Interleukin-4 induces expression of eotaxin in endometriotic stromal cells

Zhuo OuYang, M.D., Yutaka Osuga, M.D., Ph.D., Yasushi Hirota, M.D., Ph.D., Tetsuya Hirata, M.D., Ph.D., Osamu Yoshino, M.D., Ph.D., Kaori Koga, M.D., Ph.D., Tetsu Yano, M.D., Ph.D., and Yuji Taketani, M.D., Ph.D.

Department of Obstetrics and Gynecology, Faculty of Medicine, University of Tokyo, Tokyo, Japan

Objective: To study the relationship between eotaxin and interleukin-4 (IL-4) in the pathophysiology of endometriosis.

Design: Comparative and laboratory study.

Setting: University teaching hospital reproductive endocrinology and infertility practice.

Patient(s): Ectopic endometrial tissues were collected from women with endometriosis.

Intervention(s): Ectopic endometrial stromal cells (ESCs) were isolated and cultured with IL-4. Ectopic endometriotic tissues were immunostained for eotaxin and IL-4.

Main Outcome Measure(s): Gene expression of eotaxin was determined by standard and real-time reverse-transcriptase polymerase chain reaction. Secretion of eotaxin from ESC was measured using specific ELISA. The immunostained sections were examined.

Result(s): Interleukin-4 (IL-4) increased mRNA expression and protein secretion of eotaxin from ESC in a dose-dependent manner. Immunohistochemical analysis showed that eotaxin-positive cells colocalized with IL-4–positive cells and accumulated around the blood vessels in the stroma of endometriotic tissue.

Conclusion(s): IL-4 induces eotaxin in ESCs, which might promote angiogenesis and the subsequent development of endometriosis. (Fertil Steril® 2010;94:58–62. ©2010 by American Society for Reproductive Medicine.)

Key Words: Endometriosis, IL-4, eotaxin

Endometriosis, defined by the presence of viable endometriotic tissue outside the uterus, is an enigmatic disease. It deteriorates the health of women of reproductive age, causing pain and infertility (1, 2). Multiple lines of evidence suggest that inflammation and immune responses play a pivotal role in the pathogenesis of endometriosis (3, 4). Recently, T lymphocytes have been identified as playing important roles in immunoinflammatory reactions associated with endometriosis (5, 6). In particular, Th2-type immune responses are activated in women with endometriosis (7, 8) and the level of IL-4, a typical Th2 cytokine, is increased in endometriotic tissues (9). Interleukin-4 stimulates the proliferation of endometriotic stromal cells (ESCs) (6), suggesting that IL-4 is a key player in the development of endometriosis.

Eotaxin, also called CCL11, is a chemokine with a potent chemoattractive activity for eosinophils, basophils, and Th2 lymphocytes (10), and is implicated in allergic responses (11). Eotaxin also has an angiogenic activity (12). Eotaxin is present in the endometrium and in endometriotic lesions, and the concentration of eotaxin is elevated in the peritoneal fluid of women with endometriosis (13). Taken together, these findings imply that eotaxin may have a role in the progression of endometriosis. Because IL-4 induces eotaxin secretion in human airway smooth muscle cells (14), dermal fibroblasts (15), and nasal fibroblasts (16), we speculated that IL-4 may also regulate eotaxin expression in endometriotic cells and contribute to the progression of endometriosis.

In the present study, we investigated the possible relationship between IL-4 and eotaxin in the pathogenesis of endometriosis by studying the effect of IL-4 on eotaxin secretion by ESCs and by examining the localization of IL-4 and eotaxin in endometriotic tissues.

MATERIALS AND METHODS

Reagents and Materials

Dulbecco's minimum essential medium (DMEM)/Ham's F12 (F12) medium, 0.25% trypsin–ethylenediamineteraacetic acid (EDTA) and deoxyribonuclease I were from Invitrogen (Rockville, MD). Charcoal-stripped fetal bovine serum (FBS) was purchased from Hyclone (Logan, UT). Type I collagenase and antibiotics (mixture of penicillin, streptomycin, and amphotericin B) were purchased from Sigma (St. Louis, MO). Recombinant human IL-4, mouse antihuman eotaxin antibody (MAB320) and mouse antihuman IL-4 antibody (MAB304) were from R&D Systems (Minneapolis, MN). Mouse antihuman von Willebrand factor antibody (M0616)

Received December 5, 2008; revised January 22, 2009; accepted January 23, 2009;

Z.O. has nothing to disclose. Y.O. has nothing to disclose. Y.H. has nothing to disclose. T.H. has nothing to disclose. O.Y. has nothing to disclose. K.K. has nothing to disclose. T.Y. has nothing to disclose. Y.T. has nothing to disclose.

Partly supported by grants from the Ministry of Health, Labour and Welfare, and the Ministry of Education, Culture, Sports, Science and Technology.

Reprint requests: Yutaka Osuga, M.D., Ph.D., Department of Obstetrics and Gynecology, Faculty of Medicine, University of Tokyo, 7-3-1, Hongo, Bunkyo-ku, Tokyo, 113-8655, Japan (FAX: +81-3-3816-2017; E-mail: yutakaos-tky@umin.ac.jp).

0015-0282/$36.00

doi:10.1016/j.fertnstert.2009.01.129

and isotype mouse IgG_1 (X0931) were from Dako (Glostrup, Denmark).

