Original article

Role of prostaglandin E2 in bacterial growth in women with endometriosis

Khaleque Newaz Khan[1*], Michio Kitajima[1], Naohiro Yamaguchi[2], Akira Fujishita[3], Masahiro Nakashima[4], Tadayuki Ishimaru[5], Hideaki Masuzaki[1]

[1]Department of Obstetrics and Gynecology, [2]Department of Molecular Microbiology and Immunology, Nagasaki University Graduate School of Biomedical Sciences; [3]Saiseikai Nagasaki Hospital, Nagasaki; [4]Department of Tumor and Diagnostic Pathology, Atomic Bomb Disease Institute, Nagasaki; [5]Sasebo Chuo Hospital, Nagasaki, Japan

Running title: PGE2 promotes growth of *E.coli*

[*]**Address for correspondence and reprint request**:
Khaleque Newaz Khan, MD, PhD
Department of Obstetrics and Gynecology
Graduate School of Biomedical Sciences, Nagasaki University
1-7-1 Sakamoto, Nagasaki 852-8501, Japan
Tel: +81-95-819-7363
Fax: +81-95-819-7365
Email: nemokhan@nagasaki-u.ac.jp

Abstract

Title: Role of prostaglandin E2 in bacterial growth in women with endometriosis.

Study question: To investigate the role of bacteria in endometriosis.

Summary answer: Prostaglandin (PG) E2 in involved in the bacterial growth in women with endometriosis.

What is known already: Menstrual blood of women with endometriosis is highly contaminated with *Escherichia coli (E.coli)* than in non-endometriosis and *E.coli*-derived lipopolysaccharide (LPS) promotes the growth of endometriosis.

Study design, size and duration: This is a case-controlled biological research with prospective collection of body fluids and endometrial tissues from women with and with endometriosis and retrospective evaluation.

Participants/materials, setting, methods: Peritoneal fluid (PF) and sera were collected from 58 women with endometriosis and 28 women without endometriosis in an academic research laboratory. Menstrual blood was collected from a proportion of these women. Macrophages (Mϕ) from PF and stromal cells from eutopic endometria were isolated in primary culture. Exogenous effect of PGE2 on the replication of *E.coli* was examined in a bacteria culture system. Levels of PGE2 in different body fluids and in the culture media

of Mϕ and stromal cells was measured by ELISA. Effect of PGE2 on the growth of peripheral blood lymphocytes was examined.

Main results and the role of chance: PGE2 level was 2-3 times higher in the menstrual fluid (MF) than in either sera or in PF. A significantly higher level of PGE2 was found in the MF and PF of women with endometriosis than in control women ($p<0.05$ for each). Exogenous treatment with PGE2 dose-dependently increased *E.coli* colony formation when compared with non-treated bacteria. Prostaglandin E2-enriched MF was able to stimulate the growth of *E.coli* in a dilution-dependent manner and this effect was significant in women with endometriosis than in control women ($p<0.05$). PGE2 levels in the culture media of LPS-treated Mϕ/stromal cells was significantly higher in women with endometriosis than in non-endometriosis ($p<0.05$ for each). Direct application of PGE2 and culture media derived from Mϕ or stromal cells were able to significantly suppress phytohemagglutinin (PHA)-stimulated growth of peripheral blood lymphocytes. A higher PGE2 level in the menstrual fluid of women with endometriosis may be involved in the growth of *E.coli* either by its direct growth promoting effect or by its indirect immunosuppressive effect.

Limitations, reasons for cautions: Further studies are needed to examine the association between PGE2-stimulated growth of *E.coli* and endotoxin level and to investigate the possible occurrence of sub-clinical infection within vaginal cavity.

Wider implications of the findings: Still claiming as a mysterious disease, our findings may provide some new insights to understand the physiopathology or pathogenesis of endometriosis and may have new therapeutic potential.

Study funding/competing interest(s): This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Sports, Culture, Science and Technology of Japan. There is no conflict of interest related to this study.

Trial registration number: not applicable.

Key words: endometriosis, menstrual fluid, peritoneal fluid, PGE2, *Escherichia coli*, lymphocytes.

