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III. 研究成果の刊行物・別刷

Drospirenone induces decidualization in human eutopic endometrial stromal cells and reduces DNA synthesis of human endometriotic stromal cells

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Objective: To investigate the in vitro effect of drospirenone on human eutopic endometrial (EuSC) and ectopic endometriotic stromal cells (EcSC).
Design: Comparative and laboratory study. The experimental procedures were approved by the Institutional Review Board of the University of Tokyo (registration no. 0324-4).
Setting: University research laboratory.
Patients(s): Eight patients undergoing hysterectomy for benign gynecologic disease and 19 patients undergoing cystectomy or adnectomy for endometriosis.
Intervention(s): EuSC and EcSC were treated with drospirenone.
Main Outcome Measure(s): For the analysis of decidualization of EuSC, cells were observed using microscopy, and the production of PRL was measured using enzyme immunoassay. For the analysis of DNA synthesis of EcSC, 5-bromo-2'-deoxyuridine incorporation was measured by ELISA. Cells in apoptosis were detected and measured by flow cytometry.
Result(s): Drospirenone induced decidualization in EuSC, and the induction was negated by RU486. Drospirenone reduced DNA synthesis on EcSC, and this reduction was negated by RU486 or P receptor silencing, but not by aldosterone or mineralocorticoid receptor silencing. Drospirenone did not cause EcSC to undergo apoptosis.
Conclusion(s): Our study demonstrates the direct effects of drospirenone: decidualization of EuSC and reduced DNA synthesis of EcSC, but it does not cause EsSC apoptosis. (Fertil Steril® 2015;104:217–24. ©2015 by American Society for Reproductive Medicine.)
Key Words: Drospirenone, endometriosis, endometrium, decidualization, oral contraceptive(s)

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Drospirenone is a synthetic progestin that is similar to the natural hormone P and is a potent inhibitor of mineralocorticoid activity (1, 2). Similar to P, drospirenone displays high affinity for P receptors and mineralocorticoid receptors and low affinity for androgen receptors. Unlike P, which has considerable affinity for glucocorticoid receptor, drospirenone exhibits low binding to this receptor and is devoid of either glucocorticoid or antiglucocorticoid activity (1, 2). Clinically, drospirenone has been mainly used as a progestin in oral contraceptives (OCs) (3) and is expected to control endometriosis. As a constituent of OCs, 3 mg drospirenone significantly reduced the size of endometrioma and dysmenorrhea

Received November 5, 2014; revised March 23, 2015; accepted March 24, 2015; published online April 29, 2015.
M.M. has nothing to disclose. K.K. has nothing to disclose. G.I. has nothing to disclose. T.M. has nothing to disclose. A.H. has nothing to disclose. Y.H. has nothing to disclose. T.H. has nothing to disclose. M.H. has nothing to disclose. T.F. has nothing to disclose. Y.O. reports a grant from Bayer Healthcare.
This work was supported by grants (26462478) from the Ministry of Health, Labor and Welfare, the Ministry of Education (to K.K., A.H.), and the Nakatomi Foundation (to K.K.).
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Fertility and Sterility® Vol. 104, No. 1, July 2015 0015-0282/$36.00
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http://dx.doi.org/10.1016/j.fertnstert.2015.03.023

among patients with endometriosis (4). When used in the postoperative period, drospirenone-containing OC decreased anatomical symptoms and recurrence rates of endometrioma (5). Drospirenone also functions on the endometrium, reducing proliferation and thereby improving menorrhagia symptoms. Drospirenone has been shown to reduce Ki-67 expression in the human endometrium (6) and proliferation of mouse epithelial cells (7) when orally administered with and without E_2.

Despite the reported clinical impacts on the endometrium and endometriosis, the direct effects of drospirenone on the endometrium or endometriosis have not been well clarified.

To the best of our knowledge, the effect of drospirenone on the endometrium has been investigated in a single study. To investigate uterine bleeding in long-acting progestin users, Hampton et al. found that drospirenone, similar to other progestins, reduced MMP-1 and MMP-3 expression (8). The aim of this study was to clarify the direct effect of drospirenone on endometrial and endometriotic stromal cells, especially whether it induces decidualization in the endometrium and the anti-DNA synthesis effect on endometriosis.

