of sensitivity of the EIA was 5 ng/mL. The intra- and interassay coefficients of variation were 3.5% and 5.1%, respectively.

Treatment of EcSC with Drospirenone

When EcSC reached 70%–80% confluence (1 or 2 days), media were removed and replaced with fresh media containing 2% charcoal/dextran-stripped FBS and antibiotics. After culturing for an additional 24 hours, the cells were ready for use in experiments. To evaluate the effect of drospirenone on DNA synthesis, media were replaced with fresh medium containing 2% charcoal/dextran-stripped FBS and antibiotics and EcSC were incubated for 24 hours with drospirenone (10^{-5} to 10^{-7} mol/L) or P (10^{-7} mol/L). We have confirmed that drospirenone at the concentration of 10^{-5} to 10^{-7} mol/L did not have a toxic effect using propidium iodide (PI) staining.

Treatment of EcSC with P Receptor Antagonist RU486 and Mineralocorticoid Receptor Agonist Aldosterone

Because drospirenone acts as a P receptor agonist, P receptor antagonist, RU486 (10^{-8} mol/L), was added to culture media containing drospirenone to determine whether P receptors were involved in the effect of drospirenone. Drospirenone also acts as a mineralocorticoid receptor antagonist in the presence of mineralocorticoid receptor agonist. Although the culture media are steroid free, it is still possible that intracellular cortisol acts on mineralocorticoid receptors. Therefore, aldosterone (mineralocorticoid receptor agonist 10^{-8} mol/L) was added to culture media containing drospirenone to determine whether mineralocorticoid receptors were involved in the effect of drospirenone.

Silencing of P Receptor and Mineralocorticoid Receptor Using Small Interfering RNA (siRNA)

In addition to above-mentioned experiments using antagonist and agonist, we further conducted experiments where these receptors were silenced by transfecting siRNA as we reported elsewhere (12, 16). SiRNAs were obtained as ON-TARGET plus SMART pool human P receptor (L-003433-00-0005) and human mineralocorticoid receptor (L-003425-00-0005) from Dharmacon. The nontargeting siRNA control, ON-TARGET plus nontargeting pool (D-001810-10-05), was also obtained from Dharmacon. EcSC were transfected with 30 nM of siRNA for 7 hours in Opti-MEM I (Gibco) using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's protocol. The cell viability was not decreased by the siRNA transfection, which was verified using cell counting kit-8 (Dojindo). The silencing of P receptor and mineralocorticoid receptor was confirmed by real-time quantitative polymerase chain reaction carried out using a LightCycler (Roche Diagnostic) according to the manufacturer's instructions. Expression of each mRNA was normalized for RNA loading using human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA as an internal standard.

P receptor primers (sense, 5′-CAATGGAAGGGCAGCA CAAC-3′; antisense, 5′-ACAGCATCCAGTGCTCTCAC-3′) were chosen to amplify a 290-bp fragment.

Mineralocorticoid receptor primers (sense, 5′-CTGAGTTCCTTTCCTCCTGTC-3′; antisense, 5′-GCCACAGGT GACTACCCCAT-3′) were chosen to amplify a 225-bp fragment.

GAPDH primers (sense, 5′-ACCACAGTCCATGCCATCAC-3′; antisense, 5′-TCCACCACCCTGTTGCTGTA-3′) were chosen to amplify a 450-bp fragment.

The PCR conditions were as follows:

For P receptor, 40 cycles of 95°C for 10 seconds, 60°C for 30 seconds, and 72°C for 18 seconds.

For mineralocorticoid receptor, 30 cycles of 95°C for 10 seconds, 61°C for 10 seconds, and 72°C for 10 seconds.

For GAPDH, 30 cycles of 95°C for 10 seconds, 65°C for 10 seconds, and 72°C for 18 seconds.

All PCR conditions were followed by melting curve analysis.

After transfection, the medium was removed and replaced with fresh medium containing 2.5% charcoal/dextran-stripped FBS and antibiotics. EcSC were then treated with drospirenone for 24 hours. After the treatment, the 5-bromo-2′-deoxyuridine (BrdU) incorporation assay was performed.

The BrdU Incorporation Assay

The BrdU incorporation assay was performed as reported elsewhere (10), using the Biotrak cell proliferation ELISA system (GE Healthcare) according to the manufacturer's instructions. Briefly, EcSC were treated with 5% FBS with drospirenone for 24 hours, and then 10 μL BrdU solutions were added and incubated at 37°C for an additional 2 hours. After removing the culture medium, the cells were fixed and the DNA denatured by the addition of fixative (200 μL/well). The immune complexes (peroxidase-labeled anti-BrdU bound to BrdU incorporated in the newly synthesized, cellular DNA) were detected by the subsequent substrate reaction, and the resultant color was read at 450 nm in an Epoch Microplate Spectrophotometer (BioTek).

