Down-Regulation of Interleukin-1 Receptor Type 1 Expression Causes the Dysregulated Expression of CXC Chemokines in Endometriotic Stromal Cells: A Possible Mechanism for the Altered Immunological Functions in Endometriosis

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To evaluate the involvement of chemokines in the pathogenesis of endometriosis, we investigated the expression of CXC chemokines in cultured ovarian endometriotic cyst stromal cells (ECSC), endometrial stromal cells with endometriosis (ESCwE), and normal endometrial stromal cells (NESC). Using ELISA, TNF-α significantly enhanced the production of IL-8, growth-related oncogene α, and epithelial neutrophil-activating peptide-78 in all cases of ECSC (n = 10), ESCwE (n = 6), and NESC (n = 10). IL-1β did not affect the production of these chemokines in eight of 10 cases of ECSC. In contrast, IL-1β significantly enhanced the expression of these chemokines in all cases of ESCwE (n = 6) and NESC (n = 10). Western blot analysis revealed down-regulation of expression of IL-1 receptor type 1 (IL-1-R1) in all cases of ECSC with low response to IL-1β (n = 8). In contrast, significant IL-1-R1 expression was detected in all cases of NESC. Although IL-1-R1 expression was detected in all cases of ESCwE (n = 6), its expression in ESCwE tended to decrease compared with that in NESC. Moreover, phosphorylation of inhibitor α was detected in all cases of ESCwE and NESC after stimulation with IL-1β, but not in ECSC with low response to IL-1β (n = 8). In contrast, significant IL-1-R2 expression was detected in all cases of ECSC, ESCwE, and NESC. The present findings suggest that the dysregulation of IL-1/IL-1-R system relates to immunological dysfunction in endometriosis. The alteration of the CXC chemokines expression may be important for elucidation of the pathogenesis of endometriosis. (J Clin Endocrinol Metab 89: 5094–5100, 2004)

ENDOMETRIOSIS IS A common chronic inflammatory disease affecting 3–10% of reproductive women; it is associated with chronic pelvic pain, dysmenorrhea, and infertility (1). Infertility is often determined during examination of the patients with endometriosis and is a critical problem for these patients. In addition to the obstruction of fallopian tubes due to adhesion, endometriosis negatively affects embryo implantation and development in the early stage and sperm motility, which suggests the involvement of immunological changes associated with this disease (1–5). However, the underlying fundamental mechanisms of altered immunological status are still unknown.

The most widely accepted theory about the pathogenesis of endometriosis is that it is a consequence of implantation of viable endometrial tissues in the pelvis via retrograde menstruation (6, 7). It has been suggested that the i.p. environment is important in the formation of endometriotic lesions (1, 7, 8). Estrogen encourages the development of endometriotic lesions, and progesterone inhibits disease progression (9). Besides these ovarian steroid hormones, recent studies have reported that a variety of cytokines, such as IL-1 (10), IL-6 (11), IL-8 (12), interferon-γ (IFN-γ) (13), and TNF-α (14), form complex networks and play important roles in the growth and expansion of endometriotic lesions.

Chemokines are a large superfamily of structurally and functionally related molecules with chemotactic activity targeted at specific leukocyte populations. They are 70–90 amino acids in length and are divided into four major subfamilies based on the relative positions of their cysteine residues (CC, CXC, C, and CX3C) (15–17). The CXC chemokine subfamily includes IL-8, growth-related oncogene α (GROα), GROβ, GROγ, epithelial neutrophil-activating peptide 78 (ENA-78), granulocyte chemotactic protein-2, and neutrophil-activating protein-2, many of which have been shown to chemoattract and activate neutrophils (15–18). Normal endometrial stromal cells (NESC) have been reported to produce and secrete various CXC chemokines, including IL-8 (19), GROα (20), and ENA-78 (21), especially after stimulation with proinflammatory cytokines, such as IL-1 and TNF-α. The expression of these chemokines has been suggested to be important in menstruation and implantation and in the maintenance of early pregnancy (22). These chemokines are also reported to be expressed in endometriotic tissues (23, 24).

Many studies have reported the immunological disorders...
in the pelvic cavity of endometriosis. A variety of cytokines, such as IL-1 (10), IL-6 (8), IL-8 (12), and ENA-78 (25), have been reported to be increased in the peritoneal fluid of endometriotic patients. These chemokines are considered very important in the pathogenesis of endometriosis, but there have been few reports on the expression of chemokines in eutopic and ectopic endometrial tissue of endometriotic patients.

