p38 MAPK, p42/44 MAPK, phospho p42/44 MAPK, stress-activated protein kinase (SAPK)/c-Jun N-terminal kinase (JNK), and phospho SAPK/JNK were purchased from Cell Signaling Technology (Beverly, MA). MAPK inhibitors SB202190, PD98059, and SP600125 were from Calbiochem (La Jolla, CA). Collagenase was obtained from WAKO (Osaka, Japan). The antibiotic mixture of penicillin, streptomycin, amphotericin B phorbol 12-myristate 13-acetate (PMA), and ionomycin were purchased from Sigma (St. Louis, MO). Charcoal-stripped fetal bovine serum (FBS) was from Hyclone (Logan, UT). DMEM/Ham's F12 (DMEM/F12) and deoxyribonuclease I were from Invitrogen (Rockville, MD).

Patients and samples

Endometriotic tissues and PF were obtained from patients with ovarian endometriomas undergoing laparoscopy. The severity of the disease was determined according to the revised American Society for Reproductive Medicine classification. The final diagnosis was confirmed by histopathological examination. Laparoscopic excision of ovarian endometrioma was performed as follows. After inspection of the pelvis, the ovary was freed from any adhesions. The cyst wall of endometrioma was stripped away from the normal ovarian tissue gently and completely. Endometriotic tissue were obtained from the excised cyst wall of ovarian endometrioma and transported to the laboratory in DMEM/F12 on ice under sterile condition. The PF was obtained from the patients, who were diagnosed as stage III or IV. All patients had regular menstrual cycles, and none had received hormonal treatment for at least 6 months before surgery. The tissues collected under sterile conditions were processed for the generation of primary cell cultures. The peritoneal fluid was collected under sterile conditions before any manipulative procedure. PFMCs were collected as previously described (13, 14). Briefly, the collected PF was centrifuged at 200 × *g* for 5 min, and the supernatants were removed. The cell pellet was resuspended in PBS, layered onto Ficoll-Paque (Amersham Biosciences, Piscataway, NJ), and centrifuged at 150 × *g* for 30 min. PMFCs were recovered from the interface.

The experimental procedures were approved by the Institutional Review Board of the University of Tokyo and signed informed consent for use of the endometriotic tissue was obtained from each patient.

Immunohistochemistry

Paraffin-embedded specimens were sliced at a 5-μm thickness. These slide sections were deparaffinized and rehydrated. Antigens were retrieved by buffer at 98 C. Endogenous peroxidase was blocked by incubation for 20 min with a solution of 1% hydrogen peroxidase. Immunohistochemical tissue labeling was performed using the avidin-biotin peroxidase methods. After blocking with normal rabbit serum (Vector Laboratories, Burlingame, CA), the sections were incubated with 1 μg/ml anti IL-17A antibody or goat IgG for 60 min at room temperature and incubated with avidin-biotin peroxidase complex (Vectastain Elite; Vector Laboratories), according to the manufacture's instructions. The pattern of immunoreactivity was visualized using Vector VIP (Vector Laboratories) as substrate. All sections were counterstained with hematoxylin and evaluated under a light microscope.

Flow cytometric analysis

PFMCs were resuspended in 10% FBS RPMI 1640 medium. The cells were stimulated with PMA (50 ng/ml) and ionomycin (1 μg/ml) for 5 h in the presence of Goldistop. Cells were firstly stained extracellularly with anti-CD3 and anti-CD4 antibodies, then fixed and permeabilized with Perm/Fix solution (eBioscience), and finally stained intracellularly with anti-IL-17A antibody. Samples were analyzed using FACSCalibur (BD Bioscience) and Cell Quest Pro (BD Bioscience).

Isolation and culture of ESCs

The isolation and culture of human ESCs were performed as described previously (15, 16). Fresh endometriotic tissue collected in sterile medium was rinsed to remove blood cells. The tissue was minced into small pieces and incubated in phenol-red free DMEM/F12 containing type I collagenase (0.25%) and deoxynuclease I (15 IU/ml) for 120 min at 37 C. The resultant dispersed endometriotic cells were separated by filtration through a 100-μm nylon cell strainer (Becton Dickinson and Co., Franklin Lakes, NJ) and 70 μm nylon cell strainer. Stromal cells remaining in the filtrate were collected by centrifugation, resuspended in phenol-red free DMEM/F-12, plated onto 100-mm dishes (Iwaki, Asahi technology Co., Tokyo, Japan), and allowed to adhere at 37 C for 12 h. At the first passage, the cells were plated into six-well plates at 4×10^5 cells/well, 12-well plates at 2×10^5 cells/well, or 48-well plates at 1×10^5 cells/well. Once the cells reached confluence, in 2 or 3 d, they were used for experiments. The purity of ESCs was greater than 95%, according to positive cellular staining for vimentin and negative cellular staining for cytokeratin or CD45, CD68, and von Willebrand factor.

Treatment of the cells

First, to examine the effect of IL-17A on IL-8 production, the cells were incubated for 24 h in 5% FBS DMEM/F12 medium with varying doses of IL-17A. Second, to examine the effect of the anti-IL-17RA antibody, ESCs were preincubated in 5% FBS DMEM/F12 with the antibody for 30 min and then stimulated with 10 ng/ml IL-17A for 24 h. Third, to evaluate the effect of IL-17A on MAPK phosphorylation in ESCs, the cells were incubated with 5% FBS media with IL-17A (10 ng/ml) for different time periods. Fourth, to evaluate the effect of MAPK inhibitors, the cells were preincubated with each MAPK inhibitor for 1 h before the addition of IL-17A and then incubated for 24 h. Fifth, to evaluate the synergic effect of IL-17A and TNFα on IL-8 secretion, the cells were stimulated with varying doses of IL-17A (1–100 ng/ml) with or without TNFα (1 ng/ml). Finally, for time-course experiments examining the expression of IL-8 and cyclooxygenase (COX)-2 mRNA, ESCs were incubated with 5% FBS medium with IL-17A (10 ng/ml) for different time periods up to 24 h.

RNA extraction, reverse transcription, and PCR of IL-17A, IL-17RA, IL-8, and COX2

We extracted total RNA from endometriotic tissues and PFMCs by the acid guanidinium-phenol-chloroform method using ISOGEN (Nippongene, Toyama, Japan). Using an RNeasy minikit (QIAGEN, Hilden, Germany), we extracted total RNA from ESCs cultured in a 12-well plate. One microgram of total RNA was reverse transcribed in a 20-μl vol using an RT-PCR kit (TOYOBO, Osaka, Japan). Standard PCR was performed using Rever Tra Dash (TOYOBO) according to the manufacturer's instructions. Human glyceraldehyde dehydrogenase (GAPDH) primers (TOYOBO) were used as a positive control for RNA levels. Primer pairs for IL-17A, IL-17RA, IL-8, and COX2 used in PCR are shown in Table

TABLE 1. Primer pairs used for PCR analysis

mRNA		Oligonucleotide sequences (5′–3′)	Size (bp)
IL-17	Sense	ACTCCTGGGAAGACCTCATTGG	462
	Antisense	GGCCACATGGTGGACAATCG	
IL-17R	Sense	ACACCCAACAAGGAGACCTG	430
	Antisense	ATTCGTTCCACAGGGTGAAG	
IL-8	Sense	ACTTCCAAGCTGGCCGTGGCTCTCTTGGCA	295
	Antisense	TGAATTCTCAGCCCTCTTCAAAAACTTCTC	
COX2	Sense	TTCAAATGAGATTGTGGGAAAATTGCT	306
	Antisense	AGATCATCTCTGCCTGAGTATCTT	
GAPDH	Sense	ACCACAGTCCATGCCATCAC	452
	Antisense	TCCACCACCCTGTTGCTGTA	