Assessment of Apoptosis

Flow-cytometric analysis was performed as reported elsewhere (17). Apoptotic EcSC were double stained with annexin V and PI using the Annexin V-EGFP Apoptosis detection kit (Abcam) according to the manufacturer's instructions. Briefly, EcSC were detached by 0.25% trypsin-EDTA, washed twice with phosphate-buffered saline, and resuspended in 1 × binding buffer at a concentration of 1×10^6 cells/mL. Each sample solution was transferred to a 5-mL culture tube; 2 μL of annexin V-FITC and 2 μL of PI were added, and the tubes incubated for 10 minutes at 4°C in the dark, followed by filtration through a 40-mm nylon mesh (BD Biosciences) to remove cell clumps. After incubation, the samples were analyzed by FACS Calibur and Cell Quest Pro (BD Biosciences). According to the manufacturer, annexin V–positive cells are regarded as apoptotic cells.

Statistical Analysis

The differences among multiple samples were calculated using analysis of variance and Tukey's test. The differences between two samples were calculated using Student's *t*-test. $P<.05$ was considered statistically significant. The data were expressed as the mean (±SEM). Data analysis was conducted using Jmp software (ver. 10.0, SAS Institute Inc.).

RESULTS

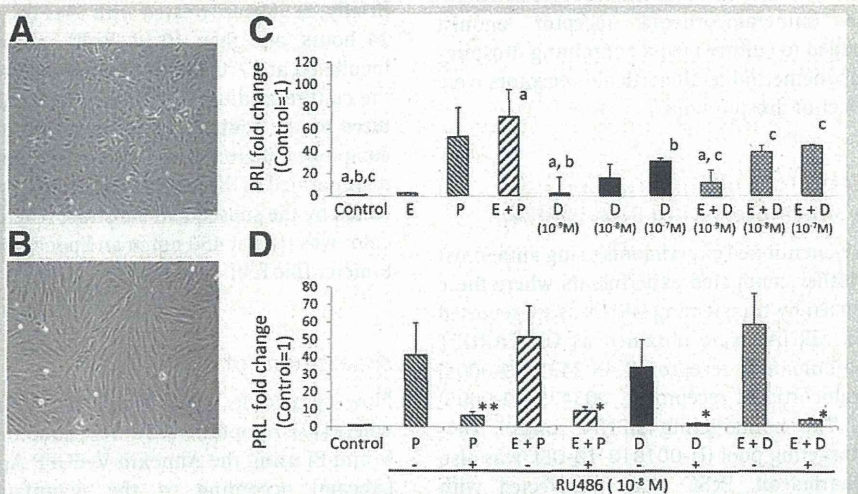
Effect of Drospirenone on the in Vitro Decidualization of EuSC

The EuSC became spindle-like in shape after 8 days of treatment with E_2 (10^{-8} mol/L) and drospirenone (10^{-9} to 10^{-7} mol/L), similar to EuSC treated with E_2 (10^{-8} mol/L) and P (10^{-7} mol/L; Fig. 1A and B). Drospirenone combined with E_2 significantly elevated the concentration of secreted PRL protein in a dose-dependent manner ($P<.05$; Fig. 1C). Drospirenone alone significantly elevated the concentration of secreted PRL protein ($P<.05$). PRL concentrations from EuSC treated with E_2 and drospirenone were slightly higher than EuSc treated with drospirenone only, although the difference did not reach statistical significance. The addition of RU486 to culture medium significantly reduced the secretion of PRL protein, which suggests that drospirenone induces decidualization of EuSC by binding P receptors (Fig. 1D).

Effect of Drospirenone on EcSC DNA Synthesis

Drospirenone at 10^{-7} and 10^{-5} mol/L decreased BrdU incorporation in EcSC (78.4 ± 4.4 and 63.5% ± 2.7% of untreated controls, respectively, mean ± SEM; n = 6; $P<.05$) and induced similar responses to P (10^{-7} mol/L, 83.3% ± 4.1%, mean ± SEM; n = 6; $P<.05$; Fig. 2A). The addition of RU486 to culture medium neutralized the decrease of BrdU incorporation induced by P or drospirenone (117.7 ± 8.4 and 124.6% ± 9.7% of controls [RU486 absent], respectively, mean ± SEM; n = 4; $P<.05$), which suggests that drospirenone inhibits DNA synthesis of EcSC through P receptors (Fig. 2B). On the contrary, aldosterone, a mineralocorticoid agonist, did not cancel drospirenone effects on BrdU incorporation (83.6% ± 9.3% and 80.6% ± 7.1% of untreated controls, respectively, mean ± SEM; n = 4), which suggests that mineralocorticoid receptors seem not to be involved in the anti-DNA synthesis effects of drospirenone (Fig. 3). We further conducted experiments where these receptors were silenced by transfecting siRNA. Transfections of siRNA targeting P receptor and mineralocorticoid receptor successfully reduced the mRNA expression of each molecule (Supplemental Fig. 1). Knockdown of P receptor partially but significantly canceled drospirenone effects on BrdU incorporation (109.7% ± 3.9% of EcSC transfected with negative control siRNA and treated with drospirenone, mean ± SEM; n = 3; $P<.05$), whereas knockdown of mineralocorticoid receptor did not cancel drospirenone effects on BrdU