In this study we investigated the production of CXC chemokines (IL-8, GROα, and ENA-78) in cultured endometriotic cyst stromal cells (ECSC), eutopic endometrial stromal cells with endometriosis (ESCwE), and NESC and the expression of IL-1 receptor type 1 (IL-1-R1), IL-1-R2, TNF receptor type 1 (TNF-R1), and TNF-R2 in these cells, and we discuss the involvement of altered immunological function in the pathogenesis of endometriosis.

Materials and Methods
ECSC, ESCwE, and NESC isolation procedures and cell culture conditions

NESC (n = 10) were obtained from premenopausal patients who had undergone hysterectomies for leiomyoma. ESCwE (n = 6) were obtained from premenopausal patients who had undergone hysterectomies for endometriosis. ECSC were obtained from premenopausal patients who had undergone salpingo-oophorectomy or evisceration for ovarian endometriotic cysts (n = 10). All patients had been free of any hormonal treatments before the operation. All specimens were diagnosed as being in the mid to late secretory phase using a standard histological examination of endometrial tissues. This study was approved by the institutional review board of the Faculty of Medicine, Oita University, and written informed consent was obtained from all patients.

ECSC were isolated from endometriotic tissues according to the method described by Osteen et al. (26). Briefly, the tissues were minced in Hanks’ balanced salt solution and digested with 0.5% collagenase (In VitroGen Life Technologies, Gaithersburg, MD) in DMEM (In VitroGen Life Technologies) at 37 C. The dispersed cells were filtered through a 70-µm pore size nylon mesh to remove the undigested tissue pieces. The filtrated fraction was separated from epithelial cell clumps by differential sedimentation at unit gravity as follows. The cells were resuspended in 2 ml culture medium and layered slowly over 10 ml of the medium in a centrifuge tube. Sealed tubes were placed in an upright position at 37 C in 5% CO2 in air for 30 min. After sedimentation, the top 8 ml medium were collected. Finally, the medium containing stromal cells was filtered through a 40-µm pore size nylon mesh. Final purification was achieved by allowing stromal cells (which attach rapidly to plates) to adhere selectively to culture dishes for 30 min at 37 C, followed by the removal of nonadhering epithelial cells. ESCwE and NESC were also isolated by digesting the endometrial tissues fragments with 0.5% collagenase, as previously described (21). Isolated ECSC, ESCwE, and NESC were cultured in DMEM supplemented with 100 IU/ml penicillin (In VitroGen Life Technologies), 50 mg/ml streptomycin (In VitroGen Life Technologies), 2.5 µg/ml amphotericin B (Sigma-Aldrich Corp., St. Louis, MO), and 10% heat-inactivated fetal bovine serum (FBS; Invitrogen Life Technologies) at 37 C in 5% CO2 in air.

ECSC, ESCwE, and NESC were in monolayer culture after the third passage were more than 99% pure, as analyzed by immunocytochemical staining with antibodies to vimentin (V9; Dako, Copenhagen, Denmark), cytokeratin (Dako), factor VIII (Dako), and leukocyte common antigen (2B11+P97/26, Dako) and were used for the following experiments.

Detection by ELISA of IL-8, GROα, and ENA-78 in culture media of ECSC, ESCwE, and NESC

To study the production of IL-8, GROα, and ENA-78 by ECSC, ESCwE, and NESC, we plated 5 x 104 cells on six-well culture plates (Corning Glass, Corning, NY) and cultured the cells until they were fully confluent. The supernatant was then replaced with 1 ml fresh culture medium containing various amounts of recombinant human IL-1β (0.001–1 ng/ml; R&D Systems, Minneapolis, MN) and recombinant human TNF-α (0.1–100 ng/ml; R&D Systems). Under these conditions, the supernatant was collected 24 h after stimulation and stored at −70 C until assay. Isolated cells from individual patients were used in each experiment, and each experiment was performed in triplicate. All experiments were performed in the presence of 10% heat-inactivated FBS. The concentrations of IL-8, GROα, and ENA-78 were determined in each supernatant with commercially available ELISA kits (R&D Systems). The sensitivities of the assay were 10 pg/ml for IL-8, 10 pg/ml for GROα, and 15 pg/ml for ENA-78.

