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Epigenetic-mediated upregulation of progesterone receptor B gene in endometrial cancer cell lines

Yuning Xiong[a], Sean C. Dowdy[a], Jesus Gonzalez Bosquet[a], Ying Zhao[a], Norman L. Eberhardt[b,c], Karl C. Podratz[a], Shi-Wen Jiang[a,b,*]

[a]*Department of Obstetrics and Gynecology, Mayo Clinic and Foundation, 200 First Street, SW, Rochester, MN 55905, USA*
[b]*Department of Medicine, Division of Endocrinology, Mayo Clinic and Foundation, Rochester, MN 55905, USA*
[c]*Department of Biochemistry and Molecular Biology, Mayo Clinic and Foundation, Rochester, MN 55905, USA*

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Abstract

Objectives. To determine if epigenetic interference can restore progesterone receptor-B (PR-B) expression in PR-B negative endometrial adenocarcinoma cell lines, and to characterize the kinetics of PR-B induction mediated by DNA methyltransferase and histone deacetylase inhibitors.

Methods. The PR-B negative endometrioid cancer cell lines KLE and HEC-1B were used as study models. PR-B mRNA and protein expression levels were measured using real-time PCR and Western blot analysis, respectively. DNA methylation levels of the PR-B promoter were determined by methylation-specific PCR. Dose–response correlations and the duration of response to aza-deoxycytidine (ADC) and trichostatin A (TSA) were characterized. Cell responses to prolonged and repeated drug treatment were also examined.

Results. Relatively low concentrations of ADC and TSA over a 24-h period induced PR-B expression. Furthermore, ADC and TSA acted synergistically to reactivate PR-B expression. Depending on the cell line used, PR-B mRNA was induced 10–110 fold. This elevated PR-B expression continued for 48 h after drug withdrawal. Sustained upregulation of PR-B mRNA and protein was observed during prolonged and repeated drug treatment.

Conclusion. The epigenetically silenced PR-B gene remains sensitive to changes in DNA demethylation and histone acetylation in uterine adenocarcinoma cell lines. Treatment with ADC and/or TSA results in a robust and sustainable PR-B upregulation. These small molecule epigenetic modifying agents may be used to sensitize poorly differentiated, PR-B negative endometrial cancers to progestational therapy.

Keywords: Progesterone receptor; DNA methylation; Epigenetics; Endometrial cancer

Introduction

The availability of endogenous progesterone is important to protect the endometrium from the hyperplastic effects of estradiol [1]. In the case of exogenous estrogen administration, progesterone is routinely given in combination with estrogen to prevent the development of endometrial malignancies [2]. Progestins have also been used effectively to treat early stage endometrial cancers in women wishing to preserve fertility [1,3]. Progesterone exerts its regulatory effects by binding to the progesterone receptor (PR). PR is a member of a closely related subgroup of nuclear receptors that includes the androgen, mineralocorticoid, and glucocorticoid receptors. Two isoforms of the progesterone receptor, PR-A and PR-B, are expressed in normal endometrium. These isoforms are transcribed from alternative promoters located in proximity to a single gene, and both may be required to limit the proliferation and promote differentiation of the endometrial glandular cells [4].

* Corresponding author. Department of Obstetrics and Gynecology, Mayo Clinic and Foundation, 200 First Street, SW, Rochester, MN 55905, USA. Fax: +1 507 255 4828.
E-mail address: jiang.shiwen@mayo.edu (S.-W. Jiang).

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Previous studies have indicated that the loss of one or both PR isoforms may provide certain advantages for endometrial cancer progression [4]. A deceased ratio of PR-B to total PR was found to be associated with compromised survival [5,6], suggesting an important PR-B specific action in the development of endometrial cancers. Many of the PR-negative tumors present in advanced stages, associated with deep invasion, high recurrence rates, and poor prognosis [5,6]. In addition to its role in tumorigenesis, PR negativity is also considered to be responsible for resistance to progestin treatment [7]. However, the presence of an intact PR gene in PR negative cancers raises the possibility for re-sensitizing these tumors to hormonal treatment following PR induction.