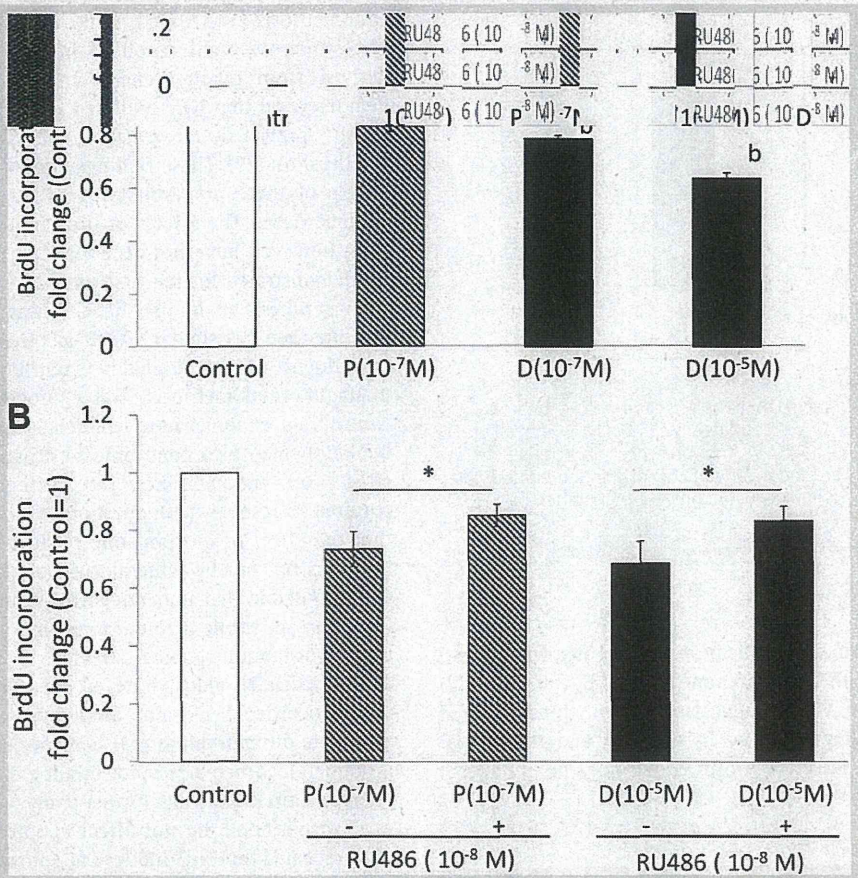
FIGURE 1



Decidualized human EuSC and the effect of drospirenone (D) on PRL secretion from EuSC. EuSC were treated with 2.5% charcoal/dextran-treated (stripped) FBS in the presence of E_2 (10^{-8} mol/L) plus P (10^{-7} mol/L) or E_2 plus D (10^{-7} to 10^{-9} mol/L) for 8 days. The number of spindle-like cells increased. (**A**) control EuSC. (**B**) EuSC treated with E_2 (10^{-8} mol/L) and D (10^{-7} mol/L). (**C**) PRL fold change in culture media of EuSC treated with E_2 (10^{-8} mol/L), P (10^{-7} mol/L), E_2 plus P, D (10^{-9} to 10^{-7} mol/L), or E_2 plus D compared with controls. The range of actual PRL concentration values in controls was from 0.07 to 1.02 ng/mL. Values are the mean (±SEM) of aggregated data from four independent experiments using different EuSC preparations. Different letters denote significant differences between groups ($P<.05$). (**D**) The effect of P receptor antagonist RU486 on in vitro decidualization induced by drospirenone. PRL concentrations in culture media of EuSC treated with P (10^{-7} mol/L), E_2 (10^{-8} mol/L) plus P, D (10^{-7} mol/L), or E_2 plus D, for the last 3 days of 8 days in the absence or presence of RU486 (10^{-8} mol/L). Values are the mean (±SEM) of aggregated data from four independent experiments using different EuSC preparations. $^{**}P<.001$ versus samples without RU486. $^{*}P<.05$ versus samples without RU486.