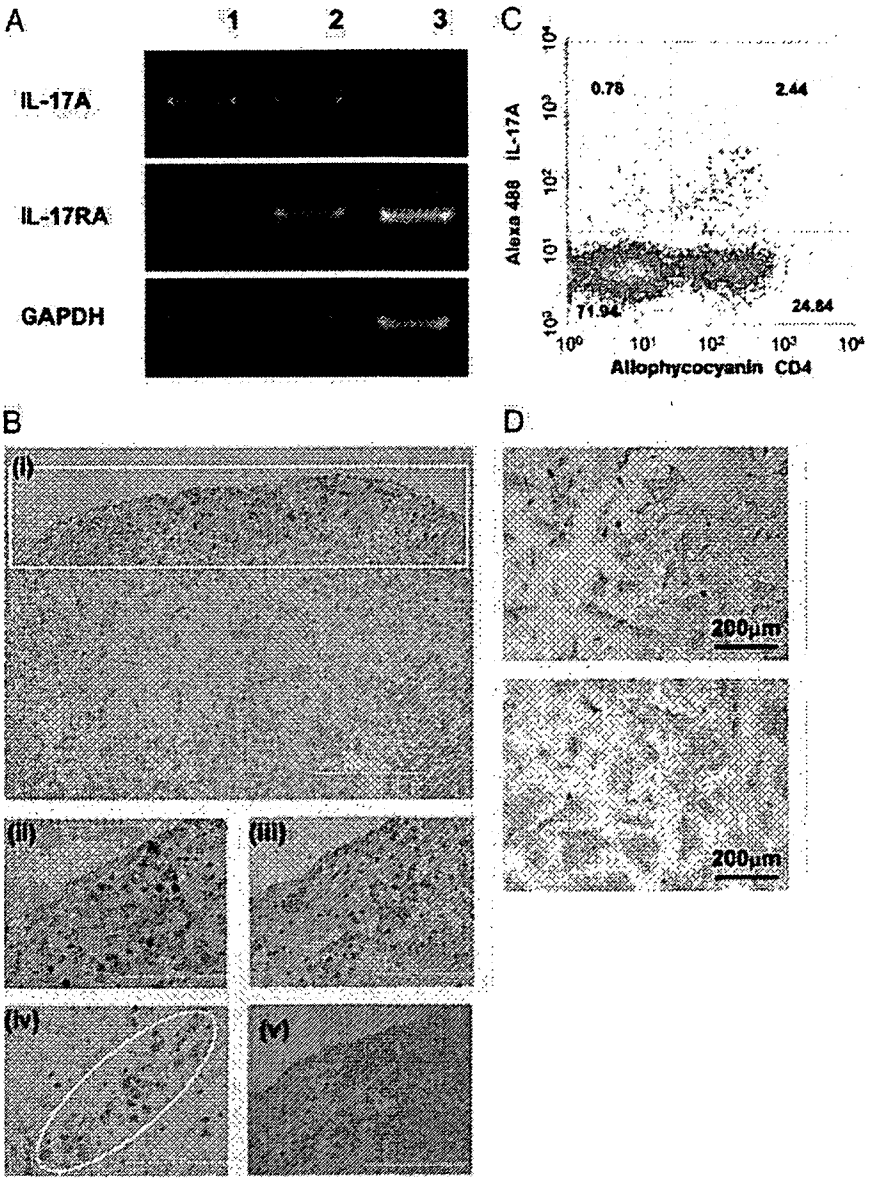

FIG. 1. A, Expression of IL-17A and IL-17RA mRNA in endometriotic tissues (lane 1), PFMCs (lane 2), and ESCs (lane 3). Endometriotic tissues, PFMCs, and ESCs without stimulation were analyzed by RT-PCR. B, Expression of IL-17A in the cyst wall of endometrioma (i, ii, iii, and iv) and ovary (v). Sections were immunostained with antihuman IL-17A antibody (i, ii, iv, and v) or goat IgG (iii). IL-17A-positive cells were detected in the stroma immediately beneath epithelium (i, *white rectangular area*). *Arrowheads* indicate IL-17A-positive cells in the stroma immediately beneath epithelium (ii). IL-17A-positive cells (*arrowheads*) were detected at the site of hemosiderin deposits (iv, *white circle*). No IL-17A-positive cells were detected in ovarian surface (v). Magnification, ×100 (i), ×400 (ii and iii), and ×200 (iv and v). *Scale bars*, 200 μm (i, iv, and v); 100 μm (ii and iii). C, Th17 cells in PFMCs of patients with endometriosis. PFMCs were stimulated with PMA (50 ng/ml) and ionomycin (1 μg/ml) and labeled with an antibody specific to lymphocytes (CD3 and CD4) and IL-17A. Th17 cells were CD4 positive and IL-17A positive. The data shown are representative of four separate experiments. D, Immunocytochemistry of IL-17RA in ESCs. Cultured ESCs were immunostained with anti-IL-17RA antibody (*upper photo*). *Lower photo* shows the control with mouse IgG1 isotype. Magnification, ×100.

1. PCR conditions for amplification were 30 cycles (for IL-17RA, IL-8, COX-2, and GAPDH) or 35 cycles (for IL-17A) at 98 C for 10 sec, 60 C for 2 sec, and 74 C for 14 sec. Each PCR product was purified with a QIAEX II gel extraction kit (QIAGEN), and the identity of PCR products was confirmed using an ABI PRISM 310 genetic analyzer (Applied Biosystems, Foster City, CA).

Real-time quantitative PCR

Real-time quantitative PCR was performed as reported previously (17). To assess IL-8 and COX2 mRNA expression, real-time quantitative PCR and data analysis were performed using Light Cycler (Roche Diagnostics GmbH, Mannheim, Germany). Expression of IL-8 and COX2 mRNA was normalized to RNA loading for each sample using GAPDH mRNA as an internal standard. The primers for IL-8 and COX2 were the same as those used for standard PCR. PCR conditions were as follows: for IL-8, 40 cycles at 95 C for 10 sec, 66 C for 10 sec, 72 C for 11 sec; for COX2, 30 cycles at 95 C for 10 sec, 66 C for 10 sec, 72 C for 13 sec; for GAPDH, 30 cycles at 95 for 10 sec, 64 C for 10 sec, 72 C for 18 sec. All PCR conditions were followed by melting curve analysis.

Counting cell numbers

Cell counting was performed using a Cell Counting Kit-8 (Dojindo, Kumamoto, Japan), according to the manufacturer's instruction.

5-Bromo-2′-deoxyuridine (BrdU) incorporation assay

BrdU incorporation assay was performed as reported previously (15, 18, 19). The effects of IL-17A and IL-8 on the proliferation of ESCs was examined by measuring BrdU incorporation into DNA using the Biotrak cell proliferation ELISA system (Amersham Biosciences) according to the manufacture's instructions. Briefly, ESCs were seeded into a 96-multiwell plate (Becton Dickinson) at a density of 5×10^4 cells/well in 100 μl of the culture medium. After 24 h, cells were stimulated with IL-17A or IL-8 for 48 h. Then 10 μl BrdU solution were added and incubated at 37 C for an additional 2 h. After removing the culture medium, the cells were fixed and the DNA denatured by the fixative. The peroxidase-labeled anti-BrdU bound to the BrdU incorporated in the newly synthesized, cellular DNA. The immune complexes were detected by the subsequent substrate reaction, and the resultant color was read

at 450 nm in the DigiScan microscope reader (ASYS Hitech GmbH, Eugendorf, Austria).

Immunocytochemistry

ESCs were cultured in 16-well chamber slides (Nunc, Naperville, IL) in a humidified 5% CO_2-95% air environment and allowed to grow to approximately 50% confluence. The cells were fixed with cold methanol/acetone at −20 C for 20 min, washed twice with PBS, blocked for 20 min with 5% bovine serum in PBS, and incubated with an anti-IL-17RA antibody (10 μg/ml in 1.5% BSA in PBS) or IgG2b mouse IgG isotype control for 40 min at room temperature. After three washes with PBS, the slides were incubated with peroxidase-conjugated secondary antibody (goat antimouse Envision plus; Dako, Glostrup, Denmark) for 30 min at room temperature. Staining was detected with the diaminobenzidine chromogen after 3 min. All slides were counterstained with hematoxylin and evaluated under a light microscope.

Western blotting

Cultured cells in 6-well plates were homogenized in lysis buffer containing 50 mM Tris-HCl (pH 6.8), 2% sodium dodecyl sulfate, 10% glycerol, 50 mM dithiothreitol, and 0.1% bromophenol blue. The lysates were further diluted with lysis buffer to give a final concentration of 1 mg total protein per milliliter. Samples were resolved using 10% SDS-PAGE. Proteins were blotted onto a nitrocellulose membrane and incubated with rabbit antibodies to p38 MAPK (1:1000), phospho-specific p38 MAPK (1:1000), p42/44 MAPK (1:1000), phospho-specific p42/44 MAPK, SAPK/JNK (1:1000), phospho-specific SAPK/JNK (1:1000) as primary antibodies, and antirabbit horseradish peroxidase antibody (1:1000) as a secondary antibody. Immune complexes were visualized by use of the ECL Western blotting system (Amersham Biosciences).

Measurement of IL-8

The concentration of IL-8 in conditioned media was measured using a specific ELISA kit (Genzyme/Techne, Minneapolis, MN). The sensitivity of the assay was 15.6 pg/ml. The intraassay and interassay coefficients of variation were less than 5%.

Statistical analysis

Data were evaluated using ANOVA with Scheffé's *post hoc* analysis for multiple comparisons and Student's *t* test for two groups. $P < 0.05$ was accepted as statistically significant.

Results

Expression of IL-17A and IL-17RA mRNA in endometriotic tissue, PFMCs, and ESCs

The expression of IL-17A mRNA was detected in endometriotic tissues and peritoneal cells, but not in ESCs, by standard RT-PCR analysis. The expression of IL-17RA mRNA was detected in endometriotic tissues, PFMCs, and ESCs (Fig. 1A).

In vivo expression of IL-17A in the endometriotic lesion

As shown in Fig. 1B, the presence of immunoreactive IL-17A was detected in the cyst wall of endometrioma. Intense IL-17A immunoreactive cells were localized in the stroma immediately beneath epithelium and at the site of hemosiderin deposits. No IL-17A immunoreactivity was visualized in endometriotic epithelial cells. Few IL-17A-positive cells were observed in the endometrium of proliferative and secretary phases (data not shown). No IL-17A-positive cells were detected in ovarian surface. No staining was observed when normal goat IgG was used as a primary antibody.

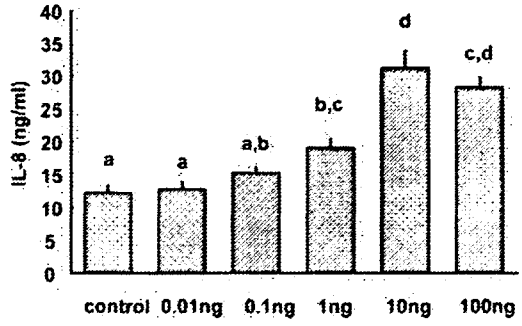

FIG. 2. IL-17A stimulates IL-8 secretion in ESCs. ESCs were cultured in 5% FBS with different doses of IL-17A for 24 h. Concentration of IL-8 in the conditioned medium was measured using a specific ELISA. Values are the mean ± SEM of pentaplicate cultures. *Different letters* denote significant differences between groups ($P < 0.05$). The result is representative of nine separate experiments using samples from different patients.

Th17 cells in PFMCs of patients with endometriosis

The presence of IL-17A-producing cells was evaluated by flow cytometry on T cells (CD3+ cells) from PFMCs after stimulation with PMA and ionomycin. As shown in Fig. 1C, IL-17A-producing T cells were detected in PFMCs of patients with endometriosis. We also found IL-17A-producing T cells were predominantly CD4+ T cells.

Expression of IL-17RA protein in ESCs

The presence of immunoreactive IL-17RA was demonstrated in ESCs (Fig. 1D). No staining was observed when mouse IgG1 was used as a primary antibody.

Effects of IL-17A on IL-8 secretion by ESCs

As shown in Fig. 2, IL-17A at 1 ng/ml and higher significantly enhanced the secretion of IL-8 from ESCs. The maximum effect was observed with IL-17A at 10 ng/ml. The magnitude of increase

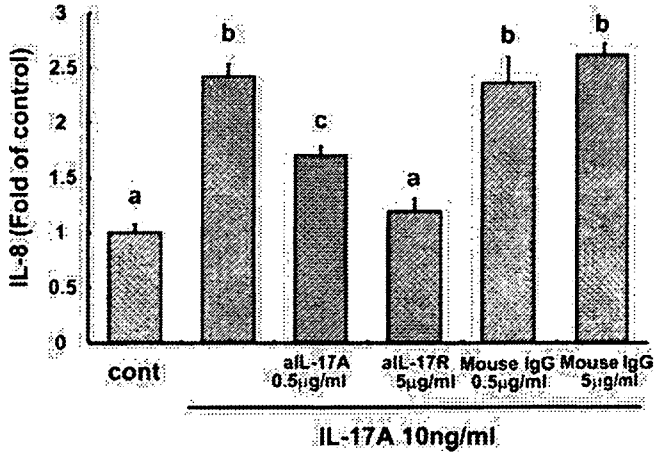

FIG. 3. Effect of anti-IL-17RA antibody (aIL-17R) on IL-17A-induced IL-8 secretion by ESCs. ESCs were preincubated in 5% FBS medium with or without mouse IgG1 and aIL-17R for 30 min and then stimulated with or without IL-17A (10 ng/ml) for 24 h. Concentrations of IL-8 in the conditioned media were measured using a specific ELISA. All value are expressed as the mean ± SEM of pentaplicate cultures. *Different letters* denote significant differences between groups ($P < 0.05$). The result is representative of four separate experiments using samples from different patients. Cont, Control.

with 10 ng/ml IL-17A varied between patients from 1.8- and 5.3-fold, with a median increase of 3.5-fold (n = 9).

