Progesterone Enhances Calcitriol Antitumor Activity by Upregulating Vitamin D Receptor Expression and Promoting Apoptosis in Endometrial Cancer Cells

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**ABSTRACT**

Human studies suggest that progesterone and calcitriol may prove beneficial in preventing or inhibiting oncogenesis, but the underlying mechanism is not fully understood. The current study investigates the effects of progesterone, calcitriol and their combination on immortalized human endometrial epithelial cells and endometrial cancer cells and identifies their targets of action. Combination treatment with both agents enhanced vitamin D receptor expression and inhibited cell proliferation through caspase-3 activation and induction of G0/G1 cell cycle arrest with associated down-regulation of cyclins D1 and D3 and p27 induction. We used mass spectrometry-based proteomics to measure protein abundance differences between calcitriol-, progesterone-, or combination-exposed endometrial cells. A total of 117 proteins showed differential expression amongst these three treatments. Four proteins were then selected for validation studies: histone H1.4 (HIST1H1E), histidine triad nucleotide-binding protein 2 (HINT2), interferon-induced, double-stranded RNA-activated protein kinase (EIF2AK2), and Bcl-2–associated X protein (BAX). Abundance levels of selected candidates were low in endometrial cancer cell lines versus the immortalized endometrial epithelial cell line. All four proteins displayed elevated expression in cancer cells upon exposure to calcitriol, progesterone or the combination. Further BAX analysis through gain or loss of function experiments revealed that upregulation of BAX decreased cell proliferation by changing the BAX:BCL-2 ratio. Knock down of BAX attenuated progesterone- and calcitriol-induced cell growth inhibition. Our results showed that progesterone and calcitriol up-regulate the expression of BAX along with other apoptosis-related proteins, which induce inhibition of endometrial cancer cell growth by apoptosis and cell cycle arrest.

**INTRODUCTION**

Endometrial cancer is the most common gynecologic malignancy in the United States. In 2012, 47,130 new cases are anticipated, resulting in 8010 deaths (1). Endometrial cancer is usually treated with surgical removal of the uterus and adjuvant therapy is administered in selected cases based on surgico-pathologic factors.
predictive of recurrence risk. For early stage disease, outcomes are quite favorable with 5-year survival rates greater than 90% (2). However, the number of women presenting with endometrial cancer in an advanced stage or a high histological grade, which is indicative of a poor prognosis, is increasing and mortality rates are rising (3, 4). Discovery of novel molecular targets for the diagnosis, prognosis and treatment of endometrial cancer is imperative to improve the management and outcome of this disease.

A strong body of clinical and epidemiologic evidence suggests that progestins are highly effective endometrial cancer preventive agents. Routine use of progestins lowers endometrial cancer risk and the protective effect increases with increasing progestin potency. In premenopausal women, use of progestin containing oral contraceptives (OCs) confers a significant reduction in endometrial cancer risk (5-7). In addition, progestin-potent OCs have enhanced endometrial cancer protective effects compared to OCs containing weak progestins (8, 9). In menopausal women, the addition of a progestin to estrogen replacement therapy decreases the risk of precancerous endometrial hyperplasias, suggesting chemopreventive effects of progestins on the endometrium (10, 11). Currently, progestins are often used as salvage therapy in recurrent endometrial cancer patients and in young patients to preserve childbearing capacity (12). Progesterone mediates its inhibitory effects on the endometrium and endometrial cancer via the progesterone receptor (PR), an intracellular steroid receptor with A and B isoforms. An increase in the response rates to progestin therapy and improved survival outcomes have been reported in tumors with a higher percentage of PR (13, 12). Progestin potency can be enhanced by either increasing the dosage of progestin or by selecting a pharmacologically potent progestin. However, both of these approaches are likely to be associated with an increase in side effects that would be undesirable for long-term chemoprevention in women. An alternative strategy would be to combine a shorter course or lower dose of progestin with a second preventive agent that has an excellent safety profile and enhances the potency of the progestin.
There is a growing body of epidemiological and laboratory evidence in support of vitamin D for the prevention of a number of malignancies including endometrial cancer (14, 15). Vitamin D3, produced in the epidermis or obtained from the diet, is metabolized to the dihydroxylated form (calcitriol, 1,25-(OH)2D3) to be biologically active. Calcitriol is well-known for its anti-proliferative roles through multiple mechanisms including the induction of cell cycle arrest, apoptosis and differentiation in a variety of cancer types, including prostate, breast cancer, colon, skin and leukemic cells (16-18). The inhibitory role of calcitriol in cellular growth and proliferation potentially provides protection from various types of cancers, as indicated by several epidemiological studies (19, 20). However, as calcitriol has been found to be efficacious in several preclinical and clinical studies, dose-limiting calcemic effects have proved a major obstacle for the use of this compound as a chemotherapeutic or chemopreventive agent (14, 15). One approach to limit the toxicity is to use lower concentrations of calcitriol in combination with other agents that enhance its anticancer activity.

