Progestins Inhibit the Growth of MDA-MB-231 Cells Transfected with Progesterone Receptor Complementary DNA

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ABSTRACT

Because progesterone exerts its effects mainly via estrogen-dependent progesterone receptor (PgR), the expression of progesterone’s effects may be overshadowed by the priming effect of estrogen. PgR expression vectors were transfected into estrogen receptor (ER)-α and PgR-negative breast cancer cells MDA-MB-231; thus the functions of progesterone could be studied independent of estrogens and ERs. Eight stable transfectant clones expressing both PgR isoform A and B were studied for their growth response to progestrone and its analogues. Although progesterone had no effect on growth in the control transfectant, the hormone markedly inhibited DNA synthesis and cell growth in all of the PgR-transfectants dose-dependently from $10^{-12}$ to $10^{-6}$ M. This growth inhibition was associated with an arrest of cells in the G0/G1 phase of the cell cycle. Progestins medroxyprogesterone acetate, Org2058, and R5020 also strongly inhibited DNA synthesis, and their doses required for maximal inhibition of 60–70% were $10^{-12}$ M, $10^{-13}$ M, and $10^{-7}$ M, respectively. Antiprogestin ZK98299 alone had no effect, but the compound was capable of counteracting the inhibitory effect of progesterone. In contrast, RU486 inhibited DNA synthesis, and it showed no further effects when it was used concurrently with progesterone. These results indicate that progestins are per se antiproliferative via a PgR-mediated mechanism in breast cancer cells. More importantly, we have shown that progesterone may exert effective inhibitory control over the cell growth if the PgR expression is reactivated in ER- and PgR-negative breast cancer cells.

INTRODUCTION

The involvement of progesterone in the growth and development of breast cancer has been increasingly recognized in recent years. However, the roles of progesterone in the growth regulation of breast cancer are still controversial. Progestins are found to stimulate growth, have no effect, or inhibit growth in breast cancer cells (1–13). The conflicting findings affect clinical decision as to whether progestins or antiprogestins would be more appropriate endocrine therapies for PgR3-positive breast cancer. Although progestins such as MPA and megestrol acetate are often effectively used in the treatment of breast cancer (14–16), a number of reports advocate that antiprogestins may be new powerful tools for treating hormone-dependent breast cancer (17–19).

The reasons for the inconsistency in reported effects of progestins are not clear, but the involvement of estrogen and ER in the demonstrated effects of progestins is an important factor to consider. Although PgRs are regulated by a number of hormones and growth factors, they are ER-dependent gene products (20–23). The cells usually need to be primed by estrogenic compounds before the effects of progesterone are studied. It is conceivable that the effects of estrogen will be present almost as long as the effects of progesterone last. Indeed, Otto (24) has shown that a pulse of 1 nm estrogen for 1 min was sufficient to partially stimulate cell proliferation for 5 days. As a result, it is often difficult to differentiate the specific effects of progesterone from that of estrogen, or the expression of progesterone’s effects may be overridden or masked by the effects of estrogens. To delineate the functions of progestins in the regulation of breast cancer cell growth, we have established ER-independent expression of PgR by stably transfecting PgR cDNA into the ER-α and PgR-negative breast cancer cell line MDA-MB-231 which has recently been reported to express ER-β mRNA (25). The effects of progestins on cell growth and cell cycle kinetics were thus studied in the PgR-positive but ER-negative cell model.

Approximately one-third of all breast cancer cases are ER-negative (26), most of which are also PgR-negative and have aggressive biological behaviors. These patients are insensitive to endocrine therapy and are generally associated with poor prognosis. As an initial step toward gene therapy, an important goal of the present study was to determine whether progesterin or antiprogestin can exert inhibitory control over growth if the PgR expression is reactivated in ER- and PgR-negative breast cancer cells.

