

Running title: ICAM-1 and KIR expression in endometriosis

**Aberrant expression of intercellular adhesion molecule-1 and killer
inhibitory receptor induce immune tolerance in women with pelvic
endometriosis**

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Capsule

Compared with control samples, women with endometriosis showed less intercellular adhesion molecule-1 expression by peritoneal macrophages and showed more inhibitory receptor expression by peripheral blood natural killer cells, favoring immune tolerance.

Abstract

Objective: To investigate host immunologic response to endometriosis in terms of intercellular adhesion molecule (ICAM)-1 expression by macrophages and killer-cell inhibitory receptor (KIR) expression by natural killer (NK) cells.

Design: Case control study of immunologic markers.

Setting: University hospital.

Patient(s): Twenty-eight Japanese women with endometriosis were studied. Control subjects were 26 women without endometriosis. Diagnoses were made at laparoscopy.

Intervention(s): Venipuncture and laparoscopic peritoneal fluid collection.

Main Outcome Measure(s): ICAM-1 expression by macrophages and KIR expression by NK cells, measured by flow cytometry.

Result(s): In women with endometriosis, expression of ICAM-1 by peritoneal macrophages was significantly lower, and expression of KIR by NK cells in peritoneal fluid and peripheral blood was significantly higher, than in control subjects.

Conclusion (s): Properties of macrophages and NK cells in women with endometriosis promote immune tolerance to implanted tissue in the peritoneal environment. Increased KIR⁺NK cells in peripheral blood may represent a risk factor for endometriosis.

Key words: intercellular adhesion molecule-1, killer inhibitory receptor, immune tolerance

Introduction

Endometriosis is a condition in which foci of hormonally responsive endometrial tissue including epithelial and stromal components are ectopically present in the peritoneal cavity and various organs. Though a reflux implantation theory by Sampson (1) and an epithelial metaplasia theory (2, 3) have been advocated, the pathogenesis of endometriosis has not been clearly understood. Recently, host immune responses have received increasing attention with respect to the pathogenesis and progression of endometriosis, considering that the ectopic endometrium in endometriosis proliferates and can show tumor like behavior (4, 5). As part of the immune response to endometriosis, immunocompetent cells (6-9) and cytokines (10-13) in peritoneal fluid (PF) have been analyzed. Cells in the PF are mainly mononuclear cells, particularly macrophages (90%); other mononuclear cells present include lymphocytes and natural killer (NK) cells. In women with endometriosis, numbers of macrophages are increased in PF (14, 15), and where increased macrophage-derived cytokines can also be found (10-13). These data indicate activation of macrophages in endometriosis. Simultaneously, functional depression of macrophages has also been reported in women with endometriosis (6, 16-18). At the same time, NK activity is decreased in mononuclear cells from peripheral blood (PB) and PF in women with endometriosis (19-21). Much remains to be understood concerning patients' immune response in endometriosis from the viewpoint of cell activation.

ICAM-1, expressed on monocytes and macrophages, is important for signal transduction and antigen presentation to surrounding immunocompetent cells. This has led to ICAM-1 being recently considered to be an index for macrophage activation (22). NK cells can express

killer-cell inhibitory receptor (KIR) that recognize major histocompatibility antigen complex (MHC) class I on the target cells and signal inhibition of NK cytotoxicity. Recently, increased KIR expression by NK cells has been observed in women with endometriosis (23).

In the present study, we investigated whether there is any difference in the ICAM-1 expression rate by PF macrophages and also in the percentage of KIR expressing NK cells in PF and PB between women with and without endometriosis.

