Effect of Medroxyprogesterone Acetate on Proliferation and Cell Cycle Kinetics of Human Mammary Carcinoma Cells

Robert L. Sutherland, Rosemary E. Hall, Grace Y. N. Pang, Elizabeth A. Musgrove, and Christine L. Clarke

Garvan Institute of Medical Research, St. Vincent’s Hospital, Sydney, N.S.W. 2010, Australia

ABSTRACT

The effect of medroxyprogesterone acetate (MPA) on breast cancer cell proliferation kinetics was investigated in ten human breast cell lines growing as monolayer cultures. Significant inhibition of growth occurred only in the estrogen receptor-positive, progesterone receptor-positive cell lines, T-47D, MCF-7, ZR 75-1, BT 474, and MDA-MB-361. Among these cell lines sensitivity to MPA varied widely; concentrations required for 20% inhibition of growth ranged from 0.04 nM for T-47D to >100 nM for ZR 75-1 cells. Furthermore, although the most sensitive line, T-47D, had the highest level of PR, sensitivity to MPA was not correlated with PR levels among the responsive cell lines.

More detailed studies were undertaken with the T-47D cell line. The growth-inhibitory response was confined to the progestins: MPA, ORG 2058, RS020, and progesterone, while androgens, estrogens, and glucocorticoids were without effect over the same concentration range (0.1–100 nM). MPA-induced growth inhibition was associated with a significant decrease in the proportion of S-phase cells with an accumulation of cells in the G0-G1 phase of the cell cycle. Cells began to accumulate in G0-G1 after 12 h of drug treatment and the effect was maximal by 24 h, i.e., maximal effects were observed during the first cell cycle following drug treatment. By contrast, significant accumulation in G0-G1 required exposure of MCF-7 cells to MPA for at least two cell cycle times, i.e., 48 h and the effect was still increasing at 96 h. Stathmokinetic studies revealed that in both cell lines accumulation in the G0-G1 phase was due to an MPA-induced increase in the G1 transit time.

These data indicate that MPA and other progestins have direct growth inhibitory effects on estrogen receptor-positive and progesterone receptor-positive human breast cancer cells in vitro and these effects can be accounted for by a decrease in the rate at which cells traverse the G1 phase of the cell cycle.

INTRODUCTION

MPA is a synthetic progestin commonly employed in the treatment of hormone-responsive tumors especially breast cancer (1–4). Whether used alone, sequentially, or in combination with the antiestrogen Tamoxifen, MPA treatment results in objective response in at least one third of patients (5). In patients with PR-positive tumors, the response to progestin therapy can be as high as 70%.

MPA is thought to inhibit tumor growth by both direct effects on tumor cell proliferation and indirect effects due to changes in the hormonal environment (2–4), and potential mechanisms of progestin mediated tumor growth inhibition have been investigated in vitro, using human breast cancer cells in culture. Direct growth inhibitory effects of synthetic progestins on proliferation in the PR-positive T-47D and MCF-7 human breast cancer cell lines have previously been demonstrated by Vignon et al. (6) and Iacobelli et al. (7) and more recently in a mutant T-47D cell line by Horwitz and Freidenberg (8). Progestin-mediated growth inhibition of normal and malignant breast epithelia has also been demonstrated (9). These studies have demonstrated varying degrees of growth inhibition by progestins. However, there are reports suggesting that progestins are able to stimulate proliferation in malignant breast cells under certain conditions (10–12), indicating that the role of progestins in the proliferative response in normal and malignant breast cells is far from clear.

The implication that responsiveness to progestins is related to PR status has not been fully investigated in vitro, and therefore the present study was undertaken to document the effect of MPA on a wide range of receptor-positive and receptor-negative human breast cancer cells in culture. The cell cycle kinetic correlates of progestin-induced inhibition of breast cancer cell proliferation in vitro have also been determined, in an effort to delineate potential cellular mechanisms for the response of breast cancer cells to progestins.