A general approach to cancer chemotherapy entails simultaneous administration of two or more chemotherapeutic drugs to patients, in anticipation that the drug combination will be more effective than any single agent. Several studies have reported that calcitriol works synergistically with chemotherapeutic drugs (21, 22). Combining progesterone with low doses of calcitriol might be a beneficial chemopreventive or therapeutic strategy in endometrial cancer. Therefore, in the present study we evaluated the effects of combinations of calcitriol and progesterone on endometrial cancer cells. The data show that combinations achieve significantly greater inhibition of tumor cell growth and enhanced anticancer activity than individual agents. Characterizing the underlying mechanisms of progesterone’s synergism with calcitriol will provide an important rationale for chemoprevention or treatment trials using this combination.
MATERIALS AND METHODS

Cell lines and culture conditions

The immortalized human endometrial epithelial cell line, EM-E6/E7-TERT, established and characterized by Dr. Satoru Kyo, (Kanazawa University, School of Medicine, Kanazawa, Japan) was kindly provided to us in 2008. This line was not authenticated by us once received in our laboratory. Human endometrial cancer cell lines HEC-1B and RL-95 were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and Ishikawa cell line was obtained from Sigma (St Louis, MO). These human-derived cell lines were authenticated by DNA short-tandem repeat analysis by ATCC and Sigma. All four cell lines were initially expanded and cryopreserved within 1 month of receipt. Cells were typically used for 3 months, at which time a fresh vial of cryopreserved cells was used. The cells were routinely tested for mycoplasma.

Immortalized epithelial endometrial (EM-E6/E7/TERT) cells established and characterized by Kyo and colleagues (23). Primary endometrial epithelial cells were transformed by the combination of human papillomavirus-16 E6/E7 expression and telomerase activation by the introduction of human telomerase reverse transcriptase (hTERT). The immortalized cells contained no chromosomal abnormalities, retained responsiveness to sex-steroid hormones, exhibited glandular structure on three-dimensional culture, and lacked transformed phenotypes on soft agar or in nude mice. The cells were grown in DMEM. Ishikawa cells were derived from a well-differentiated adenocarcinoma of the human endometrial epithelium from a 39-year-old woman, and express functional steroid receptors for estrogen, progesterone, and androgen. Cells were grown in DMEM:F12 supplemented with insulin (Invitrogen, Carlsbad, CA). HEC-1-B cells derived from a moderately-differentiated adenocarcinoma of endometrial epithelium from a 71-year-old patient were cultured in Eagle MEM (Invitrogen). RL95-2 was derived from a Grade 2 moderately-differentiated adenosquamous carcinoma of the endometrium. The cells are characteristically epithelioid with well-defined junctional complexes, tonofilaments, filopodia-like extensions, and surface microvilli. Nuclei are large, irregular, and invaginated frequently with multiple, prominent, lamellar nucleoli. The RL95-2 cells were grown in DMEM:F12 medium.
supplemented with insulin (0.005 mg/ml) and fetal bovine serum as described earlier (24, 25). The cells were treated with progesterone (20 µM P4, 99.9% pure; Sigma), calcitriol (100 nM, Sigma) or both for 24, 72 or 120 hours and collected for protein extraction. The time of treatment and doses of progesterone and calcitriol were based on our previous studies showing inhibition of cell growth and apoptosis of cancer cells (24). For a set of experiments, endometrial cancer cells were transfected with siRNA targeting BAX or scrambled siRNA and then treated with progesterone, calcitriol or both for 120 h. Protein extracts were prepared for BAX analysis.

**Cell growth and death assays**

Endometrial cells were plated at 5000 cells/cm², and fed after 24 h with fresh medium and treated with progesterone, calcitriol or both. After 120 h of these treatments, total cells were collected by brief trypsinization, and washed with PBS. Total cell number was determined by counting each sample in triplicate using a hemocytometer under an inverted microscope. Cell viability was determined using the trypan blue exclusion method. Each treatment had three independent plates. The data shown in this study are the mean of three independent experiments.

**Cell cycle analysis**

Cells were plated onto tissue culture flasks and the medium was replaced the next day with medium containing progesterone, calcitriol, combined treatment, or vehicle. Cell cycle status was measured after 24 h of exposure to treatments. Cells (1x 10⁶) were collected and centrifuged at 250 x g for 5 min. Supernatant was removed and cells were fixed by slowly adding 1.0 ml of a chilled ethanol (-20°C) while vortexing the tube at low speed. Cells were kept at-20°C until DNA staining. On the day of DNA staining, samples were centrifuged at 250 x g for 5 min. Then supernatant was removed. DNA staining buffer (1 ml containing 100µg propidium iodide, Sigma) was added to cell pellet and briefly vortexed. Cells were kept in the dark for 15 min at room temperature. The distribution of cells between phases of the cell cycle was determined using a BD LSR II Flow Cytometer (Becton Dickinson, LSR 2) and ModFit LT software (Verity Software House, Topsham, ME). Data are means ± standard deviations of 3 independent experiments.
Liquid chromatography-tandem mass spectrometry

