survivin expression persisted exclusively in ESCs after they were treated with an apoptosis-inducing drug. They further demonstrated that resistance to drug (staurosporine) -induced apoptosis was diminished by silencing survivin expression. Therefore, agents that reduce survivin expression can be used to enhance the effect of apoptosis-inducing drugs. As RVT is well known for suppressing survivin expression in various types of cells,[19,31,32] we decided to test whether RVT affects survivin expression in ESCs. Indeed, RVT dramatically reduced survivin expression in ESCs, and it is meant that, when applied clinically, RVT could be extremely useful for treating or managing endometriosis.

As an apoptosis-inducing agent, TRAIL was chosen in this study. TRAIL plays an important role in inducing apoptosis in various types of cancer cells[33]; however, several types of cancer cells are resistant to TRAIL.[34] Enhancement of TRAIL potency is considered to be critical for controlling tumor growth and disease progression.[34] Our group has previously reported that higher concentration of TRAIL is detected in peritoneal fluid in women with endometriosis than in control, whereas osteoprotegerin (OPG) expression, which prevents TRAIL from binding cells, is further enhanced in endometriosis, and that the relative decrease of TRAIL function and hence activity contributes to the pathogenesis of endometriosis.[7] Our group further demonstrated that the apoptosis-inducing effect of TRAIL on ESCs is marginal while its effect on eutopic endometrial stromal cells is meaningful, possibly because the expression of DR5, a receptor of TRAIL, is significantly lower in ESCs than in eutopic stromal cells[4]; therefore, enhancing TRAIL-induced apoptosis in ESCs is a promising approach for treating endometriosis. In the current study, we have confirmed that RVT enhances TRAIL-induced apoptosis in ESCs, which suggests that RVT could potentially contribute to the treatment of endometriosis.

The mechanism by which RVT enhances TRAIL-induced apoptosis observed in ESCs appears to hinge on its effect to suppress survivin expression given that this mechanism of action has been demonstrated in several cancer cell models.[19,35] It is possible, however, that the mechanism is more complex and that the expression of other pro-apoptotic and anti-apoptotic proteins, for example, bcl-2[36] and bcl-x,[37] or bax,[38] may contribute to RVT effects on ESCs. A complete understanding of the mechanistic pathways involved in RVT effects in ESCs requires further investigation.

This study has several limitations. Firstly, the current experiments only used ESCs and did not test the effect of RVT on eutopic endometrium. If RVT exerts its apoptosis-enhancing effect on eutopic endometrium obtained during menstrual periods, this drug could not only be therapeutic but also be preventive for endometriosis by eliminating the retrograde endometrium. Secondly, the current study does not evaluate *in vivo* effects of RVT on patients with endometriosis. Clinical studies are warranted to determine the efficacy of this therapeutics. It is also important to evaluate the safety of RVT, although several clinical studies have been assessed side-effects of RVT[39–41] and no serious adverse reaction is reported. Regarding its effect on female reproduction, RVT in theory does not interfere with ovulation and the recent study demonstrated that RVT even improves the quality of oocytes in mice,[42] but further studies are needed to verify the safety of using RVT for women who wish to conceive.

In conclusion, this study has added the apoptosis-enhancing property to the mechanism by which RVT controls endometriosis. Further related studies are needed to investigate the clinical application of RVT to manage endometriosis.

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ORIGINAL ARTICLE

Simultaneous Detection and Evaluation of Four Subsets of CD4+ T Lymphocyte in Lesions and Peripheral Blood in Endometriosis

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Problem
The proportion of CD4+ T lymphocytes, Th1, Th2, Th17, and regulatory T cells in endometriosis lesions and peripheral blood are not known.

Method of study
Lymphocytes were isolated from endometriosis lesions ($n = 10$) and endometrium ($n = 10$). Lymphocytes in peripheral blood were isolated from patients with and without endometriosis ($n = 10, 10$). The CD4+ T-lymphocyte profile was analyzed by flow cytometry.

Results
The proportion of Th1 lymphocytes was significantly lower in endometriosis tissue (59.64 ± 9.2%) in comparison with endometrial tissue (79.07 ± 8.97%), whereas the Th17 lymphocyte fraction was significantly higher in endometriosis tissue (6.66 ± 2.53%) in comparison with endometrial tissue (2.27 ± 1.51%). Analysis of peripheral blood indicated that the Th1 proportion was significantly higher in women with endometriosis (10.25 ± 2.82%) in comparison with controls (6.96 ± 4.13%).

Conclusion
The CD4+ T-lymphocyte profile in lesions and peripheral blood is altered in women with endometriosis. These findings may help better understanding of T-lymphocyte involvement in the pathophysiology of endometriosis.

Introduction

Endometriosis is an estrogen-dependent, chronic inflammatory disease that affects approximately 10 percent of women of reproductive age.[1,2] It causes pain and infertility, and remarkably deteriorates women's health. Although not fully understood, many factors such as hormonal conditions,[3] gene profile,[4] environmental,[5] and immune factors [6] are proposed to be involved in the pathogenesis of endometriosis. Immune cells are notably involved given that abnormalities in various cell types such as macrophages,[7] dendritic cells,[8] natural killer cells,[9] and lymphocytes [10,11] all contribute to the pathophysiology of endometriosis. As a consequence, a chronic inflammatory response, including enhanced production of inflammatory cytokines locally and systemically in patients with endometriosis, is reported.[12,13]

Awareness of the Th1/Th2 balance has added to our understanding of various immunological conditions,[14,15] including endometriosis. In endometriosis, it has been widely believed that the local immunological milieu is Th2-skewed and Th2 lymphocytes are reported to be present in endometriosis tissue.[16] However, robust evidence supporting a Th1/Th2

imbalance is lacking. For example, one study reported that the expression of GATA-3, a Th2 transcription factor, is higher than the expression of Tbet, a Th1 transcription factor, in endometriosis tissue[17]; however, this study did not directly examine the T-lymphocyte population. Another study characterized lymphocytes extracted from endometriosis tissue,[18] but T lymphocytes were not adequately stimulated prior to the analysis, and therefore, the results from the study may not reflect the local physiological conditions of endometriosis. To our knowledge, a simultaneous detection and evaluation of Th1 and Th2 lymphocytes to examine these Th1/Th2 balance in endometriosis has not been reported.

In addition to Th1 and Th2, and their balance, Th17 and regulatory T cells (Treg), and their balance, are also known to play a role in various immunological pathologies, especially in chronic inflammatory diseases such as rheumatoid arthritis,[19–21] Crohn's disease,[22–24] systemic lupus erythematosus,[25–27] and psoriasis.[28,29] As for endometriosis, our group previously demonstrated that IL-17A, produced by Th17 lymphocytes, was expressed in the endometriosis lesion and could contribute to disease progression, by inducing IL-8, COX-2, and cell proliferation.[30] The association between endometriosis and Treg is also described.[31] However, studies examining the balance between Th17 and Treg in the local or systemic immunological milieu in endometriosis have not been reported.

Recently, simultaneous detection of four subsets of CD4+ T lymphocytes within one sample has been performed in various physiological and pathological conditions to understand the contribution, if any, of the T-lymphocyte balance in their pathogenesis. For example, Mjosberg et al.[32] analyzed four subsets T lymphocytes in peripheral blood and in the normal decidua and found that the proportion of Treg is higher in the decidua than in peripheral blood, whereas Th2 and Th17 proportions were lower in the decidua than in peripheral blood and suggested that Treg in the implantation sites plays an important role in normal pregnancy. A Th2 and Th17 skew in peripheral blood from Henoch–Schoenlein purpura patients was also demonstrated.[33] To better understand T-lymphocyte involvement in the pathophysiology of endometriosis, we performed simultaneous detection of four subsets of CD4+T lymphocytes and evaluated the profile of each in endometriosis tissue, normal endometrium, and peripheral blood from patients with endometriosis.

Materials and methods

Subjects and Samples

The experimental procedures were approved by the Institutional Review Board of the University of Tokyo. Signed informed consent for the use of tissue and peripheral blood was obtained from each woman. All patients had not received hormones or GnRH agonist for ≥3 months before surgery.

Tissues of endometriosis lesion were obtained from ovarian endometrioma during laparoscopy (Group A, $n = 10$). Five patients were in the proliferative phase, and five were in the secretory phase at the time of surgery. Tissues of endometrium were obtained from women who underwent hysterectomy for fibroids (Group B, $n = 10$). All women in Group B were negative for endometriosis, confirmed at surgery. Five women were in the proliferative phase, and five were in the secretory phase. The age (median, range) of patients in Group A (32.5, 27–40) was significantly younger than that of patients in Group B (45.5, 42–51, $P < 0.05$).

Peripheral blood was obtained on the day before laparoscopy from patients with endometriosis (Group C, $n = 10$), and control women (Group D, $n = 10$) who were undergoing laparoscopy for benign gynecologic conditions other than endometriosis. Six women were in the proliferative phase and four were in the secretory phase (Group C), and four women were in proliferative phase and six were in secretory phase (Group D) at the time of sample collection. rASRM stage III or IV endometriosis was confirmed in all women in Group C, and the absence of endometriosis was confirmed in all control women (Group D) during laparoscopy. The age of patients in Group C (33.5, 27–43) was not significantly different from that of patients in Group D (31, 23–38).