MATERIALS AND METHODS

Cell Lines. MCF-7 cells (13) were supplied by Dr. Charles M. McGrath, Meyer L. Prentis Cancer Center, Detroit, MI. All other breast cell lines: T-47D (14), ZR 75-1 (15), BT 474 (16), MDA-MB-361 (17), BT 20 (18), Rs 0578T (19), HBL 100 (20), MDA-MB-231 (21), and MDA-MB-330 (17) were supplied by E.G. and G. Mason Research Institute, Worcester, MA, for the National Cancer Institute Breast Cancer Program Cell Culture Bank. All cell lines were maintained in RPMI 1640 medium supplemented with 5 mM glutamine, 14 mM sodium bicarbonate, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer, 20 μg/ml gentamicin, 10 μg/ml porcine insulin, and 10% FCS as previously described (22, 23). Cells were passaged once weekly to maintain almost continuous exponential growth.

Cell Growth Experiments. Except where stated otherwise, 5 × 10^4 cells in exponential growth phase were plated into 25-cm^2 flasks in 5-ml RPMI 1640 medium supplemented with the ingredients outlined above except for the FCS which was present at 5% rather than the 10% employed for stock cultures. After cells had completed one replication cycle, i.e., cell numbers had increased to approximately 10^5 cells/flask, MPA and other steroids were added to the culture medium from 1000-fold-concentrated stock solutions. This gave a final ethanol concentration of 0.1%. All drugs were added on only one occasion and were not replenished during the course of the experiments. Control cultures received ethanol alone and this concentration had no effect on the growth rate of any of the cell lines studied. At various times after drug administration cells were harvested with 0.05% trypsin-0.02% EDTA in Ca^2+-Mg^2+ free phosphate buffered saline (1.5 mM KH2PO4, 8.1 mM Na2HPO4, 2.7 mM KCl, 140 mM NaCl), counted under phase contrast on a hemocytometer and where appropriate a sample was stained for DNA analytical flow cytometry.

In some experiments cells were grown in charcoal-stripped FCS (CS-FCS) to remove the influence of steroids present in FCS. The CS-FCS was prepared as previously described (24).

Drug and Hormone Treatments. Medroxyprogesterone acetate (17α-acetoxy-6-methyl-4-pregnene-3,20-dione) was obtained from Upjohn Pty Ltd, Sydney, Australia, through the courtesy of Dr. Dudley Jacobs.
ORG 2058 (16α-ethyl-21-hydroxy-19-norpregn-4-en-3,20-dione) was purchased from Amersham Australia, Sydney, and R5020 (17α-21-dimethyl-19-norpregn-4,9-diene-3,20-dione) from Du Pont (Australia) Ltd, Sydney. All other steroids were obtained from Sigma Chemical Co., St. Louis, MO. All steroids were prepared as stock solutions (10−7–10−10 M) in analytical reagent grade ethanol and stored at −20°C.

DNA Flow Cytometry. Approximately 1 × 10⁶ cells in 2 ml of medium were stained for DNA flow cytometry with ethidium bromide-mithramycin as previously described (25). Chicken erythrocytes (approximately 10⁶) were included as an internal marker. RNase (type IA, Sigma Chemical Co.) was added directly to the stained cell preparations 5 to 15 min before analysis to yield a final concentration of 1 mg/ml. Analysis was performed on a Model ICP22 pulse cytometer (Ortho Instruments, Westwood, MA) with excitation at 360 to 460 nm and fluorescence detection at greater than 550 nm. Estimates of the cell cycle kinetic parameters, i.e., the proportion of cells in the G0-G1, S, and G2 + M phases of the cell cycle, were calculated from the resulting DNA histograms using a planimetric method of analysis (26).

Stathmokinetic Studies. The rate of efflux of cells from the G1 phase of the cell cycle was determined using stathmokinetics and flow cytometry as previously described (22, 27). ICRF 159 (Razozone) was used to prevent the re-entry of cells into G1 phase (28). This drug was supplied by ICI Pharmaceuticals Division, Macclesfield, Cheshire, UK, and was stored as a 100-fold-concentrated solution, i.e., 5–10 mg/ml in 0.4 M HCl at 4°C. Immediately following addition of 50 μl ICRF 159 solution to the medium an appropriate amount of 1 M NaOH was added to neutralize the acid.

