Interleukin (IL)-17A Stimulates IL-8 Secretion, Cyclooxygensase-2 Expression, and Cell Proliferation of Endometriotic Stromal Cells

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IL-17A is secreted from Th17 cells, a discovery leading to revision of the mechanism underlying the role of Th1/Th2 in the immune response. Strong evidence suggests that immune responses associated with inflammation are involved in the pathogenesis of endometriosis. In the present study, we first demonstrated that the presence of Th17 cells in peritoneal fluid of endometriotic women by flow cytometric analysis and IL-17A-positive cells in endometriotic tissues by immunohistochemistry. To investigate the role of IL-17A in the development of endometriosis, we then studied the effect of IL-17A on IL-8 production, cyclooxygenase-2 expression, and cell proliferation of cultured endometriotic stromal cells (ESCs). IL-17A enhanced IL-8 secretion from ESCs in a dose-dependent manner. The IL-17A-induced secretion of IL-8 from ESCs was suppressed by anti-IL-17 receptor A antibodies or inhibitors of p38 MAPK, p42/44 MAPK, and stress-activated protein kinase/c-Jun N-terminal kinase. Addition of TNFα synergistically increased IL-17A-induced IL-8 secretion from ESCs. IL-17A also enhanced the expression of cyclooxygenase-2 mRNA and proliferation of ESCs. IL-17A may play a role in the development of endometriosis by stimulating inflammatory responses and proliferation of ESCs. (Endocrinology 149: 1260–1267, 2008)
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, and phospholipid (PMA) (St. Louis, MO), charcoal-stripped fetal bovine serum (FBS) was from Hyclone (Logan, UT). DMEM/Ham’s F12 (DMEM/F12) and deoxyribonucleic acid (DNA) 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 was 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. PFMCs 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. The sections were incubated with avidin-biotin peroxidase complex (Vector Laboratories, Burlingame, CA), the sections were incubated with 5% FBS medium with IL-17A (10 ng/ml) for different time periods up to 24 h.

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 deoxyribonuclease 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 (Nippon-gene, Toyama, Japan). Using an RTnase 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 were used in PCR are shown in Table 1.
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°C 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 manufacturer’s instructions. Briefly, ESCs were seeded into a 96-well plate (Becton Dickinson) at a density of $5 \times 10^4$ 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<sub>2</sub>-95% air environment and allowed to grow to approximately 30% confluence. The cells were fixed with cold methanol/aceton 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 and evaluated under a light microscope.

**Western blotting**

Cultured cells in 6-well plates were homogenized in lysis buffer containing 50 µM 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 secretory 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.

**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...
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-
Analysis 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 immunity 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 immunity has advanced into a new era. In this context, the presence of Th17 cells in PFMCs demonstrated in our study might lead the 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 chemotraction and activation of neutrophils, angiogenesis, stimulation of proliferation, and
pendent experiments using samples from four different patients. B, The effect of IL-17A on the proliferation of ESCs is representative of four separate experiments using samples from different patients. 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. *, P < 0.05; **, P < 0.01 vs. control. The result is representative of four separate experiments using samples from different patients.

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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