Effect of anti-IL-17RA antibody on IL-17A-induced IL-8 secretion in ESCs

Treatment with the neutralizing antibodies for IL-17RA significantly diminished the IL-17A-induced increase in IL-8 secretion in a dose-dependent manner, whereas the control IgG had no effect (Fig. 3).

Effect of IL-17A on MAPK phosphorylation in ESCs

The phosphorylation of three MAPKs (p42/44 MAPK, p38 MAPK, and SAPK/JNK) by IL-17A was determined in cultured ESCs (Fig. 4A). An increase in MAPK phosphorylation was apparent after 5–15 min. Phosphorylation levels reached a maximum after 5 min for P42/44 MAPK and after 15 min for p38 MAPK and SAPK/JNK, respectively.

Effect of MAPK inhibitors on IL-17A-induced IL-8 secretion

The intracellular mechanism of IL-17A-induced secretion of IL-8 by ESCs was investigated by examining the effect of MAPK inhibitors. As shown in Fig. 4B, the addition of inhibitors for p38MAPK, p42/44 MAPK, and SAPK/JNK significantly diminished IL-17A-induced IL-8 secretion.

Synergic effects IL-17A and TNFα on IL-8 secretion in ESCs

We chose TNFα as a representative proinflammatory cytokine known to induce IL-8 secretion from ESCs (12). TNFα together with IL-17A triggered IL-8 secretion above the combined levels generated by each stimulus alone (Fig. 5). This synergistic effect was apparent when TNFα (1 ng/ml) was combined with 1 ng/ml IL-17A, and maximal synergy was obtained at the highest dose of IL-17A tested (100 ng/ml).

Effect of IL-17A on the expression of IL-8 mRNA and COX2 mRNA in ESCs

Time-course experiments were conducted to examine the effect of IL-17A on the expression of IL-8 mRNA and COX2 mRNA in ESCs (Fig. 6A). Real-time quantitative PCR anal-

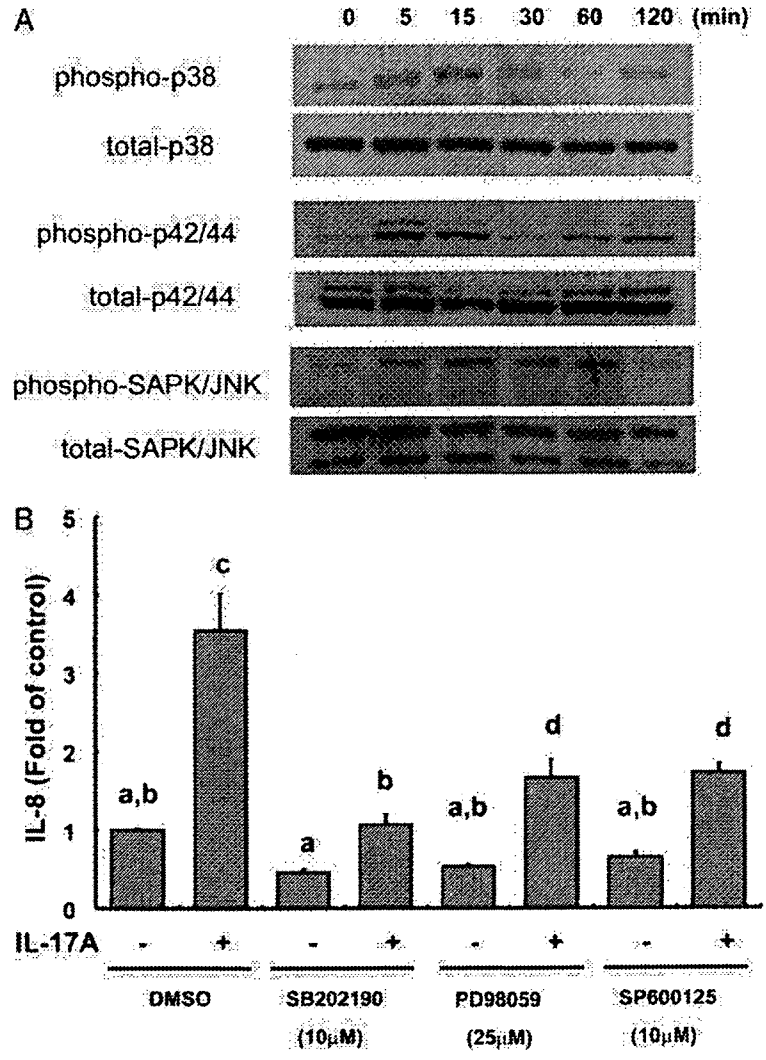

FIG. 4. Involvement of MAPKs in IL-17A induced IL-8 secretion from ESCs. A, phosphorylation of p38 MAPK, p42/44 MAPK, and SAPK/JNK induced by IL-17A in ESCs. ESCs were incubated with IL-17A (10 ng/ml) for the indicated time (0–120 min). Cell lysates were assayed for phosphorylated p38 MAPK (phospho-p38), total p38 MAPK (total-p38), phosphorylated p42/44 MAPK (phospho-p42/44), total p42/44 (total-p42/44), phosphorylated SAPK/JNK (phospho-SAPK/JNK), and total SAPK/JNK (total-SAPK/JNK) by Western blotting. The data are representative of four independent experiments. B, Effects of MAPK inhibitors on IL-17A-induced IL-8 secretion in ESCs. ESCs were pretreated with or without inhibitors of p38 MAPK (SB202190), p42/44 MAPK (PD98059), and SAPK/JNK (SP600125) for 1 h and stimulated with IL-17A (10 ng/ml) for 24 h. The conditioned medium was collected and assayed for IL-8 concentration using a specific ELISA. All values are expressed as the mean ± SEM of pentaplicate cultures. *Different letters* denote significant differences between groups ($P < 0.05$). The data are representative of four independent experiments. DMSO, Dimethylsulfoxide.

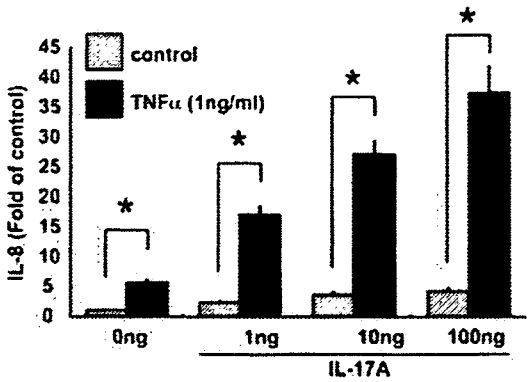

FIG. 5. Effect of IL-17A on TNFα-mediated IL-8 secretion from ESCs. ESCs were treated with IL-17A or TNFα or in combination for 24 h. The conditioned medium was collected and assayed for IL-8 concentration using a specific ELISA. All values are expressed as the mean ± SEM of pentaplicate cultures. *, $P < 0.0001$ *vs.* each control. The data are representative of four independent experiments.

ysis demonstrated that IL-17A up-regulated IL-8 and COX2 mRNA. Maximal increases in IL-8 and COX2 mRNA were observed at 4 h, followed by a decrease with time up to 24 h (Fig. 6, B and C). The maximal increase of IL-8 mRNA was 6.2-fold of the control, and that of COX2 mRNA was 13.6-fold of the control.

Effect of IL-17A on cell proliferation of ESCs

The effect of IL-17A on cell proliferation was determined in ESCs (Fig. 7A). IL-17A at 10 and 100 ng/ml significantly increased cell number by 106 and 111%, respectively, after exposure for 48 h. As shown in Fig. 7B, IL-17A at 1–100 ng/ml significantly increased BrdU incorporation into DNA in ESCs. The maximal effect (3.98-fold of control) was observed at 10 ng/ml.

Discussion

In the present study, we first demonstrated that presence of IL-17A-positive cells in the endometriotic tissue. In addition, the presence of Th17 cells in PFMCs was clearly shown by flow cytometric analysis. These findings instigated us to examine possible roles of IL-17A in endometriosis. We then showed that IL-17A stimulated the secretion of IL-8 from ESCs. ESCs expressed IL-17RA, and the anti-IL-17RA antibody inhibited IL-17A-induced IL-8 secretion. IL-17A stimulated the activation of p38 MAPK, p42/44 MAPK, and SAPK/JNK, and inhibitors of these kinases suppressed IL-17A-induced IL-8 secretion. TNFα synergistically enhanced IL-17A-induced IL-8 secretion. IL-17A also stimulated ESC proliferation and COX2 expression in ESCs.