To identify differentially expressed proteins in endometrial cancer cells exposed to progesterone, calcitriol or both, 40 μg of each total cell lysate was resolved by 1D SDS-PAGE. Twenty equivalently-sized gel slices were excised and digested using trypsin. Tryptic digests were resuspended in mobile phase A and each subjected to five recursive nanoflow liquid chromatography-MS/MS analyses (LTQ-Orbitrap Velos, ThermoFisher) as previously described (26). MS/MS spectra was searched against the UniProt human protein database using Mascot (Matrix Science) with a decoy database to result in a false peptide discovery rates of less than 1%. Differences in protein abundance between the samples were derived by spectral counting (SC) and peptides whose sequence mapped to multiple protein isoforms were grouped as per the principle of parsimony.

Silencing of BAX in Endometrial Cancer Cells

To establish that BAX is a mediator of progesterone and calcitriol-induced growth inhibition, endometrial (Ishikawa, HEC-1B and RL95-2) cancer cells were seeded in 6-well plates and transfected the following day with BAX siRNA (SC29212, Santa Cruz, CA) or scrambled siRNA (SC37007, Santa Cruz,) using the Lipofectamine 2000 reagent (Invitrogen). Transfected cells were treated with or without progesterone (20 μM), calcitriol (100 nM) or both for 5 days. Cell extracts were used to assess expression of BAX. The overall transfection efficiency for endometrial cells assessed by luciferase assay was 76% to 83%.

Cell Viability Assay.

Cell viability was evaluated after transfection of endometrial cancer cells with BAX siRNA or scrambled siRNA by using the CellTiter 96 AQeous One Solution cell viability assay (Promega) according to the instructions of the manufacturer. CellTiter 96 AQeous One Solution reagent (20 μL) was added into each well of the 96-well assay plate containing the samples in 100 μL of culture medium. Absorbance was measured at 490 nm using a microtiter plate reader. Relative cell viability was expressed as percent change of transfected cells over empty vector–transfected cells.
Western blot analysis

Endometrial cancer cell extracts from progesterone- or calcitriol-treated and non-treated control cells as well as extracts from BAX-silenced and control siRNA-transfected cells treated with or without progesterone or calcitriol were analyzed using antibodies against BCL2 associated X protein (BAX), caspase 3, cyclin D1, cyclin D3, p27 (Cell Signaling, Danvers, MA), BCL-2 (BioLegend, San Diego, CA), progesterone receptor (PgR 1294, Dako Corporation), VDR, histone H1.4 (HIST1H1E), histidine triad nucleotide-binding protein 2 (HINT2), interferon-induced, double-stranded RNA-activated protein kinase (EIF2AK2), (Santa Cruz Biotechnology, Santa Cruz, CA), and β-actin antibody (Sigma-Aldrich). Equal amounts of protein were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The enhanced chemiluminescence system was used to visualize the protein bands as recommended by the manufacturer (Pierce). Protein bands were quantified using densitometry software (BioRad, Philadelphia, PA), and normalized using actin as a loading control. To calculate the relative intensity of each band, individual bands were divided by the corresponding loading control intensity.

Statistical analysis

Data is presented as the mean of triplicate determinants with standard error of the mean. Experiments performed in triplicate were repeated at least three times. Statistically significant differences were determined between control and treatment groups using two-way ANOVA followed by Tukey post hoc test. A p-value of <0.05 was considered statistically significant. The Pearson correlation coefficient test was used to calculate the correlation between BAX expression and cell growth. Western blot data were quantified using densitometry. A representative of three immunoblots is presented in the figures along with average relative density of the bands normalized to β-actin. Statistically significant differences were determined between control and treatment groups by quantification of three immunoblots and p-values are provided in the results section.
RESULTS

**Progesterone receptors were not affected by progesterone or calcitriol treatment.**

The protein expression levels of PR-A and PR-B were studied in EM-E6/E7-TERT and three endometrial cancer (Ishikawa, HEC-1B and RL95-2) cell lines by quantification of Western blots analysis (Figure 1), using a mouse anti-human PR antibody that recognizes both human PR isoforms: PR-A (85–94kDa) and PR-B (116–120kDa). Both isoforms were expressed in endometrial cancer and in immortalized cell lines (Figure 1A). However, expression of both isoforms was reduced in cancer cell lines compared to the immortalized cell line \( p<0.05 \). We studied the effects of progesterone, calcitriol or both on PR protein expression in the same normal and endometrial cancer cell lines. No marked change was noticed in the expression of PR isoforms in immortalized cells and in endometrial cancer cells following progesterone, calcitriol or combination treatments (Figure 1A).

