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Decreased Bcl-6 and increased Blimp-1 in the peritoneal cavity of patients with endometriosis

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Summary

Purpose of investigation: The authors investigated the expression patterns of interleukin (IL)-1β and tumor necrosis factor (TNF)-α, cytokines associated with peritoneal inflammatory reactions, and of B cell leukemia lymphoma (Bcl)-6 and B lymphocyte inducer of maturation program (Blimp)-1, transcriptional factors associated with immunoglobulin (Ig) production; the concentrations of Igs, and their correlation, in patients with and without endometriosis. *Materials and Methods:* The authors analyzed the peritoneal fluid of 98 patients, 46 with endometriosis, and 52 with benign tumors. *Results:* IL-1β and TNF-α mRNAs and IgG and IgA concentrations were higher in the endometriosis group, but the differences were not statistically significant. However, Bcl-6 mRNA level was significantly lower and Blimp-1 mRNA level was significantly higher in the endometriosis group with significant correlations among transcriptional factors, Igs, and cytokines ($p < 0.05$). *Conclusion:* Peritoneal immune responses in patients with endometriosis may be due to increased IgG and IgA concentrations, as well as to changes in expression of proinflammatory cytokines and transcriptional factors.

Key words: Bcl-6; Blimp-1; Immunoglobulin; Endometriosis.

Introduction

Endometriosis is a chronic disease that causes dysmenorrhea and chronic pelvic pain, with severe endometriosis resulting in infertility. Depending on its severity, endometriosis results in the accumulation and activation of macrophages, B cells and T cells, and the secretion of various cytokines and chemokines, which induce various peritoneal immune responses [1, 2]. Macrophages and cytokines in peritoneal fluid are associated with inflammatory reactions, tissue repair, and neovascularization occurring in endometriosis. These macrophages and cytokines play an important role in the regulation of cell proliferation, activation, motility, adhesion, chemotaxis, and morphogenesis. Cytokines involved in the pathogenesis of endometriosis include interleukins (IL)-1, -2, -6, and -10; tumor necrosis factor (TNF)-α; interferon (IFN)-γ, and regulated upon activation, normal T-cell expressed and secreted (RANTES) [1, 3]. IL-1 and TNF-α, both produced by macrophages, are closely associated with inflammatory reactions to infection, and have an in vivo synergistic effect [4].

B cells present in the peritoneal cavity have been classified as CD5-positive B-1 cells and CD5-negative B-2 cells. B-1 cells are present mainly in the peritoneal and thoracic cavities, with fewer in the spleen, and none in lymph nodes and peripheral blood [5, 6]. In addition, unlike B-2 cells, B-1 cells not only secrete antibodies in the absence of external stimulation, thereby contributing to innate immunity, but also react with autoantigens, resulting in significant increases in B-1 cells in some autoimmune diseases and chronic lymphocytic leukemia [5-8]. Transcriptional factors involved in antibody production by B-cells include B cell leukemia lymphoma-6 (Bcl-6) and B lymphocyte inducer of maturation program 1 (Blimp-1). Bcl-6 is required for the generation of germinal center B cells, whereas Blimp-1 promotes differentiation by halting cell division cycle; thus, they are involved in the suppression and promotion of antibody production, respectively [9, 10].

Although previous studies investigated immune responses of various immune system cells in the peritoneal cavity, no study to date has investigated transcriptional factors involved in antibody production in patients with endometriosis. Thus, peritoneal changes associated with endometriosis have not yet been identified. The present authors therefore assayed the concentrations of IgG and IgA, immunoglobulins associated with chronic inflammation and the mucosal immune response, respectively, and the expression patterns of Blimp-1 and Bcl-6, transcriptional factors associated with the promotion and suppression of Ig production, respectively, in patients with and without peritoneal endometriosis. They also assayed the expression patterns of TNF-α and IL-

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Table 1. — *Primers for real-time RT-PCR.*

Name	Sequences	Annealing temperature	Product size (bp)
Bcl-6	F: 5`-TTCATCGTGCTCAACAGCCTCAAC-3` R: 5`-ATCTGAGTACTCAGACTGGGTCTC-3`	60	393
Blimp-1	F: 5`-CACCTGAGAGTGCACAGTGG-3` R: 5`-CACAAACTGGGTGAACTTGGC-3`	55	210
IL-1β	F: 5'TGATGGCTTATTACAGTGGCAATG-3' R: 5'-GTAGTGGTGGTCGGAGATTCG-3'	140	60
TNF-α	F: 5'-ATCTTCTCGAACCCCGAGTG-3' R: 5'-GGGTTTGCTACAACATGGGC-3'	60	51
β-actin	F: 5'-GCGAGAAGATGACCCAGATC-3' R: 5'-GGATAGCACAGCCTGGATAG-3'	60	77

