Maturitas 102 (2017) 26–33

Contents lists available at ScienceDirect

Maturitas

journal homepage: www.elsevier.com/locate/maturitas

The presence of a membrane-bound progesterone receptor induces growth of breast cancer with norethisterone but not with progesterone: A xenograft model

Yue Zhao[a], Xiangyan Ruan[a,b,*], Husheng Wang[a], Xue Li[a], Muqing Gu[a], Lijuan Wang[a], Yanglu Li[a], Harald Seeger[b], Alfred O. Mueck[a,b]

[a] Department of Gynecological Endocrinology, Beijing Obstetrics and Gynecology Hospital, Capital Medical University, Beijing, China
[b] Research Centre for Women's Health and University Hospital, University of Tuebingen, Tuebingen, Germany

ARTICLE INFO

Keywords:
PGRMC1
Breast cancer
Progesterone
Norethisterone
Xenograft

ABSTRACT

Objectives: During menopausal hormone therapy (MHT) a possible increase in breast cancer risk is thought to depend mainly on the progestogen component. In vitro studies have shown that the progesterone receptor membrane component 1 (PGRMC1) is important for tumor proliferation induced by progestogens. The primary aim of this study was to compare for the first time the natural progestogen, progesterone (P), with a synthetic progestogen, norethisterone (NET), using a xenograft model.

Methods: MCF7 cells, transfected with PGRMC1 plasmid or empty vector, were injected into nude mice and estradiol (E2) pellets were implanted. After 12 days, NET or P or placebo pellets were implanted. Tumor volumes in all groups (6 mice/group) were monitored for 6–7 weeks. Immunohistochemical expression of PGRMC1 and KI-67 was assessed. These experiments were repeated using T47D cells.

Results: Compared with the control condition, E2 and sequential E2/NET combination increased xenograft tumor growth with MCF7 and T47D cells that transgenically expressed PGRMC1 ($p < 0.01$); progesterone did not increase growth. Breast cancer cells transfected with empty vectors did not respond to either progestogen. Comparing KI-67 and PGRMC1 expression, the Pearson correlation was $r = 0.848$, $p = 0.002$.

Conclusions: E2 plus NET increases tumor growth in human breast cancer cells overexpressing PGRMC1, but there is no change with progesterone. To our knowledge, this is the first comparison of both progestogens in vivo using nude mice, which are frequently used in xenograft models. Clinical trials are needed to determine whether women with overexpression of PGRMC1 are at increased risk of breast cancer if NET instead of progesterone is used in MHT.

1. Introduction

An increased risk of breast cancer in postmenopausal women during menopausal hormone therapy (MHT) was shown in the Women's Health Initiative (WHI) study in the group using estrogen in combination with progestogen but not in the estrogen-only arm [1,2]. It was suggested in an editorial on our earlier research [3] that this finding might be explained by overexpression of progesterone receptor membrane component-1 (PGRMC1) in breast cancer cells: *"Can the increase of breast cancer observed in the estrogen plus progestin arm of the WHI trial be explained by PGRMC1?"* [4].

Only in a few observational studies has an increased risk of breast cancer been attributed to the type of progestogen. An increased risk has been seen with synthetic progestogens, but not with natural progesterone or dydrogesterone (a retro-isomer of progesterone), at least during treatment over eight years [5–8]. Since further clinical studies are lacking, the aim of our present study was to continue our experimental work on PGRMC1, much of which has already been published [3,9–16], and for the first time to compare the effect of natural progesterone with the effect of a synthetic progestogen in a xenograft model. Previous studies have reported the effect of synthetic progestogens on breast cancer xenograft tumor growth [14,17,18] but without comparison with the natural hormone, progesterone.

In earlier experiments we showed in vitro that a combination of estradiol with norethisterone (NET) exerts the strongest proliferation effects compared with other synthetic progestogens, especially when

* Corresponding author at: Department of Gynecological Endocrinology, Beijing Obstetrics and Gynecology Hospital, Capital Medical University, No. 251, Yaojiayuan Road, Chaoyang District, Beijing 100026, China.
E-mail address: ruanxiangyan@163.com (X. Ruan).

http://dx.doi.org/10.1016/j.maturitas.2017.05.007
Received 12 April 2017; Received in revised form 30 April 2017; Accepted 4 May 2017

PGRMC1 is strongly expressed, whereas progesterone was neutral in these experiments, i.e. exerting no proliferating effects [10]. Our hypothesis was that this different action also would be observed in vivo using an already validated xenograft model. Thus our primary study endpoint was the effect of estradiol on the growth of xenograft tumors with and without overexpression of PGRMC1, and to compare the effect of NET and progesterone sequentially added to estradiol, using two different breast cancer cell lines. Our secondary study endpoint was to investigate whether the expression of the well-established proliferation marker KI-67 is correlated with the expression of PGRMC1 in the xenograft tissue samples and if there is a difference between the effects of the two progestogens.

2. Materials and methods

2.1. Cell cultures

MCF7 (adenocarcinoma) and T47D (ductal carcinoma), human estrogen receptor (ER) positive primary breast cancer cell lines, were purchased from American Type Culture Collection (ATCC). Cells were routinely cultured in DMEM medium with 10% (vol/vol) heat-inactivated bovine calf serum, 25 mM HEPES and 1% penicillin/streptomycin, at 37 °C in a humid 5% CO_2 atmosphere.