Introduction

Endometriosis is an estrogen dependent chronic inflammatory disease mostly affecting women of reproductive age. Besides hormonal regulation, both secondary and initial inflammatory mediators are known to involve in the growth of endometriosis (Attar and Bulun, 2006; Khan et al., 2008, 2009). As a source of initial inflammatory mediator (lipopolysaccharide, LPS), we recently demonstrated *Escherichia coli* (*E.coli*) contamination of menstrual blood with increased levels of bacterial endotoxin (LPS) in the menstrual fluid (MF) and peritoneal fluid (PF) derived from women with endometriosis than in non-endometriosis and an LPS/Toll-like receptor 4 (TLR4) cascade in the growth of endometriosis (Khan et al., 2010). However, the mechanistic basis of this microbial contamination of menstrual blood is unclear.

Lipopolysaccharide (LPS) promoted proliferation and invasion of endometriotic stromal cells via up-regulation of cyclooxygenase 2 (COX2) and prostaglandin E2 (PGE2) has been reported (Takenaka et al., 2010). A growing body of evidence demonstrates that PGE2 regulates many pathophysiological processes including cell proliferation, anti-apoptosis, immune suppression and angiogenesis during the development of endometriosis (Wu et al., 2010). According to a literature published

previously (Kuno et al., 1986a), it has been reported that PGE2-enriched seminal plasma has the capacity to replicate HTLV-II and other HIV-associated viruses. This may explain an increase in HIV viral load either by the direct action on viral replication or indirect immunosuppressive effect of PGE2 in homosexual population (Kuno et al., 1986a, 1986b).

The local immunosuppressive effect of PGE2 has also been demonstrated to promote the growth of cancer cells (Kojima et al., 2001). Immune cells in lymphoid lineage that comprised T and B lymphocytes and natural killer cells play essential roles in determining either accept or reject survival, implantation and proliferation of endometrial and endometriotic cells. An aberrant function of these immune cells has been reported in women with endometriosis (Osuga et al., 2011). Information on the immunosuppressive effect of PGE2 on peripheral blood lymphocytes derived from women with endometriosis is limited.

Clinically, women with endometriosis are reported to complain of dysmenorrhea and pelvic pain with their association with elevated levels of PGE2/PGF2 α in MF, PF and in tissues derived from the eutopic and ectopic endometria of these

women (Benedetto, 1989; Koike et al., 1992; Wu et al., 2007). Besides its role in promoting viral replication, information regarding involvement of PGE2 in the proliferation of bacteria such as *E.coli* is lacking. We speculated that PGE2 in the MF of women with endometriosis could be responsible for the increased replication of *E.coli,* either by its direct effect or indirect immunosuppressive effect, once this common vaginal microbial flora transmigrates from the lower genital tract into the uterine cavity.

To address this question, first of all, we measured PGE2 levels in different body fluids such as in MF, PF and sera derived from women with and without endometriosis. Secondly, we investigated whether PGE2 has any direct role in the replication of *E.coli* in a bacteria culture system. Thirdly, we examined the dilution-dependent effect of MF derived from control women and women with endometriosis on *E.coli* growth and patterns of bacterial growth after neutralizing PGE2. Finally, we extended our experiment to investigate whether PGE2 exhibits growth suppression effect on peripheral blood lymphocytes derived from women with and without endometriosis.

Materials and Methods

Patient samples. The subjects in this study were women of reproductive age.

Peritoneal fluid (PF) and sera were collected from 58 women with endometriosis and cycle matched to 28 women without endometriosis during laparoscopy. Women with endometriosis aged between 20 and 42 years were recruited by either elective laparoscopy for infertility or diagnostic laparoscopy for dysmenorrhea and subsequently confirmed by histology. The control group, between 18 and 32 years old, consisted of fertile women without any evidence of endometriosis and were operated on for dermoid cyst by laparoscopy. Neither the study group nor the endometriosis-free group had been on hormonal medication in the 3 months prior to the surgical procedure. All control women and women with endometriosis had regular menstrual cycles (28-32 days). All induced menstrual cycles were excluded from the current study. All body fluid samples and biopsy specimens were collected in accordance with the guidelines of the Declaration of Helsinki and with the approval by the Institutional Review Board of Nagasaki University. An informed consent was obtained from all women.

