Progesterone Inhibits Human Endometrial Cancer Cell Growth and Invasiveness: Down-Regulation of Cellular Adhesion Molecules through Progesterone B Receptors

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ABSTRACT

Progesterone is a critical steroid hormone that controls cell proliferation and differentiation in the female reproductive tract. Progesterone acts through two nuclear receptor isoforms, progesterone receptors A and B (PRA and PRB, respectively), each with unique cellular effects. Loss of PRB has recently been linked to the development of poorly differentiated endometrial tumors, a lethal form of cancer. To study the molecular effects of progesterone, progesterone receptors were introduced into Hec50co endometrial cancer cells by adenoviral vectors encoding either PRA or PRB. Progesterone induced the cyclin-dependent kinase inhibitors p21 and p27, thereby significantly reducing the percentage of proliferating cells. Cancer cell invasion was also markedly inhibited as measured by Matrigel invasion studies. Similarly, a differentiated, secretory phenotype was induced by progesterone in cells expressing PRB. However, replicative senescence was induced by progesterone only in cells expressing PRA. Expression array analysis followed by confirmatory semiquantitative reverse transcription-PCR experiments demonstrated a significant progesterone-dependent inhibition of expression of a cadre of cellular adhesion molecules, including fibronectin, integrin α3, integrin β1, integrin β3, and cadherin 6. The level of down-regulation of adhesion molecule expression was significantly greater in the presence of the B isoform, demonstrating that progesterone acts principally through B receptors to inhibit cancer cell invasiveness modulated by adhesion molecules.

INTRODUCTION

Progesterone is a sex hormone with many effects throughout the body. The classic tissue that depends on progesterone to limit cellular growth is the uterine epithelium, where progesterone is antagonistic to estrogen-mediated cell proliferation and the principal hormone that promotes differentiation (1). Two isoforms of the progesterone receptor, PRA and PRB, are expressed in the normal endometrium, and both are likely to be required for endometrial differentiation. Down-regulation of one or both isoforms is a frequent occurrence in endometrial carcinogenesis and is a principal cause of progestin treatment failures in endometrial tumors (2, 3). The isoforms are identical except that PRB has a longer NH2 terminus consisting of 164 amino acids, called the BUS, not present in PRA. The sequence downstream of the BUS region to which coactivators bind. Both isoforms share an identical ligand-binding domain, which includes activating function-2. The BUS region encodes a third activating domain that is unique to PRB. It is proposed that the BUS affects the secondary structure of PRB, conferring different functional characteristics on the isoforms. PRB is a stronger transcriptional activator of many genes than is PRA (4, 5), but PRA apparently counters estrogen action directly by inhibiting ER function in a dominant-negative manner (6). Transgenic mice overexpressing PRA or PRB showed distinct alterations in mammary gland morphology and cell-cell adhesions (7, 8). Female knockout mice deficient in both PR isoforms, as well as those deficient in only the A isoform, also demonstrate significant endometrial hyperplasia (9, 10). The phenotype of the PRB knockout mouse model has not yet been described in the literature, but our previous work demonstrated that PRB is lost in poorly differentiated endometrial cancer cell lines such as Hec50 and KLE, supporting the hypothesis that this isoform is also important in maintaining endometrial differentiation (4, 5). Silencing of PRB isoform expression through hypermethylation has also been reported (11). This suggests that a relative change in PRs, particularly the down-regulation of the PRB isoform, may be as important as the total level of PR in the genesis of some endometrial cancers. Studies addressing the individual effects of PR isoforms are needed to clarify what the consequences of loss of either PRA or PRB may be in endometrial cancer cells.

Hec50co is a poorly differentiated endometrial cancer cell line. Our previous studies have shown that these cells lack ER and PR, and have very small amounts of endogenous PRA (4). For the present investigation, PR expression and progesterone signaling were reestablished by introducing adenoviral vectors carrying PRA (AdPRA) or PRB (AdPRB) genes. We have now characterized the phenotypic changes induced by progesterone in Hec50co cells, determined the effects of progesterone on cell cycle, and identified potential mechanisms for our findings. These studies are some of the first to demonstrate that progesterone profoundly inhibits the expression of members of the cellular adhesion molecule family that modulate endometrial cancer cell invasiveness and metastatic potential.

