Journal of Steroid Biochemistry & Molecular Biology 147 (2015) 103–110

Contents lists available at ScienceDirect

Journal of Steroid Biochemistry & Molecular Biology

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

Dienogest, a synthetic progestin, down-regulates expression of CYP19A1 and inflammatory and neuroangiogenesis factors through progesterone receptor isoforms A and B in endometriotic cells

Masayuki Ichioka [a], Shizuka Mita [a,*], Yutaka Shimizu [a], Kazunori Imada [a], Tohru Kiyono [b], Yukiko Bono [c], Satoru Kyo [c]

[a] Development Research, Mochida Pharmaceutical Co., Ltd., 722, Jimba, Gotemba, Shizuoka 412-8524, Japan
[b] Division of Virology, National Cancer Research Institute, 5-1-1, Tsukiji, Chuo-ku, Tokyo 104-0045, Japan
[c] Department of Obstetrics and Gynecology, Graduate School of Medical Sciences, Kanazawa University, 13-1 Takaramachi, Kanazawa, Ishikawa 920-8641, Japan

ARTICLE INFO

Article history:
Received 10 July 2014
Received in revised form 15 December 2014
Accepted 17 December 2014
Available online 19 December 2014

Keywords:
Dienogest
Progesterone receptor isoforms
Endometriosis

ABSTRACT

Dienogest (DNG) is a selective progesterone receptor (PR) agonist and oral administration of DNG is used for the treatment of endometriosis. DNG is considered to act on PR to down-regulate pathophysiological factors associated with endometriosis. PR exists as two major isoforms, PR-A and PR-B, and their physiological functions are mostly distinct. It was suggested that PR isoform expression patterns are altered in endometriosis, but it is unknown whether the pharmacological effects of DNG are exerted through PR-A, PR-B or both. In the present study, we investigated the pharmacological effects of DNG through these PR isoforms on the expression of CYP19A1 which encodes aromatase and inflammatory and neuroangiogenesis factors associated with the pain and progression of endometriosis. We used immortalized human endometriotic epithelial cell lines that specifically express PR-A or PR-B in a spheroid cell culture system, and treated them with DNG. We evaluated messenger RNA (mRNA) expression of CYP19A1, prostaglandin (PG)E2 synthase (cyclooxygenase (COX)-2 and microsomal PGE2 synthase (mPGES)-1), inflammatory cytokines (interleukin (IL)-6, IL-8, and monocyte chemoattractant protein (MCP)-1) and neuroangiogenesis factors (vascular endothelial growth factor (VEGF) and nerve growth factor (NGF)) using real-time polymerase chain reaction. In addition, PGE2 production was measured by enzyme immunoassay. We found that DNG down-regulated mRNA expression of CYP19A1, COX-2, mPGES-1, IL-6, IL-8, MCP-1, NGF and VEGF, and PGE2 production in human endometriotic epithelial cell lines that specifically express either PR-A or PR-B. These results demonstrate that DNG activates both PR-A and PR-B and down-regulates the expression of pathophysiological factors associated with pain and progression of endometriosis. Our results suggest that DNG exerts therapeutic efficacy against the pain and progression of endometriosis regardless of PR isoform expression patterns.

1. Introduction

Dienogest (DNG), a synthetic progestin, was demonstrated to have selective progesterone receptor (PR) agonistic activity and oral progestational potency on endometrium [1]. DNG is orally administered for the treatment of endometriosis [2]. DNG has been reported to down-regulate various factors associated with the pain of endometriosis, such as prostaglandin E2 (PGE2) [3,4], inflammatory cytokines including interleukin (IL)-6, IL-8 and monocyte chemoattractant protein (MCP)-1 [5,6], estrogen synthetase aromatase [3,4], neuroangiogenesis factors such as vascular endothelial growth factor (VEGF) [7] and nerve growth factor (NGF) [8] in endometrial or/and endometriotic cells. Endometriosis is an estrogen-dependent inflammatory disease that is characterized by the presence and proliferation of endometrium-like cells outside of the uterine cavity [9,10]. Symptoms of endometriosis include dysmenorrhea, dyspareunia, dysuria, chronic abdominal or pelvic pain, and infertility [11]. Therefore,

* Corresponding author. Tel.: +81 550 89 7881; fax: +81 550 89 8070.
E-mail address: shizuka.mita@mochida.co.jp (S. Mita).

http://dx.doi.org/10.1016/j.jsbmb.2014.12.008
0960-0760/

most patients with endometriosis show markedly reduced quality of life. It has been demonstrated that treatment with DNG effectively relieves the pain associated with endometriosis and reduces endometriotic foci [12–15].

The pharmacological mechanisms of DNG are considered to be related to anti-ovulatory activity, resulting in reduced serum estrogen levels [16] and direct anti-proliferative and anti-inflammatory effects on endometriosis tissue [2]. Similarly to other progestins, DNG acts through PR to exert pharmacological effects [2]. PR, a receptor for endogenous steroid hormone progesterone (P), is a nuclear receptor that functions as a transcription factor for P target genes. PR exists as two major isoforms, namely PR-A and PR-B. PR-A is a 94-kDa protein, whereas PR-B is a 114-kDa protein that contains an additional NH2-terminal stretch of 164 amino acids containing a region encoding a transcription function [17]. These isoforms arise as a result of initiation of translation from alternative sites in the same gene *via* transcription from alternative promoters [18].