Collection of menstrual blood. With informed consent and under strict aseptic measure, we collected menstrual blood from 20 women with endometriosis and 15

women without endometriosis on day 1 to day 3 of the menstrual cycle and as described previously (Kamiyama et al., 2004; Khan et al., 2010). Briefly, in the dorsal lithotomy position, women underwent cleansing and disinfection of the vulva and vagina. A Fr. 12-sized plastic catheter connected to a 10-mL plastic syringe was inserted into the uterine cavity transcervically, and intrauterine aspiration was performed under negative pressure induced by manual suction of a syringe pump. The materials obtained were transferred into heparinized endotoxin-free plastic containers. After serial processing and centrifugation, menstrual fluid (MF) was collected and stored. All subjects underwent a laparoscopy to confirm presence or absence of endometriosis.

All samples of sera, PF and MF were collected prospectively and stored at -80℃ for subsequent analysis.

Isolation of Mφ and endometrial stromal cells. Macrophages from the PF and stromal cells from the eutopic endometria were collected from six women each with or without endometriosis. The detail procedures of the isolation of Mφ and stromal cells were described previously (Osteen et al., 1989; Rana et al., 1996; Khan et al., 2005a, 2005b). The purity of Mφ and stromal cell preparation was more than 95%, as judged by

positive cellular staining for CD68 and vimentin, respectively and negative staining for CD45, a pan-leukocyte marker; cytokeratin, a epithelial cell marker, and von Willebrand factor, a micro-vessel marker.

Prostaglandin E2 (PGE2) assay in different body fluids. PGE2 level was measured in duplicate by ELISA (Quantikine, R & D system, Minneapolis, MN) in the sera, PF, and MF derived from women with and without endometriosis. After the first passage, macrophages and stromal cells (10^5 per well) were serum-starved for 24 hours and cultured for another 24 hours with LPS (10ng/ml) derived from *E. coli* (serotype 0111:B4; Sigma, St. Louis, MO). PGE2 level was measured in the culture media derived from basal (LPS-non-treated) and LPS-treated Mϕ and stromal cells. The antibody used in PGE2 determination does not cross-react with other cytokines. The intra-assay and inter-assay coefficients of variation were less than 10% with a detection limit of 8.25 pg/mL.

Bioculture of Escherichia coli (E.coli). We used the similar strain of *E.coli* (K-12, serotype 0111:B4, Sigma, St. Louis, MO) in a bacteria culture system that we detected in menstrual blood (Khan et al., 2010) and the cell wall extract (LPS) of which

was used for our current experiment. We cultured *E.coli* in Laria-Bertani (LB) agar plate. To induce uniform colony growth, 100 μL of *E.coli,* dissolved in dimethylsulfoxide (DMSO), was added to each of 5 mL LB liquid media and incubated in a shaking incubator (280rpm) at 37°C for 2hr, 3hr, 4hr and 6hr. We found a time-depended increase in the colony formation of *E.coli* with a minimal colony formation at 2-3 hr and with a dilution of x10^{-6} and x10^{-7}. In our next experiment, we cultured PGE2-treated and non-treated *E.coli* in LB liquid media for 3hr and then incubated overnight in LB agar plate at a dilution of x10^{-6} and x10^{-7}. Next morning, *E.coli* growth was counted using a handy colony counter (Shibata, Tokyo, Japan) and expressed as colony forming units (CFU)/mL). PGE2 was diluted in DMSO, therefore, DMSO is present in all experiments and not only in the control experiments and is controlled for all these experiments. All colonies were counted for each plate and all experiments were performed in triplicates for each dilution.

Treatment of E.coli with PGE2. We investigated dose-depended effect of PGE2 (#14010, Cayman Chemical, USA) on the growth of *E.coli* in a bacteria culture system as described above. The serially diluted *E.coli* in LB liquid media was treated with

various doses of PGE2 (1pg/mL to 100ng/mL) and incubated overnight at 37℃. The colony formation of treated and non-treated *E.coli* was examined, counted all colonies in each culture plate and expressed as CFU/mL. All experiments were performed in triplicates for each dose of PGE2.

