Distinct Molecular Pathways Mediate Progesterone-Induced Growth Inhibition And Focal Adhesion

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We have reported previously that reactivation of progesterone receptor (PR) expression in estrogen receptor (ER)- and PR-negative MDA-MB-231 breast cancer cells enabled progesterone to inhibit cell growth and invasiveness, and to induce remarkable focal adhesions. The present study addressed molecular mechanisms that mediate these anticancer effects of progesterone in the PR-transfected breast cancer cells ABC28. In response to progesterone treatment are the marked up-regulation of cyclin-dependent kinase inhibitor protein p21WAF1/CIP1 and decreased expression of cyclin A, cyclin B1, and cyclin D1 that are required for G1 progression and during cell mitosis. Progesterone also induced down-regulation of phosphorylated MAPK (p42/44 MAPK). Furthermore, this study also demonstrated that MEK inhibitor PD98059 that inhibits the phosphorylation of p42/44 MAPK (p42/44 MAPK). In the context of complex functions of progesterone in breast cancer and reproductive organs, identification of distinct pathways offers new strategies for designing therapeutic agents to target the specific pathway so as to minimize the side effects.

These results suggest that inhibition of p42/44 MAPK pathway is part of the mechanisms mediating progesterone’s growth-inhibitory effect. On the other hand, progesterone-induced focal adhesion is mediated by separate pathway. Whereas PD98059 exhibited no effects on cell adhesion, inhibitory antibody to β1-integrin was able to reverse progesterone-induced focal adhesion and progesterone-induced increase in the phosphorylation of focal adhesion kinase. On the other hand, β1-integrin antibody had no effect on progesterone-mediated growth inhibition and on progesterone-mediated expression of cyclins p21WAF1/CIP1 and phosphorylation of P42/44 MAPK. The ratio of PR-A and PR-B present in the target cells will also influence progesterone’s effect. We need to understand the progesterone-mediated signaling using various simplified models to put the jigsaw puzzle together.

Breast cancer cell line T47-D expresses both ER and PR. It has been widely used for studies of progesterone’s effect on cell cycle progression and the associated molecular mechanisms. Under experimental conditions in which slowly proliferating cells are tested, progesterone 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 them in late G1 phase of the second cycle (19). It was found that the growth arrest in the G1 phase of the second cell cycle was accompanied by a reversal of the cell cycle proteins, such as the decrease in cyclins A, B, D1, D3, and E and the induction of the cyclin-dependent kinase inhibitors p21 and p27 (20). This was echoed by another study in which progesterone not only decreased the abundance of cyclins but also inhibited the activities of the cyclins D1-Cdk4, D3-Cdk4, and E-Cdk2. In addition, cyclin E were found to be associated with Cdk inhibitors p21 and p27 in progesterone-treated cells (21). It has also been suggested that progesterone primed T47/D cells to the proliferative effect of EGF during the first cell cycle, as T47/D cells became sensitive to the mitogenic effect of EGF after progesterone treatment. This may be achieved by potentiating the growth-factor-stimulated p42/p44 MAPK, p38 MAPK, and JNK activities (22, 23). Cross-talk between ER and PR has also been identified in mediating cell signaling. In cos-7 cells transfected with the B isoform of PR-B, rapid activation of the Src/MAPK pathways by progestin depends on the cotransfection of ER (14). PR-B...
and ER were found to be associated in T47D and Cos-7 cells, and this association is essential for signaling activation by progesterone. A specific polyproline motif in the amino-terminal domain of PR was shown to mediate progesterin-dependent interaction of PR with SH3 domains of various cytoplasmic signaling molecules, including c-Src tyrosine kinases (24).

ABC28 is a subline of ER- and PR-negative MDA-MB-231 breast cancer cells transfected with PR-A and PR-B of similar concentrations (25). We have reported that progesterone induced remarkable growth inhibition and focal adhesions in ABC28 cells (25–27). Progesterone also drastically inhibited the expression of urokinase plasminogen activator, which is well-known for promoting tumor invasion. It seems, therefore, that progesterone holds excellent anticancer property in PR-transfected breast cancer cells. What are the characteristics of signaling mechanisms operated by progesterone in the context of PR-positive but ER-negative cells? Are progesterone’s effect on growth and focal adhesion associated events? These are the questions addressed in this study.