Western blotting analysis

ECSC, ESCwE, and NESC were plated in 100-mm dishes and cultured to confluence. Then TNF-α (100 ng/ml/ml) and IL-1β (1 ng/ml) were added to the dishes, which were cultured for 5 min. The time for stimulation was determined by a time-course study performed as a background experiment. After stimulation, the cells were washed with PBS, and whole cell extracts were prepared by lysing the cells in lysis buffer (50 mM Tris-HCl, 125 mM NaCl, 0.1% Nonidet P-40, 5 mM EDTA, 50 mM NaF, and 0.1% phenylmethylsulfonyl fluoride). The suspension was centrifuged at 15,000 rpm for 15 min at 4 C, and the supernatant was collected. The total protein concentration was quantified using the Coomassie protein assay reagent (Pierce Chemical Co., Rockford, IL). The whole cell protein extract was resolved with SDS-PAGE using a 10% polyacrylamide gel under reduced conditions. After transfer to Immobilon-P transfer membrane (Millipore Corp., Bedford, MA), the protein was stained with Ponceau S (Sigma-Aldrich Corp.) to verify uniform loading and transfer. Membranes were blocked with 5% skim milk (BD Biosciences, Sunnyvale, CA) in Tris-buffered saline with Tween 20 (50 mM Tris-HCl, 150 mM NaCl, and 0.1% Tween 20, pH 7.4; TBS-T) overnight and subsequently incubated with primary antibodies [IL-1-R1, IL-1-R2, TNF-R1, and TNF-R2 (Rockland, Gilbertsville, PA); nonphosphoserine inhibitor β (β-I; non-β (β-I); phosphoserine (pβ-I); Cell Signaling, Beverly, MA); and glyceraldehyde-3-phosphate-dehydrogenase (GAPDH; Ambion, Inc., Austin, TX)] with appropriate dilution for 1 h at room temperature. The membrane was washed three times with TBS-T and incubated with the appropriate horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Subsequently, the membrane was washed three times with TBS-T and analyzed by enhanced chemiluminescence (Amersham Biosciences, Arlington Heights, IL).

Preparation of IL-1-R1 cDNA fragment by RT-PCR

To evaluate the expression of IL-1-R1 mRNA in ECSC, ESCwE, and NESC by Northern blot analysis, we amplified the IL-1-R1 transcript by means of the RT-PCR method using an RNA PCR kit with AMV reverse transcriptase (Pharmacia Biotech, Piscataway, NJ) as previously described (27). Total RNA was isolated from NESC using TRIzol reagent (In VitroGen Life Technologies) according to the manufacturer’s instructions and was reverse transcribed into cDNA. To perform the PCR, primer sets for IL-1-R1 (sense primer, 5′-AAGTTGAGGATTCAGGACAT-3′; antisense primer, 5′-AGGTATCTTGGACTCCACTCA-3′) (28) were synthesized by the phosphoramidite method on a DNA synthesizer (model 8700; Biosearch, San Rafael, CA) and purified on Sephadex G-50 columns (Pharmacia Biotech, Piscatway, NJ) and by HPLC. The predicted size of the PCR product was 284 bp. The cDNA transcribed from 1 µg total RNA was amplified using a thermal cycler (model P2000; PerkinElmer, Norwalk, CT). The PCR with primer pairs for IL-1-R1 was performed for 35 cycles, with each cycle consisting of a denaturation step of 94 C for 45 sec, an annealing step of 55 C for 45 sec, and an extension step of 72 C for 60 sec. The PCR products were separated by 1.5% agarose gel (Takara) electrophoresis and visualized by ethidium bromide (Takara) staining. The PCR products were cloned using a TA cloning kit (In VitroGen Life Technologies, Leek, The Netherlands) and used as the probe for Northern blot analysis. Sequence analysis of the PCR products was also performed to confirm that the amplified cDNA was IL-1-R1.

Northern blot analysis for IL-1-R1 mRNA expression in ECSC, ESCwE, and NESC

To study the expression of IL-1-R1 mRNA in ECSC, ESCwE, and NESC, 5 x 104 cells of ECSC with low responses to IL-β (n = 4), ESCwE
(n = 4), and NESC (n = 4) were plated on 75-cm² culture flasks (Corning Glass) in 15 ml culture medium with 10% heat-inactivated FBS and cultured until fully confluent. Total RNA was isolated from ECSC, ESCwE, and NESC using TriZol reagent according to the manufacturer's instructions. Northern blotting was performed as previously described (27). The expression of β-actin mRNA was also examined as an internal control. The relative expression of IL-1-R1 and β-actin mRNA was analyzed using the public domain Image program 1.61 developed at National Institutes of Health (Bethesda, MD).

**Statistical analysis**

Data are presented as the mean ± so and were appropriately analyzed using the Mann-Whitney U test and the Bonferroni/Dunn test with StatView 4.5 (Abacus Concepts, Inc., Berkeley, CA). P < 0.05 was accepted as statistically significant.