Treatment of E.coli with menstrual fluid. In an attempt to examine the direct effect of menstrual fluid (MF) on *E.coli* growth, diluted *E.coli* ($x10^{-7}$) in LB liquid media was treated with serial dilution of menstrual fluid (1:100, 1:200. 1:300. 1:500 and 1:1000) had collected from women with endometriosis and incubated overnight at 37℃. In separate experiments, *E.coli* in LB liquid media was pre-treated with anti-PGE2 antibody (10 μ g/mL) (Sigma Chemical Co. USA), maintained in culture for 20 minutes and then further co-treated with serial dilution of MF and incubated overnight. We also performed similar experiments on the effect of MF (1:100 dilution) derived from women with and without endometriosis on the pattern of *E.coli* growth. The colony formation of MF-treated, anti-PGE2 antibody-pre-treated and MF-non-treated *E.coli* was counted and expressed as CFU/mL. All experiments were performed in triplicates for each dilution of MF.

Treatment of lymphocytes with PGE2. Total population of lymphocytes was collected from the peripheral blood of six women each with and without endometriosis by Ficoll-Paque technique (Pharmacia Biotechnology, USA). The lymphocytes (10^5 cells/mL) were treated with 0, 1, 10, 30, 50, and 100 μg/mL of phytohemagglutinin (PHA), a potent mitogen for lymphocytes, and then co-treated with PHA (50 μg/mL) and PGE2 (10, 50, 100pg/mL). Phosphate buffered saline (PBS) was used as a vehicle treatment for control experiment and to standardize lymphocyte count. The pattern in the changes of lymphocyte growth was examined by counting the number of lymphocytes for each treatment and expressed as fold increase in the growth of lymphocytes compared with non-treated cells.

Co-treatment of lymphocytes with PHA, culture media and PGE2 inhibitor. We co-treated lymphocytes with PHA and 10% culture media (100 μL) derived from separately cultured Mϕ and stromal cells had collected from women with endometriosis. In a separate experiment, the lymphocytes were pre-treated with anti-PGE2 antibody (10 μg/mL) (Sigma Chemical Co. USA), maintained in culture for 20 minutes and then further co-treated with 10% culture media derived from each of Mϕ and stromal cells to

prove that it is the PGE2 in the culture media causing suppression in the growth of PHA-stimulated lymphocytes.

Statistical analysis. The clinical characteristics of the subjects were evaluated by one-way analysis of variance. The data are expressed either as mean ± SEM or mean ± SD. Mann-Whitney *U*-test and Student's *t*-test were used to analyze differences between groups and Kruskal-Wallis test was used to analyze differences among groups. A box plot analysis of PGE2 levels in different body fluids was performed using the medians and inter-quartile range (IQR). Tukey's post-hoc test was also used to exclude bias with multiple comparisons. A p value of <0.05 was considered statistically significant.

Results

The clinical profiles of women with and without endometriosis are shown in Table 1. There were no significant differences in the mean age and other clinical characteristics between women with and without endometriosis. The distribution of peritoneal lesions in women with revised-ASRM stage I-II endometriosis and stage III-IV endometriosis are shown in Table 1. We found a predominance of mixed peritoneal lesions in both stage I-II and stage III-IV endometriosis. Two cases in stage III-IV

endometriosis had chocolate cyst only without any coexistent peritoneal lesions.

Prostaglandin E2 (PGE2) levels in different body fluids. Kruskal-Wallis test indicated that PGE2 level was the highest in the menstrual fluid (MF) among three body fluids ($p<0.001$ vs. PF and sera) (Figure 1). We found a 2-3 times higher PGE2 levels in the MF of women with and without endometriosis when compared with PGE2 levels in either PF or in sera (Figure 1). A significantly higher level of PGE2 was found in the PF and MF of women with endometriosis ($p<0.05$ for each, Figure 1) than in women without endometriosis. No apparent difference in PGE2 levels in sera was found between women with and without endometriosis.

PGE2-stimulated growth of E.coli. PGE2 appears to promote a dose-dependent increase in *E.coli* colony formation (Figure 2A). Some colonies of *E.coli* were highly responsive to grow after treatment with lower doses of PGE2 and some colonies grew in response to higher doses of PGE2 when compared with colony growth in the control (DMSO) LB agar plate ($p<0.05$, DMSO vs. PGE2 1pg/mL and 10 pg/mL; $p<0.05$, DMSO vs. PGE2 10ng/mL, 50 ng/mL and 100 ng/mL, Figure 2A). We performed similar experiment in another bacteria culture plate (McConkey agar plate) to confirm the

validity of our results and we found similar pattern of *E.coli* growth in response to PGE2.