MATERIALS AND METHODS

Cells and Reagents. Hec50 cells, from which Hec50co cells were clonally derived, were provided by Dr. Erlio Gurpide (New York University). Cells were maintained in DMEM (Life Technologies, Inc., Gaithersburg, MD) in the presence of 10% FBS (Gemini Bio Products, Inc., Calabasas, CA) and an antibiotic/antimycotic solution containing 100 units/ml penicillin-G, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B (Gemini Bio Products). Primary antibodies for actin (sc-1615), cyclin D1 (sc-246), cyclin E (sc-248), cdk2 (sc-163), cdk4 (sc-601), p21 (sc-397), and p27 (sc-528) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Adenoviral vectors were constructed as described previously (12).

Flow Cytometry. Cells were grown in 100-mm dishes and infected separately with AdCon, AdPRA, or AdPRB (adenovirus carrying no PR, PRA, or PRB genes, respectively) with a multiplicity of infection of 10. The hormone

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The abbreviations used are: PRA and PRB, progesterone receptors A and B, respectively; BUS, B-upstream sequence; ER, estrogen receptor; cdk, cyclin-dependent kinase; FBS, fetal bovine serum; TUNEL, terminal deoxynucleotidyl transferase (Tdt)-mediated nick end labeling; PAS, periodic acid-Schiff; RT-PCR, reverse transcription-PCR.
RT-PCR Quantitation. Proprietary primers for gene products of interest were ordered from Clontech, as was the RT-PCR kit used for confirmation. Total RNA was isolated from cells treated identically to those in the initial expression array experiments using the RNeasy kit, as described above. cDNA was synthesized from 1 μg of total RNA by random priming. PCR reactions were performed with the Clontech Advantage 2 PCR kit in a Perkin-Elmer (Branchburg, NJ) GeneAmp 2400 thermocycler. Reactions were run, and 5-μl samples were collected at 2-cycle intervals over a 12-cycle span determined to be the optimum for quantitation. All PCR products from a single experiment were run on a 1.5% agarose gel and stained with ethidium bromide. Each reaction included the L13Α control amplification gene to check for constancy in the amount of starting material. Fluorescence intensity photographs were captured using a Bio-Rad Fluor-S system, and the resulting bands were quantitated using volume integration with the histogram peak background correction method. Data were then plotted to ensure that L13Α levels were consistent between the control and progesterone-treated samples. When PCR is in the geometric phase of amplification the amount of product doubles with every cycle, one cycle lag corresponds to a 2-fold difference in the RNA level. Therefore, progesterone-induced fold changes in gene expression were estimated by calculating the base 2 antilog of the horizontal distance between the control and progesterone-treated lines measured in PCR cycles, corrected for any change in the expression of the control gene, L13Α.

RESULTS

Progesterone Caused Time-dependent Inhibition of Cell Cycle. Flow cytometry was performed to determine the fraction of cells undergoing active proliferation in the presence of PRA and PRB. Although expression of either PR isoform slowed cell proliferation with the addition of progesterone (Fig. 1; P ≤ 0.05 for progesterone-treated compared with vehicle-treated cells at days 1, 2, and 4), PRB showed the greatest effect, producing a 37% fall in the proliferation

Fig. 1. Progesterone inhibits endometrial cancer cell proliferation. Cells were infected with AdPRA (A) or AdPRB (B). Vehicle [ethanol (EtOH)] or progesterone (P4) was added, and cells were harvested 1, 2, or 4 days after treatment. The percentage of cells in S + G2-M was measured by flow cytometry. Bars, SE.
index at day 1 and a 77% reduction by day 4 (Fig. 1B). The effect of progesterone in cells expressing PRA remained constant beginning at day 1, with a maximal reduction of 56% in the S + G2-M phases. The data were also analyzed with respect to the number of cells in G1, which was increased by progesterone to 85% versus 34% for controls in the presence of PRB, and 72% versus 37% in the presence of PRA (P < 0.05).