**Progesterone enhances vitamin D receptors in endometrial cancer cells**

We analyzed the effect of progesterone, calcitriol or both on VDR protein expression in normal and malignant endometrial cells. As shown in Figure 1B, western blotting of cell lysates for VDR revealed a doublet at approximately 60 and 48 kDa, which likely represents multiple phosphorylated forms of the receptor. Interestingly, quantification of VDR expression data in progesterone-treated cancer cell lines resulted in a marked \( p<0.05 \) stimulation of VDR expression. Exposure to the combined treatment showed similar expression of VDR versus cells treated with only progesterone. Although calcitriol enhanced VDR expression, the enhancement was not as pronounced as that demonstrated by progesterone-treated cells. In EM-E6/E7-TERT cells, calcitriol, but not progesterone, enhanced \( p<0.05 \) VDR expression (Figure 1B).

**Progesterone and calcitriol inhibit cell proliferation and cause cell death**

We examined the dose response effects of these agents alone or in combination on cell growth and death in EM-E6/E7-TERT and three endometrial cancer lines (Ishikawa, HEC-1B and RL95-2). Cells were treated with progesterone (10, 20 or 40 \( \mu M \)), calcitriol (50, 100 and 200 nM) or the combination of the two for 120 h.
This treatment time was selected based on our previous studies, which indicated significant inhibition of cell growth after 5 days (25). At the end of treatment time, determination of total cell number as well as dead cells showed that both progesterone and calcitriol inhibit cell growth and cause cell death in a dose-dependent manner (Figure 2A and B). Combined progesterone and calcitriol treatment showed a pronounced dose-dependent decrease in cell numbers compared to either single agent in all the cell lines tested. Using the trypan blue dye exclusion method, we observed that the decrease in cell number by both the agents was accompanied by an increase in cell death. However, no treatment caused the percentage of cell death to increase beyond 20%, suggesting that the overall decrease in cell number was partially caused by the cell death-inducing effect of both the agents. In an attempt to identify the mechanism of cell death caused by each of the three treatments, we assessed the levels of cleaved caspase-3 in cells. While treatment of cells with progesterone or calcitriol alone showed moderate elevation of cleaved caspase-3, their combination substantially increased \( p < 0.05 \) caspase-3 levels (Figure 2C).

Next we investigated the effect of progesterone and calcitriol on cell cycle arrest in endometrial cancer cells to determine whether the inhibitory effect on cell proliferation is accompanied by modulation of cell cycle progression.

**Combination of progesterone and calcitriol induces G1 cell cycle arrest and modulates cell-cycle regulators in endometrial cancer cells.**

Many apoptotic stimuli induce cell cycle arrest before cell death, thereby affecting both cell cycle and apoptotic machinery. To determine whether inhibition of cell proliferation was associated with cell cycle arrest, endometrial cells EM-E6/E7-TERT, Ishikawa, HEC-1B and RL95-2 were treated for 24 h with vehicle, progesterone, calcitriol or combined treatment and analyzed by flow cytometry. The initial cell population in the G0–G1 phase was 56.39±0.42%, 36.44±0.35 %, 49.69±0.41%, and 47.14±0.14% in EM-E6/E7-TERT, HEC-1B, Ishikawa and RL95-2 cells respectively. However, after 24 hours of incubation with the combined progesterone-calcitriol treatment a significantly greater number of cells were arrested in the G1 phase (62.86±0.57%, 60.24±0.4%, 59.979±0.48% and 58.97±0.48% in EM-E6/E7-TERT, HEC, Ishikawa and RL95-2
cells respectively) compared to vehicle treated cells. In RL-95 cells, cell cycle arrest was more pronounced ($p<0.05$) following combination treatment compared to either single treatment (Figure 3 A-D and Table 1).

These results suggest a combination treatment induces G0-G1 phase arrest in endometrial cancer cells. To identify cell-cycle regulatory molecules, we examined the expression of cyclins and cyclin-dependent kinases (CDK) inhibitors. Cells were treated with progesterone, calcitriol or the combination for 5 days and cell lysates were prepared for Western blot analysis. Our results showed a marked decrease ($p<0.05$) in cyclins D1 and D3 in endometrial cancer cell lines with concomitant elevation ($p<0.05$) of p27 protein expression following treatment with progesterone, calcitriol or the combination compared with untreated cells (Figure 3E). Together, these results suggested that progesterone and calcitriol treatments in endometrial cancer cells strongly modulate cyclins and inhibit their normal regulation of cell-cycle progression.