At various times following addition of ICRF 159 to T-47D or MCF-7 cells, which had been treated with MPA for 24 and 48 h, respectively, cells were harvested, stained for DNA flow cytometry, and the proportion of cells in G0-G1, S, and G2 + M phases of the cell cycle was calculated. The proportion of cells remaining in the G0-G1 phase following correction for polyploid cells induced by treatment with ICRF 159 was calculated as previously described (22) and the log percentage G0-G1 phase cells plotted against time.

Progesterone Receptor Assays. The concentration of PR was determined using a whole cell radioligand (ORG 2058) binding technique that has been previously described (29). In brief, cells were grown to confluence in 24-well tissue culture trays in RPMI 1640 medium supplemented as outlined above but with 5% FCS. The monolayers were washed once with binding buffer (RPMI 1640 without insulin and FCS but containing 0.1% bovine serum albumin, pH 7.0) and incubated for 1 h at 37°C with 200 μl binding buffer containing [3H]ORG 2058, over the concentration range 0.25–5.0 nM, and 1 μM hydrocortisone to saturate the glucocorticoid receptor (GR). Nonspecific binding was measured in the presence of 1 μM unlabeled ORG 2058. Binding was terminated by placing the trays on ice, aspirating the supernatant, and washing the monolayer for 20 min with ice-cold PBS containing 5% bovine serum albumin. The supernatant was aspirated and the cells were then solubilized in 0.5 M NaOH-0.1%. Triton X-100, and an aliquot was taken for estimation of radioactivity. Bound and unbound ligand concentrations were calculated and these binding data were analyzed according to Scatchard (30).

RESULTS

The T-47D cell line has given rise to a number of variants in different laboratories (5, 8, 31). In this laboratory we found that the T-47D cells obtained from E.G. and G. Mason Research Institute at passage 74 remained stable in terms of response to tamoxifen and MPA for at least a further 80 weekly passages (32). Since these cells were maintained by us in almost constant exponential growth, cell population doubling times gradually decreased with passage number from 35 to 40 h (23, 24) to 22–24 h in the present study.

Effect of MPA on Cell Growth. Fig. 1 shows the effects of various concentrations of MPA on the growth of T-47D and MCF-7 breast cancer cells. T-47D cells grew exponentially for about 3 days with a mean population doubling time of approxi-
10 μM MPA was approximately 65% of control compared with T-47D cells where concentrations of ≥1 nM reduced cell numbers to 30% of control (Fig. 2).

In an attempt to further define the sensitivity of breast cancer cell lines to MPA, a series of five cell lines was exposed to increasing concentrations of MPA for a period corresponding to approximately four population doublings of the control cultures. The cell lines studied were: T-47D, MCF-7, ZR 75-1, MDA-MB-361, BT 474, MDA-MB-330, BT 20, HBL 100, HS 0578T, MDA-MB-231, and MDA-MB-330. Data points, means of six to nine flasks from two to three experiments in the case of ER+ cell lines and for ER− lines data points are the means of three flasks from each of the five lines, i.e., 15 observations/data point. Standard errors ranged from 0.3 to 11.3% of the mean and are not shown.

Table 1 Progesterone receptor levels in human breast cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Affinity* (nM)</th>
<th>Concentrationa (sites/cell)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-47D</td>
<td>1.09 ± 0.14</td>
<td>2,279,700 ± 7,550 (11)</td>
</tr>
<tr>
<td>BT 474</td>
<td>0.53 ± 0.03</td>
<td>453,840 ± 7,020 (2)</td>
</tr>
<tr>
<td>ZR 75-1</td>
<td>0.90 ± 0.08</td>
<td>405,718 ± 122,064 (7)</td>
</tr>
<tr>
<td>MDA-MB-361</td>
<td>0.61 ± 0.04</td>
<td>188,760 ± 9,710 (2)</td>
</tr>
<tr>
<td>MCF-7</td>
<td>0.95 ± 0.14</td>
<td>91,560 ± 5,121 (7)</td>
</tr>
<tr>
<td>BT 20, HBL 100, HS 0578T, MDA-MB-231, MDA-MB-330</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* The affinity (Kd) was determined according to Scatchard (30) using tritiated ORG 2058 as the ligand.
  a The concentration in sites/cell was determined from the molar concentration of ORG 2058 specifically bound and the number of cells/well.
  b Mean ± SEM.
  c Number of observations.