RT-PCR: real time-polymerase chain reaction;
Bcl-6: B-cell leukemia lymphoma-6;
Blimp-1: B-lymphocyte inducer of maturation program-1;
IL: interleukin; TNF-α: Tumor necrosis factor-α

1ß, cytokines involved in various inflammatory and immune responses, and the correlations among these Igs, transcriptional factors, and cytokines in these patients.

Materials and Methods

Materials

Peritoneal fluid samples were obtained from 98 patients who underwent laparoscopic surgery, including diagnostic laparoscopy, in the Department of Obstetrics and Gynecology at the present center between March 2010 and February 2013. Of these 98 patients, 46 had endometriosis and 52 had benign tumors. All patients provided written informed consent. Patients suspected of having inflammatory disease, lesions producing hormones, internal diseases, and immune diseases were excluded. The study protocol was approved by the institutional review boards (IRBs) of Vincent's Hospital, The Catholic University of Korea and Kyung Hee University Hospital, and informed consent was obtained from each patient (VC13TISI0057, KMC IRB 1236-02).

RT-PCR

Peritoneal fluid was collected aseptically from the Douglas pouch during surgery, taking care to avoid bleeding. Total RNA was extracted from peritoneal fluid using RNA-Bee solution kits according to the manufacturer's protocol. First-strand cDNA was synthesized by reverse transcription in 20 ml reaction mixtures containing one mg of RNA, 1x reaction buffer, one mM of each dNTP, five μM random primers, 20 units RNase inhibitor, and 20 units AMV reverse transcriptase. The reaction mixtures were incubated at 42°C for one hour, and the reactions were terminated by heating at 95°C for five minutes. Primers specific for IL-1β, TNF-α, Bcl-6, and Blimp-1 are shown in Table 1. Real-time polymerase chain reactions (PCR) were performed using a real-time system and a supermix. Each 20-μl PCR reaction mixture included two μl of cDNA, ten ml supermix, two ml of each primer, and six ml PCR grade water. The amplification protocols consisted of an initial denaturation at 95°C for 30 seconds, followed by 45 cycles of denaturation at 95°C for five seconds and annealing and extension at 55°C to 64°C for 12 seconds. The point at which expression of each of the above cDNAs crossed with that of β-actin was applied to the formula, $2^{-(\text{target gene- ß actin})}$, and the relative amounts were quantified.

Table 2. — *Bcl-6, Blimp-1, and cytokine mRNA expression in the peritoneal fluid of patients with and without endometriosis.*

	Control group (M ± SD)	Endometriosisgroup (M ± SD)	*p* value (Mann-Whitney U)
Bcl6	0.095 ± 0.452	0.039 ± 0.036	0.036
Blimp-1	0.044 ± 0.159	0.539 ± 1.748	0.006
IL1	0.030 ± 0.106	0.093 ± 0.292	0.626
TNF-α	0.078 ± 0.441	0.085 ± 0.408	0.108

Crossing point: $2^{-(\text{target gene-ß2 microglobulin})}$; Bcl-6: B-cell leukemia lymphoma-6;
Blimp-1: B-lymphocyte inducer of maturation program-1;
IL: interleukin; TNF-α: Tumor necrosis factor-α; SD: Standard deviation

Enzyme-linked immunosorbent assay (ELISA)

Peritoneal fluid collected from patients was centrifuged, and the supernatants were stored at -80°C. IgG and Ig A concentrations were measured by ELISA. Briefly, 50 μl 1:100 goat anti-human IgG and/or Ig A in coating buffer (1.59 g Na_2CO_3+2.93 g $NaHCO_3$+5% NaN_3, pH 9.6) were placed in each well of a 96-well plate and incubated overnight at 4°C. The wells were washed six times, blocking antibody was added, 50 μl of sample was added to each well, and the plates were incubated at room temperature for three hours. The wells were washed six times, purified goat anti-human IgM conjugated to horseradish peroxidase in PBS/Tween/BSA solution was added, and the plates were incubated at room temperature. The plates were washed six times, substrate solution (2,2'-AZINO-Bis) was added, and the optical absorbance was measured at 450 nm.