**Identification of novel mediators of calcitriol and progesterone signaling in endometrial cancer cells.**

We used a proteomic approach to identify additional mediators of progesterone and calcitriol signaling in endometrial cancer cells. Comparative analysis of protein expression in HEC-1B cells treated with progesterone, calcitriol or the combination for 24 h resulted in the identification of alterations in the abundance levels of 609, 387, and 519 proteins, respectively. Interestingly, between the three treatments, only 117 proteins were found in common that exhibited altered expression in HEC-1B relative to the untreated cells (Figure 4A). The list of 117 proteins is provided in a supplemental Table. The proteins that were differentially altered by these three treatments in the HEC-1B cells were categorized into different groups: oncogenes, tumor suppressors, binding proteins, membrane proteins, structural proteins, transport proteins, ribosomal proteins, receptors and transcription factors, as well as proteins involved in apoptosis and cell cycle regulation. Of the 117 proteins, four apoptosis-related proteins were selected for validation studies, HIST1H1E, HINT2, EIF2AK2, and BAX (Figure 4A). The expression levels of the selected candidates were successfully verified by Western blot to be at low levels in three endometrial cancer cell lines and their expression was significantly elevated ($p<0.05$) upon exposure to progesterone, calcitriol, or the combination (Figure 4B). We selected BAX
for further analysis and investigated the time-dependent induction of BAX and BCL-2 in response to progesterone, calcitriol or both. A time dependent increase in BAX and concomitant decrease in BCL-2 expression was observed in all cell lines (Figure 5). Quantification of the western blot images and calculation of the BAX: BCL2 ratio clearly showed a marked ($p<0.05$) increase of BAX:BCL2 following combined treatment versus either single treatment in cancer cells (Figure 5). However, in immortalized cells no significant changes were noted in BAX:BCL2 between vehicle-treated cells versus single or combination treatment cells (Figure 5).

**Knockdown of BAX enhances growth of cancer cells and restoration of BAX expression by progesterone and calcitriol attenuates proliferation.**

To confirm that the progesterone and calcitriol-induced inhibition of endometrial cancer cell growth is mediated via BAX expression, we transfected cells with BAX-targeted siRNA oligonucleotides. Cells were harvested at 24 h after transfection and BAX expression was analyzed by Western blot (Figure 6A). Whereas BAX-specific siRNA suppressed BAX synthesis almost completely, control (scrambled) siRNA had no effect. Next, we assessed the effect of BAX-specific siRNA on cell viability. Progesterone-, calcitriol- and combination-induced growth inhibition was attenuated in BAX knockdown cells compared to scrambled siRNA transfected cells (Figure 6B). BAX expression was correlated with cell proliferation using Pearson’s correlation. Strong correlation (correlation coefficient $r=0.81$; $p<.001$, HEC-1B, $r=0.90$; $p<.001$, Ishikawa, $r=0.89$; $p<.001$, RL-95) was observed between BAX expression and inhibition of cell growth.

**DISCUSSION**

Although both progesterone and calcitriol may have potential as therapeutic interventions in selected cancers, adverse side effects associated with high dosages may limit their clinical utility, especially for calcitriol, which can cause harmful hypercalcemia. These limitations could be overcome if the two agents act additively or synergistically or via a different mechanism of action, thereby allowing the use of lower doses of
each. Based on this rationale, in the present study we tested the efficacy of progesterone in combination with calcitriol against endometrial cancer. The results demonstrate that progesterone and calcitriol produced dose-dependent growth inhibition in endometrial cancer and in immortalized endometrial cells, although growth inhibition was less pronounced with calcitriol treatment alone. Of note, cells exposed to a combination of these agents showed greater inhibition than did cells exposed to either agent alone, suggesting an additive effect on tumor cell growth. Our results agree with preclinical studies demonstrating that calcitriol has additive effects when used in combination with chemotherapeutic agents including cisplatin and paclitaxel (27, 21). Such combination therapy potentially offers a therapeutic advantage over a standard dose of a single drug by enhancing the effectiveness of the therapy without increasing the dose, thus limiting dose-dependent toxic side effects.

Calcitriol has antiproliferative and antineoplastic activities, which include activation of apoptosis, induction of cell cycle arrest, differentiation, inhibition of invasion and motility, and reduction of angiogenesis (28, 29). These activities are exerted through both genomic and nongenomic pathways. The classic genomic response is believed to be the most responsible for the vitamin D action, and it is mediated through the VDR. The VDR is highly expressed in a large number of tumor tissues including ovarian, endometrial, cervical, breast, and colorectal cancer (30 -36). Herein, we showed for the first time that progesterone upregulated VDR expression in endometrial cancer but not in immortalized endometrial cells, suggesting that progesterone is able to improve calcitriol’s biological effects in cancer cells. This is corroborated by our results showing a significant decrease in cell proliferation in cells treated with a combination of progesterone and calcitriol. While progesterone-treated cells showed a dramatic increase in VDR expression, calcitriol-treated cells displayed a much more subtle increase. This suggests low basal VDR expression in the cell lines of interest, which may explain the moderate antiproliferative activity caused by calcitriol treatment in comparison with progesterone or combination treatment.
Since apoptotic stimuli often arrest growth before inducing cell death and as significant apoptosis was not evident in calcitriol-treated endometrial cells for the first 24 hours (results not shown) we opted to investigate the early effects of progesterone, calcitriol and combined treatment on the cell cycle in the first 24 hours of exposure. Our results showed G0/G1 cell cycle arrest following progesterone treatment. The combined treatment resulted in a greater number of cells undergoing cell cycle arrest in the Ishikawa cell line. However, combined treatment failed to show an additive effect on the cell cycle in EM-E6/E7-TERT, RL-95 and HEC-1B cell lines. In some cell lines the delayed effect of calcitriol and combined treatment can be attributed to the sensitivity of cell lines to treatment as 48 hr treatment (data not shown) significantly increased cell cycle arrest in immortalized endometrial cell line. In EM-E6/E7-TERT cells, increased cell cycle arrest in G0-G1 in response to calcitriol can be ascribed to increased VDR expression (Figure 2). In non-tumorigenic cells this is not surprising because vitamin D3/VDR signaling is involved in maintenance of cellular homeostasis and modulation of growth by interactions with other transcriptional regulators and cell signaling systems, including TGF-β and β-catenin, to control cell growth and differentiation.