A recent study on the Th1/ Th2 concept of T cell immunology revealed that endometriosis is an inflammatory disease with a Th2 immune response component (20, 21). The emerging concept of the Th17 pathway has challenged the conventional paradigm of Th1/Th2 hypothesis (4, 5). Together with the recent discovery of Treg, our understanding of the mechanisms underlying T cell immunology has advanced into a new era. In this context, the presence of Th17 cells in PFMCs demonstrated in our study might lead the

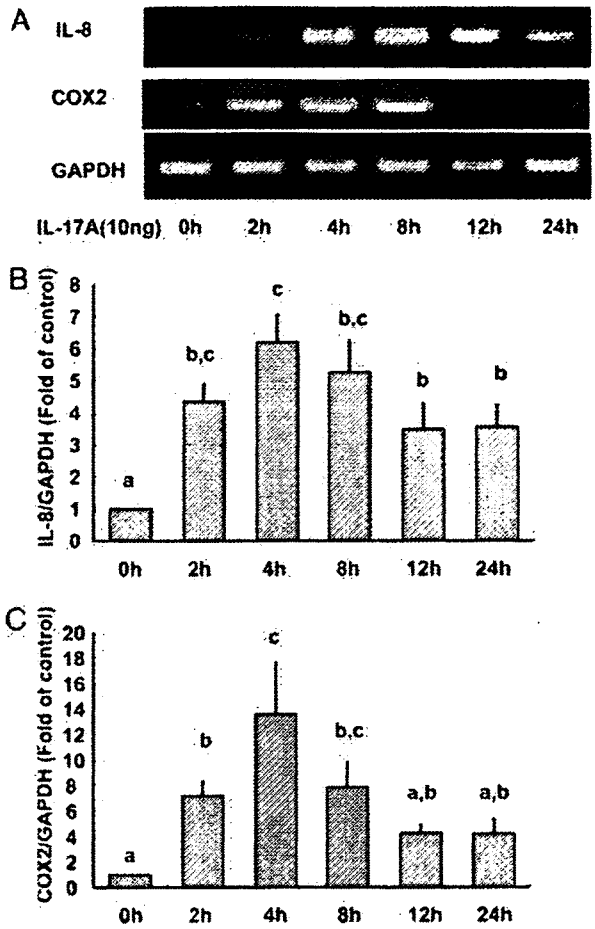

FIG. 6. Effect of IL-17A on the expression of IL-8 and COX2 mRNA in ESCs. ESCs were incubated with IL-17A (10 ng/ml) for the indicated duration. A, Expression of IL-8 and COX2 mRNA in ESCs was examined by RT-PCR. The data shown are representative of four separate experiments using samples from different women. B, Expression of IL-8 mRNA in ESCs was examined by real-time quantitative PCR. The data shown are the relative ratio (IL-8 to GAPDH) measures by real-time quantitative PCR. Data are the mean ± SEM of five independent experiments using samples from five different women. *Different letters* denote significant differences between groups ($P < 0.05$). C, Expression of COX2 mRNA in ESCs was examined by real-time quantitative PCR. The data shown are the relative ratios (COX2 to GAPDH) measured by real-time quantitative PCR. Data are the mean ± SEM of five independent experiments using samples from 5 different women. *Different letters* denote significant differences between groups ($P < 0.05$).

concept of immune response in endometriosis to a novel direction. In particular, abundant IL-17A-positive cells in the endometriotic tissue imply possible Th17 immune response therein. The present study has demonstrated multiple functions of IL-17A in ESCs. Given that IL-17A is a key effector molecule of Th17 cells, our findings form the foundation for understanding the etiology of endometriosis under the novel concept of T cell differentiation and regulation.

Substantial evidence points to IL-8 as a pivotal factor involved in the progression of endometriosis. IL-8 exerts pleiotropic functions, such as chemoattraction and activation of neutrophils, angiogenesis, stimulation of proliferation, and

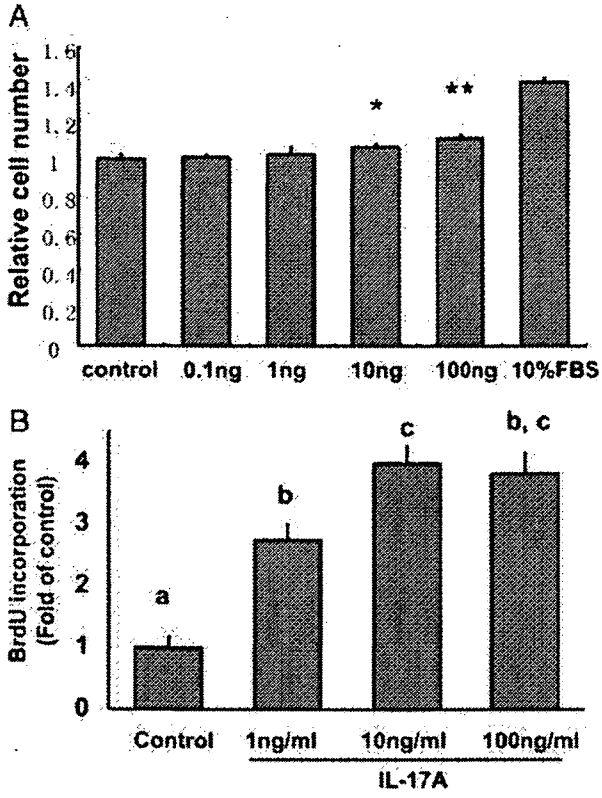

FIG. 7. Effect of IL-17A on proliferation of ESCs. A, The effect of IL-17A on proliferation of ESCs was examined by measuring cell number with cell-counting kit. ESCs were cultured in 1% FBS with different doses of IL-17A for 48 h. Values are the mean ± SEM of pentaplicate cultures. *, $P < 0.05$; **, $P < 0.01$ *vs.* control. The result is representative of four separate experiments using samples from different patients. B, The effect of IL-17A on the proliferation of ESCs was examined by measuring BrdU incorporation into DNA by using a cell proliferation ELISA. ESCs were treated with IL-17A at different concentrations for 48 h. Values are the mean ± SEM of the pentaplicate cultures. *Different letters* denote significant differences between groups ($P < 0.05$). The data shown are representative of four independent experiments using samples from four different patients.

survival of endometrial cells (1, 9). Our previous studies suggest that expression of IL-8 in endometriotic cells is regulated by various cytokines and enzymes (15, 16, 22). Notably, these IL-8-inducing molecules are derived from macrophages, neutrophils, and mast cells, which are suggested to play important roles in the development of the disease. The present study provides evidence that IL-17A is an additional stimulant of IL-8 secretion from ESCs and strongly suggests that Th17 cells participate in the development of endometriosis.

The synergistic effect of IL-17A and TNFα in stimulating secretion of IL-8 from ESCs was remarkable. TNFα is a proinflammatory cytokine that plays multiple roles in the progression of endometriosis. The importance of TNFα in endometriosis is underpinned by recent findings that TNFα-targeted suppression by specific drugs inhibits the development of endometriosis in baboons (23, 24). During the inflammatory response TNFα is secreted from various cells type, such as peritoneal macrophage, endometrial epithelial, and stromal cells. IL-17A may be an accelerator of endometriosis during chronic pelvic inflammation accompanied by increased TNFα production.

IL-17A-induced phosphorylation of p42/44MAPK, p38MAPK, and SAPK/JNK in ESCs, and the IL-17A-induced increase in IL-8 secretion from ESCs was suppressed by inhibitors of these kinases. This finding suggests that these enzymes are involved in the pathway of IL-17A-induced IL-8 secretion. A similar finding was reported for human airway smooth muscle cells (25). Interestingly, our previous study showed that IL-1β stimulated phosphorylation of these MAPKs, and IL-1β-induced IL-8 secretion from ESCs was inhibited by the same MAPK inhibitors (22). IL-1β is a well-known central player of the inflammatory condition of endometriosis, and thus, it can be speculated that cytokines promoting endometriosis, such as IL-1β and IL-17A, share similar pathways to induce IL-8 secretion from ESCs.

COX2, a key enzyme in prostaglandin biosynthesis, is up-regulated in endometriotic stromal cells (26–28). COX2 plays an important role in the inflammatory response associated with endometriosis and appears to function in the pathogenesis of endometriosis (29, 30). The present finding of IL-17A-induced COX2 expression in ESCs provides further evidence for a key role of IL-17A in endometriosis.

In the present study, IL-17A stimulated ESC proliferation. Human tumors show increased expression of IL-17A (31), and IL-17A promotes angiogenesis and tumor growth (32). IL-17A also enhances the proliferation of normal airway epithelial cells (33). Because IL-17A appears to exert a mitogenic effect on various cell types, IL-17A may stimulate progression of endometriosis as a result of this mitogenic effect, possibly in addition to other functions. Interestingly, IL-8 has been shown to stimulate the proliferation of endometrial stromal cells (34). Therefore, the proliferative effect of IL-17A on ESCs may be partially attributable to the increased production of IL-8 induced by IL-17A.

The proinflammatory and mitogenic action of IL-17A in ESCs demonstrated in the present study revealed possible roles of the molecule in the development of endometriosis. The prevailing medical treatment for endometriosis is suppression of ovarian hormones by GnRH analogs. The adverse effects of this treatment induced by the hypoestrogenic status often lead to a lack of compliance. IL-17A is well placed as a candidate target molecule for novel treatment strategies of endometriosis. Further investigation would benefit the development of an improved treatment option involving IL-17A.

In summary, the present study demonstrated that IL-17A stimulates inflammatory responses and proliferation of ESCs, suggesting a role for IL-17A in the pathogenesis of endometriosis.

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REPRODUCTIVE BIOLOGY

Dienogest inhibits BrdU uptake with G_0/G_1 arrest in cultured endometriotic stromal cells

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Objective: To investigate the effect of dienogest on the proliferation of endometriotic stromal cells.
Design: Comparative and laboratory study.
Setting: University of Tokyo Hospital.
Patient(s): Endometriotic stromal cells were isolated and cultured from ovarian endometriomas of patients undergoing surgery.
Intervention(s): Dienogest was added to the cultured endometriotic stromal cells.
Main Outcome Measure(s): 5-Bromo-2′-deoxyuridine (BrdU) incorporation into DNA of the endometriotic stromal cells was measured by ELISA. Cell cycle analysis of the cultured endometriotic stromal cells was performed by flow cytometry.
Result(s): Dienogest at concentration of 10^{-7} M and 10^{-6} M significantly inhibited BrdU incorporation into DNA at 24 and 48 hours. Dienogest significantly increased the cells in G_0/G_1 phase and reduced the cells in S phase and G_2/M phase in 24 and 48 hours.
Conclusion(s): The present study indicates that dienogest can inhibit the proliferation of the endometriotic stromal cells with G_0/G_1 arrest, suggesting a possible direct effect of dienogest in the treatment of endometriosis. (Fertil Steril® 2007;■:■-■. ©2007 by American Society for Reproductive Medicine.)

Key Words: Dienogest, endometriosis, proliferation, cell cycle

Endometriosis, defined by the presence of viable endometriotic tissue outside the uterus, is an enigmatic disease. Infertility and pain are the most common symptoms that debilitate women affected with endometriosis (1, 2). Although a most prevailing medical treatment for the disease is suppression of ovarian hormones by GnRH analogue (GnRH-a), its adverse effects caused by hypoestrogen often dismiss the compliance. Accordingly, development of better drugs is anticipated.