There was therefore a >2000-fold range in sensitivity to MPA among these ER+, PR+ cell lines. In all cases the progesterin-mediated growth inhibition could be accounted for by a drug-induced decrease in the rate of cellular proliferation as there was no evidence of MPA having cytotoxic effects on the cell lines studied.

Precise interpretation of the relative sensitivities, as expressed by IC50 values, was complicated by the shapes of the dose-response curves. In general, reduced sensitivity to MPA was accompanied by reduced maximal responsiveness, e.g., in the most sensitive cell line T-47D, maximal responsiveness resulted in a 70% inhibition of growth while in the least sensitive ZR-75-1 cells a maximum decrease in proliferation of approximately 20% was observed (Fig. 2).

Effect of Estradiol on Growth Responses to MPA. Since the experiments described in Figs. 1 and 2 were conducted in the presence of endogenous estrogen present in FCS, we measured growth responses to MPA in FCS and CS-FCS in both T-47D and MCF-7 cells. In a previous study from this laboratory (33) it was observed that growth responses to E2 in T-47D cells were maximal after 3 weekly passages in CS-FCS and at E2 concentrations between 1 and 100 nM. Thus the effects of MPA in the presence and absence of E2 were investigated in cells that had been either continuously passaged in FCS or had been passaged for the previous 3–4 weekly passages in CS-FCS. The data from these experiments are presented in Fig. 3.

In both cell lines, E2 (1, 10, and 100 nM) failed to increase the growth rate of cells previously passaged in FCS. However, under these experimental conditions E2 caused a small but significant reversal of the growth inhibitory response to MPA (P < 0.01 and P < 0.05 for MPA versus MPA + E2 in T-47D and MCF-7 cells, respectively).

When MCF-7 cells were passaged in CS-FCS their responses to MPA and E2 were identical to those in cells passaged in FCS (Fig. 3). In contrast prior passage of T-47D cells in CS-FCS reduced the growth rate by approximately 50% and this growth rate was restored by addition of 1–100 nM E2. Treatment of T-47D cells with 10 nM MPA reduced cell numbers to about 30% of control irrespective of prior culture conditions and growth rates, i.e., cell numbers in MPA-treated cultures were 31% of control in cells passaged in FCS and 28% of control in cells passaged in CS-FCS. Simultaneous addition of MPA and E2 again led to a partial reversal of the MPA-induced growth inhibition (P < 0.005 for the difference between MPA and MPA + E2). However, the percentage of increase in cell numbers in MPA + E2 treated cultures compared with MPA alone was greater in T-47D cells previously passaged in CS-FCS (47% increase) than in cells grown in FCS (17% increase).

Together these data indicate that estrogens can only induce a partial reversal of MPA-induced inhibition in T-47D and MCF-7 breast cancer cells.

Steroid Specificity of Growth Inhibition. To test that the observed effects of MPA were typical of progestins, the effects of a range of steroids on T-47D cell proliferation were investigated and the results are reported in Fig. 4. The two synthetic progestins, ORG 2058 and MPA, were by far the most potent growth inhibitors, as was R 5020 (data not shown). Progesterone was not only about 1/1,000th of the activity of the synthetic progestins but this can probably be accounted for by the extensive metabolism of this steroid which would have occurred during the 4-day growth assay (34). The androgens, testosterone and dihydrotestosterone, had some growth inhibitory activity between 10 and 100 nM (Fig. 4). Natural and synthetic estrogens (E2 and diethylstilbestrol) and glucocorticoids (hydrocortisone...
Effect of MPA on Cell Cycle Phase Distribution. The distribution of T-47D cells was cumulative with increasing time of drug exposure. As can be seen in Fig. 7 there were minimal changes in the proportions of G0-G1, S, and G2 + M phases during the first 24 h of treatment with MPA. Data points are the mean ± SEM of three to five estimates from three separate experiments. Error bars, not shown where they did not exceed the size of the symbol.