Comparison of effusion fluids

The level of each mRNA was compared in the endometriosis and non-endometriosis groups according to age, pregnancy history, and CA125 level. The authors also evaluated the correlation of these factors in the effusion fluid.

Statistical analysis

The Kolmogorov-Smirnov test was used to assess normality and Levene's test was used to assess the equality of variances between groups. Between group differences in expression were determined using independent t-tests, with correlations assessed using the Pearson correlation test. All statistical analyses were performed using SPSS version 13, with a *p*-value less than 0.05 considered statistically significant.

Results

Characteristics of patients in the endometriosis and non-endometriosis groups

The mean ages of the patients in the endometriosis and non-endometriosis groups were 36.7±9.1 years and 40.8±10.2 years, respectively ($p > 0.05$), and their mean body mass indexes (BMIs) were 21.1 ± 3.3 kg/m^2 and 22.7 ± 3.2 kg/m^2, respectively ($p > 0.05$). Fertility and history of prior surgery were also similar in the two groups ($p > 0.05$), although CA125 was significantly higher in the endometriosis than in the non-endometriosis group (51.9 ± 5.4 IU/ml vs 29.8 ± 3.2 IU/ml, $p < 0.05$).

Expression of IL-1β, TNF-α, Bcl-6, and Blimp-1 mRNA in peritoneal fluid (Table 2)

IL-1β, TNF-α, Bcl-6, and Blimp-1 mRNAs were present in the peritoneal fluid of both groups. The levels of IL-1β

Table 3. — *Concentrations of immunoglobulins in the peritoneal fluid of patients with and without endometriosis.*

	Total	control	Endometriosis	*p* value
IgG (µg/dl)	1,695 ± 30	1,674 ± 38	1,713 ± 23	0.429
IgA (µg/dl)	844 ± 41	821 ± 25	864 ± 33	0.525

*$p < 0.05$; Ig: Immunoglubulin

Table 4. — *Correlation between clinical manifestations and the expression of Bcl-6, Blimp-1, cytokines mRNA, and immunoglobulins*

		Control group		Endometriosis group	
		Pearson's Coefficients	*p* value	Pearson's coefficients	*p* value
Age	Bcl-6	- 0.083	0.631	0.197	0.249
	Blimp-1	- 0.115	0.497	- 0.067	0.698
	IL-1β	- 0.103	0.538	- 0.167	0.325
	TNF-α	0.016	0.921	0.202	0.223
	Ig G	- 0.018	0.911	0.209	0.195
	Ig A	- 0.009	0.957	0.339	0.032
Parity	Bcl-6	- 0.194	0.256	0.205	0.246
	Blimp-1	- 0.166	0.326	- 0.082	0.644
	IL-1β	- 0.207	0.213	0.168	0.333
	TNF-α	- 0.053	0.746	0.186	0.278
	Ig G	0.160	0.323	0.290	0.078
	Ig A	- 0.012	0.944	0.415	0.010
CA125	Bcl-6	0.211	0.347	0.452	0.018
	Blimp-1	0.259	0.234	0.068	0.735
	IL-1β	0.207	0.332	0.104	0.598
	TNF-α	- 0.112	0.586	- 0.011	0.957
	Ig G	0.115	0.577	0.067	0.719
	Ig A	0.446	0.029	0.432	0.022

Bcl-6:B-cell leukemia lymphoma-6;
Blimp-1:B-lymphocyte inducer of maturation program-1;
IL: interleukin; TNF-α: Tumor necrosis factor-α; Ig: Immunoglubulin

Table 5. — *Correlation between immunoglobulin concentrations, and mRNA encoding transcription factors and cytokines in peritoneal fluid.*

		Control group		Endometriosis group	
		Pearson's Coefficients	P value	Pearson's coefficients	P value
IgG	Bcl-6	-.041	.810	.034	.844
	Blimp-1	-.069	.683	-.012	.945
	IL-1β	.146	.382	-.105	.537
	TNFa	.110	.498	-.092	.582
IgA	Bcl-6	.049	.776	.131	.447
	Blimp-1	.060	.722	.179	.295
	IL-1β	-.016	.925	-.072	.672
	TNFa	-.049	.765	.137	.412

r:Pearson's correlation coefficient. Bcl-6: B-cell leukemia lymphoma-6;
Blimp-1: B-lymphocyte inducer of maturation program-1;
IL: interleukin; TNF-α: Tumor necrosis factor-α