Miyashita. Effects of drospirenone on uterus and endometriosis. Fertil Steril 2015.

FIGURE 2



Drospirenone (D) suppresses DNA synthesis of EcSC and the effect of P receptor antagonist RU486 on DNA synthesis in EcSC treated with drospirenone. (**A**) EcSC were treated with P (10^{-7} mol/L) or D (10^{-7} to 10^{-5} mol/L) for 24 hours. Values are the mean (±SEM) of aggregated data from six independent experiments using different EcSC preparations. Different letters denote significant differences between groups (a: $P<.001$; b: $P<.05$). (**B**) EcSC were treated with P (10^{-7} mol/L) or D (10^{-5} mol/L) for 24 hours in the presence or absence of RU486 (10^{-8} mol/L). Values are the mean (±SEM) of aggregated data from four independent experiments using different EcSC preparations. *$P<.05$ versus without RU486.

Miyashita. Effects of drospirenone on uterus and endometriosis. Fertil Steril 2015.

incorporation (93.6% ± 8.9% of EcSC transfected with negative control siRNA and treated with drospirenone, mean ± SEM; n = 3), which suggests that the effect of drospirenone on reducing DNA synthesis of EcSC may be mediated by P receptors but not by mineralocorticoid receptors (Supplemental Fig. 2).

Effect of Drospirenone on EcSC Apoptosis

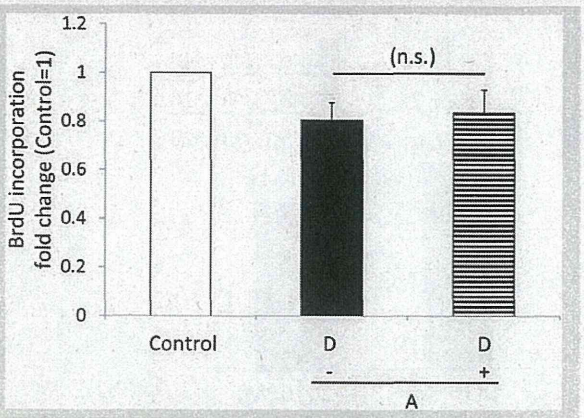
The effect of drospirenone on the induction of apoptosis in EcSC was evaluated by annexin V staining. In comparison with controls, the percentage of apoptotic cells in drospirenone-treated cells was not significantly different (Fig. 4).

DISCUSSION

This is the first study to demonstrate in vitro effects of drospirenone on human endometrial and endometriotic cells. First, we found that drospirenone induces decidualization of EuSC. Second, we demonstrated that drospirenone inhibits the DNA synthesis of EcSC. Third, we demonstrated that these drospirenone effects seem to be mediated by P receptors, not by mineralocorticoid receptors. These findings indicate that drospirenone acts on human endometrium and endometriotic tissue in a way similar to natural P and other progestins.

Many progestins such as dienogest (DNG) (18), norethisterone (NET) (19), medroxyprogesterone acetate (MPA) (19–22), and levonorgestrel (LNG) (23) induce human EuSC decidualization. In this study, we used EuSC to demonstrate the dose-dependent effect of drospirenone on decidualization indicators: morphological change and PRL secretion. We further demonstrate mediation of drospirenone effects by P receptors by showing that its effect was negated by P receptor antagonist, RU486. We also found that drospirenone induced P-responsive genes such as FKBP4 and HOXA10, which are known to be responsible for decidualization (data not shown)

FIGURE 3

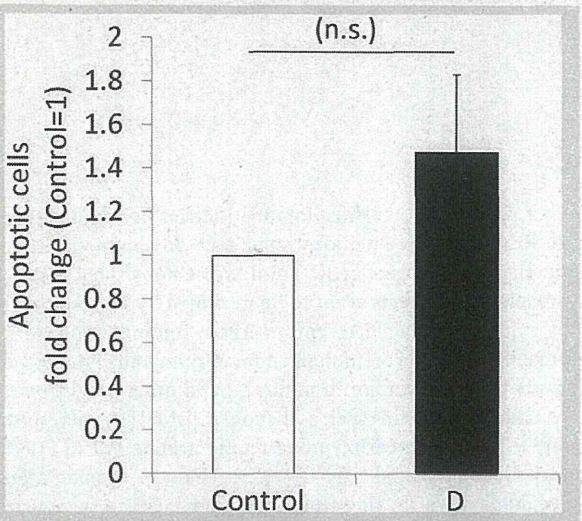


The effect of aldosterone (A) on DNA synthesis in EcSC treated with drospirenone (D). EcSC were treated with P (10^{-7} mol/L) or D (10^{-5} mol/L) for 24 hours in the presence or absence of A (10^{-7} mol/L). Values are the mean (±SEM) of the aggregated data from four independent experiments using different EcSC preparations.