To investigate in more detail the time course of changes in the proportions of G2-G1 and S-phase cells following MPA administration to T-47D cells, these parameters were measured at 3–6 h intervals during the first 48 h of drug treatment (Fig. 6). During this time period control cells maintained exponential growth as judged by a constant proportion of the cell population in G0-G1 and S phases. There was no significant change in cell cycle phase distribution during the first 12 h of treatment with 10 nM MPA but between 12 and 24 h there was a rapid decline in the percentage of S-phase cells which was paralleled by accumulation of cells in G2-G1 phase. No further change occurred with time indicating that all the effects of MPA in this cell line were apparent during the first cell cycle following drug exposure, i.e., during the first 24 h. In contrast, the effects of MPA on MCF-7 cells were cumulative with increasing time of drug exposure. As can be seen in Fig. 7 there were minimal effects of MPA on cell cycle phase distribution during the first 24 h of treatment but thereafter there was a time- and concentration-dependent accumulation of MCF-7 cells in G2-G1 phase at the expense of S phase and to a lesser extent G1 + M cells.

The decrease in cell proliferation rate induced by MPA in the other 3 ER+, PR+ cell lines, i.e., BT 474, MDA-MB-361,
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Fig. 6. Time course of changes in \( G_0-G_1 \) and \( S \) phase cells following treatment of T-47D cells with 10 nM MPA. T-47D cells in exponential growth phase were treated with 10 nM MPA, harvested at the times indicated, and the proportion of cells in the \( G_0-G_1 \) phase of the cell cycle measured by analytical DNA flow cytometry as described in "Materials and Methods." Treatments were: control (\( \bullet \)), 10 nM MPA (\( \triangle \)). Data points, means ± SEM of eight to eighteen flasks from four to six separate experiments.

Fig. 7. Effect of medroxyprogesterone acetate on the cell cycle phase distribution of BT 474, MDA-MB-361, and ZR 75-1 human breast cancer cells. Cells were harvested daily and stained for analytical DNA flow cytometry as described in "Materials and Methods." Treatments were: control (\( \bullet \)), 10 nM (\( \triangle \)), and 100 nM (\( \Delta \)) MPA. Data points, means from triplicate flasks.

Table 2. Effect of medroxyprogesterone acetate on the cell cycle phase distribution of BT 474, MDA-MB-361, and ZR 75-1 human breast cancer cells

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Control</th>
<th>10 nM MPA</th>
<th>100 nM MPA</th>
</tr>
</thead>
<tbody>
<tr>
<td>BT 474</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( G_0-G_1 )</td>
<td>71.5 ± 1.5</td>
<td>78.6 ± 0.7</td>
<td>80.7 ± 0.8</td>
</tr>
<tr>
<td>( S )</td>
<td>19.3 ± 1.3</td>
<td>14.4 ± 0.6</td>
<td>12.8 ± 0.7</td>
</tr>
<tr>
<td>( G_2+M )</td>
<td>9.2 ± 0.1</td>
<td>7.0 ± 0.2</td>
<td>6.5 ± 0.2</td>
</tr>
<tr>
<td>MDA-MB-361</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( G_0-G_1 )</td>
<td>66.1 ± 5.5</td>
<td>70.6 ± 3.7</td>
<td>72.1 ± 3.9</td>
</tr>
<tr>
<td>( S )</td>
<td>24.9 ± 3.4</td>
<td>21.1 ± 2.4</td>
<td>19.4 ± 0.9</td>
</tr>
<tr>
<td>( G_2+M )</td>
<td>9.0 ± 2.2</td>
<td>8.2 ± 1.3</td>
<td>8.4 ± 3.0</td>
</tr>
<tr>
<td>ZR 75-1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( G_0-G_1 )</td>
<td>61.8 ± 1.1</td>
<td>64.3 ± 0.5</td>
<td>69.6 ± 2.0</td>
</tr>
<tr>
<td>( S )</td>
<td>29.4 ± 0.8</td>
<td>26.8 ± 1.2</td>
<td>23.0 ± 1.5</td>
</tr>
<tr>
<td>( G_2+M )</td>
<td>8.7 ± 0.7</td>
<td>8.9 ± 0.3</td>
<td>7.3 ± 0.5</td>
</tr>
</tbody>
</table>

Fig. 8. Effect of pretreatment with medroxyprogesterone acetate on the rate of efflux of T-47D cells from the \( G_0-G_1 \) phase of the cell cycle. Cells (10^6) in exponential growth phase were treated with MPA at the following concentrations: 0 (\( \bullet \)), 0.025 (\( \triangle \)), 0.05 (\( \Delta \)), and 10 nM (\( \triangle \)). After 24 h treatment, ICRF 159 was added at a final concentration of 100 ng/ml. Replicate flasks were harvested at the times indicated, and the proportion of cells in the \( G_0-G_1 \) phase of the cell cycle computed from DNA histograms obtained by flow cytometry as described in "Materials and Methods." Data points, mean ± SEM of three to five separate determinations; error bars, not shown where they did not exceed the size of the symbol.