Table 6. — *Endometriosis group. Correlations between IgG, IgA, and IgM concentrations and mRNAs encoding Bcl-6, Blimp-1, IL-1β, -8, -12, and TNF-α.*

	IgA	IgG	TNF-α	IL-1β	Blimp-1	Bcl-6
Bcl-6	.131	.034	.314	.466**	.677**	1.000
Blimp-1	.179	-.012	.734**	.384*	1.000	.677**
IL-1β	-.072	-.105	.251	1.000	.384*	.466**
TNF-α	.137	-.092	1.000	.251	.734**	.314
IgG	.473**	1.000	-.092	-.105	-.012	.034
IgA	1.000	.473**	.137	-.072	.179	.131

*p<0.05,** p<0.01. Bcl-6: B-cell leukemia lymphoma-6;
Blimp-1: B-lymphocyte inducer of maturation program-1;
IL: interleukin; TNF-α: Tumor necrosis factor-α

and TNF-α were higher in the endometriosis group, but not significantly ($p > 0.05$ each). The Blimp-1 mRNA level was significantly higher ($p < 0.05$), while the Bcl-6 mRNA level was significantly lower ($p < 0.05$), in the endometriosis than in the non-endometriosis group.

Concentrations of Igs in effusion fluid (Table 3)

Overall mean IgG and IgA concentrations in the peritoneal cavity were 1,695 ± 30 µg/ml and 844 ± 41 µg/ml, respectively. The concentrations of IgG (1,713 ± 23 µg/ml vs 1,674 ± 38 µg/ml) and IgA (864 ± 33 µg/ml vs 821 ± 25 µg/ml) were higher in the endometriosis group, but these differences were not statistically significant ($p > 0.05$ each).

Correlations of clinical manifestations with transcription factor (Bcl-6 and Blimp-1), cytokine (IL-1β & TNF-α), and Ig (IgG and Ig A) concentrations (Table 4)

The authors observed significant correlations between clinical and demographic characteristics, including age, parity, and CA125 concentration, with IgA concentration in the two groups ($p < 0.05$ each).

Correlation of transcription factor (Bcl-6 and Blimp-1), cytokine (IL-1β & TNF-α), and Ig (IgG and Ig A) concentrations (Tables 5 and 6)

In the two groups, none of these mRNAs was correlated with the IgG and IgA concentrations ($p > 0.05$ each) (Table 5). However, significant correlations were observed between the transcription factors and cytokines in the endometriosis group ($p < 0.05$ each) (Table 6).

Discussion

Endometriosis, which is characterized by disparate morphological, histological, and biochemical properties, is an inflammatory disease resulting from changes in the pelvic environment. Ectopic lesions secrete chemotactic molecules that recruit immune cells to the peritoneal fluid, with the latter cells secreting cytokines that promote lesional proliferation, which, in turn, triggers immune responses [11]. IL-1ß and TNF-α are central to the extravasation of polymorphonuclear leukocytes (PMN) into the infected tissue. IL-1ß activates B and T cells, epithelial and fibroblast proliferation, cytokine synthesis, and histamine release, inducing fever and bone resorption [12]. TNF functions sim-

ilarly to IL-1, with the two cytokines acting synergistically [13, 14]. The present found that the levels of expression of IL-1ß and TNF-α mRNAs were higher in the endometriosis than in the non-endometriosis group, but neither of these differences was statistically significant. Differences, however, may depend on endometriosis severity, sample type (endometriosis tissue, peritoneal fluid, or serum), control group (normal subjects or those with benign tumors), disease type (diseases other than endometriosis), history of drug treatment in endometriosis patients, or genetic polymorphisms.

B cells, which play an important role in antigen recognition, antibody production, and immune system regulation, have receptors such as IgM and IgD, and surface markers such as CD19, CD20, and CD21. Stimulation of B cells by T-cell dependent and independent antigens results in B-cell proliferation or differentiation, with most of these cells undergoing apoptosis. B cells differentiate into plasma cells or memory cells that produce antibodies. Differentiated plasma cells initially produce IgM and IgD, and then produce IgG, IgA, or IgE after DNA remodeling. Thus, even in the absence of exogenous infection, various antibodies may be present in the peritoneal cavity. The present authors focused on two classes of antibodies: IgG, the class of antibody associated with chronic inflammation and auto-immune reactions; and IgA, the class of antibody associated with mucosal immunity. Previous studies showed that the concentration of specific IgG autoantibody was increased in the peritoneal fluid of patients with endometriosis and that endometrial glandular epithelial staining for both IgG and IgA was significantly increased, suggesting that endometriosis may be an autoimmune disease. Moreover, increases in IgG concentration may also suggest that patients have a precursor of endometriosis requiring treatment [11, 15, 16].