Dienogest (17α-cyanomethyl-17β-hydroxy-estra-4,9-dien-3-one) is a synthetic progestogen derived from 19-norsteroids. It has high progestational and significant antiandrogenic activity, but only moderate antigonadotrophic activity (3). Dienogest significantly decreased the volume of the endometrial implants in a rat experimental model, suggesting its effect on endometriosis (4). In humans, dienogest was highly effective on endometriotic lesions and symptoms, showing an objective endoscopic and subjective symptomatic improvement in 80% and 83%, respectively (5, 6). Furthermore, efficacy of dienogest was confirmed to be comparable with triptorelin in the treatment of endometriosis after laparoscopic surgery (7). A recent pilot study demonstrated an efficacy of long-term high-dose dienogest treatment for endometriosis (8).

The main antiendometriotic effect of dienogest has been suggested to be attributable to a central inhibition of ovulation. However, its peripheral effects are also indicated. The direct antiproliferative effect of dienogest has been shown in human eutopic endometrial stromal cells (9). In contrast, its effect on ectopic endometriotic cells remains to be elucidated. It is well known that ectopic endometrium is different from eutopic endometrium in its gene expression (10, 11). Likewise, the expression of steroid receptors differ between eutopic and ectopic endometrial tissues (12, 13). In view of these findings, the effects of dienogest may differ between eutopic and ectopic endometrial tissues.

In the present study, therefore, we examined the effect of dienogest on the proliferation of endometriotic stromal cells from ovarian endometrioma. We also examined the effect of dienogest on the cell cycle by flow cytometric analysis.

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MATERIALS AND METHODS

Tissue Collection

Endometriotic tissues were collected from the walls of ovarian endometriomas during surgery. A total of 14 patients (aged 37.6 ± 4.4 years, mean ± SD) with endometriosis were recruited in this study. All the patients had regular menstrual cycles, and none had received hormonal treatment at least 6 months before surgery. Informed consent in writing was obtained from each patient for use of the ovarian endometriotic lesion; consent forms and experimental protocols were approved by the institutional review board (IRB) of the University of Tokyo. Tissues were placed in Dulbecco's minimum essential medium (DMEM)/F12 medium (GIBCO, Grand Island, NY) on ice and transported immediately to the laboratory for separation and culture of endometriotic stromal cells. Cells obtained from each patient were used for separate experiments.

Isolation and Culture of Human Endometriotic Stromal Cells

Endometriotic stromal cells (ESC) were purified from ovarian endometriotic tissue and cultured as described previously (14, 15). The fresh endometriotic lesion collected in sterile medium was dissected free from underlying parenchyma, minced into small pieces, incubated in DMEM/F12 with 2.5 mg/mL type 1 collagenase (Sigma, St. Louis, MO) and 15 U/mL deoxyribonuclease 1 (Takara, Tokyo, Japan) for 2 hours at 37°C with agitation. The resultant suspension was separated by serial filtration. Debris was removed with a 100-μm nylon cell strainer (Becton Dickinson, Franklin Lakes, NJ), and some epithelial glands were eliminated with a 70-μm nylon cell strainer (Becton Dickinson). Stromal cells remaining in the filtrate were collected by centrifugation, resuspended in DMEM/F12, and plated onto 100-mm dishes (Iwaki, Chiba, Japan) and allowed to adhere at 37°C for 30 minutes, after which nonadhering epithelial cells and blood cells were removed with phosphate-buffered saline (PBS) rinses. The cells were cultured in DMEM/F12 reconstituted with 10% charcoal-stripped fetal bovine serum (FBS) (Hyclone, Logan, UT) and antibiotics (Sigma). The purity of ESC population was more than 95%, as confirmed by positive immunocytochemical staining for vimentin. When the cells reached confluence, they were passed into a 60- by 15-mm dish and 96-well culture plates (Becton Dickinson) at the densities of 10^6 cells/dish and 3×10^3 cells/well, respectively, in medium supplemented with 10% charcoal-stripped FBS. After 48 hours of culture, the cells were sustained with serum starvation for 24 hours and then used for experiments with 1.5% charcoal-stripped FBS in the medium.

5-Bromo-2′-Deoxyuridine Incorporation

The effect of dienogest on the proliferation of ESC was evaluated by measuring the incorporation of 5-bromo-2′-deoxyuridine (BrdU) into DNA. The BrdU incorporation was detected by using the Biotrak cell proliferation ELISA system (Amersham Pharmacia Biotech), as we reported previously (16, 17). Briefly, ESC were seeded into Falcon 96-multiwell plates at a density of 3×10^3 cells per well in 100 μL of culture medium containing 10% FBS and cultured for 48 hours. After 24 hours of serum starvation, the medium were replaced with fresh medium supplemented with 1.5% charcoal-stripped FBS containing the control vehicle or dienogest, which was dissolved in dimethyl sulfoxide and then diluted with the medium. The final concentration of dimethyl sulfoxide in the medium never exceeded 0.1%. During the last 4 hours of 24-hour and 48-hour of culture, 10 μL of BrdU solution was added and incubated at 37°C. After removing the culture medium, the cells were fixed and the DNA denatured by the addition of 200 μL/well fixative. The peroxidase-labeled anti-BrdU bound to the BrdU incorporated in the newly synthesized, cellular DNA. The immune complexes were detected by the subsequent substrate reaction, and the resultant color was read at 450 nm in the DigiScan Microplate Reader (ASYS Hitech GmbH, Eugendorf, Austria).

Flow Cytometry

The ESC were seeded into a 60- by 15-mm culture dish (Becton Dickinson) at a density of 1×10^6 cells/dish in 2 mL of the culture medium. After 48 hours of culture and later 24 hours of serum starvation, the medium was replaced with fresh medium containing 1.5% FBS and 10^{-6} M dienogest or control vehicle, and the culture was continued for an additional 24 or 48 hours. The cells were then harvested with trypsin (0.05%)/EDTA (0.02%), washed twice with ice-cold PBS (pH 7.4), and fixed with 70% ethanol at -20°C overnight. After washing twice with ice-cold PBS, the cells were incubated in 0.25 mg/mL ribonuclease solution (QIAGEN GmbH, Hilden, Germany) for 30 minutes at 37°C and stained with 50 μL/mL propidium iodide for 30 minutes, on ice, in the dark, followed by filtration through a 40-μm nylon mesh (Becton Dickinson) to remove cell clumps. A total of 2×10^5 stained cells per treatment were analyzed in the EPICS XL Flow Cytometry (Beckman Coulter, Inc., Fullerton, CA).

Statistical Analysis

The data were expressed as the mean ± SEM. Data were analyzed by Student's *t*-test for paired comparison of cell cycle analysis and one-way ANOVA with post hoc test for multiple comparisons of BrdU incorporation experiments using StatView software (SAS Institute Inc., Cary, NC). A *P* value less than .05 was accepted as statistically significant.

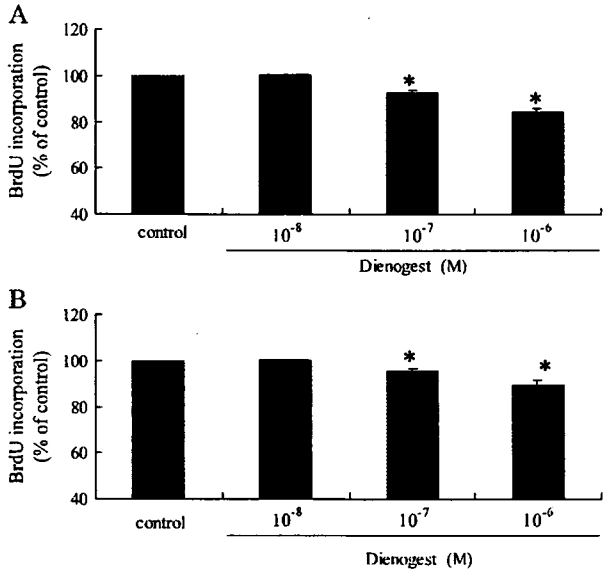
RESULTS

Effect of Dienogest on the Proliferation of ESC

The effect of dienogest on the proliferation of ESC was examined by measuring BrdU uptake in cultured ESC. As shown in Figure 1, dienogest at concentration of 10^{-7} M and 10^{-6} M induced significant suppression of BrdU incorporation into DNA at 24 and 48 hours of treatment.

FIGURE 1

Effect of dienogest on the proliferation of endometriotic stromal cells. The effect was examined by 5-bromo-2′-deoxyuridine (BrdU) incorporation using cell proliferation ELISA system. **(A)** Treatment with dienogest at concentrations between 10^{-8} M and 10^{-6} M for 24 hours. **(B)** Treatment with dienogest at concentrations between 10^{-8} M and 10^{-6} M for 48 hours. Results shown are the mean percentage of the untreated control ± SEM (bars) of hexaplicate cultures of four independent experiments. *$P<.05$ vs. control.



Fu. Antiendometriotic effect of dienogest. Fertil Steril 2007.

Effect of Dienogest on Cell Cycle in ESC

The effect of 10^{-6} M dienogest on the cell cycle in ESC was determined by flow cytometric analysis. Table 1 listed the combined data from four independent experiments. Dienogest significantly increased the cells in G_0/G_1 phase and reduced the cells in S phase and G_2/M phase in 24 and 48 hours.

DISCUSSION

In the present study, we demonstrated that dienogest inhibited the proliferation of endometriotic stromal cells in vitro. The inhibitory effect was significant from a dose of 10^{-7}, which was similar to that reported in eutopic endometrial stromal cells. It has been shown that maximum serum dienogest concentrations were reached within ~2 hours and the mean maximum serum concentrations were 0.9 x 10^{-7}, 1.7 x 10^{-7}, 3.2 x 10^{-7}, and 6.8 x 10^{-7} M after therapeutic doses of 1, 2, 4, and 8 mg, respectively (3). In addition to our finding that dienogest inhibited ESC proliferation at a dose of 10^{-7} M, dienogest at therapeutic doses conceptually yields direct suppression of the growth of endometriosis.

In recent framework of concept for etiology of endometriosis, a theory of P resistance is advocated (18). Progestins, such as medroxyprogesterone acetate (MPA) and dienogest, has been shown to inhibit the proliferation in eutopic endometrial stromal cells (9, 19, 20). However, it has also been reported that P-induced molecular changes in eutopic endometrium and ectopic endometriotic tissues of women with endometriosis are either blunted or undetectable (21–23). These observations, indicative of P resistance, were partially explained by an overall reduction of P receptors and the absence of P receptor B in the endometriotic tissue (24). In this context, it is remarkable that dienogest directly inhibited the proliferation of endometriotic cells in the present study. The present finding underscores the specific property of dienogest among progestins for endometriosis treatment.