Cells progress through the various phases of the cell cycle via the interactions of different cyclins with their respective cyclin-dependent kinase (CDK) subunits (37-39). Quiescent cells enter the cell cycle after mitogenic stimuli and upregulate cyclins D and E during the G1 phase of the cell cycle (38). Cyclins D1 and D3 are known to bind with and activate CDK4, which phosphorylates Rb protein to release E2F transcription factors to transcribe genes needed for the G1 to S transition (40). Cyclin D1 is considered an oncogene, and is overexpressed in many cancers including prostate, breast, esophagus, lung, head and neck, and colon (41-43). The Cip/Kip family of CDK inhibitors binds to and inhibits the activity of CDK-cyclin complexes that regulates G1-S and G2-M phase transitions (38, 40). The Cip/Kip family plays a critical role in the cellular response to DNA damage for cell cycle arrest (44, 45) and is upregulated in response to antiproliferative signals for cell cycle arrest (46, 47). Endometrial cancer cells treated with a combination of progesterone and calcitriol showed a decrease in cyclins D1 and D3, and an increase in p27 expression. Others (48, 49) have shown an increase in
the steady-state levels of p27 protein in prostate and endometrial cancer cells in response to calcitriol. Although cyclins are downregulated we cannot conclude from our studies that cyclins are direct transcriptional targets of calcitriol and progesterone. Cyclin suppression may have occurred as a downstream consequence of the prior modulation of direct target genes.

Induction of apoptosis in cancer cells is another approach to limit their uncontrolled proliferation (50, 51). In this process, activation of caspases is the central event (50, 51). Once activated, the executioner caspases downstream of the caspase cascade act on the key molecules inside the cells to orchestrate cell death (51). In fact, many cancer preventive and chemotherapeutic agents have been shown to activate the caspases. Cleavage of caspase 3 is considered to be a marker for apoptotic death (51). Confirming these reports, our study shows treatment of endometrial cancer cells with progesterone, calcitriol and the combination significantly enhanced levels of cleaved caspase 3. Together these observations suggest that progesterone and calcitriol inhibit cell growth through apoptosis.

Many proteins either sensitize cancer cells to apoptosis or are directly involved in apoptosis. In an effort to identify the molecular targets of progesterone and calcitriol in endometrial cancer cells we used a proteomic approach. Our data showed 117 proteins differentially regulated by progesterone, calcitriol and the combination. The four apoptosis-related proteins selected for validation were upregulated by all three treatments in endometrial cancer cells. To our knowledge, this is the first report showing BAX, HIST1H1E, HINT2, and EIF2AK2 as tumor suppressors of endometrial cancer and demonstrated their regulation by progesterone and calcitriol. These proteins have not been reported to be regulated by progesterone or calcitriol in endometrial cancer.

BAX resides in the cytosol and translocates to mitochondria upon induction of apoptosis. BAX has been shown to induce cytochrome c release and caspase activation. Its expression is considerably diminished at the mRNA and protein level in cancerous tissues. Suppression of BAX gene expression promoted tumorigenesis while its overexpression showed an inhibitory effect on cell growth (52).
In the present study, lower basal levels of BAX expression were observed in endometrial cancer cell lines than in immortalized endometrial cells. Progesterone and calcitriol were shown to upregulate BAX expression in cancer cells. Cell survival is maintained by a fine balance of the pro-apoptotic (e.g., Bad and Bax) and anti-apoptotic proteins (e.g., BCL-2 and BCL-xL), which control the process of apoptosis through release of caspases (51). Therefore, the increase in the ratio of BAX to BCL-2 proteins observed following treatment of endometrial cancer cells with a combination of progesterone and calcitriol could be responsible for the observed apoptotic effect. Experimental alterations of BAX levels disrupt cellular processes. We were able to elevate BAX protein expression via progesterone and calcitriol treatment in endometrial cancer cells and subsequently relate their effects to cellular functions such as proliferation. The highlight of this study is that BAX knockdown attenuated progesterone- and calcitriol-induced growth inhibition. Interestingly, the upregulation of BAX by combined treatment of endometrial cancer cells resulted in a greater decrease in cell proliferation compared to either progesterone or calcitriol alone. This suggests the upregulation of BAX by a combined treatment may have a cumulative effect on inhibition of cell growth.

Our results showed no significant effects of progesterone and calcitriol treatment on BAX-BCL2 expression in EM-E6/E7-TERT cells. Normal cells maintain critical balance of proteins promoting or inhibiting cell proliferation. Knocking down of BAX perturbs the balance and exposure to the two agents triggers expression of proteins that may inhibit cell growth.