In the absence of bacterial infection, the peritoneal cavity is a sterile environment, in which B cells cannot produce antibodies due to the absence of external stimulation. IgM, however, is spontaneously produced in the peritoneal cavity in the absence of external infection by B-1 cells rather than by B-2 cells [5, 6]. Since few or no B-1 cells, however, are present in cervical lymph nodes and the spleen, immunoglobulin is not produced spontaneously. Although culture of peritoneal fluid from the patients in this study detected no bacteria, IgG and IgA were secreted into the peritoneal cavities of all 98 patients. The concentrations of IgG and IgA were both higher in the endometriosis than in the non-endometriosis group, but the differences were not statistically significant, a result likely due to the patients in both groups not having infectious disease and the absence of bacteria in peritoneal fluid. The present authors found, however, that IgA concentration in the endometriosis group increased according to age and parity. Although the exact mechanism remains to be identified, peritoneal adhesion and inflammatory reactions seem to be affected by the duration and severity of endometriosis, by age-related changes in immune responses, and/or by pregnancy-associated changes in hormone concentrations.

Bcl-6 and Blimp-1 are transcriptional factors involved in antibody production by plasma cells. The present authors found that the level of Bcl-6 mRNA was lower and the level of Blimp-1 mRNA level was higher in the endometriosis group compared with the non-endometriosis group. Bcl-6 has been reported to inhibit the differentiation of plasma cells and Blimp-1 has been found to regulate secretion by B lymphocytes [17, 18]. Although the level of Bcl-6 mRNA is higher in both resting and germinal center B cells, the level of Bcl-6 protein is 3~34-fold higher in the germinal center than in resting B cells. Bcl-6, which suppresses Blimp-1, inhibits the activity of STAT3, thereby blocking the differentiation of germinal center B cells into plasma cells during an early phase. In B lymphocytes, Blimp-1 protein regulates the expression of three genes, thereby playing roles in cell cycle arrest, induction of immunoglobulin secretion, and the inhibition of germinal center functions [19]. Blimp-1 is expressed in all plasma cells, both in response to primary stimulation by T-cell dependent and independent antigens, as well as in mature plasma cells present in the bone marrow and in memory cells responsible for secondary response [20]. The present authors found that Blimp-1 mRNA level was higher in the endometriosis than in the non-endometriosis group due to the decrease in Bcl-6 mRNA level, and that the increase in Blimp-1 level resulted in increased IgG and IgA concentrations, findings consistent with those of previous studies. Thus, the higher IgG and IgA concentrations in the endometriosis group were attributable to decreased Bcl-6 and increased Blimp-1 expression, with the latter due to the increased expression of the proinflammatory cytokines, IL-1β and TNF-α, showing that inflammation-related immune responses occurred more actively in the endometriosis group than in non-endometriosis group.

This study had several limitations. For ethical reasons, its control group consisted of patients with peritoneal masses rather than healthy subjects. Thus, despite the absence of peritoneal infection, immune responses related to the mass may have occurred. In addition, the authors assessed mRNA not protein expression. Thus, these mRNAs may not have been translated into proteins. Third, they measured transcriptional factors and cytokines at the mRNA level whereas immunoglobulins were measured at the protein level. Fourth, B-1 and B-2 cells are both present in the peritoneal cavity. As these two cell types were not sorted from the peritoneal fluid, it is not clear whether the measured concentrations of IgG and IgA were attributable to spontaneous secretion by B-1 cells or stimulated secretion by B-2 cells. Fifth, clinical manifestations and immune responses in patients with endometriosis may depend on its severity. However, the authors compared patients with or without endometriosis. Sixth, although they

analyzed mRNA and protein concentrations in peritoneal fluid, they did not compare these results with expression in serum or endometriosis tissues. Thus, there were no comparisons of immune responses between tissue samples from the endometriosis and non-endometriosis groups.

In conclusion, the expression of the proinflammatory cytokines, IL-1α and TNF-α, in the peritoneal fluid of patients with endometriosis was due to peritoneal inflammatory responses and immune responses. In addition, the levels of Bcl-6 and Blimp-1 mRNAs, which are associated with the peritoneal secretion of IgG and IgA, were lower and higher, respectively, in the endometriosis than in the non-endometriosis group, indicating a vigorous B-cell immune response in patients with endometriosis.

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