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Original Articles

Progesterone receptor membrane component 1 deficiency attenuates growth while promoting chemosensitivity of human endometrial xenograft tumors

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ABSTRACT

Endometrial cancer is the leading gynecologic cancer in women in the United States with 52,630 women predicted to be diagnosed with the disease in 2014. The objective of this study was to determine if progesterone (P4) receptor membrane component 1 (PGRMC1) influenced endometrial cancer cell viability in response to chemotherapy *in vitro* and *in vivo*. A lentiviral-based shRNA knockdown approach was used to generate stable PGRMC1-intact and PGRMC1-deplete Ishikawa endometrial cancer cell lines that also lacked expression of the classical progesterone receptor (PGR). Progesterone treatment inhibited mitosis of PGRMC1-intact, but not PGRMC1-deplete cells, suggesting that PGRMC1 mediates the anti-mitotic actions of P4. To test the hypothesis that PGRMC1 attenuates chemotherapy-induced apoptosis, PGRMC1-intact and PGRMC1-deplete cells were treated *in vitro* with vehicle, P4 (1 μM), doxorubicin (Dox, 2 μg/ml), or P4 + Dox for 48 h. Doxorubicin treatment of PGRMC1-intact cells resulted in a significant increase in cell death; however, co-treatment with P4 significantly attenuated Dox-induced cell death. This response to P_4 was lost in PGRMC1-deplete cells. To extend these observations *in vivo*, a xenograft model was employed where PGRMC1-intact and PGRMC1-deplete endometrial tumors were generated following subcutaneous and intraperitoneal inoculation of immunocompromised NOD/SCID and nude mice, respectively. Tumors derived from PGRMC1-deplete cells grew slower than tumors from PGRMC1-intact cells. Mice harboring endometrial tumors were then given three treatments of vehicle (1:1 cremophor EL: ethanol + 0.9% saline) or chemotherapy [Paclitaxel (15 mg/kg, i.p.) followed after an interval of 30 minutes by *CARBO*platin (50 mg/kg)] at five day intervals. In response to chemotherapy, tumor volume decreased approximately four-fold more in PGRMC1-deplete tumors when compared with PGRMC1-intact control tumors, suggesting that PGRMC1 promotes tumor cell viability during chemotherapeutic stress. In sum, these *in vitro* and *in vivo* findings demonstrate that PGRMC1 plays a prominent role in the growth and chemoresistance of human endometrial tumors.

Introduction

Endometrial cancer is the most common gynecologic malignancy, and its incidence in the United States is predicted to reach 52,630 in 2014 with 8590 succumbing to the disease [1]. Fortunately, surgical intervention in patients diagnosed with stage 1 or 2 low-grade endometrioid endometrial cancer is highly effective as evidenced by the overall survival rate of 95% at five years. In contrast, high-grade endometrial cancers diagnosed at late stage of progression have a much poorer prognosis and an increased incidence of recurrence. Endometrial endometrioid cancer is most common among postmenopausal women; yet, 5% of women are diagnosed with the disease prior to age 40. The most routine treatment for endometrial cancer is total hysterectomy followed by radiation therapy [2,3]. For women under 40 years of age, a uterine sparing non-surgical approach is often employed with low-grade endometrial cancer confined to the uterus. This is especially true for women who wish to maintain reproductive options.

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Progestogen therapy is an effective non-toxic temporizing fertility-sparing alternative to a surgical intervention [2–4]. Progestogens are known to attenuate estrogen-induced endometrial epithelial cell proliferation while promoting differentiation of both epithelial and stromal cells. Although the response rate to progestogen therapy approaches 60% in premenopausal women with well-differentiated endometrial cancer [4,5], it is only effective in 10–15% of women with advanced or recurrent endometrial cancer [6,7]. As such, patient age, tumor histophenotype and grade greatly determine the efficacy of this approach.

Low-grade endometrioid endometrial cancer typically expresses the classical progesterone receptor (PGR). More recently it has been shown that some tumors also express non-classical progesterone receptors, such as progesterone receptor membrane component 1 (PGRMC1). PGRMC1 is a single transmembrane spanning protein that purportedly functions as a non-classical progestin receptor [8–11]. Despite its name, PGRMC1 was first isolated in microsomal fractions derived largely from the endoplasmic reticulum of liver cells [12,13]. PGRMC1 is also expressed in the plasma membrane [14,15] and nucleus [14–16].