Miyashita. Effects of drospirenone on uterus and endometriosis. Fertil Steril 2015.

(24, 25). In addition, decidualization was greater when EuSC were treated with drospirenone and E_2 than with drospirenone alone, which suggests that the expression of P receptors was up-regulated by E_2 (26, 27) and that the P receptor agonistic effect of drospirenone may be enhanced.

FIGURE 4



Drospirenone did not affect apoptosis of EcSC. EcSC were treated with drospirenone (10^{-7} mol/L) for 24 hours. Annexin V–positive cells were regarded as apoptotic. The percentage of apoptotic EcSC in drospirenone-treated cells was not significantly different from controls. Values are the mean (±SEM) of aggregated data from six independent experiments using different EcSC preparations.

Miyashita. Effects of drospirenone on uterus and endometriosis. Fertil Steril 2015.

Taken together, it seems that drospirenone acts on the human endometrium via the same receptor as other progestins.

Various progestins are known to inhibit the growth of endometriosis (18, 28–32). Bono et al. reported that NET or LNG suppress proliferation of immortalized epithelial cells derived from ovarian endometrioma (32). Minami et al. demonstrated that NET inhibited EcSC proliferation (31). Our group previously revealed that DNG reduces EcSC proliferation (29). These findings may explain the therapeutic effects of these progestins when they are used to manage endometriosis. The effects of drospirenone on endometriotic cells, however, have not been clarified. In the present study, we demonstrated for the first time that drospirenone inhibits DNA synthesis in human EcSC. These results indicate that drospirenone has similar effects as other progestins and as a constituent of OC probably contributes significantly to managing endometriosis. Other progestin effects such as controlling endometriotic epithelial cells and suppressing ovulation may also contribute to drospirenone's therapeutic effects on endometriosis, but further investigations are required to resolve these questions. Our study also suggested that the effect of drospirenone seemed to be mediated by P receptors but not by mineralocorticoid receptors, although it is still possible that mineralocorticoid receptors are involved in the drospirenone action. Interestingly, Caprio et al. report that drospirenone, when treated with mineralocorticoid agonist such as aldosterone or dexamethasone, can act as mineralocorticoid receptor antagonist and inhibit in vitro adipocyte differentiation (33). Similar effects of drospirenone on mineralocorticoid receptor might exist in endometriosis, a phenomenon that awaits further study.

Drospirenone did not affect apoptosis of EcSC. Minami et al. revealed that NET induced apoptosis of EcSC but natural P did not (31). They speculated that the difference between NET and natural P may be due to off-target effects via steroid receptors rather than P receptors. Whereas natural progestin exerts its effects only through the P receptor, synthetic progestins can also act on other steroid receptors such as androgen and mineralocorticoid receptors. Actions via these receptors may cause apoptosis or other effects that are not induced by natural P. In contrast to NET, drospirenone does not stimulate these receptors, and this may explain why drospirenone does not induce apoptosis in EcSC. This difference may be also caused by off-target effects rather than via binding P receptor, although further studies are warranted.

This is the first study to demonstrate the possible mechanism by which drospirenone controls endometriosis. The anti-DNA synthesis effect of drospirenone on EcSC demonstrated in this study alludes to the possible mechanism by which drospirenone-containing OC reduces the size of endometriomas. Other progestins can have anti-inflammatory or antiangiogenic effects. For instance, DNG inhibits aromatase and cyclooxygenase-2 expression and prostaglandin E_2 production in human EcSC (34). DNG reduces TNF-α-induced interleukin-8 (35) and nerve growth factor (NGF) (36) expression in EcSC. Progestins such as MPA, NET, LNG, and DNG inhibit estrogen-induced vascular endothelial growth factor in EuSC (37). Further investigations will determine whether drospirenone has similar anti-inflammatory and antiangiogenic effects on EcSC.

In conclusion, our study demonstrates that drospirenone induces decidualization of EuSC and reduces the DNA synthesis of EcSC. Further studies to explore the other effects of drospirenone on EuSC and EcSC are warranted to fully appreciate its actions on the human endometrium and endometriosis.