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3 The abbreviations used are: PgR, progesterone receptor; MPA, medroxyprogesterone acetate; ER, estrogen receptor; PgR-B, PgR isoform B; PgR-A, PgR isoform A; EIA, enzyme immunoassay; DCC, dextran-coated charcoal; DCC-FCS, DCC-treated FCS; BrdUrd, bromodeoxyuridine; PI, propidium iodide.
MATERIALS AND METHODS

Cell Culture. MDA-MB-231 cells and MCF-7 cells were obtained from American Tissue Culture Collection in 1995 at passages 28 and 147, respectively. MDA-MB-231 cells were cloned using 96-well plates by plating 0.5 cell/well. Clone 2 (known as MDA-MB-231-CL2) was selected for transfection studies. All of the cells were routinely maintained in phenol-red containing DMEM supplemented with 5% FCS, 2 mM glutamine, and 40 μg/ml gentamicin.

Chemicals. Progesterone, MPA, and estradiol-17β were obtained from Sigma Chemical Co (St. Louis, MO). Org2058 and R5020 were purchased from Amersham (England) and NEN (Boston, MA), respectively. RU486 (mifepristone) and ZK98299 (onapristone) were from Sintiwest Holdings, Inc. All of the tissue culture plastics and reagents were obtained from Life Technologies, Inc.

Transfection. PgR expression vectors hPR1 and hPR2 were generous gifts of Professor P. Chambon, Institute of Genetics and Molecular and Cellular Biology, Strasbourg, France. Vectors hPR1 and hPR2 contain human PgR cDNA coding for PgR-B and PgR-A, respectively, in pSG5 plasmid (27). Vector pBK-CMV (Stratagene) containing the neomycin-resistant gene was cotransfected with hPR1 and hPR2 into MDA-MB-231-CL2 cells using Lipofectin reagent (Life Technologies, Inc.). Neomycin-resistant clones selected in medium containing G418 (500 μg/ml) were further screened for vector pSG5 sequence by PCR using primers flanking the regions of nucleotide 182–405 bp, which have little sequence homology to the vector pBK-CMV. The PCR product of expected size was further confirmed by digestion with restriction enzyme CMV. The PCR product of expected size was further confirmed by digestion with restriction enzyme Neol. Levels of PgR in pSG5-positive transfectants were determined using the PgR EIA kit from Abbott Laboratories. Cells stably transfected with both vector pBK-CMV and pSG5 plasmid were used as transfection controls.

Western Blotting Analysis. Total proteins were extracted from the transfected cells by three cycles of freezing (liquid nitrogen) and thawing (37°C water bath) in buffer containing 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 1 mM DTT, and a cocktail of protease inhibitors for serine, cysteine, and metalloproteases (Boehringer Mannheim, Mannheim, Germany). Cytosols containing 50 μg of protein were separated by 7.5% SDS-PAGE and transferred to nitrocellulose membranes. After blocking overnight in Tris-buffered saline containing 0.05% Tween-20 and 3% BSA, the membrane was probed using PgR antibodies from Abbott Laboratories. The specific bands corresponding to PgR-A (Mr 90,000) and PgR-B (Mr 120,000) were detected with enhanced chemiluminescence (ECL) kit (Amer sham).

Cell Growth. Cells (2 × 10⁴) in their late exponential growth phase were seeded onto the 12-well plates in phenol red-free DMEM supplemented with 2 mM l-glutamine, 40 mg/l gentamicin, and 5% (DCC-FCS; Test Medium). The treatment of FCS with DCC was to remove from FCS the endogenous steroid hormones that may complicate the effects of progestins and antiprogestins. Two days later, the medium was replaced with fresh medium containing test compounds that were added from 1000-fold stock in ethanol. This gave a final concentration of ethanol of 0.1%. Treatment controls received 0.1% ethanol only. The medium containing test compounds was changed every other day, and the cell numbers were determined by counting on a hemocytometer after 5 days’ treatment.

DNA Synthesis. Cells (3 × 10⁴) were seeded onto 96-well plates in Test Medium and were treated with test compounds 2 days later. The effect on DNA synthesis after 48-h treatment was measured by an ELISA kit of BrdUrd incorporation (Boehringer Mannheim) according to the manufacturer’s instructions.

DNA Flow Cytometry: PI Staining. Cells (1 × 10⁴) in 6-well plates were grown in Test Medium for 3 days before they were treated with the test compounds for 24 h. The cells were then harvested and stained with PI in Vindelov’s (28) cocktail [10 mM Tris-HCl (pH 8), 10 mM NaCl, 50 mg PI/l, and 10 mg/l RNase A, and 0.1% NP40] for 20 min in the dark. The stained cells were analyzed in FACS Calibur flow cytometer (Becton Dickinson) with excitation wavelength of 488 nm. The resulting histograms were analyzed by program MODFIT (Becton Dickinson) for cell distribution in cell cycle phases. The average coefficient of variation (CV) is within 5%.