Given the abundant and endocrine regulated expression of PGRMC1 in the endometrium [10,14,17–20], the observation that PGRMC1 expression is elevated in various cancers such as ovarian and breast [21–26], and that P4 elicits actions in cells that lack expression of the classical PGR [9], the present study was undertaken to assess the role of PGRMC1 in endometrial cancer. Specifically, the objectives of this study were to: 1) evaluate PGRMC1 expression in endometrial cancer cell lines; 2) assess mitosis and cell death following treatment with P4 and/or chemotherapy in PGRMC1-intact and PGRMC1-deplete endometrial cancer cells; and 3) evaluate the onset of tumor formation and response to chemotherapy treatment *in vivo* following subcutaneous and intraperitoneal inoculation of PGRMC1-intact and PGRMC1-deplete endometrial cancer cells in immunocompromised mice.

Materials and methods

Development of PGRMC1-intact and PGRMC1-deplete Ishikawa cell lines

Ishikawa cells derived from the 3H12 clone which lack the classical PGR (*i.e.*, EV3 Ishikawa cells) [27]; were cultured in phenol red free RPMI-1640 medium (Mediatech, Inc., Manassas, VA) supplemented with 10% fetal bovine serum (FBS, HyClone, Logan, UT), 100 U/ml penicillin G, 292 mg/ml L-glutamine, 100 μg/ml streptomycin and 2.5 μg/ml amphoterocin B (Invitrogen, Carlsbad, CA) at 37 °C in a humidified atmosphere of 5% CO_2. These culture conditions were also used for Ishikawa cells expressing the classical progesterone receptor. The pLKO.1 vector harboring five different hairpin sequences for targeted knockdown of human *PGRMC1* was packaged into lentiviruses at the Molecular Profiling Facility at the Massachusetts General Hospital Center for Cancer Research in association with the RNAi Consortium of the Broad Institute (Cambridge, MA) [28] as described in detail [29]. Control virus containing the pLKO.1 vector harboring a hairpin sequence (TRCN0000061298) for *PGRMC2* was also generated. The PGRMC2 hairpin was ineffective at knocking down PGRMC1 or PGRMC2 and thus served as an effective control (*i.e.*, PGRMC1-intact) for PGRMC1-deplete cells (see Fig. 2B and Supplementary Fig. S1). Infection titers were first established by infecting HEK293T cells grown on 96-well microtiter plates with 25 μl of diluted transfected supernatants containing lentiviral particles and 25 μl polybrene (Sigma; 48 mg/kg). The estimated multiplicity of infection for each virus was 1–2, which resulted in most transformed cells containing no more than one viral integrant [29]. The Ishikawa cells were then transformed using conditions as determined in HEK293T cells. After 24 h, culture medium containing viral particles was removed and cells demonstrating stable integration of the respective plasmids were selected by culturing cells for 72 h in puromycin (2 μg/ml). PGRMC1 levels were determined by RT-PCR and Western blot analysis upon expansion of selected clones. Subsequent cell lines used for experiments are hereafter referred to as PGRMC1-intact and PGRMC1-deplete Ishikawa cells.

Cell culture experiments

For assessment of apoptosis in response to chemotherapeutic stress, Ishikawa cells were rinsed with and converted to serum free medium one day prior to each experiment. PGRMC1-intact and PGRMC1-deplete cell lines were seeded in triplicate at equal densities (1×10^5 cells/well) in 24 well culture plates. At 50% confluence, PGRMC1-intact and PGRMC1-deplete cells lacking the classical progesterone receptor were treated with vehicle (0.03% ethanol), doxorubicin (Dox; 2 μg/ml, Alexis Biochemicals, San Diego, CA), P4 (1 μM), or P4 for 30 min followed by Dox. The number of cells showing evidence of nuclear condensation or fragmentation was recorded as a percent of the total cells counted following fixation with 4% paraformaldehyde and Hoechst staining as previously described [30]. For evaluating the effects of P4 treatment on mitosis, PGRMC1-intact and PGRMC1-deplete cells were again cultured to 50% confluence, converted to serum free conditions as before and treated with P4 (0, 1, 10, 100, or 1000 nM) for 6, 24, 48 or 72 h. Following fixation and Hoechst staining the number of mitotic cells was recorded as a percentage of the total cells counted in five fields of view.

