

Anti-endometrial IgM autoantibodies in endometriotic patients: a preliminary study

T. Tanaka, N. Umesaki, K. Mizuno, Y. Fujino, S. Ogita

Department of Obstetrics & Gynecology, Osaka City University Medical School, Osaka (Japan)

Summary

Japanese herbal medicines (Kampo medicines) are usually the third most popular choice among medicines for treatment of endometriosis in Japan. This traditional therapy is used to improve various signs and symptoms of endometriosis without decreasing serum estradiol levels or causing menstrual disorders. We used flow cytometry to examine and compare the effects of the Kampo therapy and danazol on anti-endometrial humoral immunity. Autoantibodies against endometrial epithelial cell lines and endometrial stromal cells were detectable in all the examined sera of men and women irrespective of the presence of endometriosis. Moreover, no significant increase in anti-endometrial antibodies was found in endometriotic patients. Anti-endometrial antibodies included Ig- γ chain, Ig- μ chain, Ig- κ chain, and Ig- λ chain indicating polyclonal B cell activation in the endometriotic patients. Absorption tests of nonspecific antibodies with cervical cancer cells or ovarian cancer cells revealed that endometriotic patients had higher levels of endometrium-specific autoantibodies than did non-endometriotic healthy women. IgM fractions from endometriotic patients and healthy women differed in their effect on growth of endometrial adenocarcinoma cells. Therapy with the herbal compounds Keishi-bukuryo-gan but not danazol therapy, gradually decreased the tissue-specific anti-endometrial IgM antibody levels. These results indicate that tissue-specific anti-endometrial IgM may be a useful therapeutic marker for endometriotic patients treated with Keishi-bukuryo-gan and that endometrial tissue-specific immune disorders play specific roles in the pathogenesis or development of endometriosis.

Key words: Endometriosis; Anti-endometrial antibody; Herbal Medicine; Keishi-bukuryo-gan; Danazol.

Introduction

Endometriosis is an estrogen-dependent benign proliferative disease of unknown cause. The incidence of the disease has increased strikingly in developed countries during this decade. The most common medical therapies for endometriosis are gonadotropin releasing hormone agonist (GnRHa) and oral danazol. However, these therapies can decrease serum estradiol to levels that cause amenorrhea and menopausal symptoms. Herbal medicines are usually the third most popular type of treatment for endometriosis in East Asian countries such as Japan, Korea, and China. This traditional therapy can be used to improve various signs and symptoms characteristic of endometriosis without affecting serum estradiol levels or causing menstrual disorders. This indicates that, unlike GnRHa and danazol, the anti-endometriotic herbal medicines can improve endometriosis by correcting key pathogenic abnormalities that may cause the disease.

Various autoantibodies are detectable in endometric patients [1]. Clinical findings have revealed higher levels of anti-endometrial autoantibodies in the sera of endometriotic patients than in that of healthy women [2-4]. Endometriotic patients show polyclonal B cell activation and abnormal clonality of activated B cell repertoires [4]. Research also shows that pelvic endometriosis may result from a break in specific T cell tolerance rather than nonspecific polyclonal activation of responder lymphocytes [5].

Furthermore, the anti-endometriotic agent danazol improves certain autoimmune diseases and decreases autoantibody levels in patients with autoimmune diseases [1, 5]. These findings suggest that an unknown autoimmune mechanism is associated with the pathogenesis of endometriosis [6-12]. However, no investigators have clarified antigen specificity [13-15], the function of anti-endometrial antibodies [16, 17], or the changes in treated patients [1, 18, 19]. To examine the effects of the herbal medicine therapy on anti-endometrial humoral immunity in endometriosis, we used flow cytometry to detect cell surface antigens specific to endometrial cells. Moreover, to detect any biological activity of the antibodies, we characterized effects of IgM fractions purified from endometriotic patient sera and healthy women on endometrial cell growth.

Materials and Methods

Patients and Sera

To examine serum anti-endometrial levels in patients with sterility, sera were obtained immediately before ovulation from patients with endometriosis (n=13) or with oviductal obstruction without endometriosis (n=4) who were treated with in vitro fertilization and embryo transfer (IVF-ET). Endometriosis was diagnosed by laparoscopy.

Changes in specific anti-endometrial antibodies during medical treatment were also examined as described below. Sera were obtained from endometriotic patients before and after treatment with Keishi-bukuryo-gan Extract Granules for Ethical Use (KBG) (7.5 g/day) (Tsumura & Co., Tokyo, Japan) or danazol (400 mg/day) (Tokyo-Tanabe Co., Tokyo, Japan).