**Results**

Levels of IL-8, GROα, and ENA-78 in the culture medium without cells were below the levels of detection. Low levels of IL-8, GROα, and ENA-78 protein were detected in the culture medium of nonstimulated ECSC, ESCwE, and NESC incubated for 24 h. As shown in Fig. 1, the concentrations of these three chemokines stimulated by TNF-α (10 ng/ml) were higher (>2.5-fold) than those without stimulation in all specimens of ECSC (n = 10), ESCwE (n = 6), and NESC (n = 10). With IL-1β stimulation (0.1 ng/ml), the chemokine concentrations were increased more than 2.5-fold compared with those under nonstimulated conditions in all ESCwE (n = 6) and NESC (n = 10) specimens, whereas only two of 10 ECSC specimens showed more than a 2.5-fold increase in the chemokine concentration.

As shown in Fig. 2, levels of IL-8, GROα, and ENA-78 were increased in parallel with the addition of TNF-α in all cases of ECSC (n = 10). In contrast, IL-1β did not affect the production of IL-8, GROα, or ENA-78 in eight of 10 specimens of ECSC (low response group). The results for the remaining two specimens (high response group) were similar to those of ESCwE and NESC (date not shown). Levels of IL-8, GROα, and ENA-78 increased with the addition of increasing amounts of TNF-α and IL-1β in all cases of NESC (n = 10) and ESCwE (n = 6).

To analyze the underlying mechanisms of the above findings, we evaluated the signal pathways of IL-1β- and TNF-α-stimulated CXC chemokine production in ECSC, ESCwE, and NESC. As shown in Fig. 3, significant expression of IL-1-R1 was detected in all cases of NESC (n = 10), but only a faint expression of IL-1-R1 was observed in all cases of ECSC with low response to IL-1β (n = 8). IL-1-R1 expression...
in ECSC with high response to IL-1β (n = 2) and ESCwE (n = 6) was also detected; however, its expression in these cells tended to decrease compared with that in NESC. Significant expression of IL-1R2, TNF-R1, and TNF-R2 was detected in all cases of ECSC (n = 10), ESCwE (n = 6), and NESC (n = 10). The expression of these four receptors after 24-h stimulation by TNF-α and IL-1β was unchanged compared with that under unstimulated conditions (date not shown). As
shown in Fig. 4, nonstimulated ECSC, ESCwE, and NESC revealed significant non-pIκB-α and faint pIκB-α protein expression. pIκB-α protein was not detected after 5-min stimulation by IL-1β (1 ng/ml) in ECSC with a low response to IL-1β (n = 8), whereas a significant increase in pIκB protein was observed in ECSC with a high response to IL-1β (n = 2), ESCwE (n = 6), and NESC (n = 10). In contrast, after stimulation by TNF-α (100 ng/ml), a significant amount of pIκB-α protein was detected in all cases of ECSC (n = 10), ESCwE (n = 6), and NESC (n = 10). GAPDH was detected in all samples at almost equal quantities.

As shown in Fig. 5, significant expression of IL-1R1 mRNA in ESCwE (n = 4) and NESC (n = 4) was observed. However, the expression of IL-1-R1 mRNA was significantly decreased in ECSC with a low response to IL-1β (n = 4). IL-1-R1 mRNA expression tended to decrease in ESCwE compared with that in NESC. β-Actin mRNA was detected in all samples at almost equal quantities.

Discussion

CXC chemokines are believed to be involved in the pathogenesis of endometriosis (1–5). However, only IL-8 expression has been evaluated in endometriotic tissues (23). In the present study we demonstrated that the other CXC chemokines, GROα and ENA-78, are also expressed in cultured ECSC. Furthermore, the expression of IL-8, GROα, and ENA-78 was up-regulated in ECSC, ESCwE, and NESC after stimulation by TNF-α. Phosphorylation of pIκB-α was detected after stimulation with TNF-α in ECSC, ESCwE, and NESC. The effects of TNF-α on ECSC and ESCwE were similar to those on NESC. IL-1β also enhanced the expression of these three chemokines by ESCwE and NESC, but IL-1β stimulation did not affect the expression of these three chemokines in most cases of ECSC. These results are supported by the observation that the expression of IL-1-R1 mRNA and protein was detected in NESC, but significantly decreased in ECSC. Furthermore, phosphorylation of IκB-α after IL-1β stimulation, an important intracellular signaling procedure in CXC chemokine production (29), was detected in ESCwE and NESC, but not in ECSC.