Isolation of Lymphocytes

To obtain mononuclear cells, tissues were minced into small pieces and incubated in DMEM/F-12 with type I collagenase (2.5 mg/mL) and deoxyribonuclease I (15 IU/mL) for 1–2 hr at 37 °C, then filtered through a 70-μm nylon cell strainer. The cell suspension was washed in phosphate-buffered saline (PBS) and resuspended with 40% Percoll solution ($d = 1.052$) and poured on to 70% Percoll solution ($d = 1.087$). After centrifugation at 580G for 18 min, cells in the interlayer were collected. To obtain

mononuclear cells from peripheral blood, standard Ficoll–Paque Plus gradient centrifugations were performed at 400G for 30 min according to the manufacturer's recommendations.

After isolating mononuclear cells from tissue or peripheral blood, cells were washed with PBS and were resuspended in RPMI 1640 containing 10% FBS and incubated in a plastic dish for 12–24 hr to exclude adherent cells (such as macrophages and monocytes), and floating cells are used as lymphocytes.

Flow Cytometry

Lymphocytes from tissues or peripheral bloods were divided into two parts. The first part was used for the detection of Th1, Th2, and Th17 and processed for intracellular cytokine staining. The other part was used for the detection of Treg and processed for cell surface staining. Cells that were positive for both CD4 and intracellular IFN-γ were defined as Th1, cells that were positive for both CD4 and intracellular IL-4 as Th2, and cells that were positive for both CD4 and intercellular IL-17A as Th17. Cells that were positive for CD4, CD25, and FOXP3 were defined as Treg. At least 10^4 cells were counted in each sample.

To analyze Th1, Th2, and Th17, isolated lymphocytes were cultured with phorbol 12-myristate 13-acetate (50 ng/mL) and ionomycin(1 μg/mL)for 5 hr. Protein transport inhibitor (BD PharMingen, San Diego, CA, USA) was added 1 hr prior to sample collection in order to prevent cytokine secretion. Lymphocytes were collected and firstly incubated with APC-conjugated anti-CD4 antibody. Cells were then fixed and permeabilized using BD fixation/permeabilization solution (BD Biosciences, Franklin Lakes, NJ, USA) and incubated with Alexa 488-conjugated anti-IFN-γ, Alexa 488-conjugated anti-IL-4, or Alexa 488-conjugated anti-IL-17A, respectively (eBioscience, San Diego, CA, USA).

To analyze Treg, the Human Treg Flow kit (Biolegend, San Diego, CA, USA) was used according to the manufacturer's recommendations. In short, isolated lymphocytes were incubated with PE-cy5-conjugated anti-CD4 antibody and PE-conjugated anti-CD25 antibody, then fixed and permeabilized using FOXP3 Fix/Perm solution, and further incubated with Alexa 488-conjugated anti-FOXP3 antibody.

Samples were analyzed using FACSCalibur (BD Bioscience San Jose, CA, USA) and FLOW JO (Tree Star, Ashland, OR, USA). The data were presented as the percentage of double-positive (CD4 and IFN-γ for Th1, CD4 and IL-4 for Th2, and CD4 and IL-17A for Th17) or triple-positive (CD4, CD25, and FOXP3 for Treg) cells in total CD4+ cells.

Data Analysis

Statistical analyses were conducted using JUMP® ver.8.0 (SAS Institute, Cary, NC, USA). Results are presented as the mean ± S.D. Wilcoxon analysis was used to compare the results of two groups. Spearman correlation regression test was used to analyze the correlation between age and percentage of lymphocyte subsets. A P value of <0.05 was considered as statistically significant.

Results

CD4+ T Lymphocyte Profile in Endometriosis Lesion and Endometrium

The percentage of Th1 was significantly lower in endometriosis lesions (59.64 ± 9.2%) in comparison with endometrium (79.07 ± 8.97%, $P = 0.0012$). On the contrary, the percentage of Th17 was significantly higher in lesions (6.66 ± 2.53%) in comparison with endometrium (2.27 ± 1.51%, $P = 0.0015$). With regard to Th2 and Treg, significant difference was not found between their proportions in endometriosis lesions and endometrium. When the subgroup analysis was performed for both proliferative and secretory phages, the tread was similar. The percentages of four CD4+T-lymphocyte subsets in the tissue of endometriosis lesion and endometrium are presented in Table I and Table SI. As the age of patients in Group A was significantly younger than that of patients in Group B, the effect of age on the T-lymphocytes profile was evaluated. No significant correlation was detected between the age of patients and the percentage of each subset in both Group A (P values were 0.81, 0.83, 0.25, and 0.63, for Th1, Th2, Th17, and Treg, respectively) and Group B (0.54, 0.51, 0.48, and 0.89) (Fig. 1).

CD4+ T-Lymphocyte Profile in Peripheral Blood

The percentage of Th1 was significantly higher in peripheral blood from women with endometriosis (10.25 ± 2.82%) in comparison with controls (6.96 ± 4.13%, $P = 0.041$). With regard to the other subsets (Th2, Th17, and Treg), no significant

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Table I Distributions of CD4+ T-lymphocyte subsets in endometriosis lesion and endometrium

Tissue sample	Th1	Th2	Th17	Treg
Endometriosis lesion (n = 10)	59.64 ± 9.28	9.07 ± 3.43	6.66 ± 2.53	7.51 ± 4.49
Endometrium (n = 10)	79.07 ± 8.97	11.2 ± 5.58	2.57 ± 1.51	6.41 ± 3.41
P value	<0.05 (0.0012)	NS (0.29)	<0.05 (0.0015)	NS (0.67)

Values are percentages in all CD4+ cells (mean ± S.D).

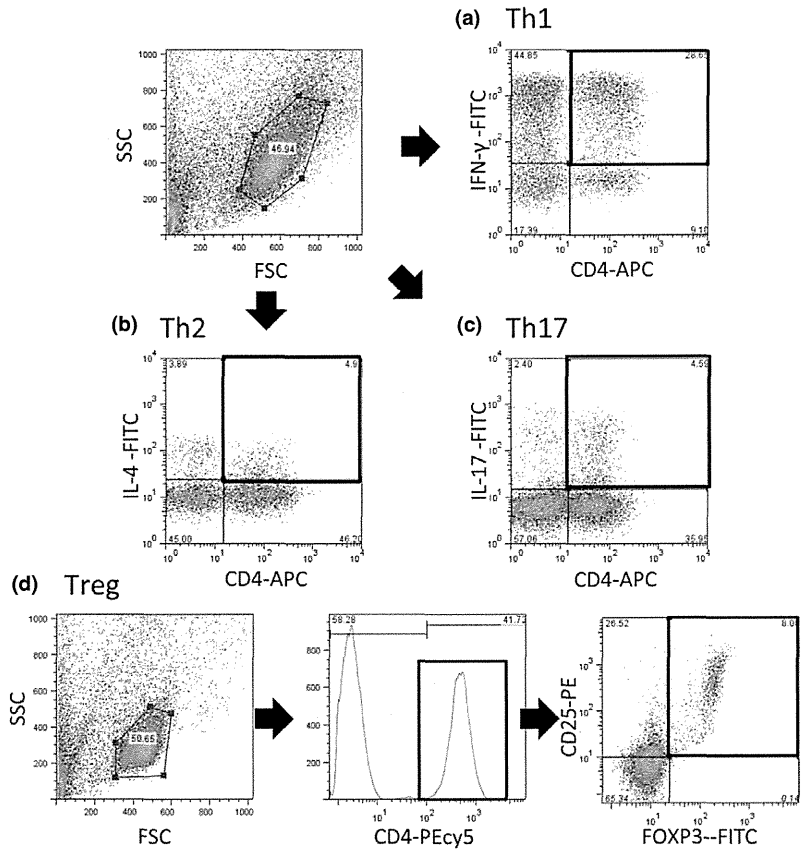

Fig. 1 Representative flow cytometry data of Th1 (a), Th2 (b), and Th17 (c) in total mononuclear cells and regulatory T cells (d) in total CD4+ T lymphocytes. Mononuclear cells isolated from the endometrium, endometrioma, and the peripheral blood were stained. For Th1, Th2, and TH17, lymphocytes were firstly gated on FSC/SSC scatter graph, and then were identified as double-positive cells (CD4 and IFN-γ for Th1, CD4 and IL-4 for Th2, and CD4 and IL-17A for Th17). For Treg, lymphocytes were firstly gated on FSC/SSC scatter graph and then were identified as triple-positive cells (CD4, CD25, and FOXP3).

difference was found between women with endometriosis and controls. When the subgroup analysis was performed for both proliferative and secretory phages, the tread was similar. The percentages of four CD4+ T-lymphocyte subsets in peripheral blood from women with endometriosis and controls are presented in Table II and Table SII.

Discussion

This is the first report of simultaneous detection of intracellular cytokines: IFN-γ, IL-4, IL-17A, CD25, and FOXP3 in CD4+ T cells and analysis of four CD4+ T-lymphocytes subsets (Th1, Th2, Th17, and Treg) in tissues of endometriosis lesions and endometrium. The proportion of Th1 was significantly lower in endometriosis lesions than in endometrium, whereas the proportion of Th17 was significantly higher in endometriosis lesions than in endometrium. The proportion of CD4+ T-lymphocyte subsets in peripheral blood were analyzed in the same manner, and we discovered that the Th1 fraction was significantly higher in patients with endometriosis in comparison with women without endometriosis.