**Materials and Methods**

**Cell line and reagents**

MDA-MB-231 cells were obtained from American Tissue Culture Collection (Manassas, VA) in 1995 at passages 28. They were cloned, using 96-well plates, by the method of single cell dilution, and clone 2 (MDA-MB-231-C2) was transfected with PR expression vectors hPR1 and hPR2 that contain human PR cDNA coding for PR isoform B and isoform A, respectively, in pSG5 plasmid (28). Details of transfection and characterization were described previously (25). PR-transfected cell clone ABC28 was used in the present study. ABC28 cells expressed approximately 660 fmol PR/mg protein, as determined by enzyme immunoassay (Abbott Laboratories, Abbott Park, IL). Cells stably transfected with vector alone were used as transfection controls, and progesterone had no effect on transfection control cells. ABC 28 cells were routinely maintained in DMEM with phenol-red and supplemented with 7.5% fetal calf serum (FCS), 2 mm glutamine, and 40 mg/liter gentamicin. For all experiments, cells were grown in phenol-red free DMEM supplemented with 5% dextran charcoal-treated FCS to remove the endogenous steroid hormones that might interfere with the analysis. Cells were treated with progesterone from 1000-fold stock in ethanol. This gave a final concentration of ethanol of 0.1%. Treatment controls received 0.1% ethanol only.

Antibodies for cyclins A, B1, D1, E, p21 CIP1/WAF1, focal adhesion kinase (FAK), phospho-FAK, and MAPK were obtained from BD PharMingen (San Diego, CA). Phospho p44/p42 MAPK was from Cell Signaling Technology (Beverly, MA). MEK 1 inhibitor PD98059 was from PharMingen (San Diego, CA). Phospho p44/42 MAPK was from Cell Signaling Technology (Beverly, MA). Antibodies for cyclins A, B1, D1, E, p21 CIP1/WAF1, focal adhesion kinase (FAK), phospho-FAK, and MAPK were obtained from BD PharMingen (San Diego, CA). Phospho p44/p42 MAPK was from Cell Signaling Technology (Beverly, MA). Inhibitory antibody for β1 integrin 5D1 was a generous gift from Dr. Li Liu of the Department of Medicine, University of Washington.

**Western blotting analysis**

A total of 1 × 10⁴ cells were grown on 100-mm Petri dishes for 48 h before they were treated with 0.1% ethanol or 0.1 μM progesterone for various periods of time. The treated cells were lysed with 200 μl cold lysis buffer (50 mM HEPES, 150 mM NaCl, 1% Triton X-100, 5 μg/ml pepstatin A, 5 μg/ml leupeptin, 2 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 100 mM sodium fluoride, and 1 mM sodium vanadate, pH 7.5). After standing on ice for 20 min, the protein supernatants were collected by centrifugation at 13,000 × g for 20 min, and the protein concentrations were determined. Twenty micrograms of the protein were analyzed by Western blotting using ECL kit (Amersham, Little Chalfont, Buckinghamshire, UK) with antibodies against specific proteins.

**Immunofluorescence microscopy**

Cells were grown on glass coverslips in six-well plates for 48 h before receiving treatment with control vehicle (0.1% ethanol), 0.1 μM progesterone, or 0.1 μM progesterone plus β1-integrin inhibitory antibody 5D1. After rinsing with PBS, the cells were fixed in 4% paraformaldehyde and permeabilized with 0.2% Triton X-100. This was followed by incubation with 2% FCS in PBS for 1 h to block nonspecific binding. All the subsequent incubations with Ab were carried out in PBS containing 2% FCS. For staining of F-actin and paxillin, antibody to paxillin was incubated with the cells overnight at 4°C, followed by incubation with fluorescein isothiocyanate (FITC)-conjugated sheep antimouse IgG and FITC-phalloidin at room temperature for 1 h. After washing in PBS, the coverslips were mounted on slides with fluorescence mounting media from Dako (Carpinteria, CA). Stained cells were viewed and photographed using Carl Zeiss (Jena, Germany) confocal laser scanning microscope model LSM 510.

**Cell cycle analysis by flow cytometer**

Cell proliferation was measured by flow cytometer analysis of the cell cycle phase distribution, such as S-phase and G0/G1 phase fraction. A total of 5 × 10⁴ cells in six-well plates were grown in test medium for 2 d before they were treated with test compounds for various time intervals. The cells were then harvested and stained with propidium iodide in Vindelov’s (29) cocktail [10 mM Tris HCl (pH 8), 10 mM NaCl, 50 mg propidium iodide/liter, and 10 mg/liter ribonuclease A, and 0.1% Nonidet P-40 (v/v)] for 20 min at 37°C. The stained cell were analyzed in a FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA) with excitation wavelength of 488 nm. The resulting histograms were analyzed by program MODFIT (Becton Dickinson) for percentage of different cell cycle phases. The average coefficient of variation was within 5%.