Collection of Tissues

Endometriotic tissues were obtained from patients with ovarian endometriomas who had undergone laparoscopy or laparotomy. Written, informed consent was obtained from each patient and the study protocol was approved by the institutional review board of the University of Tokyo. These patients had not received hormone or GnRH agonist treatment for at least 3 months before surgery. Endometriosis was classified as stage III and IV. Endometriotic tissue was obtained from the cyst wall of the ovarian endometrioma under sterile conditions and transported to the laboratory on ice in DMEM/F12 medium.

Isolation, Purification, and Culture of ESC

The isolation and culture of human ESC were performed as described previously (17, 18). Briefly, endometriotic tissue was minced into small pieces, incubated in DMEM/F12 with type I collagenase (2.5 mg/mL) and deoxyribonuclease I (15 U/mL) for 1 to 2 hours at 37°C, and then filtered sequentially through 100-μm and 70-μm nylon cell strainers. Stromal cells remaining in the filtrate were centrifuged at $200 \times g$ for 5 minutes, washed with phosphate-buffered saline (PBS), resuspended in DMEM/F12, plated onto 100-mm dishes, and then allowed to adhere at 37°C for 30 minutes. Nonadhering epithelial cells and blood cells were then removed by rinsing the cells with PBS. The adhering ESC were cultured in DMEM/F-12 containing 5% FBS, 100 U/mL penicillin, 0.1 mg/mL streptomycin, and 0.25 μg/mL amphotericin B. When the cells became confluent in 2 or 3 days, they were dissociated with 0.25% trypsin–EDTA, harvested by centrifugation at $200 \times g$ for 5 minutes, replated in six-well plates at 2×10^5 cells/well, and then incubated at 37°C in a humidified 5% CO_2/95% air environment for 24 hours. The complete media was then removed and replaced with fresh serum-free media with varying doses of IL-4 (0.1–10 ng/mL), and the cells were cultured for an additional 24 hours. At the end of the incubation, the media was collected to assay for eotaxin and the cells were collected for RNA extraction. Purification of the stromal cell population was determined by immunocytochemical staining before confluency for the following antibodies: vimentin (stromal cells), cytokeratin (epithelial cells), CD45 and CD68 (monocytes and other leukocytes), and von Willebrand factor (endothelial cells). The purity of the stromal cell was >98%, as judged by positive cellular staining for vimentin and negative cellular staining for cytokeratin, CD45, CD68, and von Willebrand factor.

Standard Reverse Transcription-Polymerase Chain Reaction (RT- PCR)

Total RNA was extracted from ESCs using the RNA easy Mini Kit (Qiagen, Hilden, Germany). Reverse transcription reactions were performed using a Rever Tra Ace-a kit (Toyobo, Tokyo, Japan). One microgram of total RNA was reverse-transcribed in a total volume of 20 μL, and cDNA was PCR amplified using oligonucleotide primers. Eotaxin primers (sense, 5′-AGAAACCACCACCTCTCACG-3′; antisense,5′-CACAGCTTTCTGGGGACATT-3′) were chosen to amplify a 233-bp fragment. Reverse transcriptase-polymerase chain reaction using primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Toyobo) were used as a control for the amount and quality of RNA. The thermocycling conditions for PCR amplification of eotaxin were: 25 cycles at 98°C for 10 seconds, 60°C for 4 seconds, and 74°C for 10 seconds. The PCR products were purified with a QIAEX II gel extraction kit (Qiagen), and the identity of the PCR products was confirmed by means of an ABI PRISM 310 genetic analyzer (Applied Biosystems, Foster City, CA).

Real-Time Quantitative PCR

To assess mRNA expression of eotaxin, real-time quantitative PCR and data analysis were performed using the Light Cycler (Roche Diagnostics GmbH, Mannheim, Germany). Expression of eotaxin mRNA was normalized to RNA loading for each sample using GAPDH mRNA as an internal standard. The primers for eotaxin and GAPDH were the same as those used for standard PCR. The thermocycling conditions for PCR amplification were as follows: for eotaxin, 40 cycles at 95°C for 10 seconds, 61°C for 10 seconds, and 72°C for 10 seconds; for GAPDH, 35 cycles at 95°C for 10 seconds, 64°C for 10 seconds, and 72°C for 18 seconds. PCR amplifications were followed by melting curve analysis.

Measurement of Eotaxin

The concentration of eotaxin 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 <5%.

Immunohistochemistry

Endometriotic tissue samples were washed in PBS, embedded in OCT compound (Sakura, Tokyo, Japan), and snap frozen in liquid nitrogen. Serial sections were cut at a 6-μm thickness and mounted onto poly-L-lysine-treated slides. Sections were fixed in acetone for 20 minutes on ice and then washed in PBS for 5 minutes twice. Sections were treated with 3% H_2O_2 for 15 minutes to eliminate endogenous peroxidase. After blocking with nonspecific staining blocking reagent, the sections were incubated with 10 μg/mL mouse antihuman eotaxin antibody, 10 μg/mL mouse antihuman IL-4 antibody, 10 μg/mL mouse antihuman von Willebrand factor antibody (1:50) or 10 μg/mL mouse IgG_1 isotype control for 60 minutes at room temperature and then incubated with peroxidase-conjugated goat antimouse secondary antibody (Dako) for 30 minutes. Staining was detected with the vector novaRED substrate kit (Vector,