2.2. Transfection of MCF7 cells and T47D cells and cell proliferation

MCF7 cells were stably transfected with expression vector pcDNA3.1 containing hemeagglutinin-tagged (3HA) PGRMC1 [19] (described in text and figures as MCF7-HA-PGRMC1). Briefly, cells were transfected with plasmids. G418 (600 ug/ml) was supplemented after 48 h. Surviving cells after 3-week culture were selected as single clones. Stable transfection was verified by PCR using chromosomal DNA and primers spanning pcDNA3.1 vector and PGRMC1 CDS to distinguish integrated PGRMC1 cDNA from the endogenous chromosomal sequence. MCF7/PGRMC1 was used for experiments and MCF7 transfected with 3HA for control experiments (described in text and figures as MCF7-HA-Vector).

T47D cells were stably transfected using the same method as above.

All proliferation assays were conducted using complete medium (medium with 10% fetal bovine serum; Thermo). Briefly, cells were seeded in 96-well plates and proliferation was determined by MTT (Promega) assay at the indicated time points according to the manufacturer's instructions.

2.3. Hormone pellets

Sixty-day release pellets containing estradiol (0.72 mg), progesterone (10 mg), norethisterone (10 mg) or placebo were obtained from Innovative Research of America. After implantation, steady-state blood levels of the injected hormones reach 2–10 ng/ml.

2.4. Animal experiment

We conducted all animal studies in accordance with the guidelines approved by the Animal Experiments and Experimental Animal Welfare Committee of Capital Medical University, China (AEEI-2015-112). These guidelines comply with the ARRIVE guidelines, in accordance with the National Institute of Health guide for the care and use of laboratory animals (NIH Publications No.8023, 1978). Female athymic BALB/c nude mice (aged 5–6 wk, 18–20 g), often used as a model for research on breast cancer risk [14,18], were purchased from the Vital River Laboratory Animal Technology Co. Ltd. All experiments were performed using age-matched mice, and all experiments were blinded. Only animals in excellent health were used. Animals were randomly assigned to treatment groups using a random number generator. E2 pellets were implanted 24 h before tumor cell injection to supplement the endogenous E2 production, as MCF7 xenograft growth in nude mice requires the presence of estrogen pellets. Subsequently, 1×10^7 MCF7-HA-PGRMC1 or MCF7-HA-Vector cells were re-suspended in 0.15 ml DMEM-F12 medium, and bilaterally injected subcutaneously into the flanks of each mouse (0.15 ml/injection). When the mean tumor volume reached approximately 100 mm^3 (about 12 days after tumor cell injection), progesterone or NET pellets or placebo pellets were inoculated subcutaneously (n = 6 mice/group). This is referred as the sequential regimen. Electronic calipers were used to measure the length and width of each tumor twice per week, and tumor volume was estimated by applying the equation: volume = length × $width^2$. At the end of the treatment period (45 days, as indicated in the figures), the mice were sacrificed and the tumors harvested. Fresh tumor tissue was immediately placed in 4% paraformaldehyde for immunohistochemical analysis (IHC).

2.5. Immunohistochemical assays

Tumor tissues were fixed overnight in 4% paraformaldehyde, followed by paraffin infiltration and embedding. Sections 5 μm thick were mounted close together on precleaned glass slides (Superfrost Plus, Fisher Scientific, Pittsburgh, PA), stained with H & E, and examined for cellularity by light microscopy. IHC was carried out for PGRMC1 and KI-67 as proliferation markers. To detect PGRMC1, sections were incubated at 4 °C overnight with a goat polyclonal raised antibody (8.0 μg/ml; ab48012; Abcam, Cambridge, MA). Within 24 h, all sections were incubated with appropriate biotinylated secondary IgG (donkey anti-goat IgG) for 30 min. The semi-quantitative HSCORE was determined by a pathologist who was blind to treatment and calculated using the formula H-SCORE = $\Sigma Pi\ (i + 1)$, where i is the staining intensity, scored as 1, 2 or 3 (weak (+), moderate (++), or strong (+++)), and Pi is the percentage of cells in each staining category, relative to total cell count [20].

2.6. Statistical analysis

All in vitro experiments were performed in triplicate and were repeated at least three times. Each in vivo experiment was independently replicated 8–10 times to measure tumor volume, with different mice being used in each experimental replicate, and tumor volume data were analyzed with one-way analysis of variance (ANOVA) or t-test with repeated measurements over time. The assumption of ANOVA was examined and nonparametric measurements based on rank were used, as needed. Values were reported as mean (SEM) from replicated experiments as analyzed with GraphPad Prism statistical software package (version 4.0). Analysis for differences in immunohistochemical expression levels among each of the three study groups in MCF7-HA-PGRMC1 and MCF7-HA-Vector xenografts were performed by ANOVA, and associations between PGRMC1 and KI-67 expression (determined by immunohistochemistry) were evaluated using Pearson's correlation coefficient. Group assignment of mice was random and statistical significance was set at $p < 0.05$.

Sample size was calculated using a formula for repeated measurements using our own pilot data and published data on breast cancer risk and progestogen action performed with the same xenograft model (nude mice) [17,18]. The sample size calculation for a power of 90% related to the primary study endpoint of tumor volume indicated a sample size of 5 mice per group. We assigned 6 mice to each group to ensure adequate power.

3. Results

3.1. Stable overexpression of PGRMC1 promotes MCF7 and T47D in vitro proliferation

Overexpression of HA-PGRMC1 in MCF7 and T47D was confirmed