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Keishi-bukuryo-gan, an Anti-endometriotic Kampo Medicine

We used the herbal compound Keishi-bukuryo-gan (KBG), which is a mixed pro-drug of five herbs consisting of cinnamon bark (the bark of the trunk of *Cinnamomum cassia* Blume or other species of the same genus (*Lauraceae*)), peony root (the root of *Paeonia lactiflora* Pallas or allied plants (*Paeoniaceae*)), peach kernels (the seeds of *Prunus persica* Batsch or *Prunus persica* Batsch var. *daurica* Maximowicz (*Rosaceae*)), hoelen (the sclerotium of *Poria cocos* Wolf (*Polyporaceae*)), usually from which the outer layer has been mostly removed), and mountain bark (the root bark of *Peonia suffruticosa* Andrews (*Paeonia mountain* Sims) (*Paeoniaceae*)). It has been traditionally given to endometriotic patients in East Asia to relieve their dysmenorrhea and hypermenorrhea. According to the manufacturer's drug information book, 7.5 g of TSMURA Keishi-bukuryo-gan Extract Granules for Ethical Use contains 1.75 g of dried extract obtained from mixed raw herbs in the following ratio: 3.0 g of cinnamon bark, 3.0 g of peony root, 3.0 g of peach kernels, 3.0 g of hoelen, and 3.0 g of mountain bark.

Cell Lines and Cell Culture

In this study, four human gynecologic cancer cell lines were used to assay the tissue-specific anti-endometrial autoantibodies. HHUA [20], a highly differentiated endometrial adenocarcinoma cell line, was obtained from Riken Cell Bank (Tsukuba, Japan). ME180 [21], a cervical squamous cell carcinoma cell line, and HEC-1 [22], a moderately differentiated endometrial adenocarcinoma cell line, was obtained from Japan Resources of Cell Bank (JCRB) (Tokyo, Japan). Ovk18 [23], an endometrioid adenocarcinoma cell line of the ovary, was obtained from The Aging Institute of Tohoku University (Sendai, Japan). All cell lines were cultured in Opti-MEM medium (GIBCO-BRL, Gaithersburg, MD, USA) with 5% v/v of fetal bovine serum. Nonendometriotic patients with uterine leiomyomas gave informed consents for obtaining normal human endometrial stromal cells from their surgically excised uteri [24]. These stromal cells were also cultured with Opti-MEM medium with 5% v/v of fetal bovine serum.

Flow Cytometric Assay of Total Anti-endometrial Antibodies

Serum anti-endometrial autoantibodies were semiquantitatively assayed by indirect flow cytometry against normal human endometrial stromal cells or differentiated endometrial adenocarcinoma cell lines, HHUA and HEC-1. Cell surface antigens on their viable cells were examined, and titrations of the anti-endometrial antibodies were evaluated as relative mean fluorescence intensities. Briefly, cultured cells were detached from the culture flask by phosphate buffered saline (PBS)/5 mM EDTA and stained as follows. Cells (1×10^5) were incubated with 30 μ l of the diluted patient serum for 20 minutes at 4° C and the washed twice with washing buffer (PBS, 2% FCS, 0.1% NaN_3 , 5 mM EDTA). Next, the cells were reacted with an excessive dose of FITC-conjugated goat antihuman IgG/IgA/IgM (Organon Teknika Corp., West Chester, PA, USA) for 20 minutes at 4° C, washed twice, and finally suspended in 200 μ l of the washing buffer for analysis with a Coulter-Epics Flow Cytometer. To examine immunoglobulin subclasses of the anti-endometrial antibodies, the following second antibodies were used for flow cytometric analyses: FITC-conjugated goat anti-human Ig- γ (Organon Teknika Corp.), FITC-conjugated goat anti-human Ig- μ (Organon Teknika Corp.), FITC-conjugated goat anti-human Ig- κ (Biosource Int., Camarillo, CA, USA),

and FITC-conjugated goat anti-human Ig- λ (Southern Biotech. Assoc. Inc., Birmingham, AL, USA). The data are expressed as mean \pm SD and were analyzed with the Student's t-test.