Materials and Methods

Subjects

Twenty-eight women with endometriosis (5 with stage I disease according to the revised classification of the American Fertility Society's classification of endometriosis (r-AFS); 6, stage II; 8, stage III; and 9, stage IV) were studied, as were 26 women without endometriosis (control group; 12 with benign ovarian cysts, 7 with uterine myoma, 4 with infertility, 2 with paraovarian cysts, and 1 with carcinoma in situ of uterine cervix). All patients were examined laparoscopically in the Department of Obstetrics and Gynecology of Kochi Medical School between April 1999 and August 2000. Mean ages for endometriosis and control groups were 32.8 ± 7.5 and 35.0 ± 8.9 , respectively (no significant difference). Women with a history of pregnancy within 3 years or a history of treatment with gonadotropin-releasing hormone analogues within 3 years, as well as those with complications of apparent pelvic inflammatory disease, were excluded from analysis. Laparoscopy was performed during the early follicular

phase. Informed consent for obtaining PF samples during laparoscopy was obtained before the procedure. This study was approved by the institutional review board of Kochi Medical School Hospital.

Monoclonal antibodies (mAb)

Fluorescein isothiocyanate (FITC)-labeled anti-CD3 mAb and anti-CD4 mAb, as well as PE-labeled anti-CD8 mAb, were used to identify the T cell marker. Phycoerythrin (PE)-labeled anti-CD19 mAb was used as a B cell marker. FITC-labeled anti-CD16 mAb was used as an NK cell marker. FITC-labeled anti-CD14 mAb was used as a monocyte/macrophage marker (all from Beckman-Coulter). PE-labeled anti-CD54 (ICAM-1) mAb was used as a marker for monocyte/macrophage activation, and PE-labeled anti-CD158a, anti-CD158b, and CD94 were used as markers for killer-cell inhibitory receptor (KIR) (both from Beckman-Coulter). Peripheral blood mononuclear cells (PBMC) and peritoneal fluid mononuclear cells (PFMC) were allowed to react with the mAbs and were evaluated by flow cytometry to determine differences between women with and without endometriosis.

Flow cytometry

PB (2 ml) was collected in heparinized Hank's buffer before laparoscopy. PF identified within the abdominal cavity was collected in heparinized Hank's buffer at laparoscopy. Each mAbs (5μ l) was added to a 200μ l aliquot of peripheral blood sample and allowed to react at room temperature for 1 hour. PF cells were suspended in phosphate-buffered saline (PBS) after pelleting by centrifugation at 1500 rpm for 7 minutes; then each mAb was added as for PB and allowed to react at room temperature for 1 hour. After the mAb reaction, erythrocytes were hemolyzed in ammonium chloride; then each cell sample was centrifuged and resuspended in 0.5

ml of PBS. An EPICS ELITE flow cytometer (Beckman-Coulter) was used for analysis. PBMC and PFMC fractions in each sample were gated with forward-scattered and laterally scattered light, and fluorescence intensities of cell populations in this region were measured.

ICAM-1

The expression of CD14⁺ICAM-1 in monocytes in PBMC and expression in macrophages in PFMC were measured by flow cytometry. Anti-CD54 mAb was used for detection of ICAM-1. Mean fluorescence intensity for CD54 was measured. The mean CD54 fluorescence intensity in macrophages in PFMC was divided by that in monocytes in PBMC, as an index of ICAM-1 activation potential (ICAM-1 intensity ratio; Fig. 1).

KIR

The percentage of KIR⁺NK cells among all NK cells was measured in PBMC and PFMC by flow cytometry. Anti-CD158a mAb was used for detection of KIR2DL1 (KIR subfamily), and anti-CD158b mAb was used for detection of KIR2DL2 (KIR subfamily) (Fig. 2).

Results

The absolute numbers of T cells, B cells, NK cells, and monocytes in peritoneal fluid were increased in women with endometriosis compared to controls. Simultaneous increase of peritoneal fluid was found in the peritoneal cavity, however, the concentration of each cell was not significantly increased compared to control (data not shown). Then proportions of each cell in PFMC were analyzed. These proportions did not differ significantly in PFMC nor in PBMC

between women with and without endometriosis (Table).