DNA Flow Cytometry: BrdUrd-DNA Analysis. Cells (2 × 10⁴) were grown in 60-mm Petri dishes in medium A for 3 days before they received 30 min pulse labeling with 20 μM BrdUrd. The cells were then washed and treated with 1 mM progesterone for 6, 16, and 30 h. At the designated time, the cells were harvested and trypsinized, fixed in 70% ice-cold ethanol, and the DNA was denatured into single-stranded molecules in 2 N HCI/0.5% Triton X-100. After neutralization with 0.1 M sodium tetraborate (pH 8.5), the cells were stained with FITC-labeled anti-BrdUrd (Becton Dickinson) for 30 min at room temperature in PBS containing 0.5% Tween-20/1% BSA. After washing with PBS/0.5% Tween-20/1% BSA, the cells were stained with 5 μg/ml of PI and analyzed on FACS Calibur flow cytometer for incorporated BrdUrd (FITC) and total DNA content (PI) simultaneously.

Statistical Analysis. Differences between treatments were tested by ANOVA. When significant differences were detected by ANOVA, multiple comparisons among means were performed by the least significant difference test. Correlation analysis was performed using the program in Microsoft Excel.

RESULTS

Characterization of Transfectants. ER-α- and PgR-negative breast cancer cells MDA-MB-231-CL2 were transfected with PgR expression vectors hPR1 and hPR2. Vector pBK-CMV was cotransfected with PgR expression vectors because it contains neomycin-resistant gene as selection marker for initial screening. Eight transfectant clones expressing both PgR-A (Mr 90,000) and PgR-B (Mr 120,000) were generated as shown by Western blotting analysis (Fig. 1). The identity of the M r 80,000 band is not clear. Because the band is absent in PgR-negative control transfectant CTC15 cells, it is possible that it represents the proteolytic fragments of PgR. The ratios of the two isoforms expressed differ among the transfectant clones. The transfectant clones ABC3, ABC4, ABC5, ABC13, ABC21, ABC24, ABC28, and ABC79 expressed 456, 815, 149, 517, 1429, 1992, 657, and 488 fmol/mg protein, respectively, as analyzed by EIA. By comparison, the levels of PgR reported for

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T47-D cells were 1000–3000 fmol/mg protein, and for estradiol-treated MCF-7 cells were 136–326 fmol/mg protein (1, 7, 9). Clone CTC15 transfected with both pSG5 and pBK-CMV plasmids was used as transfectant control. No PgR were detected in CTC15 by either EIA or Western blotting analysis.