Flow Cytometric Assay of Tissue-specific Anti-endometrial Antibodies

Tissue-specific anti-endometrial autoantibodies were semiquantitatively assayed by indirect flow cytometry against HHUA cells. Cell surface antigens on viable HHUA cells were examined. Nonspecific anti-endometrial antibodies were completely absorbed by incubating 1 ml of patient serum with more than 3×10^6 cells of ME180 cells or Ovk18 cells suspended in PBS/0.1% NaN_3 /5 mM EDTA for 2 hours at 4° C. Titrations of the anti-endometrial antibodies were evaluated as relative fluorescence intensities. The cell staining procedure for flow cytometry is described above. During treatment with KBG (7.5 g/day) or danazol (400 mg/day), changes in serum tissue-specific anti-endometrial IgG and IgM titers against HHUA cells were examined by the indirect flow cytometry. The patient sera for the assays were incubated with ME180 cells or Ovk18 cells to remove non-specific anti-endometrial antibodies before reacting the sera with HHUA cells for the flow cytometry. The data are expressed as mean \pm SD and were analyzed with the Student's t-test.

Cell Proliferation Effects of Serum IgM-rich Fractions from Endometriotic or Nonendometriotic Women

To examine cell proliferation effects of serum IgMs from endometriotic patients on human endometrial cells, serum IgM fractions were partially purified by 50% ammonium sulfate precipitations and Sephacryl S-300 (Pharmacia, Uppsala, Sweden) gel filtration column chromatographies from two endometriotic patients and two healthy women.

Briefly, 2 ml of patient sera was diluted with 8 ml of PBS and gently incubated overnight with 10 ml of 100% ammonium sulfate solution at 4° C. After the solution was centrifuged at 12,000 rpm at 4° C for 20 minutes, the pellet was resuspended in 2 ml of PBS and dialyzed overnight against 2,000 ml of PBS/0.1% NaN_3 at 4° C. The dialyzed solution was centrifuged at 12,000 rpm at 4° C for 20 minutes to remove insoluble fragments, and then applied to a Sephacryl S-300 gel filtration column (2.0 cm \times 96.0 cm) equilibrated with PBS/0.1% NaN_3 at 4° C. IgM-rich fractions (MW 700-1100 kDa) were collected and concentrated by Centriprep (Amicon Inc, Beverly, MA, USA).

To clarify any IgM-specific effects on the endometrial cell growth, we purified IgM-deprived fractions from each of the purified IgM-rich fractions described below. Each IgM-rich fraction was applied to an anti-human IgM-conjugated Sepharose 4B gel column (1.0 cm \times 2.0 cm) equilibrated with PBS/0.1% NaN_3 at 4° C. The flow-through fractions (IgM-deprived fractions) were collected and adjusted to an absorbance of 1.0 at 280 nm to prepare the same protein concentration as the other IgM-deprived fractions.

The anti-human IgM-conjugated Sepharose 4B gels used in the study were prepared by coupling sheep anti-human IgM antibody (Wako Corp., Tokyo, Japan) and CNBr-activated Sepharose 4B (Pharmacia) according to the manufacturer's protocol. Removal of IgM from the IgM-rich fractions by the anti-human IgM-conjugated Sepharose 4B gel affinity chromatography was confirmed by assaying IgM concentrations in the IgM-rich fractions and IgM-deprived fractions (data not shown).

Protein concentrations of the IgM-rich fractions and the IgM-deprived fractions were adjusted to an absorbance of 1.0 at 280

mm. To examine whether or not they had any effect on endometrial cell proliferations, the IgM-rich fractions and the IgM-depleted fractions were stepwise-diluted and then added to cultures of HHUA cells.

Viable cell numbers were assayed with a non-RI colourimetric assay kit, XTT (Boehringer-Mannheim, Mannheim, Germany), which quantitated enzyme activity in viable cells for absorbances at 450 nm. Our preliminary studies showed that the absorbance at 450 nm was proportional to viable cell numbers (data not shown). The stimulatory effect of the diluted IgM-rich fractions or the diluted IgM-depleted fractions on the cell growth of HHUA cells was assayed as follows. On day 1, HHUA cells in the log phase were detached in 0.25% trypsin/1 mM EDTA (GIBCO-BRL) and cultured overnight in 96 well-culture plates (5,000 cells/well). On day 2, the diluted IgM-rich or IgM-depleted fractions were added to the cells. On day 3, viable cells were counted with the kit. All the experiments were performed three times to verify the results. The data are expressed as means and were analyzed with the Student's t-test ($n=5$).

