears to be specifically regulated during the cell cycle (33, 34), it is possible that the induction of DHFR in these MTX<sup>®</sup> MCF-7 cells results indirectly as a consequence of the mitogenic effect of the hormone on these cells. However, it is also possible that estrogen exerts a positive effect on the expression of the DHFR gene which is independent of the hormonal influence on cell growth. In either case, this DHFR gene-amplified, hormone-responsive cell line represents a unique model system in which to study the effects of steroid hormones on the regulation of an essential housekeeping gene.

As alluded to earlier, since the rate of MTX transport is relatively slow and its subsequent binding to DHFR rapid and stoichiometric, the level of DHFR enzyme is a critical determinant of MTX cytotoxicity in vitro. Indeed, in vivo studies indicate that only a few additional copies of the DHFR genes and small increases in DHFR levels may result in clinical resistance to this drug (18-21). Conversely, relatively small reductions in DHFR levels in tumor cells should increase the therapeutic effectiveness of MTX. Thus, these studies on the regulation of DHFR levels in MCF-7 cells suggest a possible rationale for the clinical use of MTX in combination with antiestrogen therapy for the treatment of breast cancer. In addition to the inhibitory effect of antiestrogen therapy on the growth of breast cancer cells, tamoxifen might potentiate the cytotoxicity of MTX through its inhibition of DHFR enzyme synthesis and reduction in enzyme levels in tumor cells.

These studies may have other therapeutic implications as well, since in addition to its effect on DHFR, estrogen has also been shown to induce the activity of a variety of other housekeeping enzymes in breast cancer cells including: thymidine kinase (46); thymidylate synthase (47); aspartate transcarbamylase (47); and orotate phosphoribosyl transferase (47, 48). The same mechanisms involved in the hormonal modulation of DHFR may also, in some coordinated fashion, effect the expression of these other housekeeping genes as well. Some of these enzymes, such as thymidylate synthase, are themselves target enzymes for antineoplastic drugs (e.g., fluorodeoxyuridine), thus indicating the potential for drug synergism with antiestrogens. In contrast, physiological doses of estrogen might augment the cytotoxicity of chemotherapy, not only by synchronizing the growth of breast cancer cells, thus rendering them more susceptible to cell cycle-
zymes such as orotate phosphoribosyl transferase which are involved in the intracellular conversion of antineoplastic prodrugs, such as 5-fluorouracil, to their active intermediates (48-50). Thus, these studies on the hormonal regulation of DHFR gene expression may have implications for the interaction of hormonal manipulations with a variety of chemotherapeutic agents.

Studies in vitro have demonstrated synergistic effects of antiestrogens with cytoktotic drugs, such as 5-fluorouracil, on breast cancer cell lines (51). However, despite the efficacy of both hormonal therapies and chemotherapy in the treatment of breast cancer, clinical trials which combine these 2 modalities have not, in general, produced results significantly better than that achieved by chemotherapy alone (37). On the other hand, the results from recent clinical trials suggest that new approaches in which specific endocrine manipulations were used sequentially with chemotherapy may potentiate the cytotoxicity of the latter and may prove clinically useful (52-54). Further studies on the metabolic and pharmacological consequences resulting from the interactions of these 2 modalities may help to provide a more rational basis for the use of hormonal therapy with cytotoxic drug therapy for the treatment of breast cancer.

REFERENCES


HORMONAL MODULATION OF DHFR GENE EXPRESSION

Effects of Estrogen and Tamoxifen on the Regulation of Dihydrofolate Reductase Gene Expression in a Human Breast Cancer Cell Line


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