Effect of MPA on the Rate of Efflux of Cells from \( G_0-G_1 \) Phase. In order to assess if the MPA-induced accumulation of T-47D cells in \( G_0-G_1 \) phase was due to arrest of cells in this phase or to an increase in the time required to traverse \( G_1 \) phase, the rate of efflux of cells from \( G_1 \) phase, following 24 h of pretreatment with various concentrations of MPA, was measured using techniques previously developed for the study of tamoxifen (22). In control cultures cells left \( G_0-G_1 \) with a single exponential rate of decay with \( t_0 \) of approximately 7 h while pretreatment with MPA led to a concentration-dependent decrease in the proportion of T-47D cells leaving \( G_0-G_1 \) during the 48 h of study (Fig. 8). At the highest concentration tested, i.e., 10 nM, cell cycle progression appeared to be almost completely arrested for 36 h with a subsequent small decline in \( G_0-G_1 \) cells between 36 and 48 h. Similarly, 0.05 nM MPA induced arrest of cell cycle progression for 12–24 h and this was followed by a loss of cells from \( G_0-G_1 \) indicating a resumption of cell cycle progression albeit at a rate appreciably slower than that observed in control cultures. Finally at the 0.025 nM concentration, complete arrest of cell cycle progression was short lived, i.e., about 3 h, but the rate at which cells were able to subsequently progress through \( G_1 \) phase was significantly retarded. The simplest interpretation of these data is that in this cell line MPA, and presumably other progestins, cause a transient concentration-dependent arrest of cell cycle progression followed by a concentration-dependent decrease in the rate at which cells can traverse the \( G_1 \) phase of the cell cycle.

Similar experiment were undertaken with MCF-7 cells which had been pretreated with MPA for 48 h (Fig. 9). In agreement with our previous observations the rate of efflux of MCF-7 cells...
from the $G_0$-$G_1$ phase of the cell cycle was best described as the sum of two exponential components which have previously been defined functionally as "slowly cycling" and "rapidly cycling" pools (22). As with T-47D cells pretreatment of MCF-7 cells with MPA led to a concentration-dependent decrease in the rate of efflux from $G_0$-$G_1$ phase. There was no evidence of complete arrest of cell cycle progression in this cell line and as with the effects of tamoxifen the decrease in growth rate could be largely explained by an increase in the proportion of "slowly cycling" cells which increased in a concentration-dependent way from 13% in control cultures to 28% in cells pretreated with 100 $\mu$M MPA.

**DISCUSSION**

The present study has clearly demonstrated that MPA and other progestins can directly inhibit the growth of human breast cancer cell lines in culture. The ER- and PR-positive cell lines were far more sensitive as a group to the growth inhibitory action of MPA than the receptor-negative cell lines. This *in vitro* observation correlates well with the clinical picture, in which higher numbers of responders to progestin therapy are found among patients with PR-positive tumors (5). Among the cell lines expressing the PR, the sensitivity to MPA and the maximum inhibition induced varied markedly, despite the likelihood that most of the observed effects were mediated via the PR. There was little relationship between sensitivity to MPA and PR levels in these cell lines. Sensitivity was in the order: T-47D > BT 474 > MCF-7 > MDA-MB-361 > ZR 75-1 while the PR concentrations, as assessed by whole cell radioligand exchange assays, were in the order: T-47D > BT 474 > ZR 75-1 > MDA-MB-361 > MCF-7. Although the exquisite sensitivity of T-47D cells was accompanied by the highest levels of receptor measured, the ZR 75-1 line had about 1/5th the number of receptors but 1/2,000th of the sensitivity. The lack of correlation between the sensitivity to growth inhibition by MPA and PR concentration suggests that responsiveness is related to PR status but not to PR concentration, and points to aberrations in receptor function or post-receptor events in some of these cell lines. In particular it would be of interest to know the relationship between estrogen inducibility of PR and progestin sensitivity in these cells. Such studies will form part of further experiments aimed at identifying the molecular basis of progestin sensitivity in breast cancer cells.