Fluorescence intensity of CD14⁺ICAM-1 expression in PB monocyte was not significantly different between women with and without endometriosis ($p=0.08$ by Kruskal-Wallis test). The ICAM-1 intensity ratio in samples from women with early endometriosis (stages I and II) was significantly lower than those from controls (2.9 ± 1.1 , 1.8 ± 0.7 , for controls and women with early endometriosis, respectively, $p=0.03$ by student T-test; Fig. 3). The ICAM-1 intensity ratio from women with advanced endometriosis (stages III and IV) was 2.6 ± 1.3 , not significantly different compared to controls (Fig. 3).

The percentage of KIR2DL1⁺NK cells among CD16⁺NK cells from PB in women with both early and advanced endometriosis were significantly higher than in controls (controls 11.7 ± 5.5 , early endometriosis 17.3 ± 8.3 , and advanced endometriosis 17.5 ± 8.7 , $p=0.008$ by Kruskal-Wallis test; Fig. 4). In women with advanced endometriosis, the percentage of KIR2DL1⁺NK cells among CD16⁺NK cells from PF was significantly higher than controls (11.6 ± 6.8 , 24.3 ± 15.6 , for controls and women with advanced endometriosis, respectively, $p=0.008$ by student T-test; Fig. 4). The percentage of KIR2DL1⁺NK cells among CD16⁺NK cells from PF from women with early endometriosis was 11.9 ± 9.3 , not significantly different compared to controls ($p=0.70$; Fig.4). There was no statistically significant difference in the percentage of KIR2DL2⁺NK cells from PB and PF among women with and without endometriosis (PB, $p=0.20$, PF, $P=0.18$ by Kruskal-Wallis test; Fig. 5).

Discussion

ICAM-1 is a transmembrane adhesion molecule present on macrophages that can bind to lymphocyte function-associated antigen (LFA)-1 and Mac-1. ICAM-1, after induced by cytokines, plays an important role in the immune response by macrophages with surrounding T cells, NK cells, and macrophages that express LFA-1 and Mac-1 (7-9). Especially, in antigen presentation by macrophages, ICAM-1 is particularly important as a costimulatory molecule that functions together with the MHC and T cell receptor (7-9). Because of these immunologic functions, ICAM-1 is considered to be an index for macrophage activation (22).

To examine the individual activation potential of macrophages that had migrated into the peritoneal cavity from peripheral blood, ICAM-1 fluorescence intensity of macrophages from PF was divided by that in monocytes in PBMC (ICAM-1 intensity ratio). Decreased ICAM-1 intensity ratio in women with early endometriosis indicates relative inactivation of macrophages in the peritoneal cavity and may predict a decreased immune response against foreign body (ectopic endometrial tissue) in that site. Increased ICAM-1 intensity ratio in advanced endometriosis, but no significantly different compared to controls, is possibly as a result of repeated episodes of inflammation.

KIR2DL1 and KIR2DL2 (both KIR subfamilies) belong to the immunoglobulin superfamily and function as receptors expressed on the surface of NK cells and part of T cells. Upon recognizing the different C-locus of HLA class I of the target cell, both KIR inhibit NK cell activation signaled from CD16, CD2, and LFA-1 (24). In this study, the percentage of KIR2DL1⁺NK among CD16⁺NK cells in PB was significantly increased in both early and advanced endometriosis, while that of KIR2DL2⁺NK cells was not. Furthermore, the percentage

of KIR2DL1⁺NK among CD16⁺NK cells in PF was significantly increased in advanced endometriosis. Because increased percentage of KIR2DL1⁺NK among CD16⁺NK cells in PB was observed even in early stage endometriosis, and NK cells were over 1000 times more abundant among PB than PF, increased KIR2DL1⁺NK cells among PBMC probably should not be affected by intraperitoneal condition. Instead, originally increased KIR2DL1⁺NK cells in PB more likely migrate into the peritoneal cavity in advanced endometriosis.