**Light microscopy**

A total of 2 × 10⁴ cells were grown in six-well plates and received different treatment for the required period of time before they were viewed and photographed under a ZEISS AXIOVERT 35 phase contrast microscope.

**Gene expression by real-time PCR**

**cDNA synthesis.** Total RNA was extracted from the cells by Trizol reagent (Life Technologies, Inc., Gaithersburg, MD) with an extra step of acid-phenol extraction. Five micrograms of total RNA from each sample were used for RT using random hexamers and the SuperScript First-Strand Synthesis System (Life Technologies, Inc.).

**Real-time PCR.** Real-time PCR was performed with SYBR Green Master Mix on an ABI Prism 7700 Sequence Detection System according to the manufacturer’s protocol (Applied Biosystems, Foster City, CA). The primers for cyclin B1 are 5'-agg tgt ggc tcc ttc tat ctc act-3' (forward) and 5'-cgg att gga ata ctt gtg ctt gag agt caa-3' (reverse). The primers for cyclin D1 are 5'-ggc atg ggt gca gta gtc tct-3' (forward) and 5'-tgg ggt tgg gca gtc tgc tag gtt ac-3' (reverse). The primers for p21WAF1/CIP1 (forward) and 5'-att aag aag ccc aca aca agg cag caa t-3' (forward) and 5'-cgg tga aag ccc aca aca agg cag caa t-3' (reverse). The CDNAs were amplified by incubation for 10 min at 95°C to activate the Hot Start AmpliTaq Gold DNA polymerase, followed by 35 cycles of denaturation at 95°C for 30 sec, annealing at 60°C for 1 min, and extension at 72°C for 1 min. The PCR for each gene fragment was performed in triplicate. Melting curves were generated after amplification to check the PCR specificity. Amplion size and reaction specificity were further confirmed by electrophoresis on a 1% agarose gel, and a single PCR product with expected size should be observed. The changes in fluorescence of the SYBR Green I dye in each cycle were monitored by the ABI 7700 system; and the threshold cycle, which is defined as the cycle number at which the amount of amplified target reaches a fixed threshold, was obtained for each gene in each sample. The relative amount of PCR products generated from each primer set was determined on the basis of the threshold cycle value. Primer sets for 36B4 gene were included in each plate of PCRs as control for normalizing the quantity of cDNA used.
Statistical analysis

All experiments were repeated at least twice. Differences between treatments were tested by ANOVA when applicable. Where significant differences were detected by ANOVA, comparisons between means were performed by t test.

Results

It has been reported in detail previously (25) that progesterone markedly inhibited the growth of PR-transfected MDA-MB-231 cells ABC28 by arresting cells at the G0/G1 phase of the cell cycle, with concurrent reduction of cells in S-phase fraction. Progesterone also induced remarkable cell spreading and focal adhesion in these cells.

Progesterone induced remarkable up-regulation of p21\textsuperscript{CIP1/WAF1} protein (Fig. 1).

The up-regulation of p21\textsuperscript{CIP1/WAF1} by progesterone began as early as 1 h after treatment, and the effect was increased, with time, over the 72 h examined. On average, the up-regulation was 19-fold between 8 h and 72 h after progesterone treatment. At 72 h after treatment, p21\textsuperscript{CIP1/WAF1} expression in progesterone-treated cells was 26-fold higher than vehicle-treated controls.

Progesterone markedly decreased the protein levels of cyclin A, cyclin B1, and cyclin D1 in ABC28 cells (Fig. 2).

The reduction of cyclin A, B1, and D1 at the protein level was evident after 16 h of progesterone treatment, and the effect persisted at the subsequent time points tested (24 h, 48 h, and 72 h). Whereas the reduction of cyclin D1 by progesterone was between 2- and 4-fold, the cyclin A and cyclin B1 protein levels were reduced by at least 20- to 40-fold. On the other hand, the level of cyclin E protein remained unchanged for the first 24 h after progesterone treatment. There were 35% and 80% increases of cyclin E protein after 48 h and 72 h of progesterone treatment, respectively.

Progesterone induced prolonged reduction of the level of phospho-p42/p44 MAPK (Fig. 3).

p42/p44 MAPK or ERK function in a protein kinase cascade that plays critical roles in the cell growth and differentiation. The activation of these proteins is mediated by the phosphorylation of Thr/Tyr residues. Both phosphorylated p42 and p44 MAPK were detectable but phospho-p42 MAPK was the major protein detected in ABC28 cells. p44 MAPK was not visible in the blot probed by anti-PAN ERK, which is specific for total p42/p44 MAPK. Progesterone caused a prolonged reduction in phospho-MAPK from 1- to 48-h period examined. The reduction of phospho-p42/p44 MAPK was as much as 5-fold after 8 h of progesterone treatment. On the other hand, there was no evident change in the level of total MAPK.