Table II Distribution of CD4+ T-lymphocyte subsets in the peripheral blood from women with or without endometriosis

Peripheral blood	Th1	Th2	Th17	Treg
Women with endometriosis (n = 10)	10.25 ± 2.82	2.00 ± 0.96	1.26 ± 1.39	4.16 ± 1.55
Women without endometriosis (n = 10)	6.96 ± 4.13	0.96 ± 0.65	0.63 ± 0.32	5.25 ± 1.56
P value	<0.05 (0.041)	NS (0.37)	NS (0.81)	NS (0.16)

Values are percentages of CD4+ cells (mean ± S.D).

In the current study, we found that the proportion of Th1 in endometriosis lesion was significantly lower than in endometrium, but the proportion of Th2 was not different between groups. These findings indicate that in endometriosis lesions, the Th1/Th2 balance skews toward Th2, and this is consistent with a previous proposal that defined endometriosis as a Th2 disease. The mechanism for the Th2 skew may result from increased Th2 migration from circulation, although no evidence is currently available. A local induction of Th2 from naïve T cells may also explain the skew. In this context, it is interesting to introduce our previous study showing that thymic stromal lymphopoietin (TSLP), which activates dendritic cells inducing a Th2 response, is expressed in endometriosis lesions.[34]

Previously, our group demonstrated for the first time the presence of Th17 in endometriosis lesion.[35] The current study demonstrates that the proportion of Th17 in endometriosis lesion is higher than in the eutopic endometrium. Th17 accumulation at the focus of disease has been shown in other various inflammatory diseases such as Crohn's disease,[36] psoriasis,[37] and rheumatoid arthritis.[38] Our previous study demonstrated that the expression of CCL20, a chemokine that attracts Th17, is enhanced in endometriosis lesions, and this may explain the accumulation of Th17 at the disease focus. It is also possible that not only the attraction of Th17 from periphery, but also the local induction of Th17 from naïve T cells is increased in the endometriosis lesion, given that the production of Th17 inducing cytokines, namely IL-6, increases in the endometriosis lesion in comparison with normal endometrium.[39]

Treg and Th17 are known to express and regulate local immunity in a reciprocal manner, and Treg has a suppressive effect on inflammation. In contrast to the increased proportion of Th17 in endometriosis tissue, the proportion of Treg did not significantly differ between endometriosis lesion and endometrium, although it is possible that higher Treg infiltration was occurred in endometriosis lesion but the difference did not reach statistically significant due to the small sample size,[10] as reported previously.[40] Taken together with our findings for Th17, the current study suggests that the balance of Th17 and Treg in the local environment in endometriosis is skewed toward Th17.

Regarding the systemic Th1/Th2 and Th17/Treg balance, this is the first report of an approach to simultaneously evaluate four subsets of CD4+ T lymphocyte in peripheral blood in women with and without endometriosis. Previous similar studies have not evaluated circulating Th1 and Th17 populations in patients with endometriosis. The proportion of Th2 cells was examined in two studies, but results are inconsistent; one study demonstrated an elevation in endometriosis,[18] while the other showed no elevation.[41] As for Treg, results have been also inconsistent, one study found no difference[11] and the other found a decrease[42] in endometriosis. In the current study, where the profile for four CD4+ T-lymphocyte subsets was simultaneously analyzed in each sample, we found that the proportion of Th1 was significantly higher in patients with endometriosis in comparison with women without endometriosis, while no difference was found between groups for proportions of Th2, Th17 and Treg.

There are discrepancies between our systemic and local data. The T-lymphocyte balance skews toward Th2 and Th17 in the local immunological milieu of endometriosis tissue, but the peripheral T-lymphocyte balance in endometriosis patients was Th1-skewed. Similar discrepancies have been reported in other conditions. For instance, in rheumatoid arthritis, one study demonstrated that the T-lymphocyte balance in synovial fluid showed a Th1 skew, but in peripheral blood, lymphocytes were Th2-skewed.[43] Another study showed that the proportion of Th17 was increased in synovial fluid, but a similar increase was not observed in peripheral blood.[44] In endometriosis, mononuclear cells in the peritoneal cavity secrete high levels of IL-4 and low levels of IFN-γ, but cells in peripheral blood secrete high

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levels of IFN-γ and low levels of IL-4.[45] These results, together with our findings, indicate that the systemic immunological milieu does not necessarily reflect the local milieu, possibly because there are different regulators in each environment.

Recently, therapeutic strategies targeting particular T lymphocytes have been attempted for several diseases such as psoriasis[46] and rheumatoid arthritis.[47] Local Th2- and Th17-skewed milieus could be targeted to treat endometriosis, although further studies are warranted to identify the causal factors that contribute to the immunological imbalance. It may be of interest to note that novel subsets of T lymphocytes, such as T-lymphocytes double positive for IL-17A and IFN-γ, have been recently discovered and shown to be involved in immune diseases such as Crohn's disease.[48] These novel populations of T lymphocytes may also contribute to the etiology of endometriosis and further investigations on this can be expected.

In summary, simultaneous analysis of the CD4+ T-lymphocyte profile in endometriosis has revealed that the Th1/Th2 and Treg/Th17 balance is altered in lesions and peripheral blood; low Th1 and high Th17 proportion in lesions, and high Th1 proportion in peripheral blood. These findings enhance our understanding of CD4+ T-lymphocyte involvement in the pathophysiology of endometriosis.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Distributions of CD4+ T-lymphocyte subsets in endometriosis lesion and endometrium in proliferative phase.

Table S2. Distributions of CD4+ T-lymphocyte subsets in peripheral blood in proliferative phase.

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Effects of low dose oral contraceptive pill containing drospirenone/ethinylestradiol in patients with endometrioma

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ABSTRACT

Objectives: Low dose oral contraceptive pills (OCPs) that contain synthetic estrogen and progestin are often used to relieve chronic pelvic pain associated with endometriosis. We sought to evaluate the efficacy of drospirenone/ethinylestradiol (DRSP/EE) with low-dose estrogen in treating endometrioma.
Study design: A prospective clinical study in six hospitals and one clinic in Japan was conducted. Forty-nine 23- to 45-year-old patients who suffered from endometriosis-associated dysmenorrhea were included in the study. The primary endpoint was the change in size of ovarian endometrioma as measured by transvaginal ultrasonography. The secondary endpoint was the change in dysmenorrhea as evaluated by VAS (visual analog scale) scores before treatment and at 3 and 6 cycles of treatment. In addition, serum CA125, anti-mullerian hormone (AMH), interleukin (IL)-6, and IL-8 were evaluated after 6 cycles of treatment.
Results: The maximum diameter and volume of the ovarian endometrioma significantly decreased after 3 and 6 cycles compared with pretreatment. VAS scores of dysmenorrhea pain were also reduced after 1, 3 and 6 cycles. A significant correlation between the reduced size of the endometrioma and the decline of VAS scores was found. The levels of serum CA125 and AMH concentration were decreased after 6 cycles. No significant changes were observed in serum IL-6 and IL-8.
Conclusion: Low dose DRSP/EE therapy is a promising treatment not only to reduce the size of endometrioma but also for dysmenorrhea.

Introduction

Endometriosis, a chronic gynecologic disease, is defined as ectopic growth of endometrial tissues outside the uterine cavity. This disorder causes dysmenorrhea, infertility, and chronic pelvic pain. The prevalence of endometriotic disease seems to be approximately 5%, with a peak between 25 and 35 years of age [1,2]. A 0.1% annual incidence of endometriosis among women aged 15–49 years has been reported [3]. Endometriosis is often the cause of chronic pelvic pain in adolescents [4]. A prospective, multicenter survey conducted in 10 European countries demonstrated that the average annual cost per patient with endometriosis in 2008 was almost 10,000 Euro, including health care and loss of productivity costs [5]. Ovarian endometrioma is a common endometriotic lesion, affecting 55% of patients with endometriosis [6]. Management of endometrioma includes surgical and medical treatment, taking into consideration symptoms, childbearing desire, lesion dimension, and ovarian reserve [7]. The commonly used medications to treat endometriosis are non-steroidal anti-inflammatory drugs, low dose oral contraceptive pills (OCPs), GnRH agonists (GnRHa), danazol, progestins, and recently, the levonorgestrel intrauterine system.

OCP as a first choice treatment is a simple and effective way to manage endometriosis. OCPs containing synthetic estrogen and progestin are effective for pelvic pain relief and control of endometriotic lesions. OCPs suppress ovulation and reduce the growth of endometrial tissue, thus reducing both menstrual flow and prostaglandins production [8]. Recently, OCPs were also shown to down-regulate cell proliferation and increase apoptosis in the eutopic endometrium of women with endometriosis

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[9]. GnRHa and progestins are effective in relieving the growth of endometriosis and its associated pain, but this pain relief appears to be relatively short term. OCPs' possible side effects, such as headache, nausea, and atypical uterine bleeding, are generally well tolerated and much safer than those of other hormonal agents [10]. A critical issue in managing endometriosis is chronic or recurrent symptoms that require long-term or repeated courses of medication.

A randomized, controlled trial (RCT), which demonstrated the efficacy of a monophasic OCP (norethisterone/ethinylestradiol: NET/EE) for dysmenorrhea associated with endometriosis and the reduction of the endometrioma volume larger than 3 cm after 4 cycles of OCP, has already been reported [11]. Recently, an OCP containing drospirenone/ethinylestradiol 3 mg/20 μg (DRSP/EE: Yaz®), administered in a 24/4 regimen (24 days of active pills followed by 4 days hormone free) was proposed for the treatment of dysmenorrhea. Mabrouk et al. also described the possible beneficial effect of this regimen in patients with endometriosis [12]. The OCP with DRSP/EE possesses the advantages of the anti-mineralocorticoid activity reflected clinically in less fluid retention [13]. The beneficial effects of OCP with DRSP/EE on moderate acne [14] and premenstrual dysphoric disorder (PMDD) are well known and highly satisfactory [15].