Results

First, we examined total serum anti-endometrial antibody titers in IVF-ET patients with or without endometriosis. No significant increase in total anti-endometrial antibodies (IgG/IgM/IgA) occurred in endometriotic patients (Figure 1).

Second, we used indirect flow cytometry to examine immunoglobulin subclasses of the anti-endometrial antibodies. Figure 2 shows a typical result of an endometriotic patient. All sera from the sterile patients examined (7 endometriotic patients and 6 nonendometriotic patients) included Ig- γ , Ig- μ , Ig- κ , and Ig- λ chains, and both antibodies against endometrial epithelial cells (HHUA, HEC-1) and endometrial stromal cells. Both autoantibodies against endometrial epithelial cells and endometrial stromal cells were detectable in all the examined sera irrespective of the presence of endometriosis.

Third, we assayed tissue-specific anti-endometrial antibody levels by flow cytometry to examine differences in B cell clonalities activated in endometriotic patients and

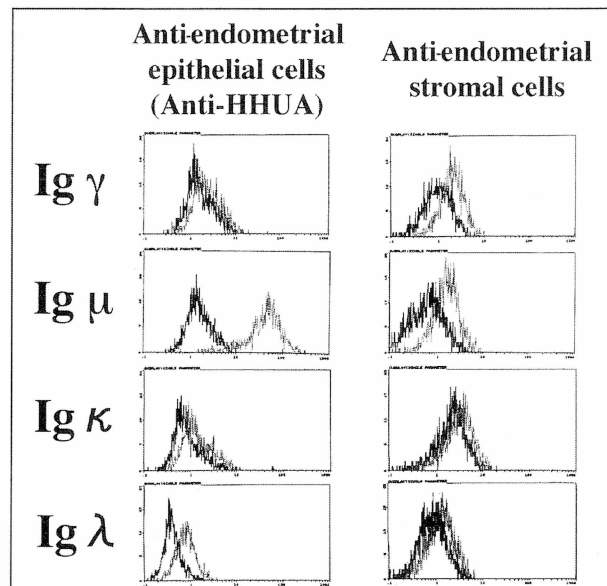


Figure 2. — Polyclonal B cell activation in endometriotic patients. Flow cytometry for 7 endometriotic patients and 6 nonendometriotic patients detected anti-HHUA antibodies and anti-endometrial stromal cell antibodies. Thick lines show controls (second antibody alone) and thin lines show positive stainings.

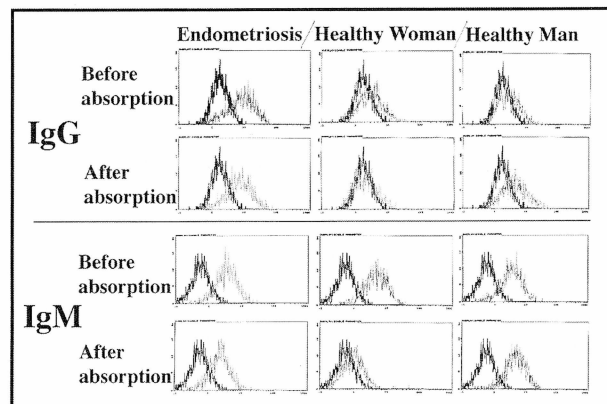


Figure 3. — Detection of tissue-specific anti-endometrial antibodies by absorption tests. Nonspecific anti-endometrial antibodies were absorbed by incubating the examined sera with ME180 cells before flow cytometric analyses. The figure shows typical flow cytometric patterns of anti-HHUA IgGs and anti-HHUA IgMs from an endometriotic patient, a healthy woman, and a healthy man. Thick lines show controls (second antibody alone) and thin lines show positive stainings.

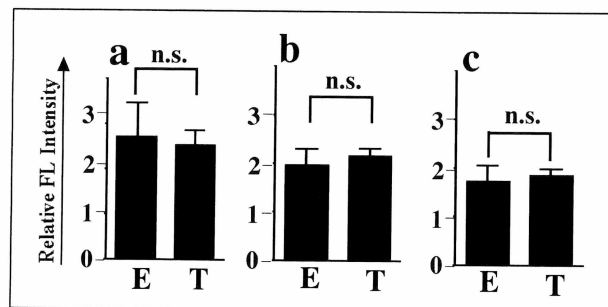


Figure 1. — Total serum anti-endometrial autoantibody titers in sterile patients with or without endometriosis. Total anti-endometrial antibody titers (IgM/IgG/IgA) were examined in IVF-ET patients with endometriosis ($n=13$) or with oviductal obstruction without endometriosis ($n=4$). a: antibody titers against HHUA cells, b: antibody titers against HEC-1 cells, c: antibody titers against endometrial stromal cells. E: patients with endometriosis, T: non-endometriotic patients with oviductal obstruction. n.s.: not significant.

