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Low-dose SKA Progesterone and Interleukin-10 modulate the inflammatory pathway in endometriotic cell lines

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ABSTRACT

Objective: To study the efficacy of low-dose SKA Progesterone and IL-10 in modulating the inflammatory pathway in endometriosis.
Design: Experimental basic science study.
Setting: Reproductive biology laboratory.
Models: Immortalized human endometriotic epithelial cells (12Z) derived from active red peritoneal lesions, immortalized human endometriotic stromal cells (22B) derived from active red peritoneal lesions and immortalized human endometrial cell line T-Hesc (ATCC collection).
Methods: Cells were treated with SKA-Progesterone and SKA-IL10 at low doses (10 pg/ml and 10 fg/ml respectively). Medroxyprogesterone 17-acetate (MPA) was used at a dose of 10 μM as reference treatment.
Main outcome measure(s): Modulation of HSD17B1 levels by WB analysis after low-dose SKA Progesterone and MPA; Modulation of IKBα protein levels and NF-kB p65 nuclear levels by WB analysis after low-dose SKA-Progesterone, low-dose SKA-IL10, low-dose SKA-Progesterone and low-dose SKA-IL10 (combined treatment), MPA.
Results: Low-dose SKA Progesterone was effective in the inhibition of HSD17B1 expression in endometriotic epithelial (12Z) and stromal (22B) cell lines. Low-dose of SKA Progesterone and low-dose of SKA-IL10 inhibit NF-kB p65 nuclear localization and DNA binding in endometriotic epithelial (12Z) cells, stromal (22B) cells line and in endometrial cell line T-Hesc. The combined treatment showed an additive effect, namely increasing the inhibition of nuclear localization of NF-kB p65 and DNA binding as result of single treatments.
Conclusion: Our data suggest that the use of a combination of low-dose SKA Progesterone and IL-10 may represent an opportunity for the development of new therapies in the clinical management of endometriosis.

1. Introduction

Endometriosis is a chronic gynecological inflammatory disease characterized by the presence of functional endometrial glands and stroma outside of the uterine cavity [1]. It affects 7–10% of women of reproductive age, up to 50% of women with infertility and up to 60% of women with dysmenorrhea [2]. Endometriosis is known to be an estrogen-dependent disease and progesterone resistance has also been documented as a mechanism of progression of endometriosis [3–6]. In healthy pre-menopausal women, the primary source of estradiol (E2) is the ovary, while in women with endometriosis, in addition to endocrine E2 production, E2 can be synthesized locally in the endometrial lesions from inactive adrenal precursors, such as dehydroepiandrosterone sulfate, dehydroepiandrosterone and androstenedione (A), via the aromatase pathway [7–9]. Aromatase (CYP19A1) catalyzes the conversion of ovarian or adrenal testosterone (T) to E2, as well as A to a weak estrogen estrone (E1). Increased aromatase expression has been shown in peritoneal, ovarian and deep endometrial implants in different studies [10,11]. E1 can then be further converted into E2, by the action of the 17β-hydroxysteroid dehydrogenase type 1 (HSD17B1), and, to a lower extent, also by 17β-hydroxysteroid dehydrogenases type 5, 7 and 12. Several studies have demonstrated the presence or elevated

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expression of HSD17β1 in ovarian and deep infiltrating endometriosis [11–13].

Recent studies provided evidences that endometriosis is characterized by a strong inflammatory component which is one of the key factors in its onset and progression [14,15].

Progestins are used for the treatment of endometriosis, since progesterone is a powerful anti-inflammatory and anti-proliferative hormone in the female reproductive tract [16,17]. Shimizu et al. showed that the progestin dienogest (DN) decreases aromatase expression in immortalized human endometrial epithelial cells [18]. Moreover, Fechner et al. reported a decrease in aromatase transcription after medroxyprogesterone acetate (MPA) and dydrogesterone (D) injection in mice with implanted human endometrium [19], suggesting that these progestins can regulate local estrogen biosynthesis in endometriosis.

Several literature data have been provided about the involvement of the NF-κB pathway in endometriosis pathophysiology [20–25]. In vitro studies have shown an increased encoding of growth factors and proinflammatory and antiapoptotic proteins mediated by NF-κB activation in human endometrial and endometriotic cells [26–41]. Human endometrial cells have been shown to activate NF-kB pathway in response to IL-1β and TNF-α in vitro [28], inducing a consequent increasing of IL-8 production [32].

Although progestins at pharmacological dose are effective in the control of endometriosis, they may cause breakthrough bleeding as a common side effect, often leading to the discontinuation of therapy [45]. Furthermore, as medical therapies control but do not cure the disease, long periods of pharmacologic treatment may be needed.

IL-10 plays a significant role in down-regulating the inflammatory environment in patients with endometriosis [52–54] and it might also provide a therapeutic modality for endometriosis.

The role of sequential kinetic activation (SKA) signaling molecules oral administration in inflammatory status management is demonstrated [46]. If effective in the control of the disease, low-dose molecules, prepared by SKA, might be an opportunity for the long-term treatment of patients with endometriosis.