These results provide evidence that calcitriol and progesterone produce cell death in endometrial cells by apoptosis. All investigations into vitamin D and cell death to this point have pointed to an apoptotic mechanism. Although caspase cleavage and the action of other apoptosis-related molecules usually occurs within 24 h or less, it has been shown that vitamin D acts much more slowly to effect apoptosis. For example, Zhang and colleagues (53, 54) have shown the strongest apoptotic response at 6 days following treatment using a vitamin D analog in several cell lines. Mathiasen et al (55) also demonstrated peak vitamin D-induced apoptosis at 6-7 days post-treatment. The latter group also concluded that vitamin D-induced apoptosis may not be caspase-
dependent. While we have not conducted caspase inhibition studies, it should be noted that cleaved caspase-3 increased primarily in response to progesterone treatment, particularly in combination with calcitriol. The cumulative evidence presented herein points strongly to an apoptotic pathway for cell death in these endometrial cancer cell lines in response to progesterone and calcitriol.

Induction of chromatin and condensation and cleavage of DNA are characteristic steps of apoptosis, leading to the formation of oligomers of nucleosomes. HIST1H1E is essential for the generation of condensed structures and initiation of the subsequent internucleosomal cleavage of DNA, which is a hallmark of programmed cell death. During early apoptosis, induction of internucleosomal DNA cleavage and increase in HIST1H1E expression is a prerequisite for DNA accessibility and/or endonuclease. Similar to our findings in human leukemic cells, HIST1H1E is low and expression increases when cells are exposed to an apoptosis-inducing monoclonal anti-CD95 antibody (56).

HINT proteins are adenosine monophosphate–lysine hydrolases. HINT2 is a mammalian-specific, nuclear-encoded mitochondrial HINT hydrolase. We have shown low expression of HINT2 in endometrial cancer cells compared to control cells and expression of HINT2 was upregulated by progesterone and calcitriol treatment. These results are in agreement with a study suggesting that HINT2 overexpression sensitizes hepatocellular carcinoma to apoptosis (57).

EIF2AK2 is a member of the eukaryotic initiation-factor 2 alpha kinase family. EIF2AK2 has been shown to play a significant role in signaling pathways involved in other cellular processes such as cell proliferation, differentiation, metabolism, DNA repair, tumor suppression and apoptosis (58). EIF2AK2 regulates TNF-β-induced p53-dependent apoptosis in U937 cells. In addition, it can interact directly with p53 and phosphorylate it (58). The identification of these new progesterone and calcitriol molecular targets in endometrial cancer cells offers unique opportunities for future development of novel strategies for endometrial cancer therapies.
In summary, we conclude that progesterone combined with calcitriol enhances expression of pro-apoptotic (BAX, HIST1H1E, HINT2, and EIF2AK2) proteins and causes apoptosis and cell cycle arrest in endometrial cancer cells.

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REFERENCES


FIGURE LEGENDS

**Figure 1**  Expression of progesterone receptor (PR) and VDR in normal immortalized human endometrial (EM-E6/E7/TERT) and endometrial cancer (Ishikawa, HEC-1B and RL95-2) cell lines. Whole-cell extracts of progesterone (20 μM), calcitriol (100 nM) or combination-treated cell lines containing an equal amount of protein (100 μg) for progesterone receptors (A) and (20 μg) for VDR (B) were separated by electrophoresis, and immunoblots were probed with 3 mg/ml of mouse monoclonal anti-human PR antibody from DAKO Corporation or VDR antibody at 4°C. The immunoblots shown here are representative of three independent experiments with similar results. The values above the bands represent relative density of the bands normalized to β-actin. The upper values represent quantification of PR B and lower represent PR A. The quantitation of VDR includes both the upper and lower bands.

**Figure 2:** Effect of progesterone, calcitriol and combination treatment on cell growth and death in human immortalized and endometrial cancer cells. Cells were treated with ethanol (control) or 10, 20 and 40 μM progesterone or 50, 100 or 200 nM of calcitriol or the combination for 120 h. At the end of the treatment period, both floaters and attached cells were collected and processed for (A) total cell number determination and (B) dead cell count using trypan blue exclusion method. The data shown are mean ± SEM of three samples for each treatment. These results were similar in three independent experiments. Two-way ANOVA and the Tukey post-hoc test were used to determine differences between treatment groups. Three asterisks (***), represent statistically significant difference across the entire cell line, calculated by two way ANOVA (p<0.05). a and b show statistically significant difference from the lowest dose within a treatment group at (p<0.01) and (p<0.05) respectively. #, denotes a significant interaction between PROG and Calcitriol combination group compared to single agent groups.
(C) Representative Western blots and quantification of caspase-3 protein in progesterone (20 µM), calcitriol (100 nM) and combination-treated endometrial cells. The relative expression of caspase-3 protein was analyzed using β-actin as a control. The quantitation of caspase-3 includes both the upper and lower bands.