Although antiproliferative effect of dienogest on eutopic endometrial cells has been reported, the mechanism has not been understood. Concerning this, a notable finding in the present study was that antiproliferative effect of dienogest on endometriotic stromal cells could be attributable to arrest of cell cycle in G_0/G_1. This finding seems to be similar to that P-inhibited proliferation of rat aortic smooth muscle cells with arrest of cell cycle in G_0/G_1 (25). Interestingly, P suppresses E_2-induced cell proliferation, increasing p27 protein expression in endometrial glandular cells (26). It can be thus speculated that the inhibition of the proliferation of eutopic endometrial stromal cells is also associated with G_0/G_1 arrest. Recent studies have shown that G_0/G_1 arrest of ESC was also induced by other drugs (27, 28).

TABLE 1

Effect of dienogest on the number of endometriotic stromal cells in the respective phase of the cell cycle.

	G_0/G_1	S	G_2/M
24 h	102.04% ± 0.89%[a]	92.90% ± 3.21%[a]	89.39% ± 3.91%[a]
48 h	101.88% ± 0.75%[a]	89.51% ± 5.64%[a]	88.69% ± 3.55%[a]

Note: Values are the mean ± SEM of the control from four independent experiments using samples from different women.
[a] $P<.05$ vs. control.

Fu. Antiendometriotic effect of dienogest. Fertil Steril 2007.

Except for the antiproliferative effect demonstrated in the present study, direct effect of dienogest on endometriotic stromal cell has been hitherto poorly understood. There is only one report that dienogest attenuated tumor necrosis factor-α-induced interleukin-8 production (29). Further study would be warranted to understand additional direct effects of dienogest on endometriotic lesion.

In summary, we demonstrated that dienogest inhibited proliferation of endometriotic stromal cells in vitro with G_0/G_1 cell cycle arrest. The finding subserves the understanding of the antiendometriotic effect of dienogest, a new therapeutic alternative to GnRH-a and conventional progestins.

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CORRESPONDENCE

High soluble CD44 concentration in peritoneal fluid in endometriosis

The concentration of the soluble form of CD44, a competitive inhibitor for CD44 and hyaluronic acid binding, is higher in the peritoneal fluid of patients with endometriosis than in the peritoneal fluid of patients without endometriosis. (Fertil Steril® 2007;■:■-■. ©2007 by American Society for Reproductive Medicine.)

Endometriosis is an enigmatic disease that affects women of reproductive age, causing a decline in health and often resulting in infertility (1). Implantation of endometrial tissues in retrograde menstrual flux is a widely accepted etiology of the disease. From this perspective, adhesion molecules that form connections between endometrial and mesothelial cells may play a pivotal role in the pathogenesis of endometriosis. The adhesion molecule CD44 is a type I transmembrane protein that interacts with hyaluronic acid (HA) in a wide variety of physiological and pathological processes including lymphocyte homing, cell migration, and cancer cell metastasis (2). The soluble form of CD44 (sCD44), which consists of the ectodomain of CD44, has recently emerged as a key regulator of CD44 action. Soluble CD44 can also bind to HA, acting as a competitive inhibitor for membrane-bound CD44. Although there is a growing body of evidence that CD44 is involved in endometriosis (3, 4), the importance of sCD44 is still unknown. As a first step, we investigated the sCD44 expression in the peritoneal fluid (PF) of patients with endometriosis.

A total of 63 women of reproductive age with (n = 46; age, 34.4 ± 5.4 years) and without (n = 17; age, 33.9 ± 6.0 years) endometriosis participated in this study. Informed consent was obtained from each woman before laparoscopy. All of the women had regular menstrual cycles. None of the women took medications that affect hormonal and/or immunological status in the 3 months before laparoscopy, and no patient had undergone surgical treatment for endometriosis within 1 year. At the time of surgery, 35 women were in the proliferative phase, and 28 were in the secretory phase. The stage of endometriosis was evaluated according to the revised American Society for Reproductive Medicine (r-ASRM) classification (5). The distribution of the stage of endometriosis was as follows: stage I, n = 12; stage II, n = 5; stage III, n = 12; stage IV, n = 17. PF was collected via a laparoscopic cannula introduced into the cul-de-sac before any manipulative procedure. The fluid was centrifuged at 400 *g* for 10 minutes, and the supernatants were frozen and stored at −80°C until assay. Soluble CD44 concentrations in the PF were measured using a specific enzyme-linked immunosorbent assay for sCD44 (Kamiya Biomedical, Seattle, WA). The data were described as median and interquartile range (IQR). The Mann-Whitney test was used for the statistical analysis.

The sCD44 concentrations in PF showed no significant difference between the proliferative phase and the secretory phase (data not shown). The data were then combined and analyzed regardless of the menstrual phases.The sCD44 concentrations in the PF of the women with endometriosis (median, 423ng/mL; IQR, 292–638) were significantly higher than those of the women without endometriosis (257 ng/mL, 196–337; P=.0004). As shown in Figure 1A, a positive correlation between the sCD44 concentrations of PF and r-ASRM score was observed (r = 0.536; P<.0001). The women with endometriosis were then subdivided into those with stage I/II and those with stage III/IV endometriosis. As shown in Figure 1B, the concentrations of sCD44 in stage III/IV endometriosis (460 ng/mL, 311–758) were significantly higher than those in stage I/II endometriosis (310 ng/mL, 261–442; P=.0144) and those in non-endometriosis (257 ng/mL, 196–337; P<.0001). The sCD44 concentrations in stage I/II endometriosis were also significantly higher than those in non-endometriosis (P=.0439).

This study is the first to demonstrate the presence of sCD44 in the PF of patients with endometriosis. We found a significant increase in sCD44 concentration in the PF of patients with endometriosis compared with patients without endometriosis. Furthermore, the expression of sCD44 was positively correlated with the stage of endometriosis.

It has been reported that CD44 is expressed not only in endometrial cells but also in endometriosis lesions (3, 4). Moreover, Dechaud et al., using the in vitro adhesion model, reported that interaction between endometrial cell–associated CD44 and mesothelial cell–associated HA might be crucial for the initiation of endometriosis (6).

In the light of recent evidence on the importance of soluble receptors in various physiological settings and in the pathophysiology of various diseases (7–9), it is tempting

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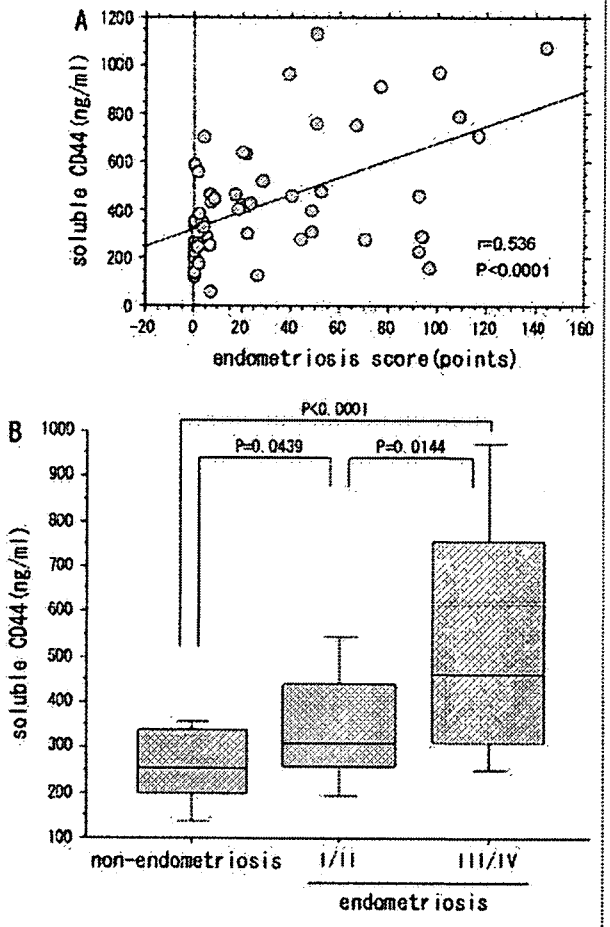
FIGURE 1

(A) Correlation between endometriosis score and soluble CD44 concentration in PF. Simple regression analysis showed that endometriosis score was positively correlated with the soluble CD44 concentration; $r = 0.536$, $P < .0001$, N = 63 (n = 17 patients without endometriosis; n = 46 patients with endometriosis). (B) Concentration of soluble CD44 in PF of women without endometriosis (n = 17), women with I/II endometriosis (n = 17), and women with III/IV endometriosis (n = 29). Boxes represent the distance between the first (25%) and third (75%) quartiles, and horizontal lines in the boxes represent medians.



Hasegawa. Soluble CD44 in endometriosis. Fertil Steril 2007.

to speculate that sCD44 in PF may have a role in the pathogenesis of endometriosis. CD44 expressing endometrial cells is delivered into peritoneal cavity via retrograde menstruation. Given that sCD44 can act as a competitive inhibitor for the interaction of membrane-bound CD44 and HA, up-regulation of sCD44 in women with endometriosis may inhibit attachment of influxed endometrial cells to the peritoneal mesothelial cells as a defense mechanism against disease progression. Because CD44 is expressed in most human cell types (2), the source of sCD44 in PF is unclear. Nevertheless, in the PF, which reflects the peritoneal environment, sCD44 is expressed at a high concentration in patients with endometriosis. In view of this finding, one can hypothesize that the relative proportion of CD44 and sCD44 in PF might be important for initiation and progression of the disease. Further study is needed to elucidate the mechanism of sCD44 up-regulation in patients with endometriosis and to understand the action of sCD44 in the pathogenesis of endometriosis.

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CASE REPORT

A case of hydrosalpinx associated with the menstrual cycle

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Objective: To describe a case report of hydrosalpinx that changed dramatically in size during the menstrual cycle.
Design: Case report.
Setting: University teaching hospital reproductive endocrinology and infertility practice.
Patient(s): A 32-year-old woman with a history of medical and surgical treatments of endometriosis who sought infertility treatment.
Intervention(s): Transvaginal ultrasonography performed sequentially during menstrual cycles.
Main Outcome Measure(s): Size of hydrosalpinx-like image.
Result(s): The size of the hydrosalpinx-like image in the left adnexal region varied; it peaked during the ovulatory period and then remarkably diminished in a cyclic manner. Laparoscopy revealed a dense adhesion between the left tubal fimbriated end and the posterior uterine wall, which led to terminal obstruction of the tube.
Conclusion(s): Change in the volume of the hydrosalpinx in this case was speculated to reflect the normal tubal fluid production regulated by ovarian hormones. (Fertil Steril® 2007;■:■–■. ©2007 by American Society for Reproductive Medicine.)