Local inflammatory reaction in the peritoneal environment is one of the contributing factors in the pathogenesis of endometriosis. We previously demonstrated the presence of increased levels of proinflammatory cytokines, such as interleukin (IL)-6 and IL-8, in the peritoneal fluid (PF) of patients with endometriosis [16,17]. We also showed that IL-6 and IL-8 were produced in the endometriotic stromal cells derived from ovarian endometrioma [18,19] and are indispensable factors related to the development of endometriosis. In addition, it is well known that anti-mullerian hormone (AMH) is produced by the granulosa cells of primary to small antral follicles to prevent depletion of the primordial follicle pool. Recently, serum AMH is regarded as a reliable marker of ovarian reserve, which is menstrual cycle-independent and is significantly unaffected by the use of OCP or GnRHa [20]. Considering this, we investigated changes in IL-6, IL-8, and AMH concentration in this study.

We analyzed whether OCP containing DRSP/EE diminishes the size of ovarian endometriomas, alleviates dysmenorrhea, synthesizes proinflammatory cytokines, and preserves ovarian reserve.

Methods

Patients and study design

This study was approved by Tottori University institutional review board; written informed consent was obtained from each patient. We recruited 49 women with unilateral ovarian endometriomas from August 2012 to December 2013. Patient inclusion criteria included women 23–45 years old; regular menstrual cycles (28 ± 2 days); ovarian endometrioma (larger than 3 cm in diameter diagnosed by ultrasound or magnetic resonance imaging); normal cervical and endometrial smear cytology; moderate or severe dysmenorrhea (evaluated by a modified pain scale); and no medical or surgical treatment for endometriosis within 8 weeks of entry into this study, including hormonal agents, such as OCPs, GnRHa, progestin, and danazol. The degree of dysmenorrhea was evaluated according to a visual analog scale (VAS) from 0 mm "no pain" to 100 mm "the worst pain you could imagine".

This study was conducted in 6 hospitals and 1 private clinic in Japan. An OCP containing DRSP/EE 3 mg/20 μg in a 24/4 regimen was administered for 6 cycles. The use of other hormonal treatments for pain or vaginal bleeding was not allowed. The characteristics of this study population are described in Table 1.

Table 1
Characteristics of patients.

Patients (n = 49)	Average or number
Age (range)	33.3 years (23–45)
BMI (range)	20.3 kg/m^2 (15.9–26.6)
Parity	
(−)	38
(+)	11
Cycle length (range)	28.8 days (25–32)
Adenomyosis	8
Pre-treatment drugs	
(−)	42
(+)	7 (GnRHa; 2, LEP/OCP; 5, overlapping: Medium dose OCP; 1, Dienogest; 1, Danazol; 1)
Use of analgesics	
(−)	29
(+)	20 (Loxoprofen; 14, Ibuprofen; 6, overlapping: Acetoaminofen; 2, Aspirin; 2)

GnRHa, GnRH agonist; LEP/OC, low dose estrogen-progestin/oral contraceptive pill.

Four kinds of analgesics in addition to the OCP were used as needed in 20 patients: Loxoprofen, Ibuprofen, Acetoaminofen, Aspirin.

Patient monitoring

In our study protocol, each patient underwent a pre-recruitment evaluation, consisting of a general medical and gynecologic history, physical and pelvic examination, patient evaluation of pain, and review of the menstrual records. Blood was drawn for pretreatment clinical laboratory determinations, including hematology (white blood cells, platelet, red blood cells, hemoglobin, and hematocrit), coagulation tests (fibrinogen, plasminogen, prothrombin time: PT, and activated partial thromboplastin time: APTT), biochemical test (glutamic oxaloacetic transaminase: GOT; glutamic pyruvic transaminase: GPT; gamma-glutamyl transpeptidase: GTP; and alkaline phosphatase: Al-P), CA125, AMH, IL-6, and IL-8. At this point, eligibility for entry into the study was determined, pain assessed, and the drug provided. Patients were followed every 4 weeks to evaluate pain, side effects, and other health concerns. After 6 cycles of treatment, efficacy was evaluated.

Main outcome measures were the final measurement of the volume of the ovarian endometrioma and the VAS score during treatment with DRSP/EE. The measures recorded at the time of the therapy prescription were considered the baseline. Clinical and ultrasound examinations were performed at baseline (pretreatment) and after 1, 3, and 6 months of treatment. Maximum diameter of the endometrioma (Fig. 1A) was measured by ultrasound, and the total volume of the endometrioma (Fig. 1B) was obtained by calculating the three-dimensional diameters on a longitudinal and sagittal scan.

ELISA

Blood samples were obtained from the patients at the time of prescription (pretreatment) and after 6 months of treatment. The serum was separated from whole blood, transferred to sterile polypropylene tubes, and stored at −80 °C, until assayed. The concentration of serum IL-6 or IL-8 was determined by ELISA kits (R&D Systems, Minneapolis, MN, USA) and AMH Gen II ELISA kit (MBL, Osaka, Japan) according to the manufacturer's instructions. Absorbance was measured with a microplate reader. The ratio to mean value of control (% of control) was used for comparison.

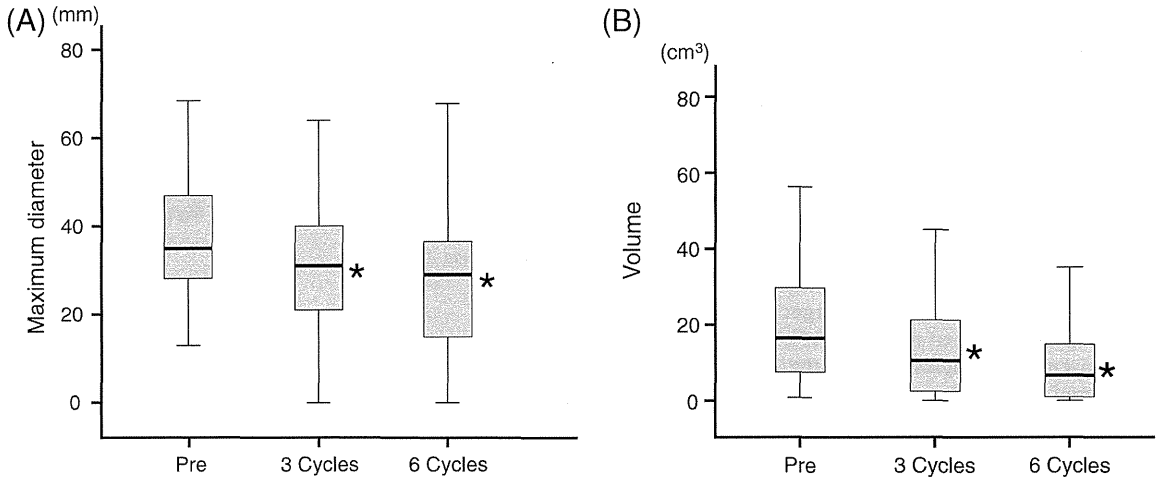

Fig. 1. Comparison of (A) maximum diameter and (B) volume of ovarian endometriomas in 49 patients taking the DRSP/EE during 6 cycles. The boxes represent the interquartile ranges and the whiskers extend to the maximum and minimum values. The bars within the boxes show the median values. Pre: pretreatment *$p < 0.001$ vs. pretreatment.

Statistical analysis

Size of endometriomas and VAS score were analyzed using Wilcoxon signed rank test with Bonferroni correction. To assess serum CA125, AMH, IL-6, and IL-8, Wilcoxon signed-rank test was used. Central horizontal lines indicate the median. The boxes represent the interquartile ranges, and the whiskers extend to the maximum and minimum values. Spearman rank correlation was used to test the association between two ranked variables. All statistical analyses were carried out using IBM SPSS Statistics Ver.21 (IBM Japan, Tokyo, Japan). $P < 0.05$ was accepted to indicate statistical significance.

Results

The maximum diameter of ovarian endometrioma treated with DRSP/EE after 3 (median: 31.0 mm vs. pretreatment: 35.0 mm) and 6 cycles (median: 29.0 mm vs. pretreatment) was significantly decreased ($P < 0.001$) (Fig. 1A). Similarly, the volume of endometrioma was also reduced after 3 (Median: 10.6 cm^3 vs. pretreatment: 16.5 cm^3) and 6 months (6.7 cm^3 vs. pretreatment) ($P < 0.001$) (Fig. 1B). The medians of VAS scores of dysmenorrhea pain gradually declined after 1 (27 mm), 3 (10 mm) and 6 (10 mm) cycles compared with pretreatment (68 mm) ($P < 0.001$) (Fig. 2). According to the Spearman rank correlation test, correlations between the reduction of maximum diameter or volume of endometrioma, and that of VAS scores after 6 months treatment were found ($P = 0.039$: the maximum diameter: Fig. 3A) ($P = 0.025$: the volume: Fig. 3B).