healthy women. Nonspecific anti-endometrial antibodies were removed from the sera by absorption with ME180 cells. Absorption tests were performed for sera obtained from three endometriotic patients who had endometrial cysts of their bilateral ovaries, three nonendometriotic healthy women, and three healthy men. As shown in Figure 3, anti-endometrial antibody levels of the healthy women dramatically decreased after absorption, while those of the endometriotic patients hardly decreased. Results of the absorption tests are summarized in Figure 4.

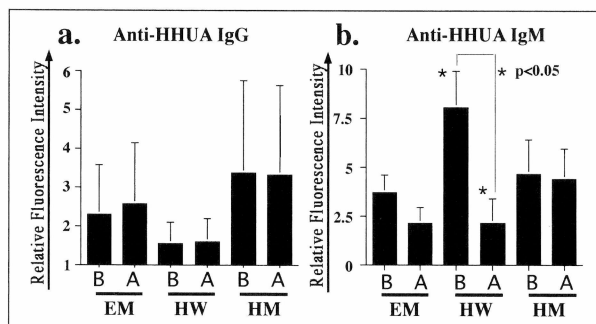


Figure 4. — Changes in anti-HHUA antibody titers after absorption tests. Nonspecific anti-endometrial antibodies were absorbed by incubating the examined sera with ME180 cells before flow cytometric analyses. Absorption tests were performed for sera obtained from 3 endometriotic patients (EM), 3 non-endometriotic healthy women (HW), and 3 healthy men (HM). Data are mean \pm SD. Significant decrease in anti-HHUA IgM antibody titers were found in nonendometriotic healthy women. B: before absorption, A: after absorption.

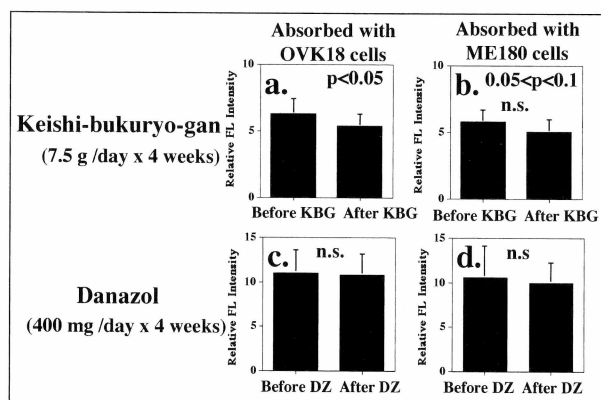


Figure 5. — Effects of Keishi-bukuryo-gan therapy and danazol therapy on tissue-specific anti-endometrial IgM antibodies in endometriotic patients. Three endometriotic patients were treated with Keishi-bukuryo-gan (KBG) and 3 were given danazol (DZ) for 4 weeks. Serum tissue-specific antibodies before and after the treatments were analyzed simultaneously by flow cytometry. Nonspecific anti-endometrial antibodies were absorbed by incubating the examined sera with ME180 cells or OVK18 cells before flow cytometric analyses. n.s.: not significant.

Fourth, we examined changes in tissue-specific anti-endometrial antibodies in endometriotic patients who were treated for four weeks with KBG (7.5 g/day) or danazol (400 mg/day). We detected no significant changes in tissue-specific IgG levels during either treatment. However, significant decreases in tissue-specific anti-endometrial IgM levels occurred in endometriotic patients treated with KBG, while those patients treated with danazol showed no significant changes in the IgM levels (Figure 5).