MEK inhibitor PD98059 mimicked the effect of progesterone in decreasing the levels of phospho-MAPK and cyclin D1 (Fig. 4).

Recent evidence indicated that the expression of cyclin D1 is the down-stream event of p42/p44 MAPK pathway (30). Because the down-regulation of phospho-MAPK by progesterone was also associated with decreased cyclin D1 level, it would be interesting to know whether progesterone-regulated cyclin D1 expression was mediated by MAPK pathway. As is shown in Fig. 4A, MEK inhibitor PD98059 inhibited the activation of p42/p44 MAPK but had no effect.
on the total p42/p44 MAPK level. Interestingly, PD98059 also decreased cyclin D1 level. A quantity of 50 μM PD98059 reduced cyclin D1 by an average of 2.5-fold among samples analyzed after 6, 12, and 24 h treatment. In contrast, PD98059 had no effect on the protein level of cyclin A and cyclin B1.

PD98059 also significantly (P < 0.01) reduced the S-phase fraction of ABC28 cells in a concentration-dependent manner (Fig. 4B). There was 35% reduction of cells in S-phase fraction after 24 h treatment with 50 μM PD98059, as compared with 70% decrease of S-phase fraction in progesterone-treated cells. It seems, therefore, that the reduction of cyclin D1 by progesterone was partly attributable to the inhibition of p42/p44 MAPK activity. In contrast, PD 98059 had no detectable effect on the morphology and focal adhesion of ABC28 cells (Fig. 4C).

Inhibitory antibody of β1-integrin reversed progesterone’s effect of cell spreading and focal inhibition but had no effect on progesterone-mediated growth inhibition

Integrins are major adhesion molecules that mediate interactions between cytoskeleton and extracellular matrix proteins. We have reported previously (27) that β1-integrin antibody mAb13 (Becton Dickinson) was able to inhibit progesterone-induced cell spreading and focal adhesion, whereas monoclonal Ab to other integrin subunits β1, β4, α2, α3, α4, α5, and α6 had no effect. Because the antibody mAb13 has been withdrawn from the market, β1-integrin inhibitory antibody 5D1 tested in this study was kindly provided by Dr. Li Liu of the Department of Medicine, University of Washington. As is shown in Fig. 5, 5D1 significantly inhibited...
Progesterone-induced cell spreading and focal adhesion. Cells receiving antibody 5D1 together with progesterone (C and F) were obviously much rounder and less spread with little lamelipodia than cells treated with progesterone alone (B and E). More interestingly, /H9252 1-integrin antibody 5D1 was also able to reverse progesterone’s effect on the phosphorylation of FAK (Fig. 5G). Progesterone induced an increase in phosphorylated FAK after 12 h and 24 h treatment. This increase of phospho-FAK was completely reversed by /H9252 1-integrin antibody 5D1 after 24 h treatment. It is to be noted that the lower band of the FAK blot corresponds to the phospho-FAK. The identity of the upper band is not clear at this stage.

In contrast, the /H9252 1-integrin inhibitory antibody did not reverse progesterone-mediated growth inhibition, as is revealed by flow cytometry analysis of S-phase fraction (Fig. 6). However, 5D1 together with progesterone caused slight reduction in S-phase fraction of the cells, compared with progesterone treatment alone. This further reduction was also demonstrated by another integrin /H9252 4-antibody (clone mAb13) and a /H9252 4-integrin antibody (clone AA3), all belonging to the rat IgG 2 subtype (Becton Dickson). Whereas integrin /H9252 1-antibody mAb13 also reversed progesterone-induced spreading and focal adhesion, integrin /H9252 4-antibody showed no detectable effect (data not shown). This suggests that the small reduction of S-phase fraction by antibody 5D1 may be some nonspecific effect intrinsic to the IgG subtype.
Furthermore, β1-integrin inhibitory antibody 5D1 had no notable effect on progesterone-regulated expression of cell-cycle regulatory proteins such as cyclin D1, cyclin B1, and p21WAF1/CIP1 (Fig. 7). The result is further supported by real-time RT-PCR studies that showed no effects of 5D1 on the progesterone-mediated regulation of cyclin D1, cyclin B1, and p21WAF1/CIP1 at the mRNA level (Fig. 8). Similarly, the antibody 5D1 also had no significant effect on progesterone-mediated down-regulation of phospho-p42/p44 MAPK (Fig. 7).