That the growth inhibitory effects of MPA were mediated by the PR was only convincingly demonstrated in T-47D cells where studies with a number of different steroids showed that responsiveness was closely related to affinity for the PR (29). Similar observations were made in MCF-7 cells where 1 $\mu$M of several progestins resulted in significant growth inhibition while the same concentration of dexamethasone or hydrocortisone was without effect. It is possible that in the other responsive cell lines, *i.e.*, BT 474, MDA-MB-361, and ZR 75-1 the effects of MPA on proliferation could be mediated via its known ability to interact with the GR. This, however, appears unlikely in view of the fact that all the ER-, PR-negative cell lines failed to respond to MPA despite the presence of significant concentrations of GR.

The effects of progestins on the growth of human mammary carcinoma are controversial. Clinical studies have clearly demonstrated the effectiveness of progestin therapy in advanced breast cancer, and its use as first-line therapy for patients with PR-positive tumors has been suggested (35). Studies *in vitro* and *in vivo* have also shown inhibitory effects of progestins on normal and malignant breast cells (9), and this study has extended these observations to show that the responsiveness of a series of 10 human breast cancer cell lines was related to their PR status. All these studies have provided convincing evidence that progestins have growth inhibitory effects in human breast cancer.

In contrast, animal models have been used to demonstrate that progestins can act either as tumor growth promoters or antagonists in carcinogen-induced tumors, depending upon the time during the natural history of the tumor at which these agents are administered (5). Furthermore, progestin pretreatment of animals has been shown to be protective against the development of carcinogen-induced tumors (9), yet progestins can also act as cocarcinogens in the presence of estrogen (5). It has been clearly demonstrated that progestins can antagonize the mitotic effects of estrogen in the uterus, but there has also been a suggestion that progestins can provoke uterine epithelial mitoses without prior estrogen treatment (36). This apparently paradoxical effect of progestins in animal systems may not be applicable to human breast cancer, although it must be noted that a recent brief report has suggested that progestational contraceptive steroids stimulate growth of malignant but not normal human breast tissue (10). Furthermore it is difficult to extrapolate from studies *in vitro* to those *in vivo* and it may well be that the diversity of effects observed *in vivo* reflect quite different physiological situations arising from changes in other circulating hormones known to modulate the effects of progestins, *e.g.*, estrogens.

The involvement of estrogens in the growth inhibition by progestins is not well understood. Studies from Rochefort's laboratory have indicated that the growth inhibitory response of human breast cancer cells to progestins is dependent upon estrogens and that progestins are ineffective in cells not stimulated by estrogen (6). A more recent report has shown in contrast that R 5020 stimulated growth of MCF-7 cells in phenol red-free media (11), and it has also been shown that MCF-7 cells adapted to grow in high concentrations of progestin are growth stimulated by this agent (12). Our data have
demonstrated that both T-47D and MCF-7 cells are growth inhibited by MPA when grown in CS-FCS. These cells were free from the influence of estrogen normally found in FCS, but not from the estrogenic effects of phenol red in the medium (37). Nevertheless, T-47D cells were still sensitive to added E₂, which restored the growth of CS-FCS-treated cells to that observed in FCS-treated cells, yet was unable to reverse the progesterin-mediated growth inhibition. MCF-7 cells were not sensitive to added E₂ in terms of increased growth, although some reversal of the progesterin response by simultaneous administration of estrogen was observed. The estrogen reversible component of the response in both T-47D and MCF-7 cells was minor. These data support the view that growth inhibitory responses to progestins can occur independently of estrogen action. This was previously shown most convincingly in a mutant T-47D cell line which was unresponsive to estrogen and antiestrogen but maintained high sensitivity to the growth inhibitory effects of R5020 (8).