**Effect of Progesterone on the Growth of PgR-transfected Cells.** We focused our study on PgR-transfected clone ABC28 initially because it expressed a moderate number of PgRs. The effects of progesterone on other PgR-transfected clones will be reported in the latter part of the text. Fig. 2 illustrates the effect of various concentrations of progesterone on BrdUrd incorporation (BrdUrd incorporation will also be referred to as DNA synthesis in the rest of the article) and cell growth of PgR-transfected clone ABC28 cells and the controls. The results are expressed as percentage of vehicle–treated controls. Measured 2 days after treatment, progesterone significantly (P < 0.01) inhibited BrdUrd incorporation of ABC28 cells from 10^{-11} M, and the inhibitory effect increased with increasing concentrations of the hormone (Fig. 2a). Maximal inhibition (80%) of DNA synthesis was achieved with 10^{-5} M. Similarly, progesterone also reduced the growth of the PgR transfectants measured by a cell-counting experiment in a concentration-dependent fashion (Fig. 2b). Maximal inhibition of cell growth was observed with 10^{-9} M, and the cell number was reduced by 70% after 5 days’ treatment. In contrast, progesterone had no significant effect on cell growth in transfected control clone CTC15 cells and the parental cell line MDA-MB-231-CL2 cells. In a, DNA synthesis was measured by ELISA of BrdUrd incorporation. Cells were plated onto 96-well plates (3000 cells/well) in phenol red-free DMEM supplemented with 5% DCC-FCS. Progesterone containing medium was added to the cells 2 days later at various concentrations in 0.1% ethanol. BrdUrd incorporation was measured 48 h later by ELISA according to manufacturer’s instructions. In b, cell growth was determined by cell counting. Cells (2 × 10^4) were plated onto 12-well plates in 1 ml of phenol red-free DMEM supplemented with 5% DCC-FCS. Progesterone-containing medium was added to the cells 2 days later at various concentrations in 0.1% ethanol. The medium containing progesterone was changed every 2 days. The cells were harvested after 5 days of treatment, and cell numbers were determined by counting on a hemocytometer. The results are expressed as percentages of vehicle-treated controls (mean ± SE, n = 4). c, the effect of progesterone on the growth of PgR-transfected clone ABC28 during 9 days of culture. Cells were plated in 24-well plates at 10,000/well in 0.5 ml of phenol red-free DMEM supplemented with 5% DCC-FCS. Progesterone (P)-containing medium was added to the cells 2 days later at concentrations of 0, 0.1 nM, and 0.1 μM in 0.1% ethanol. The medium containing progesterone was changed every 2 days. The cell numbers were determined by counting on a hemocytometer at 0, 2, 4, 7, and 9 days after treatment. The results are expressed as mean ± SE, n = 4.
Progesterone at $10^{-9}$ M and $10^{-6}$ M significantly ($P < 0.01$) inhibited DNA synthesis (Fig. 3a) and cell growth (Fig. 3b) in all of the PgR-transfectants. The inhibition varied from about 15% to 75% depending on the clones tested. More interestingly, significant ($P < 0.05$) correlation was observed between the extent of growth inhibition by progesterone and PgR concentrations analyzed by EIA.

Effect of Progesterone on Cell Cycle Kinetics. The growth-inhibitory effect of progesterone in PgR-transfected MDA-MB-231-CL2 cells was associated with dose-dependent reductions of the percentage of S phase cells, accompanied by an increase in G0-G1 phase cells (Fig. 4). To investigate in more detail the time course of changes in the distribution of cell cycle phases, ABC28 cells were analyzed for the distribution of cell cycle phases at 3–6-h intervals for a 48-h period after progesterone treatment (Fig. 5). As expected, vehicle-treated cultures displayed cyclic changes in distribution of cell cycle phases, most obviously seen in the percentage of S phase cells. Progressive reduction of S phase cells began at 12 h after treatment. Maximal reduction (65%) of S phase cells was achieved by 36 h. The S phase cells in progesterone-treated cultures was maintained at 40–50% of the vehicle-treated control during the 6-day period, during which time the medium was changed every 2 days (Fig. 6). The reduction of S phase cells was paralleled by the accumulation of cells in G0-G1 phase. The proportion of cells in G0-M phase in progesterone-treated cells began to decrease after 36 h of treatment. This decrease in G2-M phase cells is likely the consequence of the accumulation of G0-G1 phase cells.

The reductions of S phase cells in progesterone-treated ABC28 cells were also demonstrated in low serum condition under which the cells were slowly proliferating. As is shown in Table 1, the S phase fractions in progesterone-treated ABC28 cells grown in phenol red-free medium containing 1% DCC-FCS were reduced to approximately 12.5% after 15 h and 18 h of treatment, and to 10% after 24 h and 48 h of treatment. This is similar to the S phase fractions in vehicle-treated control cells were approximately 20–30% lower in medium containing 1% serum as compared with the cells in medium containing 5% serum.