**Progesterone Inhibited Endometrial Cancer Cell Invasion.** To assess the effects of progesterone on cell invasiveness and metastatic potential, cells were seeded on a Matrigel invasion chamber after infection with AdCon, AdPRA, or AdPRB and treatment with progesterone or vehicle alone. Cells that successfully invaded through the Matrigel and the pores on the filter were stained with crystal violet (Fig. 2A). Of note was the dramatic inhibition of invasiveness caused by progesterone treatment in cells infected with PRB virus (Fig. 2Af; P < 0.001). Little inhibition of invasiveness occurred for cells treated with progesterone but infected with AdCon (Fig. 2Ab) or with AdPRA. Therefore, cell invasiveness is controlled by progesterone through PRB.

**Progesterone Induced a Secretory Phenotype through PRB.** In the normal endometrium, progesterone induces a differentiated, secretory phenotype in epithelial cells. Accordingly, we noted that in HeC50co cells expressing PR, progesterone induced visible intracellular inclusions that we hypothesized represented secretory products. To confirm this hypothesis, the cells were stained with PAS reagent to identify glycogen. PAS-positive, pink-staining secretions were increased in AdPRA- and AdPRB-infected cells after progesterone treatment (Fig. 2B, d and f); however, the effect was particularly dramatic in the presence of PRB. Hence, a differentiated, secretory phenotype can be reinstated in highly proliferative endometrial cancer cells by progesterone in the presence of PR and PRB is the principal inducer of this effect.

**Progesterone Induced Replicative Senescence through PRA.** β-Galactosidase staining under acidic conditions is a biomarker that identifies senescent human cells in culture and in aging skin in vivo (14). Senescent cells no longer have the capacity to enter the cell cycle and do not divide. The senescence-associated enzyme β-galactosidase was induced by progesterone in the presence of PR, particularly PRA (Fig. 2C). PRB had little or no effect.

**Progesterone and Growth Factor Deprivation Induced Apoptosis.** To determine whether increased apoptosis could be a growth regulatory mechanism in endometrial cancer cells exposed to progesterone, cells infected with AdCon, AdPRA, or AdPRB were treated with progesterone or vehicle for 48 h and subjected to TUNEL assays. In complete medium containing 10% FBS, apoptosis was not induced by progesterone (data not shown). However, for cells infected with AdPRB and grown for an additional 48 h in medium lacking serum, apoptosis was clearly induced in response to progesterone, as shown in the right panel of Fig. 3, in which cells undergoing apoptosis have dark brown nuclei and are clearly seen. Approximately 11% of the cells were positive for apoptosis. Cells grown in the absence of progesterone continued to grow and to pile up, as indicated by the methyl green staining seen in the left panel of Fig. 3, and no apoptosis was apparent. Therefore, withholding serum from the medium alone did not induce apoptosis; progesterone and PRB had to be present as well. However, with growth factor deprivation, progesterone treatment clearly sensitized the cells to programmed cell death through a PRB-dependent mechanism.

**Progesterone Inhibited the Cell Cycle by Up-Regulating cdk Inhibitors p21 and p27.** As shown above, the cell cycle was inhibited by progesterone through both PRA and PRB (Fig. 1, A and B). To determine the primary mechanism leading to this effect, cell protein extract was obtained 24 h after progesterone or vehicle treatment, and Western blots were performed for factors that control the G1-to-S transition (Fig. 4). In general, progesterone treatment did not change the levels of cyclin D1, cdk2, or cdk4 proteins. In contrast, the cdk inhibitors p21 and p27 were up-regulated by progesterone in cells infected with either AdPRA or AdPRB. Cells were harvested and loaded onto Matrigel chambers. After incubation, invading cells were stained with crystal violet and photographed at ×100 magnification. B, progesterone induces a secretory phenotype in endometrial cancer cells. Cells were treated with vehicle (Veh) or progesterone (P4) after infection with AdCon, AdPRA, or AdPRB. Staining with PAS reagent was performed, and the cells were photographed at ×100 magnification. C, progesterone induced replicative senescence in PRA-expressing cells. Cells were incubated in β-galactosidase staining solution. The arrows indicate positive cells.