MATERIALS AND METHODS

Reagents and Materials

Type I collagenase and deoxyribonuclease I were purchased from Wako. Antibiotics (a mixture of penicillin, streptomycin, and amphotericin B), 17β-estradiol, P, and aldosterone were obtained from Sigma. Dulbecco's modified Eagle's medium (DMEM)/F12 medium, 2.5% trypsin, HEPES, and 0.25% trypsin-EDTA were from Gibco. Charcoal/dextran-stripped fetal bovine serum (FBS) was from HyClone. RU486 (Mifepristone) was from Cayman Chemical. Drospirenone was provided by Bayer HealthCare.

Patients and Samples

The Institutional Review Board of the University of Tokyo approved this study, and written informed consent for use of the tissue was obtained from each woman. Endometrial tissues were obtained from endometrial tissue during hysterectomies on eight patients (aged 45 $\pm$ 1.07 years, mean $\pm$ SD). In each case, hysterectomies were for managing benign gynecological conditions such as uterine fibroid without endometrial pathologies. All patients had experienced regular menstrual cycles and had not received hormone therapy for at least 6 months before surgery. The specimens were dated according to each patient's menstrual history and standard histological criteria, according to Noyes et al. (9).

Endometriotic tissues were obtained from patients with ovarian endometriomas (n = 19, ages 36.9 $\pm$ 1.38 years, mean $\pm$ SD) who were undergoing laparoscopy or laparotomy. These patients had not received hormones or GnRH agonist for $\geq$3 months before surgery. Endometriotic tissue samples were obtained from the cyst wall of ovarian endometriomas under sterile conditions and transported on ice in DMEM/F12 to the laboratory.

Isolation and Culture of Human Eutopic Endometrial Stromal Cells (EuSC) and Ectopic Endometriotic Stromal Cells (EcSC)

Isolation and culture of human EuSC were as described elsewhere (10). Endometrial tissue was minced and incubated in DMEM/F12 containing 0.25% type I collagenase, 15 U/mL deoxyribonuclease I, 0.006% trypsin, and 0.02 mol/L HEPES for 60 minutes at 37°C. The resultant dispersed endometrial cells were separated by filtration through a 40-μm nylon cell strainer (BD Biosciences); dispersed EuSC passed through the strainer into the filtrate. EuSC in the filtrate were centrifuged and resuspended in DMEM/F12 containing 5% FBS and antibiotics. EuSC were plated in a 100-mm culture plate and maintained at 37°C in a humidified 5% CO_2/95% air atmosphere. At the first passage, the cells were plated at a density of 2×10^5 cells/well into 6-well culture plates.

EcSC were prepared as reported elsewhere (11–13). Endometriotic tissue was minced into small pieces, incubated in DMEM/F12 with 0.25% type I collagenase, 15 U/mL deoxyribonuclease I, 0.006% trypsin, and 0.02 mol/L HEPES for 1–2 hours at 37°C and filtered through 100-μm (aperture size) and then 70-μm nylon cell strainers. EcSC were cultured in DMEM/F12 containing 5% FBS and antibiotics. The purity of the stromal cells was >98%, as judged by positive cellular staining for vimentin and negative cellular staining for cytokeratin and CD45. More specifically, immunostaining with anti-CD10 antibody identified >95% of the cells as endometriotic stromal cells (13). At the first passage, the cells were plated at a density of 2×10^5 cells/well into 6-well culture plates or 96-well plates at 1×10^4 cells/well and incubated at 37°C in a humidified 5% CO_2/95% air environment.

In Vitro Decidualization of EuSC

To examine the effect of drospirenone on decidualization of EuSC, in vitro decidualization was achieved as described elsewhere (14). Briefly, after EuSC had reached 70% confluence in 6-well culture plates, they were rinsed and treated with 2.5% charcoal/dextran-stripped FBS in the presence of either E_2 (10^{-8} mol/L), P (10^{-7} mol/L), drospirenone (10^{-7} to 10^{-9} mol/L), E_2 (10^{-8} mol/L) plus P (10^{-7} mol/L), or E_2 (10^{-8} mol/L) plus drospirenone (10^{-7} to 10^{-9} mol/L). Previous studies have shown that P induces decidualization of EuSC in a dose-dependent manner (15), and in this study P was used as a positive control. The culture was terminated on the eighth day, when cells in the positive controls start to decidualize. We have confirmed that 8-day treatment of these hormones did not affect the cell number. To block binding to P receptors, EuSC were treated with P receptor antagonist RU486 (10^{-8} mol/L). Culture media were collected and replenished every 3 or 4 days. To evaluate decidualization, cells were observed using microscopy and the concentration of PRL in culture media collected on day 8 was determined.