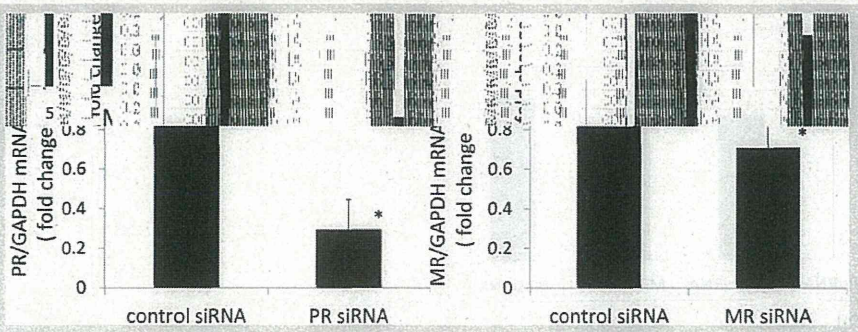
Acknowledgments: The authors thank medical colleagues at the University of Tokyo Hospital for collecting samples and Dr. Kate Hale for editing the manuscript.

REFERENCES

1. Fuhrmann U, Krattenmacher R, Slater EP, Fritzemeier KH. The novel progestin drospirenone and its natural counterpart progesterone: biochemical profile and antiandrogenic potential. Contraception 1996; 54:243–51.
2. Muhn P, Fuhrmann U, Fritzemeier KH, Krattenmacher R, Schillinger E. Drospirenone: a novel progestogen with antimineralocorticoid and antiandrogenic activity. Ann N Y Acad Sci 1995;761:311–35.
3. Oelkers W, Foidart JM, Dombrovicz N, Welter A, Heithecker R. Effects of a new oral contraceptive containing an antimineralocorticoid progestogen, drospirenone, on the renin-aldosterone system, body weight, blood pressure, glucose tolerance, and lipid metabolism. J Clin Endocrinol Metab 1995;80:1816–21.
4. Del Forno S, Solfrini S, Ferrini G, Zannoni L, Bertoldo V, Monti G, et al. Do low-dose oral contraceptives have an effect on ovarian endometrioma diameter and endometriosis symptoms? J Endometr Pelvic Pain Dis 2013;5:151–8.
5. Mabrouk M, Solfrini S, Frasca C, Del Forno S, Montanari G, Ferrini G, et al. A new oral contraceptive regimen for endometriosis management: preliminary experience with 24/4-day drospirenone/ethinylestradiol 3 mg/20 mcg. Gynecol Endocrinol 2012;28:451–4.
6. Maia H Jr, Casoy J, Athayde C, Valente Filho J, Coutinho EM. The effect of a continuous regimen of drospirenone 3 mg/ethinylestradiol 30 microg on Cox-2 and Ki-67 expression in the endometrium. Eur J Contracept Reprod Health Care 2010;15:35–40.
7. Otto C, Fuchs I, Altmann H, Klewer M, Walter A, Prelle K, et al. Comparative analysis of the uterine and mammary gland effects of drospirenone and medroxyprogesterone acetate. Endocrinology 2008;149:3952–9.
8. Hampton AL, Nie G, Salamonsen LA. Progesterone analogues similarly modulate endometrial matrix metalloproteinase-1 and matrix metalloproteinase-3 and their inhibitor in a model for long-term contraceptive effects. Mol Hum Reprod 1999;5:365–71.
9. Noyes RW, Hertig AT, Rock J. Dating the endometrial biopsy. Am J Obstet Gynecol 1975;122:262–3.
10. Kodama A, Yoshino O, Osuga Y, Harada M, Hasegawa A, Hamasaki K, et al. Progesterone decreases bone morphogenetic protein (BMP) 7 expression and BMP7 inhibits decidualization and proliferation in endometrial stromal cells. Hum Reprod 2010;25:751–6.
11. Yoshino O, Izumi G, Shi J, Osuga Y, Hirota Y, Hirata T, et al. Activin-A is induced by interleukin-1beta and tumor necrosis factor-alpha and enhances the mRNA expression of interleukin-6 and protease-activated receptor-2 and proliferation of stromal cells from endometrioma. Fertil Steril 2011; 96:118–21.
12. Urata Y, Koga K, Hirota Y, Akiyama I, Izumi G, Takamura M, et al. IL-1beta increases expression of tryptophan 2,3-dioxygenase and stimulates tryptophan catabolism in endometrioma stromal cells. Am J Reprod Immunol 2014;72:496–503.
13. Hirota Y, Osuga Y, Hirata T, Harada M, Morimoto C, Yoshino O, et al. Activation of protease-activated receptor 2 stimulates proliferation and interleukin (IL)-6 and IL-8 secretion of endometriotic stromal cells. Hum Reprod 2005;20:3547–53.
14. Koga K, Osuga Y, Tsutsumi O, Yano T, Yoshino O, Takai Y, et al. Demonstration of angiogenin in human endometrium and its enhanced expression in endometrial tissues in the secretory phase and the decidua. J Clin Endocrinol Metab 2001;86:5609–14.
15. Irwin JC, Kirk D, King RJ, Quigley MM, Gwatkin RB. Hormonal regulation of human endometrial stromal cells in culture: an in vitro model for decidualization. Fertil Steril 1989;52:761–8.
16. Urata Y, Osuga Y, Akiyama I, Nagai M, Izumi G, Takamura M, et al. Interleukin-4 and prostaglandin E2 synergistically up-regulate 3beta-hydroxysteroid dehydrogenase type 2 in endometrioma stromal cells. J Clin Endocrinol Metab 2013;98:1583–90.