Fig. 2. A, progesterone inhibits endometrial cancer cell invasion. Cells were treated with vehicle (Veh) or progesterone (P4) after infection with AdCon, AdPRA, or AdPRB. Cells were harvested and loaded onto Matrigel chambers. After incubation, invading cells were stained with crystal violet and photographed at ×100 magnification. B, progesterone induces a secretory phenotype in endometrial cancer cells. Cells were treated with vehicle (Veh) or progesterone (P4) after infection with AdCon, AdPRA, or AdPRB. Staining with PAS reagent was performed, and the cells were photographed at ×100 magnification. C, progesterone induced replicative senescence in PRA-expressing cells. Cells were incubated in β-galactosidase staining solution. The arrows indicate positive cells.
Progesterone Down-Regulated the Expression of Cellular Adhesion Molecules. To define potential gene products involved in the effects of progesterone on cell invasiveness and adhesion, we performed expression analysis using the Clontech Atlas Array Human 1.2-1 and Cancer 1.2 blots. Hec50co cells were screened for the expression of 1900 genes. Cells were infected with AdCon, AdPRA, or AdPRB and treated with vehicle (Veh) or progesterone (P4). Genes that met our criteria for (a) being expressed at levels that were at least twice the blot background and (b) being up- or down-regulated at least 2-fold by progesterone through PRA and/or PRB were further evaluated. Semiquantitative RT-PCR was used to confirm the blot data, and our initial focus was on gene products likely to be associated with the phenotypic effects observed with progesterone treatment. The five gene products listed in Table 1 were confirmed to be regulated by progesterone. To compare our data across experiments, a control housekeeping gene, L13A, was identified, quantitated by RT-PCR, and found not to be significantly regulated by progesterone (Fig. 5). In contrast, the integrin β1 gene product, for example, demonstrated a profound inhibition by progesterone in cells infected with AdPRB (Fig. 5). The progesterone-treated sample lagged the control by eight cycles in this case, or by more than two orders of magnitude ($2^7 = 128$), the most dramatic inhibition of expression found in this study. The results of the fold reduction in expression for the five genes investigated are shown in Table 1. Differences between the PR isoforms were evident: in general, PRB had a greater inhibitory effect on cellular adhesion molecule expression compared with PRA. Only PRB significantly inhibited the expression of fibronectin and the integrins α3 and β1, but both PR isoforms inhibited cadherin 6 and integrin β3.

Table 1  Gene expression changes induced by progesterone in cells overexpressing PRA or PRB

<table>
<thead>
<tr>
<th>Gene</th>
<th>Down-regulation by progesterone (fold)</th>
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<tr>
<td></td>
<td>PRA</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>None</td>
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<tr>
<td>Integrin α3</td>
<td>None</td>
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<tr>
<td>Integrin β1</td>
<td>None</td>
</tr>
<tr>
<td>Integrin β3</td>
<td>8</td>
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<tr>
<td>Cadherin 6</td>
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Fig. 3. Progesterone sensitized endometrial cancer cells to growth factor deprivation. Cells were infected with AdPRB. Vehicle (Veh) or progesterone (P4) was given for 2 days. Serum was removed from the culture medium for the next 2 days. TUNEL assays were performed to detect apoptosis. The left panel shows absence of apoptosis in vehicle-treated cells. The arrows in the right panel indicate apoptotic cells in the presence of progesterone and the absence of serum.

Fig. 4. Effect of progesterone on cell cycle regulators. Cells were infected with AdCon, AdPRA, or AdPRB and treated with vehicle (Veh) or progesterone (P4). Immunoblotting was performed with antibodies to the indicated factors. Bottom, immunoprecipitation was carried out using an anticyclin E antibody (Ab), and the complex was probed with an anti-p21 antibody.

Fig. 5. Semiquantitative RT-PCR. Top, amplification of the housekeeping gene L13A in Hec50co cells infected with AdPRB with vehicle (ctrl) or progesterone (P4) and the resulting quantitation plot for expression of L13A in vehicle- (●) and P4-treated (●) cells. Bottom, amplification of integrin β1 in vehicle- (ctrl) and progesterone-treated (P4) cells and the quantitation plot for expression of integrin β1 in vehicle- (●) and P4-treated (●) cells.
The mechanisms by which progesterone reverses premalignant endometrial hyperplasia and successfully limits the spread of some endometrial cancers (those with PR) have not been well characterized (16). Many of the effects of progesterone are attributable to its ability to oppose the action of estrogen. Progesterone inhibits ER gene expression and enhances degradation of ER proteins (17). Progesterone may also oppose ER-mediated gene-regulatory events, probably through sequestration of transcription factors that are essential for estrogen action (17) or by direct dominant-negative effects of PRA on ER transcriptional function. Nonetheless, some of the effects of progesterone are likely to be independent of estrogen (1). Our studies have now identified pathways by which progesterone limits endometrial cancer cell growth and metastatic potential in a highly malignant cell line that does not express ER. In these investigations, the cell cycle was inhibited in late G1 (Fig. 1) by induction of p21 and p27 (Fig. 4); cells could no longer invade through a Matrigel chamber (Fig. 2A); cells underwent conversion to a more differentiated, secretory phenotype (Fig. 2B); replicative senescence was induced (Fig. 2C); and apoptosis ensued when growth factors were removed from the medium (Fig. 3). These effects were dependent on the expression of PR. Although both PR isoforms were involved in cell cycle inhibition, the PRA isoform predominated as an inhibitor of invasion and an inducer of differentiation, whereas the PRA isoform promoted cell senescence.