Finally, we preliminarily examined endometrial cell growth effects of the partially purified IgM-rich fractions from endometriotic patient sera since certain IgM autoantibodies might cross-link and stimulate cell surface receptors for growth signals. The IgM-rich fractions from endometriotic patients apparently differed in effect on

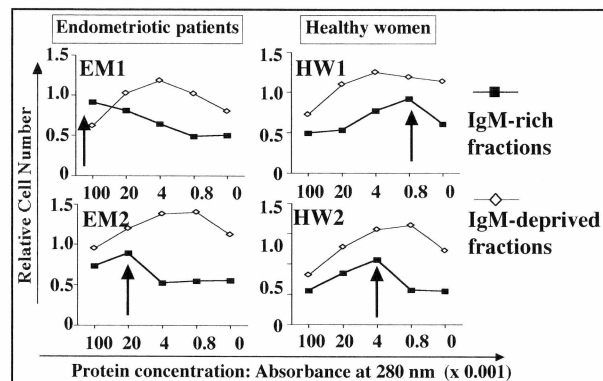


Figure 6. — Effects of the IgM-rich fractions and the IgM-depleted fractions on HHUA cell proliferation. The IgM-rich fractions and the IgM-depleted fractions were purified from 2 endometriotic patients (EM1 and EM2) and 2 healthy women (HW1 and HW2) according to the procedure described in the Materials and Methods. Thick lines with closed squares show dose-dependent effects of the IgM-rich fractions on HHUA cell growth, and thin lines with open diamonds show dose-dependent effects of the IgM-depleted fractions on the cells. Arrows indicate maximum stimulating effects of the diluted IgM-rich fractions on cell proliferation.

growth of HHUA cells compared with the IgM-rich fractions purified from healthy women and men (Figure 6). Moreover, no differential growth effects on HHUA cells from endometriotic patients and healthy women were found in their IgM-depleted fractions (Figure 6).

Discussion

Our present study indicates that generation of anti-endometrial antibodies is never specific to endometriotic patients, although anti-endometrial autoantibodies (Figure 1) and polyclonal B cell activation (Figure 2) were detected in all the endometriotic patients examined. Total anti-endometrial antibody concentrations in endometriotic patients were not higher than those in healthy women (Figure 1). However, our absorption test data revealed that clonalities of anti-endometrial autoantibodies in endometriotic patients apparently differ from those in nonendometriotic women (Figures 3 and 4). This finding indicates that a certain clonal immunological response has some relation to the pathogenesis of endometriosis.

It has been reported that danazol suppresses various autoantibody levels in patients with autoimmune disease [1, 25]. As shown in Figure 5, however, danazol treatment for four weeks did not suppress the tissue-specific anti-endometrial antibodies, while KBG treatment did suppress tissue-specific anti-endometrial IgM antibodies of the endometriotic patients. Our results suggest that KBG has a certain immunoregulatory effect on autoimmune responses in endometriosis. Our study may be the first to explore a pharmacological mechanism of KBG on endometriosis.

As reported in a previous paper, anti-endometrial antibody levels in an endometriotic patient were not affected by GnRHa therapy, which suppressed endogenous estradiol production and CA 125 expression for five months [26]. However the specific anti-endometrial IgM titers in

the patient decreased during treatment with KBG but not with GnRHa. These results suggest that one of the pharmacological mechanisms of KBG in endometriosis is an immunomodulatory function independent of endocrine regulation. Serum CA 125 is well known to be a useful clinical marker for endometriosis and adenomyosis [27]. The case data show a close relation between serum estradiol levels and serum CA 125 levels and indicate that serum CA 125 levels had no relation to endometriotic symptoms of the patients who were treated with KBG. KBG suppressed both endometriotic symptoms and specific anti-endometrial IgM levels but did not affect serum CA 125 levels [26]. The results suggest that serum CA 125 is indeed a marker for growth of ectopic endometrial tissues but not a therapeutic marker for endometriotic patients treated with KBG. Thus, specific anti-endometrial IgM may play a pathogenic role in endometriosis, and the specific IgM titer could be a novel clinical marker for endometriosis.

Although various autoantibodies have been found in sera of endometriotic patients, the biological significance has not been analyzed. Given that IgM antibodies against cell surface antigens can cross-link the antigens and cause proliferation/differentiation signals in the cells, IgM type autoantibodies may induce abnormal proliferation/differentiation of the target cells. In this preliminary study we have first revealed a possibility that anti-endometriotic IgM antibodies in endometriotic patients may have differential growth effects on the human endometrial epithelial cell line compared to their effects in healthy women. These results also suggest that anti-endometrial IgM antibodies may be a causative pathogen in endometriosis and thus that endometriosis is a potential autoimmune disorder.

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Address reprint requests to:
TETSUJI TANAKA, M.D., Ph.D.
Assistant Professor
Department of Obstetrics & Gynecology
Osaka City University Medical School
1-4-3 Asahi-machi, Abeno-ku, Osaka 545-8585
Osaka (Japan)