In addition, serum CA125 and AMH levels after 6 cycles were significantly lower than those pretreatment (CA125: 20.3 U/ml vs. pretreatment: 13.2 U/ml) ($P < 0.005$), AMH: 3.2 U/ml vs. pretreatment: 2.7 ng/ml) ($P < 0.001$) (Fig. 4A and B). On the other hand, no significant changes in the serum IL-6 (0.68 pg/ml vs. pretreatment: 0.63 pg/ml) and IL-8 (9.5 pg/ml vs. pretreatment: 9.8 pg/ml) concentration were observed after treatment (Fig. 4C and D). On serum IL-6 or IL-8 concentration, the use of the anti-inflammatory agents did not influence the rate of change after DRSP/EE treatment as well as the values before and after treatment. No new ovarian endometriomas occurred in this period. No serious adverse events related to this drug use occurred.

Comments

Our present data revealed that low dose OCP with DRSP/EE was effective at treating dysmenorrhea, and that it reduced the size of ovarian endometriomas in Japanese women. To the best of our knowledge, 2 other research groups described the reduction of endometrioma size with the preoperative use of GnRHa for 3 or 6 months [21,22]. In one recent study, a significant reduction in the diameter of endometrioma after 6 months of DRSP/EE 3 mg/20 μg treatment was shown [12]. Moreover, after 18 months of therapy, the mean diameter and the dysmenorrhea VAS score in that study were significantly diminished. The continuous regimen seems to be associated with a greater reduction than the cyclic one. In Japan, a RCT comparing the OCP (4 cycles of NET/EE) with placebo in women suffering from endometriosis-related dysmenorrhea was conducted [11]. The volume of endometrioma (larger than 3 cm in diameter) was significantly decreased in the OCP group. From the first cycle through the end of treatment, dysmenorrhea in the OCP

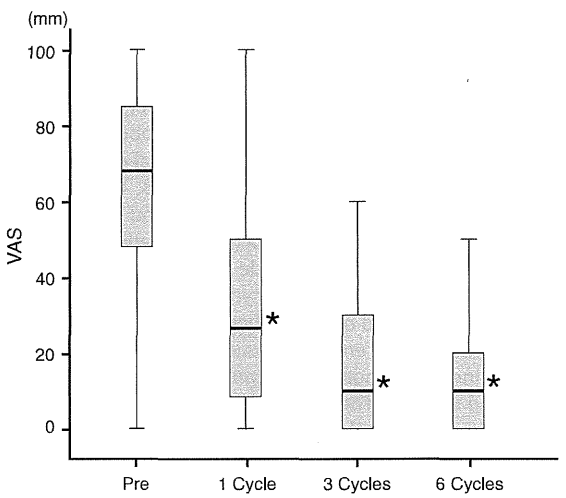

Fig. 2. Comparison of VAS score in dysmenorrhea during 6 cycles. The boxes represent the interquartile ranges and the whiskers extend to the maximum and minimum values. The bars within the boxes show the median values. Pre: pretreatment *$p < 0.001$ vs. pretreatment.

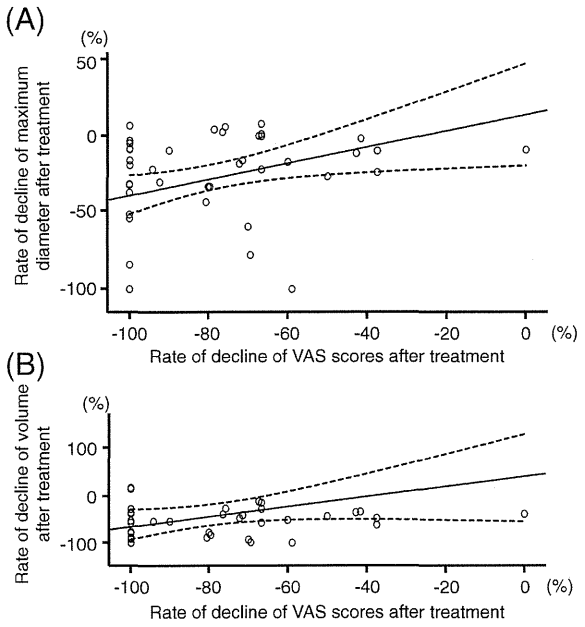

Fig. 3. Correlations between the reduction of (A) maximum diameter or (B) volume of endometrioma, and that of VAS scores after 6 months treatment. Spearman rank correlation test was performed.

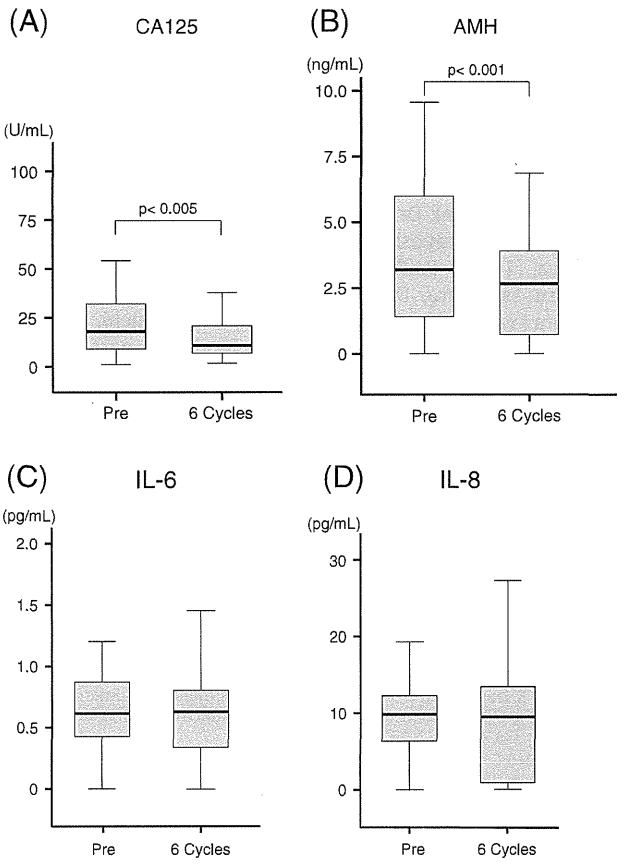

Fig. 4. Serum (A) CA125, (B) AMH, (C) IL-6, and (D) IL-8 concentration were compared between pretreatment and after 6 cycles. The boxes represent the interquartile ranges and the whiskers extend to the maximum and minimum values. The bars within the boxes show the median values. Pre: pretreatment.

group was milder than in the placebo group. Vercellini et al. also performed a RCT a 6-month treatment with a GnRHa (3.6 mg goserelin) or a low dose cyclic OCP (0.02 mg EE and 0.15 mg desogestrel) in 57 endometriosis patients with mild to severe pain symptoms. They reported that reduction in deep dyspareunia and non-menstrual pain associated with endometriosis was observed in both groups at 6 months of treatment, and that low dose OCPs may be a valuable alternative for the treatment of endometriosis [23].

Endometriosis-associated intraperitoneal inflammation is thought to promote propagation of disease and impair fecundity. Numerous proinflammatory cytokines released by peritoneal macrophages and endometriotic cells are up-regulated by the nuclear factor-kappa B (NF-κB) [18,24]. We demonstrated that the levels of IL-6 and IL-8 increased in the PF of patients with endometriosis [16]. We also showed that similar levels of IL-6 were produced in the endometriotic stromal cells (ESCs) derived from ovarian endometrioma and by macrophages [18]. In addition, we verified that IL-8 enhances mitogenic activity, and that TNFα, as a pluripotent mediator in the early events of tumorigenesis, upregulates IL-8 expression through NF-κB activation in ESCs [19]. These data suggest that IL-6 and IL-8 maybe the crucial factors related to the pathogenesis of endometriosis. Although the OCP treatment with DRSP/EE did not change the serum IL-6 and IL-8 concentration (Fig. 4C and D), these data might reflect the quite low values during the examination or the limitations of the small sample size.

Laparoscopic excision of ovarian endometriomas using the stripping technique is the widely recommended common surgical procedure. However, the surgery risks damaging ovarian reserve through inadvertent removal of healthy ovarian tissue adjacent to the cyst wall and causing vascular injury [25]. Nevertheless, it has also been shown that the endometrioma per se may cause damage to ovarian reserve and function. The thickness of ovarian tissue removed surgically seems to increase proportionally with cyst diameter [26]. Therefore, the reduction in volume of the endometrioma by low dose OCPs is crucial to preserve fecundity.

OCPs suppress the hypothalamic–pituitary–ovarian axis and thereby potentially influence ovarian reserve. AMH is considered a good predictor of ovarian response to ovarian induction [27]. In fact, it remains controversial whether AMH concentration is influenced by OCP utilization [28,29]. It seems likely that weak study design and low sample size underlies this confusion. The decrease in serum AMH levels during the use of OCPs probably reflects decreased numbers of small antral follicles. High androgenic OCP exposure appears to suppress functional ovarian reserve and oocyte yields [30]. Li et al. reported that serum AMH level was not significantly different between pre- and post-treatment of OCPs measurement [31]. At 1 year after cystectomy for ovarian endometriomas, the decreased AMH level in patients was restored [32]. Moreover, serum AMH value on day 7 of the hormone-free interval after the use of OCPs was recovered on cycle days 2–5 of two subsequent natural cycles [33]. Hence, while DRSP/EE treatment for 6 months diminished the AMH level (Fig. 4B), we predicted this decreased AMH would recover.

Current data suggest that low dose OCP containing DRSP/EE is effective in the treatment for endometriosis. DRSP/EE treatment is a promising treatment not only for endometriosis-associated dysmenorrhea but it also reduces chocolate-cyst size without damaging ovarian reserve.

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Conflict of interest

The authors have nothing to disclose regarding conflict of interest.