The arrest in G0-G1 phase is further illustrated by the analysis of BrdUrd/DNA distributions measured for ABC28 cells at several time points after progesterone treatment (Fig. 7).
Vehicle-treated controls are displayed in parallel with progesterone-treated samples at each time point. The cells were labeled with 20 μM BrdUrd for 30 min before treatment. Two populations of G₀-G₁ cells are seen at 16 h after treatment; the top portion are BrdUrd-labeled cells that were in S phase at the time of BrdUrd pulse; the population at the lower portion are unlabeled cells that were in G₀-G₁ or G₂-M phase at the time of BrdUrd addition. It can be seen that many fewer unlabeled cells progressed into S phase in progesterone-treated cells than in controls at 16 h after treatment. On the other hand, at this time (16 h) BrdUrd-labeled cells could progress to G₂-M phase and subsequently into G₀-G₁ phase normally in progesterone-treated sample as compared with the controls. At 30 h after treatment, the cells had completed one cycle after the addition of progesterone and the second cycle began as indicated by the labeled cells progressing into S phase. There were considerably fewer number of cells progressing to S phase in progesterone-treated cells when compared with the controls at 30 h after treatment in both the BrdUrd-labeled and -unlabeled populations. Consequently, there was also a reduction of G₂-M phase cells in progesterone-treated cells.

Fig. 5 Time course of progesterone-induced changes in the distribution of cell cycle phases in PgR-transfected clone ABC28. Cells (1 × 10⁶) in 6-well plates were grown in phenol red-free DMEM supplemented with 5% DCC-FCS for 3 days before they were treated with 0.1% ethanol (○) or 1 nm progesterone in 0.1% ethanol (●). Cells were collected for cell cycle analysis at various times after the addition of progesterone. Methods are as described in Fig. 5. Results are expressed as mean ± SE, n = 4.

Fig. 6 Progesterone-induced changes in cell cycle distribution of PgR-transfected clone ABC28 cells during 6 days in culture. Cells (1 × 10⁶) in 6-well plates were grown in phenol red-free DMEM supplemented with 5% DCC-FCS for 3 days before they were treated with 0.1% ethanol (○) or 1 nm progesterone in 0.1% ethanol (●). Cells were collected for cell cycle analysis after 0, 2, 4, and 6 days of treatment with progesterone. Methods are as described in Fig. 5. Results are expressed as mean ± SE, n = 4.

Effect of Various Progestins, Antiprogestins, and Estriol on DNA Synthesis of PgR-transfectant. Our study with eight clones of PgR-transfected cells has shown that the inhibitory effect of progesterone on BrdUrd incorporation correlated well (P < 0.001) with that of cell number determination. BrdUrd incorporation was, therefore, used to measure the effect on the growth of various synthetic progestins and antiprogestins in PgR-transfectant clone ABC28. Progestins MPA, Org2058, and R5020 inhibited DNA synthesis of PgR–transfectant ABC28 cells, and the inhibition is concentration-dependent (Fig. 9a). The potencies were in the order: MPA > Org2058 > progesterone > R5020. MPA was by far the most potent growth inhibitor; the inhibitory activity begins with 10⁻¹⁷ M, and this is 100,000 times more potent than the natural compound progesterone. Of special mention is that all of the four progestins inhibit BrdUrd incorporation to a maximum of 70% of the vehicle-treated controls, although their effective doses may vary by several thousand fold.
On the other hand, antiprogestins RU486 and ZK98299 showed different effects on the growth of PgR-transfectants. ZK98299 alone was without any effect over the concentration range of 10^{-12} – 10^{-6} M, but the compound was capable of countering the inhibitory effect of progesterone as illustrated in Fig. 9b. In contrast, RU486 inhibited DNA synthesis from 10^{-12} – 10^{-6} M, and it had no further effects when it was concurrently used with progesterone. Estradiol-17β at concentrations of 10^{-12} – 10^{-6} M showed no effect on DNA synthesis in ABC28 cells.

It is also interesting to note that estradiol-17β at concentrations of 10^{-12} – 10^{-6} M did not modify the growth inhibitory effect of progesterone on ABC28 cells (Fig. 9a). There appeared to be, therefore, no ER-β-mediated events in PgR-mediated growth inhibition of ABC28 cells.