Measurement of PRL

Concentrations of PRL in the media were measured using a specific enzyme immunoassay (EIA) kit (Cayman). The limit

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

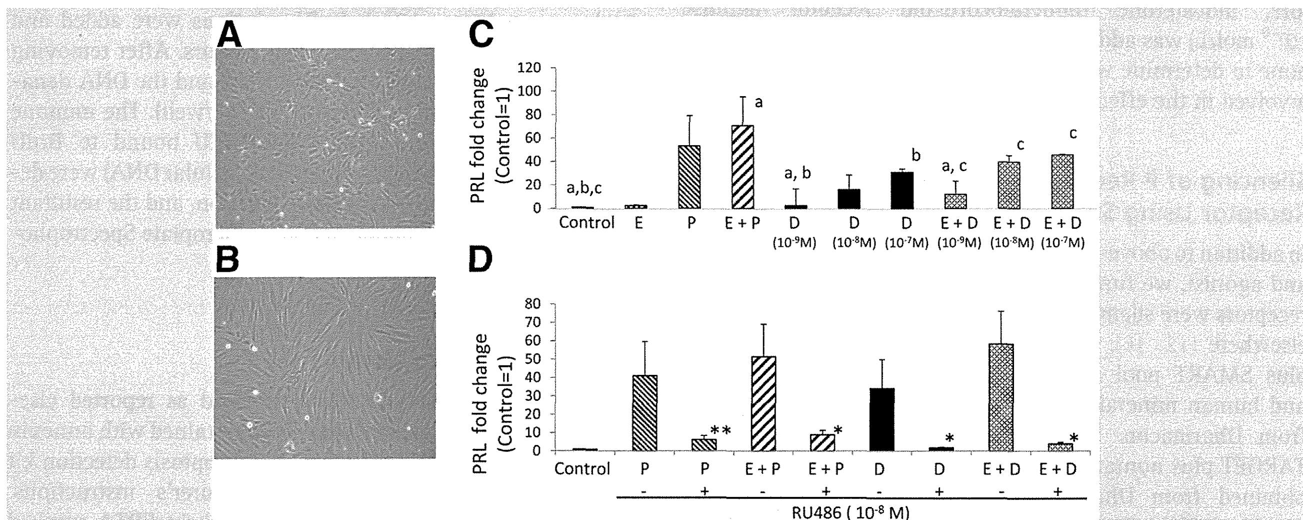
Effect of Drospirenone on the in Vitro Decidualization of EuSC

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.

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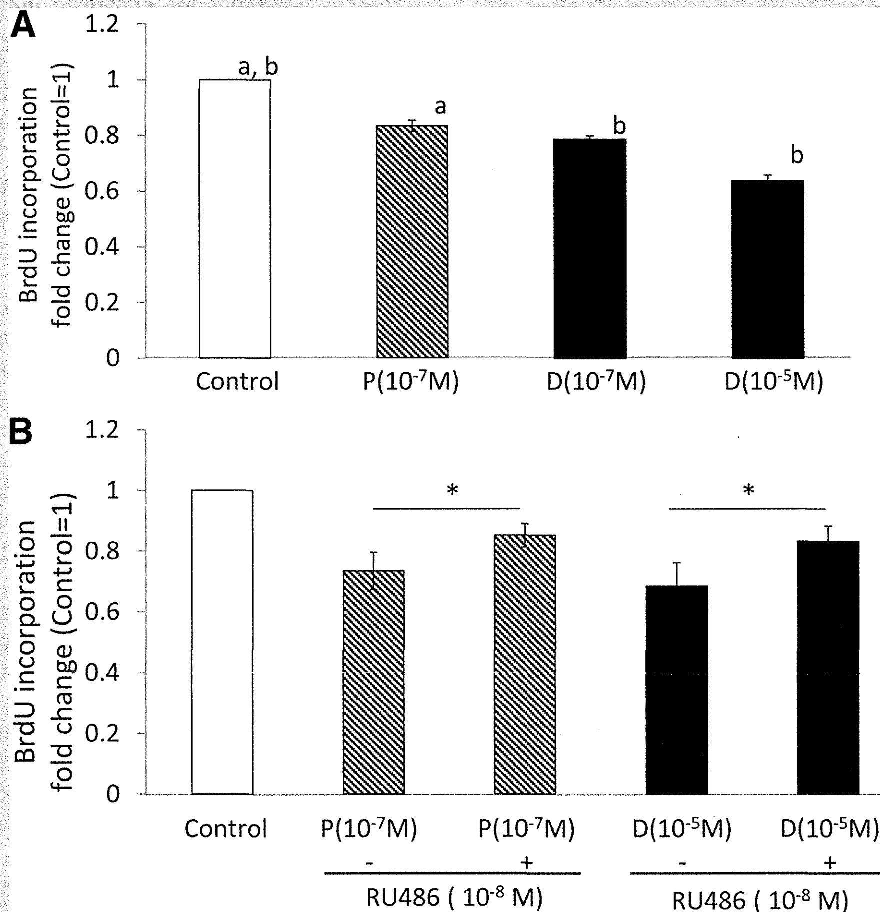
FIGURE 2