**Figure 3.** Progesterone and calcitriol induce cell cycle arrest in endometrial cancer cells. (A-D) Cells were treated with progesterone (20 µM), calcitriol (100nM), or a combination for 24 h and stained with propidium iodide. DNA content was analyzed using flow cytometry. Results are shown as the percent of cell population in G1, S, and G2-M phases of the cell cycle. (E) Cell cycle regulatory proteins expression in progesterone- and calcitriol-treated cells. The immunoblots shown are representative of three independent experiments with similar results. The values above the bands represent relative density of the bands normalized to β-actin.

**Figure 4.** Identification and validation of new progesterone and calcitriol targets in endometrial cancer cells. (A). Total number of proteins altered in endometrial cancer cells by progesterone, calcitriol and combination treatment. Common proteins differentially regulated by all treatments are shown in the center and four proteins selected for validation are listed. (B). Verification of a subset of progesterone- and calcitriol-regulated proteins in endometrial cancer cell lines by Western blotting. Representative Western blots of three independent experiments with similar results are shown. The values above the bands represent relative density of the bands normalized to β-actin.

**Figure 5.** Upregulation of BAX and downregulation of BCL-2 in endometrial cancer cells by progesterone and calcitriol. (A) Normal immortalized EM-E6/E7/TERT and endometrial cancer cells (Ishikawa, HEC-1B, and RL95-2) treated with progesterone (PROG, 20µM), calcitriol (100 nM), or a combination for 24, 72 and 120
hours were evaluated by Western blot for expression of BAX and BCL-2. β-Actin was used as a loading control. Representative Western blots of three independent experiments with similar results are shown. The values at the top of the blots represent the ratio of BAX to BCL-2 after normalization to β-Actin as determined by densitometric scanning. BAX and BCL-2 density values are the average of the three independent immunoblot densities. *, denotes a significant difference between combined PROG and Calcitriol treatment and single agent groups. ns denotes p values not statistically significant between the three treatment groups.

**Figure 6.** Effect of BAX silencing on protein expression and growth of normal and malignant cells. Cells were transfected with BAX siRNA (5 μM) or control siRNA (5 μM) using Lipofectamine 2000. After 24 h, cells were treated with progesterone or calcitriol or a combination for 5 days. (A). Expression of BAX was analyzed by Western Blot. Representative Western blots of three independent experiments with similar results are shown. The values above the bands represent relative density of the bands normalized to β-actin. (B). Cell proliferation was evaluated by MTS assay. Data shown are means ± SEM of values from 3 independent experiments. *** denotes p < 0.05 (statistically significant) between the control and the three treatment groups.

Table 1. Effect of progesterone and calcitriol on cell cycle arrest.
### TABLE 1. Effect of progesterone and calcitriol on cell cycle

| EM-66/TET: TERT | HEC-1B |
|----------------|--|------------------|------------------|
|                | G1/G0 | S | G2/M | G1/G0 | S | G2/M |
| Control        | 56.59±0.43 | 24.58±0.56 | 19.03±0.05 | 36.84±0.35 | 45.28±0.84 | 18.28±0.58 |
| PROG           | 59.19±0.84 | 23.70±0.73 | 13.11±0.17 | 60.94±0.45 | 30.54±0.53 | 8.53±0.16  |
| Calcitriol     | 63.71±0.37 | 21.34±0.73 | 14.95±0.44 | 38.01±0.57 | 43.05±0.26 | 18.94±0.42 |
| PROG+Calcitriol | 62.86±0.57 | 20.04±0.46 | 17.11±0.11 | 60.24±0.47 | 30.38±0.83 | 9.38±0.48  |

| Unknown       | G1/G0 | S | G2/M | G1/G0 | S | G2/M |
|---------------|--|------------------|------------------|
| Control        | 49.69±0.41 | 34.58±0.05 | 15.74±0.37 | 47.14±0.42 | 37.15±0.23 | 15.71±0.63 |
| PROG           | 55.42±0.74 | 30.13±0.47 | 14.45±0.32 | 57.01±0.76 | 35.66±0.67 | 7.33±0.22  |
| Calcitriol     | 49.51±1.03 | 35.34±1.05 | 15.15±0.31 | 52.47±2.51 | 32.49±0.37 | 13.01±0.35 |
| PROG+Calcitriol | 56.95±0.60 | 29.02±0.2 | 11.92±0.71 | 58.97±0.68 | 33.35±0.33 | 7.68±0.24  |

* Denotes statistically significant difference across the entire cell line, calculated by two way ANOVA (p<0.05).

* Denotes a statistically significant difference from PROG, calculated by Tukey HSD (p<0.01).

b Denotes a statistically significant difference from Calcitriol calculated by Tukey HSD (p<0.05).
Cancer Prevention Research

Progesterone Enhances Calcitriol Antitumor Activity by Upregulating Vitamin D Receptor Expression and Promoting Apoptosis in Endometrial Cancer Cells

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