Key Words: Hydrosalpinx, ultrasound, endometriosis, menstrual cycle, tubal fluid

Hydrosalpinx is usually found as a consequence of chlamydia or other infections in the fallopian tube (1). Cases of hydrosalpinx that appear during ovarian stimulation, though very rare, have also been reported (2, 3). Here we report a unique case of hydrosalpinx, which developed in the absence of infection and hormonal treatment and changed size dramatically with the menstrual cycle. No specific institutional review board approval was sought because the patient was not enrolled in a research protocol.

CASE REPORT

The patient was a 32-year-old woman who had had endometriosis since the age of 24 years. She underwent ethanol sclerotherapy of bilateral ovarian endometrioma at age 26 years and laparoscopic cystectomy for the right endometrioma at age 30 years. She received GnRH analogue treatments at 24 and 26 years of age. She was seen for infertility treatment after 1 year of attempting to get pregnant. Her basal body temperature was biphasic, with adequate duration of high phase. Serum anti-*Chlamydia trachomatis* IgA and IgG antibodies were negative. Hysterosalpingography conducted just after menstruation showed terminal obstruction of the left fallopian tube. During ultrasonography workups to check follicular growth and ovulation, we noticed a hydrosalpinx-like structure at the left adnexal area, which varied in size and appearance with each observation. We then observed this structure sequentially by ultrasonography during menstrual cycles. Interestingly, the size of the structure was minimal during the menstrual period but increased and reached a maximum size at ovulation, to then shrink again (Fig. 1A and B). Laparoscopy revealed a dense adhesion between the left tubal fimbriated end and the posterior uterine wall, presumably caused by previous surgery and endometriosis, which subsequently led to terminal obstruction of that tube (Fig. 2). Salpingectomy was undertaken with the hope of improving IVF-ET outcome. Histologically, the structural integrity of tubal epithelium was maintained, and endometriosis was found at the serosa of the tubal wall.

DISCUSSION

This is a unique case of hydrosalpinx with menstrual cycle–dependent change during normal menstrual cycles. Although there are several reports of hydrosalpinx that enlarge during ovarian stimulation (2, 3), there has been no report of hydrosalpinx with enlargement and shrinking during menstrual cycles. Another unique aspect of this case is that no evidence of pathological damage was found within the tubal epithelium.

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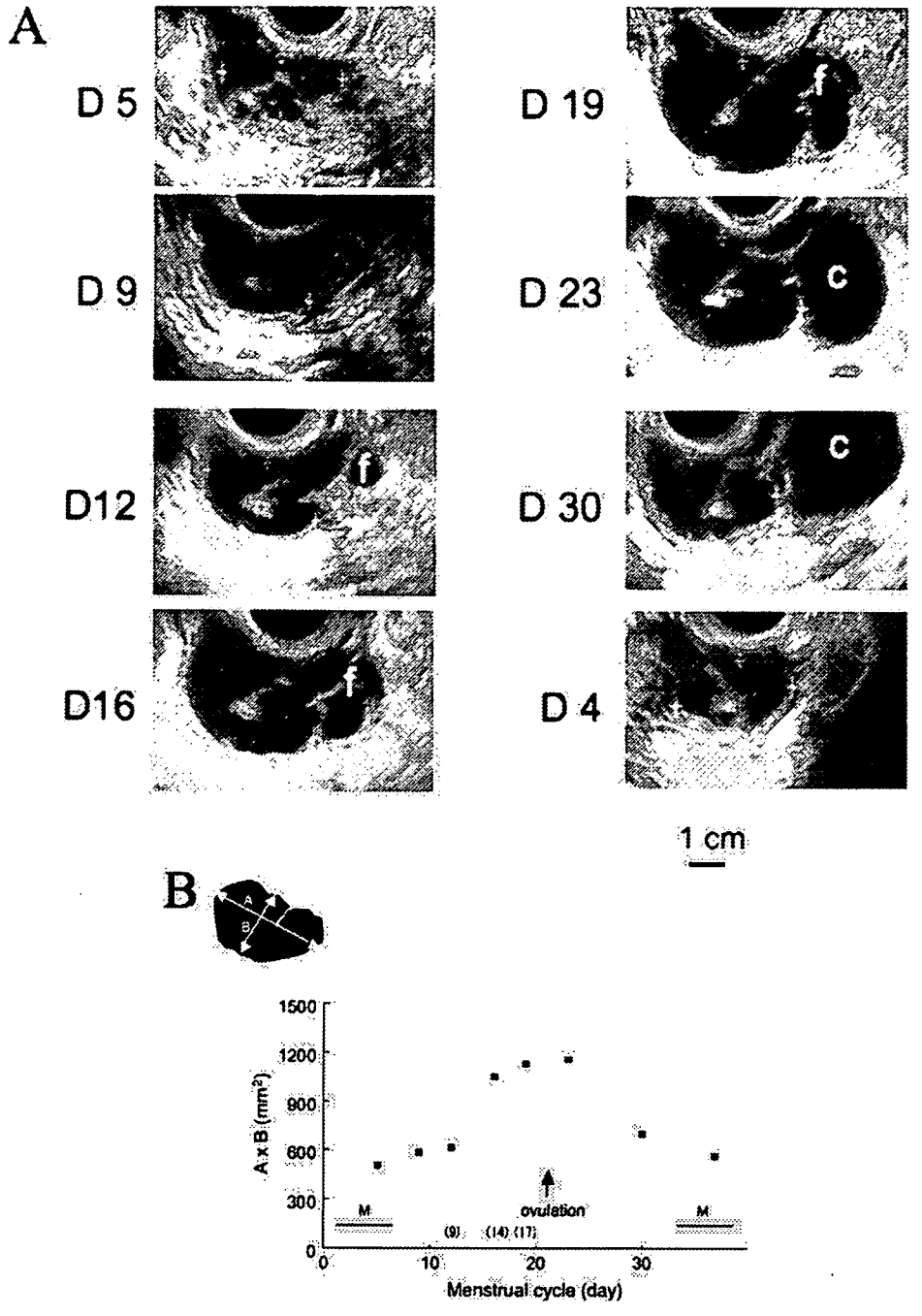
FIGURE 1

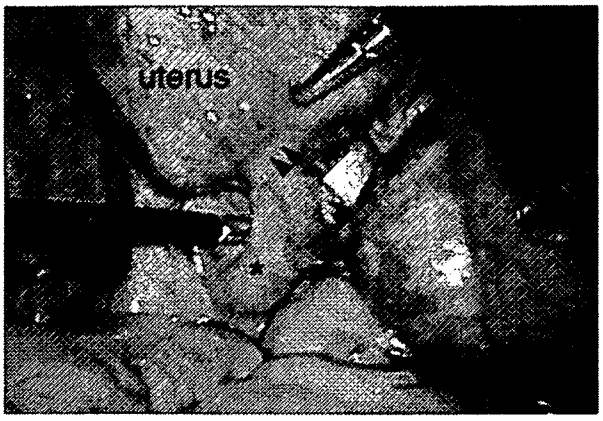
Sequential change of ultrasonographic images of the left fallopian tube in a representative menstrual cycle. **(A)** The left adnexal area was scanned by transvaginal ultrasonography on various menstrual days. A cystic part on the right side of the hydrosalpinx at day (D)12 and D16 are follicles (f), whereas D23 and D30 are corpus luteum (c). Ovulation occurred at D20, confirmed by basal body temperature. **(B)** The size of the hydrosalpinx area imaged by ultrasonography was plotted. M = menstruation. Values in parenthesis show the mean diameter (in millimeters) of the dominant follicle.



Osuga. Cyclic enlargement of hydrosalpinx. Fertil Steril 2007.

FIGURE 2

Laparoscopic finding of the left tube. A dense adhesion between the left tubal fimbriated end and the posterior uterine wall was found (*arrowheads*), which subsequently led to terminal obstruction of the left tube (*asterisk*).



Osuga. Cyclic enlargement of hydrosalpinx. Fertil Steril 2007.

Hydrosalpinx is often a sequel of chlamydial infection, which affects fallopian tube epithelial transporters and ion channels, particularly the cystic fibrosis transmembrane conductance regulator, resulting in increased epithelial secretion and decreased fluid absorption, hence an accumulation of hydrosalpinx fluid (4). In our case, however, the serologic chlamydial test was negative and the microscopic structure of the tubal epithelium was normal, suggesting that the capacity of fluid secretion and absorption in the affected tube was maintained (5). Given these observations, what was the underlying cause of the tubal fluid retention and its cyclic volume change?

It is well established in animal models that the fluid production by the fallopian tube is controlled by ovarian hormones and that the volume is highest during the ovulatory period. A recent study suggests that an ovarian hormone–regulated water channel, aquaporin 9, located in the oviductal epithelium, is involved in tubal fluid balance (6). In the human, the tubal fluid may also be regulated hormonally, reaching maximum levels during the ovulatory period. However, the fluid constantly empties into the peritoneal cavity, so that changes are not detected by ultrasonography. Only in patients undergoing ovarian stimulations, in whom ovarian hormone levels are superphysiological, fluid production is excessive and detectable by ultrasonography, when it is not effectively discharged from the tube. The resulting fluid retention could explain the etiology of hydrosalpinx during ovarian stimulation, as reported previously. On the other hand, in the present case, obstruction of the tubal terminal end would lead to fluid accumulation or a hydrosalpinx as seen by ultrasonography. Indeed, increased volume during the ovulatory period may reflect normal tubal fluid production, regulated by ovarian hormone.

In summary, we have reported a unique case of hydrosalpinx that changed size during menstrual cycles. This change is proposed to reflect a normal tubal epithelial function, producing fluid under ovarian hormone regulation.

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1 **A case of hydrosalpinx associated with the menstrual cycle**

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The authors present a unique case of hydrosalpinx that changed size during menstrual cycles, peaking during the ovulatory period and then remarkably diminishing in a cyclic manner.