17. Hasegawa A, Osuga Y, Hirota Y, Hamasaki K, Kodama A, Harada M, et al. Tunicamycin enhances the apoptosis induced by tumor necrosis factor-related apoptosis-inducing ligand in endometriotic stromal cells. Hum Reprod 2009;24:408–14.
18. Okada H, Nakajima T, Yoshimura T, Yasuda K, Kanzaki H. The inhibitory effect of dienogest, a synthetic steroid, on the growth of human endometrial stromal cells in vitro. Mol Hum Reprod 2001;7:341–7.
19. Levin JH, Tonetta SA, Lobo RA. Comparison of norethindrone and medroxyprogesterone acetate with natural progesterone and estradiol in stimulating prolactin production from cultured endometrial stromal cells. Am J Obstet Gynecol 1990;163:1932–8.
20. Matsumoto H, Sakai K, Iwashita M. Insulin-like growth factor binding protein-1 induces decidualization of human endometrial stromal cells via alpha5beta1 integrin. Mol Hum Reprod 2008;14:485–9.
21. Brosens JJ, Hayashi N, White JO. Progesterone receptor regulates decidual prolactin expression in differentiating human endometrial stromal cells. Endocrinology 1999;140:4809–20.
22. Mak IY, Brosens JJ, Christian M, Hills FA, Chamley L, Regan L, et al. Regulated expression of signal transducer and activator of transcription, Stat5, and its enhancement of PRL expression in human endometrial stromal cells in vitro. J Clin Endocrinol Metab 2002;87:2581–8.
23. Critchley HO, Wang H, Jones RL, Kelly RW, Drudy TA, Gebbie AE, et al. Morphological and functional features of endometrial decidualization following long-term intrauterine levonorgestrel delivery. Hum Reprod 1998;13:1218–24.
24. Yang H, Zhou Y, Edelshain B, Schatz F, Lockwood CJ, Taylor HS. FKBP4 is regulated by HOXA10 during decidualization and in endometriosis. Reproduction 2012;143:531–8.
25. Das SK. Regional development of uterine decidualization: molecular signaling by Hoxa-10. Mol Reprod Dev 2010;77:387–96.
26. Tseng L, Zhu HH. Regulation of progesterone receptor messenger ribonucleic acid by progestin in human endometrial stromal cells. Biol Reprod 1997;57:1360–6.
27. Prange-Kiel J, Rune GM, Zwirner M, Wallwiener D, Kiesel L. Regulation of estrogen receptor alpha and progesterone receptor (isoform A and B) expression in cultured human endometrial cells. Exp Clin Endocrinol Diabetes 2001;109:231–7.
28. Fu L, Osuga Y, Morimoto C, Hirata T, Hirota Y, Yano T, et al. Dienogest inhibits BrdU uptake with G0/G1 arrest in cultured endometriotic stromal cells. Fertil Steril 2008;89:1344–7.
29. Miyashita M, Koga K, Takamura M, Izumi G, Nagai M, Harada M, et al. Dienogest reduces proliferation, aromatase expression and angiogenesis, and increases apoptosis in human endometriosis. Gynecol Endocrinol 2014;30: 644–8.
30. Shimizu Y, Takeuchi T, Mita S, Mizuguchi K, Kiyono T, Inoue M, et al. Dienogest, a synthetic progestin, inhibits the proliferation of immortalized human endometrial epithelial cells with suppression of cyclin D1 gene expression. Mol Hum Reprod 2009;15:693–701.
31. Minami T, Kosugi K, Suganuma I, Yamanaka K, Kusuki I, Oyama T, et al. Antiproliferative and apoptotic effects of norethisterone on endometriotic stromal cells in vitro. Eur J Obstet Gynecol Reprod Biol 2013;166:76–80.
32. Bono Y, Kyo S, Kiyono T, Mizumoto Y, Nakamura M, Maida Y, et al. Concurrent estrogen action was essential for maximal progestin effect in oral contraceptives. Fertil Steril 2014;101:1337–43.
33. Caprio M, Antelmi A, Chetrite G, Muscat A, Mammi C, Marzolla V, et al. Antiadipogenic effects of the mineralocorticoid receptor antagonist drospirenone: potential implications for the treatment of metabolic syndrome. Endocrinology 2011;152:113–25.
34. Yamanaka K, Xu B, Suganuma I, Kusuki I, Mita S, Shimizu Y, et al. Dienogest inhibits aromatase and cyclooxygenase-2 expression and prostaglandin E(2)