The aim of the study was to evaluate the efficacy of low-dose SKA Progesterone and IL-10 in the modulation of the inflammatory response in endometriotic cell lines. We carried out closer investigation to evaluate whether low-dose SKA Progesterone (10 pg/ml) was effective as well in the modulation of HSD17B1 levels in the endometriotic epithelial cell lines 12Z [49] and in the endometriotic stromal cell lines 22B [49]. We moreover evaluated the effect of both Low-dose SKA Progesterone (P) and low-dose SKA IL-10 (as isolated and combined treatment) in modulating the nuclear translocation of NFkB-p65 and the consequent putative anti-inflammatory and/or anti-apoptotic effects.

2. Methods

Institutional Scientific Board approved the study. Since the study was performed in cell lines, without involving patients or human samples, our IRB declared the study exempt from IRB approval.

2.1. Cell culture

Immortalized human endometriotic epithelial and stromal cells (12Z and 22B respectively) and immortalized were maintained in Dulbecco's modified Eagle's medium (DMEM)/F12 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin G and 100 g/ml streptomycin (Life Technologies,NY, U.S.A.) in a humidified atmosphere of 5% CO_2–95% air at 37 °C. Immortalized human endometrial cell line T-Hesc (ATCC collection) was maintained in Dulbecco's modified Eagle's medium (DMEM)/F12 medium supplemented with 10% fetal bovine serum, 1% ITS liquid media supplement (Sigma, Saint Louis, Missouri, U.S.A.), 100 U/ml penicillin G and 100 g/ml streptomycin (Life Technologies,NY, U.S.A.). For the experiments with Progesterone treatments, cells were starved from hormones for 48 h replacing normal fetal bovine serum with charcoal stripped fetal bovine serum (Life Technologies,NY, U.S.A.).

2.2. Reagents

Low-dose SKA Progesterone (GUNA-Progesterone) and low-dose SKA IL10 (GUNA-INTERLEUKIN 10) were prepared by GUNA Laboratories (GUNA S.p.a, Milan, Italy) using a standardized method [50].

The preparations were supplied at a concentration of 10 pg/ml for Progesterone and 10 fg/ml for IL-10. Medroxyprogesterone 17-acetate (MPA) (Sigma, Saint Louis, Missouri, U.S.A.) was used at a concentration of 10 μM.

2.3. Western blot analysis

Cells were washed with ice-cold PBS and extracted in RIPA protein lysis buffer (50 mM Tris–Cl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40,0.5% Na desoxicholate, 0.1% SDS, 1 mM EDTA) supplemented with a cocktail of protease inhibitors (Boehringer-Germany). Protein concentration was determined by Bradford assay. All SDS–PAGE were transferred onto PVDF membranes (Millipore). Membranes were developed using the enhanced chemiluminescence (ECL™ Amersham-UK) by chemiluminescence imaging system, Alliance 2.7 (UVITEC, Cambridge-UK) and quantified by the software Alliance V_1607. The following primary antibody were used: polyclonal rabbit anti-NFkBp65 (R&D- Minneapolis,US), polyclonal rabbit anti-Sp1 (Santa Cruz-Dallas,US), monoclonal mouse anti-α-tubulin (Sigma), monoclonal mouse anti-HSD17B1 (R&D), monoclonal mouse anti-Human IL10-Receptor-α (R&D), monoclonal mouse anti-Progesterone Receptors A and B (ThermoFisher-Massachusetts, US) monoclonal mouse anti-IKBalpha (ThermoFisher- Massachusetts, US), monoclonal mouse anti-Bcl2 (Dako), polyclonal rabbit anti-Bax (Dako).

2.4. Isolation of nuclear/cytoplasmic fractions

Nuclear extracts were prepared as follows: cells, scraped off the plate with PBS, were resuspended for 15 min in hypotonic lysis buffer (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA) added with protease inhibitors (Boehringer-Germany). After resuspension, NP-40 was added to a final concentration of 0.6%, vortexed for 15 s and the nuclei were isolated by centrifugation at 10 000 r.p.m. for 30 s at 4 °C. After removal of the supernatant (i.e. the cytoplasmic extract), nuclei were resuspended in nuclear extract buffer (20 mM HEPES pH 7.9, 25% glycerol, 0.4 M NaCl, 0.1 mM EDTA, 0.1 mM EGTA), rocked for 15 min at 4 °C and then recovered by centrifugation at 14 000 r.p.m. for 5 min at 4 °C.

2.5. NF-kB p65 transcription factor assay (DNA binding activity)

Freshly prepared cells nuclear extracts were quantified with Bradford reagent and used for NF-kB p65 Transcription factor Assay that was performed according to manufacturer's instruction (Abcam-Cambridge-UK).

2.6. IL-1β immunoassays

IL-1β levels were measured in cell culture supernatants obtained from 12Z cells by an enzyme-linked immunoassay (ELISA) according to manufacturer's instructions (USCN Life Science Inc. and Cloud-Clone Corp.USA).