The observation that maximal growth inhibition was 70% for T-47D cells and only about 20% in ZR 75-1 cells indicates that progesterin controlled pathways only account for part of the overall control of cell proliferation in these cells. This is sharply contrasted with the effects of antiestrogens which have been shown to completely arrest cell proliferation in the same cell lines (22–24). It is perhaps interesting to note however, that when MCF-7 cell growth is antagonized with high affinity antiestrogen analogs the ER-mediated component of the dose-response curve results in only 60–70% growth inhibition (38, 39). When assayed for the effects of MPA under similar culture conditions MCF-7 cells were maximally inhibited to only 30–35% illustrating that in this line, at least, maximal sensitivity via PR was markedly less than sensitivity via ER and supporting the concept that even in "hormone-responsive" cell lines ER and PR only control a part of the proliferation process.

Progestin-induced growth inhibition of T-47D cells was associated with marked changes in cell cycle phase distribution during the first cell cycle of exposure. No effect was observed during the first 12 h and the effect was half-maximal at 18 h. This indicates that the earliest mean point of action of the progesterin is 18 h before cells enter S phase. The point of action of the drug, however, must be within G₁ phase since progression through S and G₂ + M phases was unaltered during the first 12 h of treatment as evidenced by constant cell cycle phase distribution over this period. That the effect is early in G₁ phase is supported by data on cell growth which showed insignificant differences between control and drug-treated cell numbers after 24 h indicating that almost all cells could complete the first replication cycle following administration of the drug. Since the mean length of G₁ phase in these cells was 13.5 h (calculated from a mean doubling time of 22 h and a steady state percentage of G₁ phase of 62.5%) there must be a delay of at least 4.5 h between addition of MPA and the mean point of action. This delay may represent the time required for PR-mediated modulation of specific gene transcription to be reflected in the production and/or depletion of specific gene products controlling cell cycle progression in this cell line.

A further interesting aspect of the effects of progestins on cell cycle kinetics in T-47D cells was the concentration-dependent recovery from the changes that occurred between 12 and 24 h. At 48 h after treatment with 10 nm MPA the proportion of S-phase cells was beginning to increase indicating a resumption of cell cycle progression. This conclusion is supported both by the growth curve and the stathmokinetic data which showed an almost complete arrest of growth between 24 and 48 h but only partial arrest thereafter. At lower concentrations of MPA the cell cycle progression was less markedly inhibited. The time and rate of reinitiation of cell cycle progression were concentration-dependent but normal growth rates were not restored over the period studied at any concentration investigated. This illustrates that while the cells were able to rapidly escape from the transient complete arrest of progression the effects on overall proliferation rate were long-lived. The recovery of cells from arrest by MPA may be related to the rapid depletion of PR which is known to take place following ligand binding with subsequent time- and concentration-dependent replenishment. We are currently investigating this phenomenon.

The changes in cell cycle kinetics observed with MCF-7 cells were similar but much less marked than with T-47D cells. In particular, changes in cell cycle phase distribution were increased with prolonged exposure to MPA. Since growth experiments had shown that MCF-7 cells were not only significantly less sensitive to MPA but that the maximal response was also diminished, the results might be explained by the fact that a much smaller proportion of the total population was sensitive to MPA and that the effect was maximized by multiple exposure at the point of sensitivity.

Previous studies from this laboratory have shown that progestin treatment of T-47D and MCF-7 cells results in an increase in the level of both lactogenic and epidermal growth factor receptors in these cells (29, 40). Since the ligands for both these receptors have been shown to be mitogenic in these cells (41, 42) the relationship between this effect and progestin-induced growth inhibition is unclear. Perhaps the progestin-induced increase in cell surface receptors is the result of this class of steroid interfering with receptor turnover and/or the receptor signal transduction mechanism.

The present report has investigated the effect of MPA on the proliferative response of ten human breast cancer cells lines in culture. Cell lines which were PR-positive responded to MPA with decreased growth, although the sensitivity and the magnitude of the response was unrelated to PR concentration in these cell lines. The basis for the observed effect was shown to be the progestin-mediated accumulation of cells in the G₁ phase of the cell cycle, followed by a decrease in the rate at which progestin-treated cells progressed through the cell cycle. Whether this mechanism is operative in all breast cancer cells that respond to progestins has yet to be determined.

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Effect of Medroxyprogesterone Acetate on Proliferation and Cell Cycle Kinetics of Human Mammary Carcinoma Cells

Robert L. Sutherland, Rosemary E. Hall, Grace Y. N. Pang, et al.


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