**DISCUSSION**

We have stably transfected PgR cDNA into ER and PgR-negative MDA-MB-231 cells to delineate the growth effect of progesterone in the absence of estrogen and/or ER. These PgR transfectants expressed both isoforms A and B receptors with varying A:B ratios. Our study has convincingly demonstrated that progesterone and other synthetic progestins markedly inhibited DNA synthesis from 10^{-12} – 10^{-6} M, and it had no further effects when it was concurrently used with progesterone. Estradiol-17β at concentrations of 10^{-12} – 10^{-6} M showed no effect on DNA synthesis in ABC28 cells.
provide evidence that some progestins (progesterone, Org2058, Org 30659, gestodene, levonorgestrel, 3-ketodesogestrel, and norethisterone) stimulate the growth of breast cancer cells by ER-mediated mechanisms (5, 6, 10, 13, 29). Our goal was to establish a PgR-positive but ER-negative cell model that would enable us to isolate the growth effects of progesterone from that of estrogens, and to differentiate between PgR-mediated effects and ER-mediated effects. The results using the PgR-transfected MDA-MB-231 cells have clearly shown that progestins inhibit the growth of breast cancer cells via PgRs that are expressed independent of ER and estrogens, thus supporting the notion that the growth stimulatory effect could be ER-mediated.

Although PgR concentrations in PgR-transfected MDA-MB-231 cells are similar to what was reported for T-47D cells (1, 7, 9), the effective doses (10^{-17} M, 10^{-14} M, 10^{-12} M, and 10^{-9} M for MPA, Org2058, progesterone and R5020, respectively) for the inhibition of growth of PgR-transfected cells are much lower than what was reported for T47D cells (1, 7). This may be due to the absence of the counteracting effect of estrogens or the absence of ER-mediated effects. The molecular basis for the varying potency of these progestins is not clear, and we have not performed ligand-binding studies to determine the binding affinity of each of these compounds to PgR.

Flow cytometry analysis revealed that progestin-induced cell cycle changes in PgR-transfected MDA-MB-231 cells were characterized by significant reduction of S phase fraction and an accumulation in G_0-G_1 phase beginning at 12 h after the treatment with progesterone. This response is similar to that reported by Sutherland et al. (1) for T-47D cells induced by MPA, which exhibited growth inhibitory effect. However, under experimental conditions in which slowly proliferating cells are tested, progestins caused a biphasic change in the cell cycle progression of T47-D cells: the cell growth was accelerated through the first cell cycle but arrested in late G_1 phase of the second cycle (8, 30). Groshong et al. (31) showed similar biphasic regulation by progesterone of the growth in T-47D-YA or T-47D-YB cells, which constitutively express the B or A isoform of PgR (32). These are in contrast with our findings that progesterone consistently inhibited the growth of PgR-transfected MDA-MB-231 cells when the cells were tested under low serum (1%) condition after 15 h, 18 h, 24 h, and 48 h of treatment.

It is interesting to note that antiprogestin ZK98299 reversed the growth inhibitory effect of progesterone in PgR-transfected MDA-MB-231 cells whereas antiprogestin RU486 did not. Instead, RU486 exhibited agonist activity to inhibit the growth. This finding is in accord with the theory that ZK98299 and RU486 belong to two different mechanistic types of anti-progestin (33, 34). Type II antiprogestin, represented by RU486, can switch its property from an antagonist to an agonist under the influence of a certain signaling network (32, 35) and the steroid receptor interacting proteins (36). It is known that ovarian steroid hormones play an important role in the growth and development of breast cancer. Antiestrogen tamoxifen is presently the front line therapy for ER- and PgR-positive breast cancer (37, 38). On the other hand, one-third of all breast cancer cases are presented with ER- and PgR-negative disease (26). One potential therapeutic
approach for this group of patients is to introduce exogenous ER or PgR genes to breast cancer cells so that hormonal control can be reestablished (39, 40, 41). Jiang and Jordan (42) have shown that estrogens can inhibit the growth of ER cDNA-transfected MDA-MB-231 cells. Our study also indicates that progesterones are able to exert strong inhibitory control over the growth in vitro if the PgR expression is restored in ER- and PgR-negative breast cancer cells. In the light of high inhibitory potencies of progesterones in these PgR-transfected cells, very low dose of progesterones or even circulating level of progesterone may effectively combat the cancer growth. It is to be acknowledged, however, that the results obtained in our study may only be representative of a subset of breast cancer cells. Jiang and Jordan (42) have shown that estrogen inhibited the growth of ER cDNA-transfected MDA-MB-231 cells whereas the hormone is a well known mitogen in ER-positive breast cancer cells. Studies should be conducted on other ER- and PgR-negative breast cancer cell lines transfected with PgR cDNA to generalize the findings in this report.

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