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.

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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

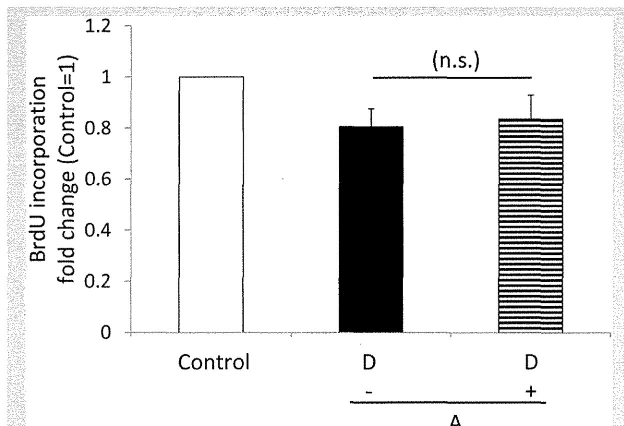
The effect of drospirenone on the induction of apoptosis in EcSC was evaluated by annexin V staining. In comparison with controls, the percentage of apoptotic cells in drospirenone-treated cells was not significantly different (Fig. 4).

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.

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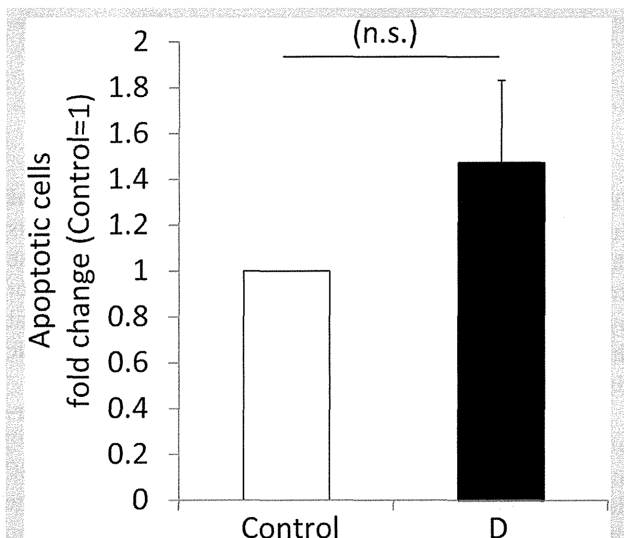
(24, 25). In addition, decidualization was greater when EuSC were treated with drospirenone and E_2 than with drospirenone alone, which suggests that the expression of P receptors was up-regulated by E_2 (26, 27) and that the P receptor agonistic effect of drospirenone may be enhanced.

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.

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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.

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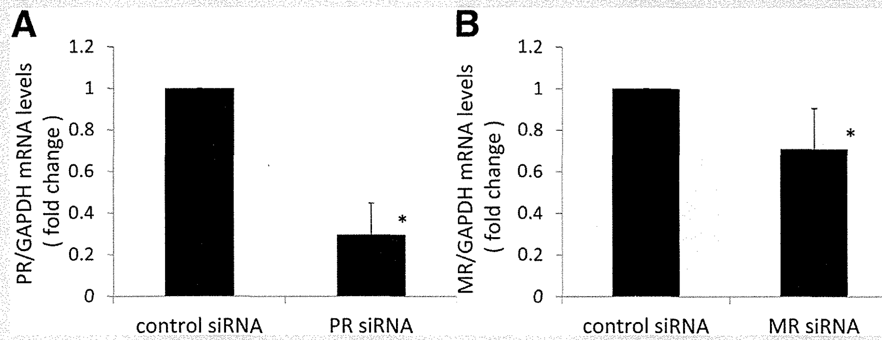
SUPPLEMENTAL FIGURE 1



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.

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