These results suggest that the regulatory mechanisms of local chemokine expression are partially altered in endometriosis. Previous studies have demonstrated that the levels of various substances in the pelvic cavity that are important in the pathogenesis of endometriosis are altered when the disease is present (1, 7, 8). Sakamoto et al. (30) reported that IL-8 was produced in endometriotic tissues, especially after TNF-α stimulation. Their findings are consistent with our present results. Lebovic et al. (31, 32) reported that the production of regulated upon activation, normal T cell-expressed and secreted (RANTES), vascular endothelial growth factor, and IL-6 was enhanced by IL-1β stimulation. Akoum et al. (33) reported that macrophage inflammatory protein-1α expression was increased by estradiol and IL-1β-stimulation. However, relatively high doses of IL-1β were used in these studies. In the present study we demonstrated that the expression of IL-1-R1 protein and mRNA was down-regulated in most cases of ECSC, and IL-1β did not affect the production of CXC chemokines in these cases of ECSC. Furthermore, we indicated that the expression of IL-1-R1 protein and mRNA in ESCwE tended to decrease compared with that in NESC. It is suggested that the alteration of this receptor expression has already begun at a eutopic location before the development of endometriotic lesions. Furthermore, this may mean that the down-regulation of the IL-1/IL-1-R system in endometriotic lesions induces changes in various cellular functions in affected cells in addition to the altered chemokine production, causes abnormality of the immune system in the pelvic cavity, and leads to infertility, especially because of implantation failure of blastocysts.

![Fig. 4. Effects of TNF-α (A; 100 ng/ml) and IL-1β (B; 1 ng/ml) stimulation on the levels of non-pIκB-α, pIκB-α, and GAPDH protein in ECSC, ESCwE, and NESC. The figure shows a representative result.](image)
Iwabe et al. (23) demonstrated that IL-8 exerts its growth-promoting actions in endometriotic cells as well as in normal endometrial cells. Like IL-8, ENA-78 and GROs belong to the CXC chemokine family, so it is expected that these chemokines may play similar roles in the development of endometriosis.

There is much evidence suggesting that cytokines such as IL-1 play a critical role in the process of blastocyst implantation (34–37). The expression of IL-1-R1 mRNA occurs during the entire menstrual cycle in normal human endometrial stromal and epithelial cells, but it is significantly increased in both the early and late secretory phases (34, 38). Several studies have reported that blocking of IL-1-R1 prevents embryo implantation. Lindhard et al. (36) reported that mice treated with an IL-1-R antagonist express a lower level of integrins that are active during the adhesion process during endometrial receptivity, and that the murine embryos treated with an IL-1-R antagonist had the capacity to implant and develop normally when transferred to normal mice. Given this finding, IL-1-R seems to be an important cytokine when pregnancy, especially implantation, occurs.

Various cytokines, such as IL-1, TNF-α, IL-8, IL-13, IL-15, and IFN-γ, have been reported to be differently expressed among eutopic and ectopic endometrial cells with endometriosis and NESC (24, 39). Sotnikova et al. (24) reported the differential expression of cytokines, including TNF-α, IL-1β, IFN-γ, and IL-8 in eutopic and ectopic endometrial cells with endometriosis and NESC. They reported that TNF-α expression in eutopic and ectopic endometrial cells with endometriosis was significantly enhanced compared with that in NESC. In contrast, IL-1β expression in ectopic endometrial cells with endometriosis was reduced compared with that in eutopic endometrial cells with or without endometriosis. These results are consistent with our present results, which indicated the decrease in IL-1-R1 mRNA levels in ECSC. However, the underlying mechanism of this phenomenon is still unclear. Additional research on genomic rearrangement is required. Akoum et al. (40) reported that the expression of IL-1-R2, which is described as an IL-1 decoy receptor and plays an important physiological role in IL-1 regulation as a competitor of IL-1-R1; in endometriotic cells, it decreases compared with the level in NESC. However, in our present study there were no differences in IL-1-R2 expression among ECSC, ESCwE, and NESC.

In summary, we demonstrated for the first time that IL-1-R1 protein and mRNA expressions are down-regulated in most cases of ECSC, leading to dysregulated expression of CXC chemokines. A poorly regulated immunological status due to the lack of IL-1-R1 in ECSC may lead to reproductive failure. At present, the significance of our findings is not fully understood, but we hope that these discoveries will be useful in elucidating the pathogenesis of endometriosis.

Acknowledgments

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