Studies using PR-A- and PR-B-specific knockout mice have shown that PR-A and PR-B exert mostly distinct, but partially overlapping, responses to P [19]. According to studies into the molecular mechanisms of PR isoforms, PR-B tends to be a stronger activator of transcription on target genes [20]. On the other hand, PR-A has weak transcription activity and functions as a transcription repressor of PR-B [17,21], as well as hormonal receptors such as estrogen receptor α [22], glucocorticoid receptor, androgen receptor [17] and mineralcorticoid receptor [21]. It has also been reported that activation of PR-A or PR-B down-regulates transcriptional activity of nuclear factor (NF)-κB and activating protein (AP)-1, which are inflammatory transcriptional factors [23–25].

Although the evidence related to PR isoform function has increased, their pharmacological functions are not fully understood. Several researchers have reported altered expression levels and patterns of PR isoforms in endometriosis, but the results are controversial [26]. For example, some groups have reported that PR-A is more commonly expressed in endometriotic tissue than PR-B [27,28]. Based on these reports, it has been proposed that endometriosis is a P-resistant disease [29]. In contrast, other reports have demonstrated that PR-B expression or PR-B/PR-A expression ratio does not differ between women with or without endometriosis [30,31].

Although DNG acts through PR to exert pharmacological effects, it has not been clarified whether both PR isoforms contribute to the expression of these effects. As the expression patterns seem to change in endometriosis, it is important to clarify the responsibilities of PR-A and PR-B to the action of DNG. In the present study, we examined the direct effects and pharmacological mechanisms of DNG and P as an endogenous control of PR activation, on the mRNA expression of CYP19A1, which encodes aromatase, and inflammatory and neuroangiogenesis factors, as well as PGE2 production in immortalized human endometriotic epithelial cell lines that specifically express PR-A or PR-B.

2. Materials and methods

2.1. Drugs and reagents

Progesterone (P), bovine serum albumin (BSA), Dulbecco's modified Eagle's medium (DMEM), and phosphate-buffered saline (PBS) were purchased from Sigma–Aldrich. Phenol red-free DMEM, fetal bovine serum (FBS), antibiotics and L-glutamine were obtained from Life Technologies. Dextran-coated charcoal-treated (DCC)-FBS was purchased from HyClone. DNG was supplied by Bayer HealthCare.

2.2. Cell culture

We used three immortalized endometriotic epithelial cell lines. The EMosis-CC/TERT1 parental cells were previously established by immortalization of an endometriotic epithelial cells from ovarian endometrioma [32]. The EMosis-CC/TERT1/PRA and EMosis-CC/TERT1/PRA−/PRB+ cell lines were established by retroviral infection of cDNAs for intact PR-A and a point-mutated PR-B gene (in which the 2nd ATG for PR-A transcription initiation was destroyed by substitution mutations) into the EMosis-CC/TERT1 parental cell line. As we only used a well-established parental cell line that was reported previously, Institutional Review Board approval was not necessary for this study. These cell lines were maintained in DMEM supplemented with 10% inactivated FBS and antibiotics (50 U/mL penicillin and 50 μg/mL streptomycin) at 37 °C in 95% air/5% CO_2.

2.3. RNA extraction and reverse transcription–polymerase chain reaction (RT–PCR)

Semi-confluent cell lines seeded in culture flasks were harvested and rinsed twice with cold PBS. Total RNA was extracted using the RNeasy Mini Kit (QIAGEN) according to the manufacturer's instructions, and concentrations were determined using the NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific). RT–PCR was performed using the QIAGEN OneStep RT–PCR Kit (QIAGEN). Primers used for amplification and PCR cycles were as follows: human PR-A/B common sequence, forward primer 5′-CCTGACACCTCCAGTTCTTTGCTGA-3′ and reverse primer 5′-GGGATCTGCCACATGGTAAGGCATA-3′, 25 cycles; human PR-B specific upstream sequence, forward primer 5′-ACACCTTGCCTGAAGTTTCG-3′ and reverse primer 5′-CTGTCCTTTTCTGGGGGACT-3′, 30 cycles, and human housekeeping gene primer set for human glyceraldehyde-3- phosphate dehydrogenase (GAPDH) (TaKaRa), 25 cycles. After the reaction, PCR products were visualized with AE-6962FC Light Capture (ATTO) following gel electrophoresis.

2.4. Western blot analysis

Cell lines were seeded into 150-mm culture dishes at a density of 2.0×10^6 cells per dish in 30 mL of medium containing 10% DCC-FBS and antibiotics for 3 days of culture. After washing and harvesting, cells were lysed in RIPA buffer containing 1% phenylmethanesulphonyl fluoride, 1% protease inhibitor cocktail, 1% sodium orthovanadate (Santa Cruz Biotechnology). After ultrasonication, cell lysates were incubated on ice for 30 min. After centrifugation at 10,000 rpm for 10 min at 4 °C, supernatant was collected as whole cell lysates. These lysates (10 μg/lane) were separated by 5–20% SDS-PAGE and were transferred to PVDF membrane (ATTO). After blocking at room temperature for 1 h, membranes were incubated with anti-PR (H-190; Santa Cruz Biotechnology), anti-PRB (C1A2; Cell Signaling Technology) and anti-β-actin (C4; Santa Cruz Biotechnology) antibodies overnight at 4 °C, followed by washing with Tween/PBS. Subsequently, membranes were incubated with horseradish peroxidase-linked anti-mouse or rabbit IgG antibodies (Cell Signaling Technology) at room temperature for 1 h, followed by washing with Tween/PBS. Protein–antibody complexes were detected using the ECL Prime Western blotting detection system and chemiluminescence was visualized using ImageQuant LAS 4000 mini (GE Healthcare Life Sciences).

2.5. Drug treatment and real-time quantitative PCR

Cell lines were seeded with phenol red-free DMEM supplemented with 10% DCC-FBS and antibiotics in U-bottom 96-well