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Original Article

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Molecular Background of Estrogen Receptor Gene Expression in Endometriotic Cells

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Abstract

The molecular background of estrogen receptor (ER) expression is important to understand the pathophysiology of the high estrogen environment in endometriosis. However, the molecular details have not been fully understood. The objective of this study is to evaluate the molecular background of ERα and ERβ messenger RNA (mRNA) expression in endometriotic cells. The following summarizes our observations: (1) ERα mRNA expression in endometriotic cells was estimated to be approximately one-tenth of that in endometrial cells. (2) Three mRNAs, which include 3 different 5′-untranslated exons tagged to an open reading frame of wild-type ERα, were detected. (3) Expression of ERβ mRNA depends mostly on 0N promoter and includes 2 open reading frames: one for a wild-type ERβ1 and another for a splice variant ERβ2. (4) Expression of ERβ1 mRNA was approximately 40-fold higher than that in endometrial cells. (5) Expression of ERβ2 mRNA was almost at a comparable level of the ERβ1. 9 (6) ERα and ERβ mRNAs are equivalently expressed in endometriotic cells. These observations show the molecular background of ER mRNA expression in endometriotic cells and provide a clue to further understanding the estrogen-dependent pathophysiology leading to clinical application in endometriosis.

Keywords

estrogen receptor α, estrogen receptor β, mRNA expression, endometriosis

Introduction

Endometriosis is an estrogen-dependent, inflammatory disease characterized by the presence of endometrium-like tissue primarily on the pelvic peritoneum and ovaries. The role of estrogen is obvious because the symptoms associated with endometriosis often disappear after menopause, and the administration of GnRH agonists or progestin relieves the pelvic lesions and associated pain. A marked upregulation of aromatase gene associated with aberrant DNA demethylation[1,2] leads to a high estrogen environment in endometriotic tissues. Its inhibition by aromatase inhibitor is effective to reduce endometriosis-associated symptoms.[3] One of the important issues to be clarified is a further understanding of the role of estrogen-dependent growth mechanism in endometriotic tissues. To explain the pathophysiology leading to clinical applications, distinct profiles of estrogen receptor (ER) expression have been documented: a higher ERβ and a lower ERα expression in endometriotic tissues, which is in the inverse relationship in endometrium.[4-7] Estrogen receptor α is the dominant receptor in the uterus and mediates a number of estrogen actions, including the stimulation of proliferation and the induction of progesterone receptor expression. In contrast, ERβ antagonizes the inflammatory and proliferative actions of ERα.[8,9] Therefore, the ER expression profile has been proposed as a major background of estrogen action in endometriosis. However, the molecular details of ER expression in endometriotic tissues have not been fully understood so far.

Recently, several splice variants of ERβ showing distinct transcriptional activities have been identified, and their roles along with the classical wild type have been extensively re-evaluated.[10-13] These findings prompted us to re-evaluate the molecular background of ER expression in endometriosis. In this study, we focused on the mRNA expression in endometriotic cells.

We show the molecular background of ER mRNA expression in endometriotic cells. We observed the expression of a splice variant ERβ2 mRNA along with the wild-type ERβ1 in endometriotic cells. Importantly, these ERβ transcripts were expressed almost at a comparable level of the ERα in endometriotic cells.

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Materials and Methods

Patients

The institutional review boards of Tottori University Faculty of Medicine approved this project. We obtained informed consent from all patients. Endometrial and endometriotic tissues were obtained from patients as described previously.[1,2,14-16] Endometrial tissues were obtained from the uteri of (n = 19) cycling premenopausal women who underwent hysterectomy for uterine leiomyoma during the proliferative or secretory phase. The chocolate cyst lining of the ovaries of (n = 21) patients with endometriosis was the source of endometriotic tissue collected during the follicular or luteal phase. These patients had received no hormonal treatment before surgery. The menstrual cycle phase was determined by measuring serum estradiol and progesterone levels as well as by histological examination.

Endometrial and Endometriotic Stromal Cells

Stromal cells were collected from endometrial and endometriotic tissues as described.[1,2,14-16] In brief, the endometrial and endometriotic tissues were minced and digested with 0.5% collagenase. The dispersed cells were filtered through a 70 μm nylon mesh to remove the undigested tissue pieces containing the glandular epithelium. The filtered fraction was separated further from epithelial cell clumps by differential sedimentation at unit gravity. The medium containing stromal cells was filtered through 40 μm nylon mesh. Final purification was achieved by allowing stromal cells, which attach rapidly to plates, to adhere selectively to culture dishes within 30 minutes at 37°C in 5% CO_2 in air. To confirm the purity of the isolated stromal cells, immunocytochemical analysis was performed using cytokeratin (DAKO Corp, Kyoto, Japan) as a marker of epithelial cells, vimentin (DAKO Corp) as a marker of stromal cells, CD14 (Nichirei, Tokyo, Japan) as a marker of activated macrophages, and factor VIII F8/86 (DAKO Corp) as a marker of endothelial cells. The results showed that the purity of the stromal cells was more than 98%. Unless otherwise indicated, cells were cultured in DMEM/F12 supplemented with 10% fetal calf serum at 37°C in 5% CO_2. At the first passage, endometrial and endometriotic stromal cells were plated at a density of 5×10^5 cells/60 mm dish and incubated to reach a confluence of 80% to 90% for RNA preparation.

Analysis of ER mRNA Expression

Total cellular RNA was prepared using the RNeasy Plus kit (QIAGEN, Tokyo, Japan) according to the manufacturer's protocol. Using 2 μg of total RNA, single-stranded complementary DNAs (cDNAs) were prepared in 20 μL of reaction mixture containing 1 mmol/L each of deoxynucleotide, 20 units of PrimeScript RTase (Takara, Kyoto, Japan), 20 units of RNase inhibitor (Takara, Kyoto, Japan), and 20 μmol/L of oligo-dT_{16} primer at 42° C for 30 minutes. Then, 1 μL of the cDNA reaction mixture was subjected to polymerase chain reaction (PCR) amplification in a 25 μL Multiplex PCR Mixture (Takara, Kyoto, Japan) containing 50 pmol each of the respective forward and reverse primers. Using ERα-F (5′-CAGGGT GGCAGAGAAA-GATTGG-3′ in exon 2) and ERα-R (5′-GGTCTGGTAGGATCATACTCGG-3′' in exon 4), a sequence of 532 bp in human ERα cDNA (Accession No: M12674) was amplified. Using ERβ-F (5′-CCTGCTGTGATGAATTACAG-3′ in exon 1) and ERβ-R (5′-TTCTCTGTCTCCGCACAAG-3′ in exon 4), a sequence of 550 bp in human ERβ cDNA (Accession No: NM001437) was amplified. The conditions for PCR were 30 seconds at 94°C, 15 seconds at 58°C (ERα) or 55°C (ERβ), and 30 seconds at 72°C for 38 cycles. At the end of the PCR cycles, 10 μL of the reaction mixture was removed and subjected to electrophoresis on a 1.8% agarose gel in Tris-Borate-EDTA buffer at a constant voltage of 100 V. Separated DNA sequence stained with ethidium bromide was visualized under UV light. As an internal control, β-tubulin mRNA was assayed in parallel.

Analysis of the Alternative 5′-Untranslated Exon

Using the cDNAs as described earlier, PCR was performed. Forward primers[17] in alternative 5′-untranslated exons of ERα are as follows: 1A-F: 5′-GGAGCTGGCGGGGGGCGTTG-3′; 1BF: 5′-CGCGTTTATTTTAAGCCCAG-3′; 1CF: 5′-CGGC CCTTGACTTCTACAAG-3′; 1DF: 5′-CTTCTTCACCTGA-GAGAGCC-3′; 1EF: 5′-CAGAGAAATAATCGCAGAGC-3′; 1FF: 5′-CCAAAACTGAAAAT-GCAGGC-3′. The reverse primer, located in exon 2, was common to all reactions: 2R: 5′-CCTTGCAGCCCTCACAGGAC-3′. The primers[18] in alternative 5′-untranslated exons of ERβ are as follows: exon 0 N, 5′-AGATTTTTTAAATTTGAGATTGGGGTTG-3′ and 5′-CT TACCTTACAAATAAACACACC-3′; and exon 0 K, 5′-GTT GGGGTTATTTYGGGGTTGTT-3′ and 5′-CCTCCAACAAA-CACATTCA-3′. The PCR condition was 1 minute at 94°C, 1 minute at 62°C, and 1 minute at 72°C for 38 cycles. At the end of the PCR cycles, 10 μL of the reaction mixture was removed and subjected to electrophoresis on a 1.8% agarose gel. The separated DNA sequence was stained with ethidium bromide and visualized under UV light.