This study did not clarify a correlation between the percentage of KIR⁺NK cells and NK activity. Decreased NK activity in PBMC has been reported in women with endometriosis (19, 20, 25). Furthermore, NK activity reportedly failed to recover after LASER cauterization of endometriotic lesions (26). This observation suggests that decreased NK activity may be a pathogenic factor in endometriosis rather than a result. Another epidemiologic observation of frequent familial occurrence of endometriosis (27-29) indicates that genetic factor are involved in pathogenesis. Demonstration of increased KIR2DL1⁺NK cells in peripheral blood may be related to these epidemiologic findings.

The immunologic properties that we observed--decreased ICAM-1 expression by macrophages in PF, and increased KIR⁺NK cells in PF and particularly in PB--suggest relative immune tolerance in women with endometriosis. Further examination of other function molecule expression and cytokine production by peritoneal macrophages, and further examination of a correlation between the percentage of KIR expressing NK cells and NK cytotoxicities against endometriotic cells are needed for understanding the pathogenesis of endometriosis.

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Legends

Fig. 1.

Calculation of the fluorescence intensity ratio for intercellular adhesion molecule (ICAM)-1 by flow cytometry in women with endometriosis. The Y-axis shows fluorescence intensity of CD14 expressing cells. The X-axis shows fluorescence intensity of ICAM-1 (CD54) expressing cells.

The term **a** indicates mean fluorescence intensity corresponding to ICAM-1 expression by CD14⁺ICAM-1⁺ monocytes in peripheral blood. The term **b** indicates mean fluorescence intensity representing ICAM-1 expression by CD14⁺ICAM-1⁺ macrophages in peritoneal fluid.

Fig. 2.

Two-color flow cytometric scattered plots for detection of killer-cell inhibitory receptor (KIR) 2DL1⁺ natural killer (NK) cells (**A**) and KIR2DL2⁺NK cells (**B**) in women with endometriosis.

Scattered plots show mononuclear cells in peripheral blood, and peritoneal fluid. FITC- labeled anti-CD16 monoclonal antibody (mAb), PE-labeled anti-KIR2DL1 (CD158a) mAb (**A**) and PE-labeled anti-KIR2DL2 (CD158b) mAb (**B**) were used respectively. **A**: zone 1+ zone 2 includes CD16⁺NK cells; zone 2 includes KIR2DL1⁺NK cells. KIR2DL1⁺NK among CD16⁺NK cells = zone 2 / (zone 1 + zone 2). The same analysis was performed for KIR2DL2⁺NK cells.

Fig. 3.

ICAM-1 intensity ratios calculated by fluorescence intensity of ICAM-1 expression of PB monocytes and PF macrophage in women without and with early and advanced endometriosis. The ICAM-1 intensity ratio in samples from women with early endometriosis was significant

($p=0.03$ by student T-test). The ICAM-1 intensity ratio from women with advanced endometriosis was not significant ($p=0.51$ by student T-test).

Fig. 4.

The percentage of KIR2DL1⁺NK cells among CD16⁺NK cells from peripheral (PB) and peritoneal fluid (PF) in women without and with early and advanced endometriosis. Data shows individual values and mean (-) of each column. The percentage of KIR2DL1⁺NK cells from PB between controls and women with both early and advanced endometriosis were statistically significant ($p=0.008$ by Kruskal-Wallis test). The percentage of KIR2DL1⁺NK cells from PF from women with early endometriosis was not significantly different compared to controls ($p=0.70$ by student T-test). In women with advanced endometriosis, the percentage of KIR2DL1⁺NK cells from PF was statistically significant ($p=0.008$ by student T-test).

Fig. 5.

The percentage of KIR2DL2⁺NK cells among CD16⁺NK cells from peripheral (PB) and peritoneal fluid (PF) in women without and with early and advanced endometriosis. Data shows individual values and mean (-) of each column. There was no statistically significant difference in the percentage of KIR2DL2⁺NK cells from PB and PF among women with and without endometriosis (PB, $p=0.20$, PF, $P=0.18$ by Kruskal-Wallis test).