An accumulating body of evidence suggests that epigenetic events, including changes in DNA methylation and/or histone acetylation, regulate gene transcription by modulating chromatin conformation [8,9]. Hypermethylated DNA and deacetylated histones in the promoter region are usually associated with downregulated or silenced gene expression. Two lines of reagents, affecting DNA methylation and histone acetylation, respectively, have been used to reactivate epigenetically silenced genes. Aza-deoxycytidine (ADC) is a modified nucleoside homolog that becomes incorporated into nascent DNA strands during cell replication [10,11]. After incorporation, ADC covalently binds to and arrests DNA methyltransferase (DNMT), leading to genome-wide demethylation [11]. Trichostatin A (TSA) inhibits histone deacetylase (HDAC), leading to the accumulation of acetylated histone [12,13]. While it was reported that PR-B is silenced by a DNA methylation mechanism and PR-B expression was upregulated by ADC treatment [14], it is not clear how HDAC inhibitors alone, or in combination with DNMT inhibitors, may affect PR-B expression. Furthermore, important features of PR-B induction by epigenetic interference, such as the time and dose response patterns, have not been determined. The aim of this investigation was to determine if PR-B expression can be restored by epigenetic interference, and if so, to characterize the kinetics of PR-B expression induced by ADC and TSA in PR-negative endometrial cancer cell lines.

Materials and methods

Cell lines and reagents

Human endometrioid cancer cell lines KLE and HEC-1B were purchased from American Type Culture Collection (ATCC, Rockville, MD). The cells were grown in DMEM/F12 containing 10% fetal bovine serum (BioWhittaker, Walkersville, MD), 100 μg/ml streptomycin, 100 units/ml penicillin, and 2 mM L-glutamine. Cells were maintained at 37°C in an atmosphere containing 5% CO_2 and 100% humidity. The rabbit PR-B antibody was purchased from Upstate (Waltham, MA) and applied following the manufacturer's recommendation. Aza-deoxycytidine and trichostatin A were purchased from Sigma (St. Louis, MO).

Cell treatment, RNA isolation, cDNA synthesis, and quantitative real-time PCR

Cells were plated in 10-cm dishes at 20% confluence. When cultures grew to approximately 50% density, ADC or TSA was added in various concentrations as indicated in the figure legends. Except for the time point study, cells were treated for 48 h prior to harvesting for RNA and DNA isolation.

Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA). cDNA was synthesized with 1 μg RNA using the SuperScript™ kit (Invitrogen, Carlsbad, CA). The 20 μl reverse transcription products were diluted to 100 μl and 2 μl used for each real-time PCR. Reactions were performed in a volume of 25 μl containing 140 ng primers and 12.5 μl SYBR green Master Mix (Stratagene, Cedar Creek, TX). The primer sequences are: PRB-F, 5′-ACTGAGCTGAAGGCAAAGGGT and PRB-R, 5′-GTCCTGTCCCTGGCAGGGC. As an input control, GAPDH (glyceraldehyde-3-phosphate dehydrogenase) mRNA levels were measured using primers: GAPDH-F, 5′-GAAGGTGAAGGTCGGAGTC and GAPDH-R, 5′-GAAGATGGTGATGGGATTTC. PCR conditions were: initial denaturing, 95°C for 5 min; 40 cycles of repeated denaturing at 95°C for 15 s; annealing at 56°C for 30 s; and extension at 72°C for 30 s.

Methylation-specific PCR

Genomic DNA was isolated using Wizard Genomic DNA Isolation Kit (Promega) following the manufacturer's instructions. 2 μg DNA was subject to sodium bisulfite conversion using an EZ DNA methylation Kit™ (ZYMO Research). The converted DNA was eluted from DNA affinity columns and 2 μl was used for PCR. PCR was performed using the following primers: PRB-MF, 5′-GATTGTCGTTCGTAGTACG, and PRB-MR, 5′-CGACAATTTAATAACACGCG for methylated DNA, and primers PRB-UF, 5′-TGATTGTTGTTTGTAGTATG and PRB-UR, 5′-CAACAATTTAATAACACACA for unmethylated DNA. PCR products were resolved in 2% agarose gels and DNA bands visualized by ethidium bromide staining.

Western blot analysis

Cell cultures were rinsed three times with cold phosphate buffered saline (PBS) and harvested by scraping with a plastic policeman in lysis buffer containing 20 mM Hepes, pH 7.2, 25% Glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, and 0.5 mM phenylmethylsulfonyl fluoride. To prevent protein degradation, the solution was supplemented with 1× protease inhibitor cocktail (Sigma, St. Louis, MO). Cell lysates were centrifuged at