The differential expression of a panel of gene products after exposure to liganded progesterone demonstrated the inhibition of a cadre of adhesion molecules. This finding provided a mechanistic link between changes in cell phenotype, as described above, and effects of progesterone on gene expression. Progesterone significantly inhibited the expression of fibronectin; integrins α3, β1, and β3; and cadherin 6 (Table 1). These effects were dependent on PR and, in some cases, were highly influenced by the PR isoform. Only PRA significantly inhibited expression of fibronectin and integrins α3 and β1, whereas both isoforms inhibited cadherin 6 and integrin β3.

Fibronectin is an extracellular glycoprotein that serves as a ligand for the integrin family of receptors. Fibronectin expression has been linked to tumorigenesis and cell metastasis (18–20). Integrins are heterodimeric proteins consisting of one α and one β subunit. On one level, the integrin dimers function as adhesion molecules forming bridges between extracellular matrix proteins, such as fibronectin and collagen, and the cytoskeleton. On a second level, integrins are involved in signal transduction with downstream effects on cell replication, differentiation, and apoptosis (21–23). Effects on the cell cycle have been reported, as integrins influence the progression of cells from the G1 phase to the S phase as well as from the G0 phase to G1 (24). Integrins are expressed in the nonmalignant endometrium (25), and the modification of integrin expression, particularly the overexpression of integrin β1, has been associated with tumorigenesis and cell invasiveness in other organs (22, 23, 26–28). The studies reported herein indicate the potential significance of integrins in malignant endometrial cells. The predominance of PRA as the active PR isoform that down-regulates the integrin receptor dimer, α3β1, and one of its ligands, fibronectin, is an important finding because it suggests that in poorly differentiated tumors with little PRB, this receptor/ligand pair may play an important role in cell metastasis.

Cadherins are calcium-dependent transmembrane cell adhesion molecules that link to actin-containing cytoskeletal elements. The cytoplasmic domains of cadherins interact with a group of intracellular proteins known as catenins, which in turn interact with members of the Wnt signaling pathway (29). Thus, cadherins form cell-to-cytoskeletal junctions that help to control tumor organization and invasiveness; cadherins also modulate important signal transduction pathways with the potential to affect cell proliferation. Previous studies in breast cancer cells have suggested that progesterone controls cell motility or invasion through the regulation of cadherins and other cell adhesion molecules (30). The morphology of breast cancer cells has been reported to be influenced by progesterone, with increased stress fibers and focal adhesion molecule expression (30). The expression of cadherins has been reported to induce cell motility (31), and overexpression of progesterone in a transgenic mouse caused a significant change in cadherin and laminin expression in mammary gland epithelium, resulting in disorganized basement membrane and decreased cell to cell adhesion (7).

The specific cadherin found to be regulated by progesterone in these studies was cadherin 6. This protein was originally identified in the kidney, where it mediates the formation of the nephrons (32). Cadherin 6 is also overexpressed in urological cancers, where it is reported to inhibit E-cadherin function and promote carcinogenesis (33). This is the first report demonstrating the expression of cadherin 6 in the uterine endometrium and its regulation by progesterone through both PRA and PRB. The down-regulation of these and other members of the cell adhesion family by progesterone strongly supports its role as an inhibitor of cell motility, invasiveness, and cell proliferation. Future studies will address the role of selective inhibition of cell adhesion molecules and progesterone reintroduction as potential therapeutic avenues in endometrial cancer.

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