Analysis of ER Isoform Expression

The single-stranded cDNAs prepared from endometrial and endometriotic cells were used. For the analysis of ERα isoform expression, PCR with the forward primer H1 (5′-ACGGAC CATGACCATGACCCT-3′ in exon 1) and the reverse primer HR5 (5′-GGTCTGGTAGGATCATACTCGG-3′ in exon 5) or HR8 (5′-TCAGACCGTGGCAGGGAAA-3′ in exon 8) was performed. For the analysis of ERβ isoform expression, PCR with primer sets of exon 7 and the variant-specific exon 8 was performed.[10] The forward primer, 5′-AGTATGTACCCTCTGGT-CACAGCG-3′ in exon 7, is shared by all the ERβ isoforms. The isoform-specific reverse primers were as follows: ERβ1: 5′-CCAAATGAGGGACCA CACAGCAG-3′ and ERβ3: 5′-GCAGTCAAGGTGTCGACAAAGGCTGC-3′. The reverse

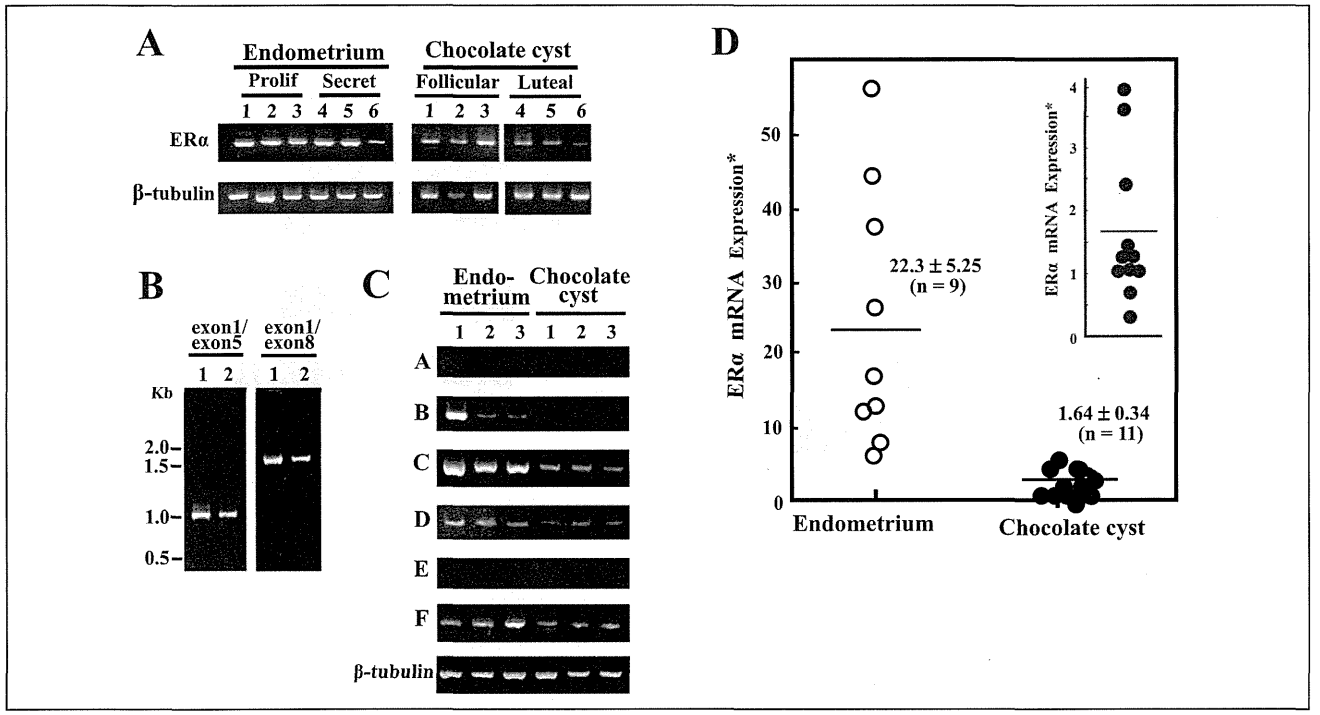

Figure 1. Estrogen receptor α (ERα) messenger RNA (mRNA) expression in endometrial and endometriotic cells. A, Analysis of ERα mRNA expression. Stromal cells were collected from 6 endometrial (3 from proliferative phase and 3 from secretory phase) and 6 endometriotic tissues (3 from follicular phase and 3 from luteal phase). Single-stranded complementary DNA (cDNA) was prepared from total cellular RNA and subjected to polymerase chain reaction (PCR). A sequence from exon 2 to exon 4 (532 bp in length) of ERα cDNA was amplified and subjected to a 1.8% agarose gel electrophoresis. As an internal control, β-tubulin mRNA was assayed in parallel. Amplified signals were visualized using ethidium bromide staining under UV light. B, Analysis of alternative splicing. Single-stranded cDNAs were prepared from endometrial (lane 1, proliferative phase) and endometriotic cells (lane 2, follicular phase). Following PCR using primer sets from exon 1 to exon 5 (1014 bp) and from exon 1 to exon 8 (1795 bp), amplified sequences were subjected to a 1.8% agarose gel electrophoresis and visualized. C, Analysis of untranslated first exons in ERα mRNA. Single-stranded cDNA was prepared from 3 endometrial (lanes 1 and 2 from proliferative phase and lane 3 from secretory phase) and 3 endometriotic cells (lanes 1 and 2 from follicular phase, and lane 3 from luteal phase). Following PCR using respective 6 forward primers in the untranslated exons, A, B, C, D, E, and F, and the reverse primer in the exon 2, amplified sequences were subjected to a 1.8% agarose gel electrophoresis and visualized. As an internal control, β-tubulin mRNA was assayed in parallel. D, Quantitative analysis of ERα mRNA expression. Single-stranded cDNAs from 9 endometrial (4 from proliferative phase and 5 from secretory phase) and 11 endometriotic cells (6 from follicular and 5 from luteal phase), which were randomly picked up from cells, were subjected to the TaqMan real-time PCR in triplicate. The specific probes (ERα mRNA, ESR1 #69, and β-tubulin #43) were selected on the website of the Universal Probe Library System. ABI PRISM 7900HT Sequence Detection System was used. Relative ERα mRNA expression was estimated using the β-tubulin mRNA expression as an internal control. *Values (mean ± SEM) are expressed as an arbitrary unit. Endometrium versus chocolate cyst, $P < .001$ inset, relative ERα mRNA expression in endometriotic cells is shown using a larger scale.

primer, 5′-CTTTAGGCC ACCGAGTTGATTAGA-G-3′, was shared by ERβ2, ERβ4, and ERβ5.

Quantitative Real-Time PCR

Expression of ER mRNA was assessed using real-time PCR. As an internal control, β-tubulin mRNA expression was estimated in parallel. One μL of each cDNA samples prepared from endometrial and endometriotic stromal cells (~50 ng) as indicated above was subjected to the TaqMan real-time PCR in triplicate. The real-time PCR was performed using the Universal Probe Library system (Roche Diagnostics, Tokyo, Japan). The specific probe (ERα #69, ERβ1 #3, and β-tubulin #43) was selected on the website of Universal Probe Library Assay Design Center (https://www.roche-appliedscience.com/sis/rtpcr/upl/adc.jsp). The FastStart TaqMan Probe Master (Roche) was used for the reaction solution. Relative expression of ERβ1, ERβ2, and ERβ5 mRNA was estimated using SYBR Premix Ex TaqII kit (Takara, Kyoto, Japan) using the respective primer sets of exon 7 and exon 8 as follows: ERβ1: 5′-CCGGCTAACCTCCTGATGC-3′ and 5′-CCAAATGAGG GACCACACAGCAG-3′, ERβ2: 5′-AGTA TGTACCCTC TGG TCACAGCG-3′ and 5′-AGGCCTTTTC TGCCCCTCGATGC-3′, and ERβ5: 5′-ATGGGATTATGT GCTCCACGGA-3′ and 5′-CTT TAGG CCACCGAGTTGAT TAGAG-3′. ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Tokyo, Japan) was used for the real-time PCR.