The Presence of Midkine and its Possible Implication in Human Ovarian Follicles

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Keywords
Follicular fluid, folliculogenesis, growth factor granulosa cells

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Problem
Ovarian follicles undergo a dynamic change to provide a mature ovum, and the process involves angiogenesis, follicular cell proliferation and leukocyte recruitment. Midkine (MK) is a heparin-binding growth factor that has angiogenic, mitogenic, and chemotactic activities. In the present study, we investigated the presence of MK and its possible role in human ovarian follicles.

Method of study
Follicular fluid (FF) and luteinized granulosa cells (LGC) were collected from women undergoing *in vitro* fertilization and embryo transfer. Expression of MK protein in FF was examined by Western blotting. Concentrations of MK, estradiol and oxygen in FF were measured. 5-bromo-2′-deoxyuridine (BrdU) incorporation assay was performed in LGC. Normal ovarian tissues were obtained surgically and used in in-situ hybridization of MK mRNA.

Results
The presence of MK protein was verified in FF. MK mRNA was expressed in both granulosa cells and theca cells of large follicles. There is a significant negative correlation between the concentrations of MK and oxygen in FF, and a significant positive correlation between the concentrations of MK and estradiol. MK promoted BrdU uptake in LGC.

Conclusion
The present findings imply that hypoxic condition, a characteristic of growing follicles, associates with the production of MK. Given that MK is involved in granulosa cell proliferation and estradiol production in developing follicles, MK may play a role as a local regulator in the human ovary.

Introduction

Folliculogenesis is a dynamic process that involves angiogenesis, follicular cell proliferation and steroid hormone production. These phenomena are under the control of general and local regulators, such as gonadotropins, steroids, cytokines, and growth factors. Substances participating in folliculogenesis are often secreted or transported into follicular fluid (FF). Several factors have been detected in human FF, and they are suggested to play roles in the human ovary.[1–4]

Midkine (MK) is a heparin-binding growth factor. MK is a basic, low molecular weight non-glycosylated

protein, composed of two peptide chains held by disulfide bridges. MK is widely expressed in adult rat and human tissues, and strongly induced during oncogenesis, inflammation, and tissue repair.[5] MK has multiple activities such as promoting angiogenesis, cell migration, and cell proliferation.[5] Receptors of MK are considered to be a molecular complex containing receptor-type tyrosine phosphatase z (PTPz), low density lipoprotein receptor-related protein 1 (LRP1), anaplastic leukemia kinase (ALK), and syndecans.[5]

MK protein has been shown to be present in bovine FF.[6] In the rat ovary, MK mRNA is located in granulosa cells, and is increased by pregnant mare serum gonadotropin (PMSG) *in vivo*, and by follicle stimulating hormone (FSH) *in vitro*.[7,8] In addition, we have reported high concentrations of MK in FF of the human ovary.[9]

In view of pleiotropic functions of MK and its presence in the ovary, we hypothesized that MK is involved in the development of human ovarian follicles. In the present study, we first verified the presence of MK protein in FF. We also studied the gene expression of MK by *in-situ* hybridization. We then assayed concentrations of MK, ovarian hormones and oxygen in FF, and analyzed their relationships. Lastly, an effect of MK on proliferation of granulosa cells was studied.

Materials and methods

Collection of FF and Measurement of Oxygen Concentration in FF

We recruited a total of 16 women, aged between 33 and 41 years (mean, 37.5 years), undergoing the treatment of *in vitro* fertilization (IVF) for tubal factor and/or male factor. The experimental procedure was approved by the institutional review board, and signed informed consent for use of FF and luteinized granulosa cells (LGC) were obtained from each woman.

The protocol for ovarian stimulation and collection of FF was described previously.[2,3] Briefly, following ovarian suppression by the gonadotropin releasing hormone (GnRH) agonist (buserelin acetate; Suprecur, Aventis Pharma, Tokyo, Japan) from the midluteal phase of the preceding cycle, a dose of 150–300 IU human menopausal gonadotropin (hMG) (Nikken, Tokyo, Japan) was given daily until the diameter of the leading follicle reached 17 mm or greater. Then, human chorionic gonadotropin (hCG) at a dose of 10,000 IU (hCG; Mochida, Tokyo, Japan) was administered and transvaginal ultrasound-guided oocyte retrieval was performed 35 hr later.

To evaluate the relationship between concentrations of oxygen, cytokines, and steroid hormones in FF, FF from each follicle was separately aspirated without flushes. Measurement of oxygen concentration was performed as reported previously.[4] After isolation of an oocyte, oxygen concentration in aspirated FF was immediately measured with a dissolved oxygen meter (DO meter) (Iijima Electronics Corporation, Aichi, Japan). The principle of this oxygen meter is based on a diaphragm-type Galvanic cell system, which is widely applied to measure dissolved oxygen in various fluid samples.[10] The gas analysis was performed within 30 s from the aspiration. The samples were confined to a constant-temperature glass bath during the analysis. Aspirated FF was then centrifuged ($300 \times g$) for 5 min, and the supernatant was divided into several aliquots and stored at −80°C until use. In total, 21 FF samples were obtained from five women.

Isolation of LGC

Isolation and culture of LGC were processed as described previously.[11] To obtain LGC, FF was aspirated with repeated flushes during the oocyte pick-up procedure. FF contained mainly mural granulosa cells and very few cumulus cells. All the follicular aspirates and flush medium from each woman were mixed and centrifuged at $300 \times g$ for 5 min to obtain a cell pellet, which was resuspended in phosphate-buffered saline (PBS) with 0.1% hyaluronidase and incubated at 37°C in a shaking water bath for 30 min. The suspension was layered onto Ficoll-Paque Plus (Amersham Biosciences, Uppsala, Sweden) and centrifuged at $150 \times g$ for 30 min. Crude LGC fractions were recovered from the interface and washed with PBS. The cells were then treated with the monoclonal anti-CD45 antibody coupled with magnetic immunobeads (Dynal A.S, Oslo, Norway) to remove white blood cells. The cells were suspended in 2 mL medium containing 2% fetal bovine serum (FBS) and incubated for 20 min at 4°C with anti-CD45 immunomagnetic beads. Subsequently, the suspension was placed into a magnetic test tube rack (Dynal A.S) for 2 min at room temperature to remove immunobeads-bound white blood cells from the cell suspension. With this method, remaining CD68 (a marker of monocyte/macrophages) positive cells were <1% in isolated LGC by immunostaining.

Heparin-Sepharose Chromatography

Midkine in FF was partially purified by heparin-sepharose affinity chromatography. Heparin-sepharose chromatography was performed as described previously.[2] The slurry of 1.0 g Heparin Sepharose CL-6B (Amersham Biosciences) was incubated with approximately 100 mL FF with gentle stirring for 3 hr at 4°C. The mixture was poured into an open column. The packed column was washed with PBS and eluted with 20 mL NaCl in stepwise concentrations of 0.2, 0.4, 0.6, 0.8, and 1.0 M. The eluted fractions were further concentrated by Centricon YM-10 (Millipore, Billerica, MA, USA) and tested for Western blotting.

Western Blotting

Samples were resolved by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Proteins were blotted onto a nitrocellulose membrane and incubated with rabbit anti-MK primary antibody (1:200), which was kindly provided by Cell Signals (Yokohama, Japan), and anti-rabbit horseradish peroxidase secondary antibody (1:1000, Amersham Biosciences). Immune complexes were visualized by use of an ECL Western blotting system (Amersham Biosciences).

RNA Extraction and RT-PCR of MK mRNA and its Receptor LRP1 mRNA

Total RNA was extracted from isolated LGC using an RNeasy Mini Kit (Qiagen, Hilden, Germany). RT-PCR was performed using Rever Tra Dash (Toyobo, Tokyo, Japan). One microgram of total RNA was reverse-transcribed in a total volume of 20 μL, and cDNA was amplified using oligonucleotide primers based on the sequences of human MK and human LRP1. The human GAPDH primers (Toyobo) were used to ensure RNA quality and amounts. MK primers (sense, 5′-CCTGCAACTGGAAGAAGGAG-3′; antisense, 5′-AGCAGACAGAAGGCACTGGT-3′) were chosen to amplify a 320 bp fragment. LRP1 primers (sense, 5′-CACCTTAACGGGAGCAATGT-3′; antisense, 5′-GTCACCCCAGTCTGTCCAGT-3′) were chosen to amplify a 240 bp fragment. PCR conditions for the amplifications of MK, LRP1, and GAPDH were 30 cycles at 98°C for 10 s, 60°C for 2 s, and 74°C for 20 s. PCR products were analyzed by agarose gel electrophoresis with ethidium bromide. Each PCR product was purified with a QIAEX II gel extraction kit (Qiagen), and their identities were confirmed using an ABI PRISM 310 genetic analyzer (Applied Biosystems, Foster City, CA, USA).

Collection of Ovarian Tissues

Tissue specimens of human ovaries were obtained from four women (age range, 28–40 years) who underwent salpingo-oophorectomy for the treatment of uterine cervical cancer under signed informed consent. All patients had normal regular ovarian cycle before operation and no histological abnormality of ovarian tissues. At the time of operation, all patients were in the late follicular phase and their ovaries had large follicles (>9 mm). The experimental procedure was approved by the institutional review board.

In Situ Hybridization

Ovarian tissues were fixed in neutral-buffered formalin and embedded in paraffin blocks. Sections were prepared from the blocks and stained with hematoxylin and eosin.

For preparation of the digoxigenin (DIG)-labeled RNA probe for MK, the 320-bp fragment of the human MK cDNA, obtained by RT-PCR with the primers described above, was subcloned into the appropriate restriction site of the PCR II-TOPO vector (Invitrogen, Carlsbad, CA, USA). After linearization of the plasmid with an appropriate restriction enzyme, the linearized vectors were used as templates for the synthesis of DIG-labeled RNA probes using SP6 or T7 RNA polymerase. In situ hybridization was performed using an ISHR starting kit (Nippon Gene, Toyama, Japan) as described previously.[12,13] Briefly, tissues collected for in situ hybridization were fixed by immersion in 10% neutral-buffered saline overnight at 4°C before routine paraffin embedding. The paraffin-embedded specimens were sliced at a 5-μm thickness. These sections were mounted on poly-l-lysine-treated slides, deparaffinized, and rehydrated. They were further digested with 5 mg/mL proteinase K for 10 min at room temperature, treated with acetic anhydride, and then subjected to treatment with pre-hybridization solution for 30 min at 42°C. Hybridization was carried out by applying the diluted probe to each slide section. Each section was incubated in a humidified chamber overnight at 42°C. Slides were washed and then treated with RNase for 30 min at