Table 1
PCR primers.

Gene name	Primer sequences
PRA	ACAGAATTCATGACTGAGCTGAAGGCAAAGGGT
	ACAAGATCTCAAACAGGCACCAAGAGCTGCTGA
PRB	ACAGAATTCATGAGCCGGTCCGGGTGCAAG
	ACAAGATCTCCACCCAGAGCCCGAGGTTT
PGRMC1	ACCTGCTGCTGCTTGGCCTCTG
	CCTGGATGCATCTCTTCCAGC
PGRMC2	AGAAGCGGGACTTCAGCTTG
	TCCCATTCTCGAACACTCTCC
PAQR7	CGGATGATCCAGCTCTTCTC
	CGTGTGCAGAGGCTCATAGA
PAQR8	TACCTCACCTGCAGCCTTCT
	GCAACAGCCAGCACAAGATA
PAQR5	ACTATGGTGCCGTCAACCTC
	TCCCAGGTGTACGGATAAGC

RNA isolation and RT-PCR

Total RNA was isolated using TriReagent from two lines of Ishikawa cells that vary in expression of the classical PGR (Sigma Chemical Co., St. Louis, MO). Samples were subjected to DNase I digestion (RQ1 RNase-free DNase; Promega, Madison, WI) to eliminate potential genomic DNA contamination. cDNA was synthesized using SuperScript II reverse transcriptase and oligo-dT primer (Life Technologies, Carlsbad, CA). Expression of various known and purported progesterone receptors was assessed by conventional RT-PCR using primer sets shown in Table 1. Each PCR product was sequenced to confirm specific amplification of the target gene. A negative control (*i.e.* mock reverse transcriptase) was also included for each mRNA sample in which reverse transcriptase was omitted to further confirm the absence of genomic DNA contamination. RT-PCR was also used to assess *PGRMC1* mRNA expression in Ishikawa cells infected with lentivirus to knock down *PGRMC1* expression.

Western blot analysis

Protein lysates were collected from Ishikawa cells and six other endometrial cancer cell lines for Western blot analysis as described in detail [31]. After electrophoretic separation using the NuPage system (Invitrogen, Carlsbad, CA), proteins were transferred (30 V, 1 h) onto polyvinylidene difluoride membranes. Nonspecific binding was blocked with 5% fat-free milk in PBST buffer (0.1% Tween 20 in PBS) for 1 h at room temperature. PGRMC1 antibody was diluted in PBST with 5% fat-free milk and applied to membranes for overnight incubation at 4 °C. Membranes were then washed (3×10 min each) in PBST buffer and incubated with biotin conjugated secondary antibodies (Cell Signaling Technology, Danvers, MA; 1:2500 dilution) for 1 h at room temperature. Membranes were washed in PBST as before, and bound antibody was detected using enhanced chemiluminescent reagents based on the manufacturer's recommendations (Amersham, Piscataway, NJ). Control blots were also completed in which primary antibody was omitted. To verify equal protein loading, membranes were then stripped [1 M glycine (pH 2.5), 1 h, 37 °C] and reprobed with beta-actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA; 1:1000 dilution).

Immunocytochemistry

PGRMC1-intact and PGRMC1-deplete Ishikawa cells were cultured in microscope slide culture chambers. Cells were fixed with 4% paraformaldehyde for 10 min at 4 °C and permeabilized with 100% methanol for 10 min at –20 °C. Cells were incubated in blocking solution containing normal donkey serum and bovine serum albumin for 1 h at room temperature. PGRMC1 antibody (Sigma Aldrich, St. Louis, MO) was applied at 1:1000 and allowed to incubate overnight at 4 °C. Alexa 546 donkey anti-rabbit (Life Technologies, Carlsbad, CA) secondary antibody was applied at a dilution of 1:2000 for 1 h. Cells were washed 3 times with PBS, DAPI was applied in mounting medium, and cells were imaged.

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