**Discussion**

This study identified molecular mechanisms mediating progesterone-induced growth inhibition and focal adhesion in ABC28 cells. We presented evidence that distinct molecular pathways mediated different functions of progesterone. The significant up-regulation of p21WAF1/CIP1 and down-regulation of cyclin A, cyclin B1, cyclin D1, and phospho-p42/p44 MAPK may contribute to progesterone’s growth inhibitory effects, because these proteins are well-known cell cycle regulators. On the other hand, β1-integrin-mediated signaling seems to be involved in mediating progesterone’s effect on focal adhesion. Inhibitory antibody to β1-integrin was able to reverse progesterone-induced cell spreading and focal adhesion. It also reversed progesterone’s effect on the increase of phospho-FAK, which is well-known for its role in mediating focal adhesion. The data suggest that β1-integrin mediates progesterone-induced focal adhesion via activation of FAK, which will then trigger the generation of other signaling molecules in the focal contacts, as is generally proposed for the model of signal transduction in focal adhesion.

It is interesting to note that reduction of phospho-MAPK by MEK inhibitor PD98059 resulted in a reduced level of cyclin D1 and reduction of S-phase fraction in ABC28 cells, which resembled the effects of progesterone. However, 2.5-fold reduction of cyclin D1 level by 50 μM PD98059 was associated with only 35% reduction of S-phase fraction. This is in contrast with an average of 2.6-fold decrease in cyclin D1 level and an associated 70% reduction of S-phase fraction by progesterone. Therefore, the growth inhibitory effect of progesterone is the coordinated effort of many cell cycle regulators, and MAPK-cyclin D1 pathway may be part of the mechanism.

The inhibitory effect of progesterone on phosphorylation of p42/p44 MAPK is in contrast with studies using other models in which progesterin contributed to the activation of MAPK. In T47D-YB cells, progesterone had no effect alone but primed the cells for the activation of p42/p44 MAPK by EGF (22, 23). In Cos-7 cells transfected with the PR-B, progesterone activated MAPK pathway only when the cells were co-transfected with ER (14). The differences reflect the diversity of PR signaling among cell models and systems. It is unlikely attributable to the presence of both isoforms of PR in ABC28 cells, because progesterone also inhibited activation of MAPK in cells expressing PR-A or PR-B alone (our unpublished observation). Instead, absence of ER in ABC28 cells may contribute to this difference, because cross-talk mechanisms between PR and ER have been shown to play an important part in the activation of MAPK. However, the regulatory effect of progesterone on the level of cyclins and p21WAF1/CIP1 is similar to that in T47-D cells. This is not surprising, because progesterone also inhibits the cell proliferation in T47-D cells after the initial phase of growth stimulation (20, 21).

Integrins are adhesion receptors that mediated cell-matrix interaction. They also control the activation of many signaling pathways through focal adhesion complexes in which numerous signaling molecules are gathered. It is widely believed that integrin-mediated cell adhesion also conducts
signals regulating cell proliferation (31–33). For example, growth factor activation of the P42/p44 MAPK cascade is enhanced when cells are adherent. In addition, MAPK, Rho GTPases, and G1-phase cyclin-dependent kinases are all regulated jointly by growth-factor receptors and integrins. Although most data were generated using fibroblast cells, recent studies using MCF-7 cells also revealed adhesion-mediated activation of p42/p44 MAPK (34, 35). Our study indicates dissociation of integrin-mediated cell adhesion and regulation of cell proliferation in PR-transfected MDA-MB-231 cells. Inhibition of β1-integrin activation had no effect on MAPK activation, and inhibition of MAPK activation exhibited no influence on cell adhesion. It is to be noted that cell adhesion is a process related to cell migration and tumor metastasis. Although the prevalence of this dissociated signaling of cell growth and adhesion is yet to be investigated, slow-growing but highly invasive tumors, or fast-growing but nonmetastatic tumors are common in clinical settings. It is tempting to speculate that our finding provides a molecular basis for these clinical conditions.

Progesterone regulates many functions in the reproductive organs, and progesterone analogs have wide clinical applications, such as hormone replacement therapy and breast cancer treatment. On the other hand, there can be many side effects associated with progestin therapy. This breast cancer treatment. On the other hand, there can be many side effects associated with progestin therapy. This study suggests that the function of the progesterone may be modulated at the pathway level; and hence, therapeutic agents may be designed for a down-stream target of PR. The advancement of genomics and proteomics technology offers the possibility of comprehensive identification of progesterone-mediated pathways in the context of a complex cellular network (36–38).

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