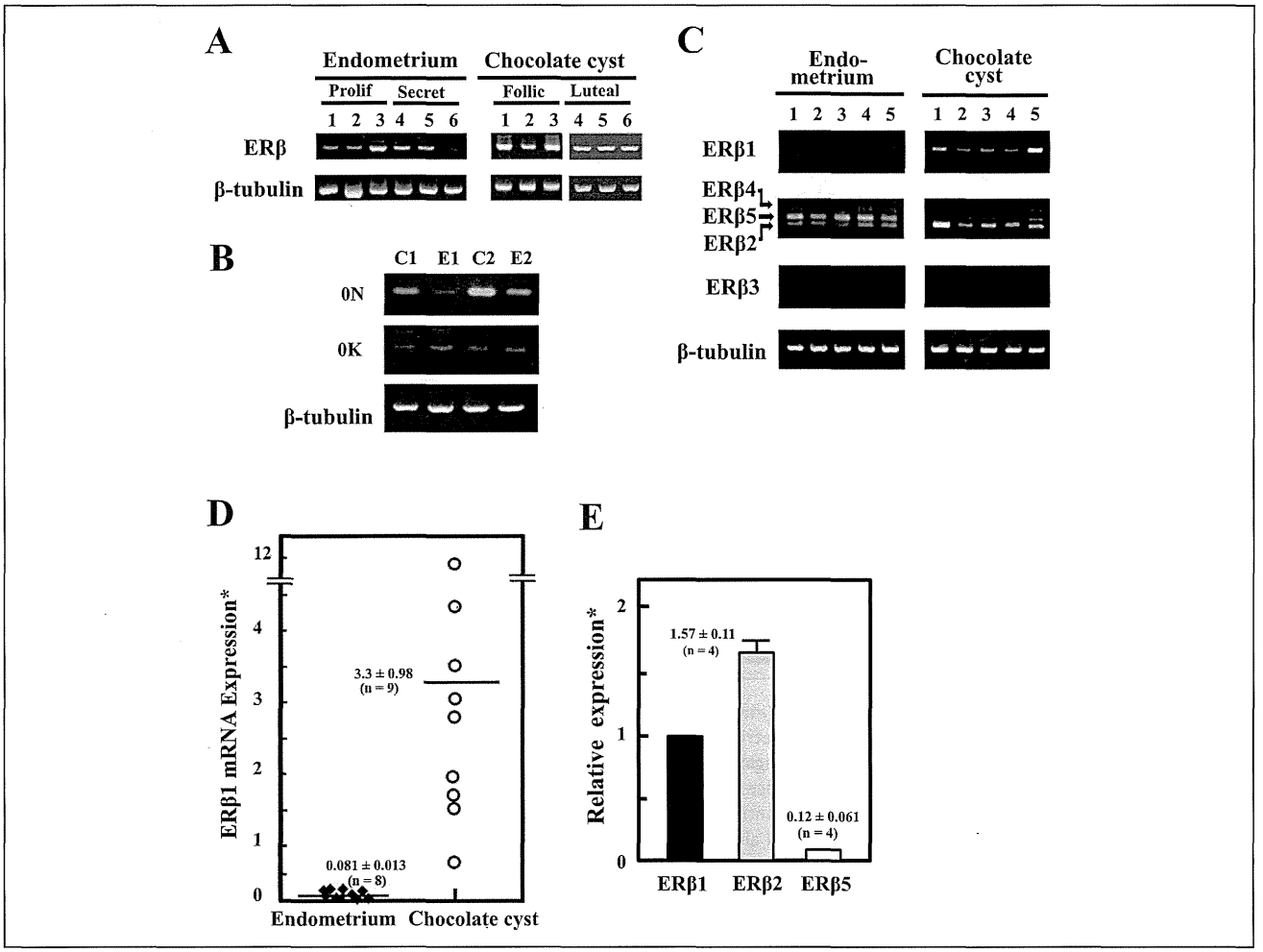

Figure 2. Estrogen receptor β (ERβ) messenger RNA (mRNA) expression in endometrial and endometriotic cells. A, ERβ mRNA expression. Stromal cells were collected from 6 endometrial (3 from proliferative phase and 3 from secretory phase) and 6 endometriotic tissues (3 from follicular phase and 3 from luteal phase). Single-stranded complementary DNA (cDNA) was prepared from total cellular RNA and subjected to polymerase chain reaction (PCR). An ERβ cDNA sequence from exon 1 to exon 4 (550 bp in length) was amplified and subjected to 1.8% agarose gel electrophoresis. As an internal control, β-tubulin mRNA was assayed in parallel. B, Analysis of the untranslated first exons. Single-stranded cDNA was prepared from endometrial (lanes E1 and E2) and endometriotic cells (lanes C1 and C2). PCR using 2 forward primers, 0 N and 0 K, and a reverse primer located in exon 2 was performed. As an internal control, β-tubulin mRNA was assayed in parallel. C, Analysis of alternative splicing. Single-stranded cDNAs were prepared from 5 endometrial and 5 endometriotic cells. Following PCR with primer sets of the variant-specific exon 7 and exon 8, amplified sequences were visualized on a 1.8% agarose gel electrophoresis. As an internal control, β-tubulin mRNA expression was assayed in parallel. Arrows indicate the location of amplified sequences corresponding to ERβ2, ERβ4 and ERβ5 mRNAs. D: Quantitative analysis of ERβ1 mRNA expression. Single-stranded cDNAs from 8 endometrial (4 from proliferative phase and 4 from secretory phase) and 9 endometriotic cells (6 from follicular phase and 3 from luteal phase) were prepared. The specific probes (ESR2 #3 for ERβ1 and β-tubulin #43) were selected using the Universal Probe Library System. ABI PRISM 7900HT System was used. Relative ERβ1 mRNA expression was estimated using the β-tubulin mRNA expression as an internal control. *Values are expressed as arbitrary unit (mean ± SEM). Endometrium versus chocolate cyst, $P < .006$. E, Relative expression of ERβ1, ERβ2, and ERβ5 mRNA in endometriotic cells. Using SYBR system with primer sets of variant-specific exon 7 and exon 8, the expression of ERβ1, ERβ2, and ERβ5 mRNAs was assayed. The expression was estimated using the β-tubulin mRNA expression as an internal control. Relative expression of ERβ2 and ERβ5 mRNA was normalized against ERβ1 mRNA expression. Values are the means ± SEM. ERβ1 versus ERβ2, $P < .002$, ERβ2 versus ERβ5, $P < .006$.

Statistical Analysis

Results were analyzed using one-way analysis of variance followed by Fisher protected least significant difference test. Values were expressed as means ± standard error of mean. $P < .05$ was considered statistically significant.

Results

Expression of ERα mRNA

Expression of ERα mRNA was observed in both endometriotic and endometrial cells (Figure 1A). We then searched for the

splice variant mRNA using reverse transcription-PCR with primer sets to amplify the open reading frame (ORF) of wild-type ERα (from exon 1 to exon 8 in ESR1). As shown in Figure 1B, only a single cDNA sequence, which includes an ORF predicting a wild-type ERα, was always amplified from cDNAs prepared from endometriotic and endometrial cells. The 5′-untranslated exons, C, D, and F, were tagged to exon 1 in endometriotic cells, while in endometrial cells, the B, C, D, and F were tagged to exon 1 (Figure 1C). Among these ERα mRNAs, the exon C-tagged mRNA was the most abundant. Relative expression of ERα mRNA in endometriotic cells was estimated to be approximately one-tenth of that in endometrial cells (Figure 1D).

Expression of ERβ mRNA

Expression of ERβ mRNA was observed in both endometriotic and endometrial cells (Figure 2A). In endometriotic cells, the expression depends mostly on 0N promoter (Figure 2B) and includes 2 ORFs: one for a wild-type ERβ1 and another for a splice variant ERβ2 (Figure 2C). Expressions of ERβ2 and ERβ5 mRNA were detectable in endometrial cells (Figure 2C). The expression of ERβ1 mRNA in endometriotic cells was approximately 40-fold higher than that in endometrial cells (Figure 2D). The ERβ2 mRNA expression was almost at a comparable level of ERβ1 in endometriotic cells, while the ERβ5 mRNA expression was at a marginal level (Figure 2E).

Discussion

The present study was undertaken to evaluate the molecular background of ER expression in endometriotic cells. So far, ER expression profile, a higher ERβ, and a lower ERα expression in endometriotic tissues, which is in the inverse relationship in endometrium,[4-7] has been proposed as a major background of estrogen action in endometriosis. Although the ER mRNA expression is low in endometriotic cells (Figure 1D, 2D, and 2E), the expression of ERα and ERβ mRNAs is almost at a comparable level. It is interesting to note that ERβ2 mRNA[10,11,18] was found to be coexpressed along with ERα and ERβ1 mRNAs in endometriotic cells. These observations suggest a pathophysiological role of the ER expression profile in endometriosis.

Receptor dimerization in response to ligand binding is one of the crucial steps in ER-dependent transactivation.[19] Homodimerization of ERα accelerates cellular proliferation.[19] On the other hand, ERβ is thought to counteract the stimulatory effects of ERα through the formation of functional heterodimer with ERα.[20,21] Experimental evidence suggests that heterodimer-induced activation of target genes is markedly different from that induced by homodimers.[22,23] However, these studies have been conducted based on the ligand-dependent dimerization of wild-type ERs.

Recently, multiple ER isoforms have been identified in human tissues and cells. The ERα variants have been identified in a number of human cell types. More recently, ERβ variants with distinct biological functions have been identified.[11-13] Markedly higher ERβ expression has been documented as one of the distinct gene expressions in endometriotic cells[4-7]; however, these observations were restricted to the expression of wild-type ERβ1, and the expression had been evaluated in comparison with that in endometrial cells.

We observed the expression of splice variant ERβ2 mRNA along with the wild-type ERβ1 in endometriotic cells (Figure 2C). More importantly, these ERβ transcripts were expressed almost at a comparable level of the ERα in endometriotic cells (Figure 1D, 2D and 2E). The observation suggests that the heterodimerization probably occurs in the cells. ERβ/ERβ and ERα/ERβ dimers are known to exhibit antiproliferative effects,[20,21] and ERα/ERβ is reported to be relatively more stable than the ERβ/ERβ homodimer.[13] Although ERβ2 exhibits no estrogen binding, ERβ1/ERβ2 heterodimer shows higher estrogen-induced transactivation than ERβ1/ERβ1 homodimer in a ligand-dependent manner.[11] To further understand the role of the ER expression profile may lead to the estrogen-dependent pathophysiology in endometriosis.

In conclusion, expression of ER isoforms, ERα and ERβ, is the major circumstance for estrogen action in endometriosis. Our results demonstrated that multiple ER isoforms, ERα, ERβ1, and ERβ2, are expressed simultaneously within endometriotic cells. However, little is known regarding the preference for formation of ER homodimer versus heterodimer and how this is affected by ER expression level and ligand occupancy. On therapeutic perspectives, promoting heterodimerization of ER isoforms using selective ER modulators may become an important strategy to design therapeutics for endometriosis.[24] To address these issues, clarifying the molecular basis of ER expression profile and the downstream ER-dependent gene expression may lead us to an avenue of further understanding the estrogen-dependent pathophysiology in endometriosis. It is interesting to note that ERβ and prostaglandin E2 integrate at Ras-like, estrogen-regulated, growth inhibitor, leading to the increase in endometriotic cell proliferation.[25]

Declaration of Conflicting Interests

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