production in human endometriotic stromal cells in spheroid culture. Fertil Steril 2012;97:477–82.
35. Horie S, Harada T, Mitsunari M, Taniguchi F, Iwabe T, Terakawa N. Progesterone and progestational compounds attenuate tumor necrosis factor alpha-induced interleukin-8 production via nuclear factor kappa B inactivation in endometriotic stromal cells. Fertil Steril 2005;83:1530–5.
36. Mita S, Shimizu Y, Sato A, Notsu T, Imada K, Kyo S. Dienogest inhibits nerve growth factor expression induced by tumor necrosis factor-alpha or interleukin-1beta. Fertil Steril 2014;101:595–601.
37. Okada H, Okamoto R, Tsuzuki T, Tsuji S, Yasuda K, Kanzaki H. Progestins inhibit estradiol-induced vascular endothelial growth factor and stromal cell-derived factor 1 in human endometrial stromal cells. Fertil Steril 2011;96:786–91.

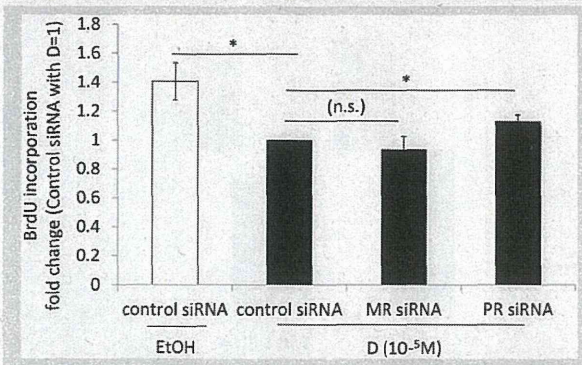
SUPPLEMENTAL FIGURE 1



Silencing of P receptor (PR; panel **A**) and mineralocorticoid receptor (MR; panel **B**) in EcSC. EcSC were transfected with PR siRNA, MR siRNA, or negative control siRNA for 7 hours. Total RNA purified from EcSC was reverse transcribed and amplified by real-time polymerase chain reaction using specific primers for PR and MR to confirm the silencing of these genes. Values are the mean (±SEM) of aggregated data from three independent experiments using different EcSC preparations. *$P<.05$ versus transfected with negative control siRNA.

Miyashita. Effects of drospirenone on uterus and endometriosis. Fertil Steril 2015.

SUPPLEMENTAL FIGURE 2



The effect of silencing of mineralocorticoid receptor (MR) or P receptor (PR) on the drospirenone-reduced DNA synthesis in EcSC. EcSC were transfected with negative control siRNA, MR siRNA, or PR siRNA for 7 hours. After the transfection, EcSC were treated with drospirenone (10^{-5} mol/L) for 24 hours. Values are the mean (±SEM) of the aggregated data from three independent experiments using different EcSC preparations. *$P<.05$ versus EcSC transfected with negative control siRNA and treated with drospirenone.

Miyashita. Effects of drospirenone on uterus and endometriosis. Fertil Steril 2015.

ORIGINAL ARTICLE

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

Masashi Takamura, Kaori Koga, Gentaro Izumi, Tetsuya Hirata, Miyuki Harada, Yasushi Hirota, Osamu Hiraike, Tomoyuki Fujii, Yutaka Osuga

Obstetrics and Gynecology, The University of Tokyo, Tokyo, Japan

Keywords
Endometrium, proportion, regulatory T cells, Th1, Th17, Th2

Correspondence
Kaori Koga, Obstetrics and Gynecology, The University of Tokyo, 7-3-1 Hongo Bunkyo, Tokyo 113-8655, Japan.
E-mail: kawotan-tky@umin.ac.jp

Submission June 21, 2015;
accepted August 18, 2015.

Citation
Takamura M, Koga K, Izumi G, Hirata T, Harada M, Hirota Y, Hiraike O, Fujii T, Osuga Y. Simultaneous detection and evaluation of four subsets of CD4+ T lymphocyte in lesions and peripheral blood in endometriosis. Am J Reprod Immunol 2015; 74: 480–486

doi:10.1111/aji.12426

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