MF-stimulated growth of E.coli. Menstrual fluid was found to significantly stimulate the growth of *E.coli* in a dilution dependent fashion when compared with DMSO-treated *E.coli* ($p<0.05$ for each dilution of MF except 1:1000, Figure 2B). We found a higher colony formation of *E.coli* in response to low dilution of MF (1:100 and 1:200) and lower *E.coli* growth in response to higher dilution of MF (1:300 and 1:500). We did not find any growth of *E.coli* with further dilution of MF (1:1000) and was similar to DMSO-treated *E.coli* growth. This MF-stimulated *E.coli* growth was significantly suppressed after pre-treatment of *E.coli* with anti-PGE2 antibody ($p<0.05$ for each dilution of MF except 1:1000, Figure 2B).

We also found a significantly higher colony formation of *E.coli* in response to MF (1:100) derived from women with endometriosis comparing to the effect of MF (1:100) derived from control women ($p<0.05$, Figure 2C). This MF (1:100)-stimulated *E.coli* growth was significantly suppressed in both control and endometriosis women after pre-treatment of *E.coli* with anti-PGE2 antibody ($p<0.05$ for each, Figure 2C).

PGE2 levels in the culture media of Mϕ and stromal cells. LPS treatment

significantly increased the secretion of PGE2 in the culture media of Mϕ derived from women with and without endometriosis when compared with non-treated cells ($p<0.05$ for each group) (Figure 3, A). Comparing to non-treated cells, LPS-treated PGE2 level was significantly higher in the culture media of stromal cells derived from women with endometriosis ($p<0.05$) but not in control women (Figure 3, B). We also found a substantial and significant increase in PGE2 levels in the culture media of LPS-non-treated cells derived from women with endometriosis than in control women ($p<0.05$ vs. control, Figure 3A, B). LPS-treated PGE2 levels in the culture media of Mϕ and stromal cells were significantly higher in women with endometriosis than in control women ($p<0.05$ for each cells, Figure 3, A and B).

Effect of PGE2 on the growth of lymphocytes. Figure 4A shows a dose-depended growth of lymphocytes, in response to PHA, derived from women with and without endometriosis without showing any significant difference between them. Co-administration of PGE2 dose-dependently (10-100pg/mL) decreased PHA-stimulated growth of lymphocytes derived from women with endometriosis ($p<0.05$ for 10pg/mL and 50pg/mL; $p<0.01$ for 100pg/mL). This growth suppression effect of PGE2 on

PHA-stimulated lymphocytes was only observed at a dose of 100pg/mL in control women (Figure 4A). In our preliminary experiment on the dose-depended effect of PGE2 (10pg to 10ng/ml) on lymphocyte growth, we found that there was so further decrease of lymphocyte growth in response to PGE2 with a dose beyond 100pg/mL (data not shown).

We used 10% culture media (10 μL) from each of basal Mϕ and stromal cells derived from women with endometriosis. We found that PHA-promoted growth of lymphocytes was significantly suppressed after application of 10% culture media derived from each of Mϕ and stromal cells ($p<0.05$ for both, Figure 4B). Pretreatment of lymphocytes with anti-PGE2 antibody (10 μg/mL) abrogated the growth suppressing effect of culture media on these cells (Figure 4B).

Discussion

We demonstrated for the first time that higher PGE2 levels in the menstrual fluid of women with endometriosis was involved in the bacterial growth such as *E.coli* in a bacteria culture system. This effect of PGE2 on bacteria may be contributed by its direct growth promoting effect on *E.coli* or by its indirect immunosuppression effect on peripheral blood lymphocytes (PBL). In fact, we found significantly higher levels of

PGE2 in the MF and PF of women with endometriosis than in control women. The experimental doses of PGE2 stimulating *E.coli* growth were within the level in MF and PF.

The direct effect of PGE2-enriched MF in promoting the growth of *E.coli* was supported by the findings that pre-treatment of MF with anti-PGE2 antibody significantly suppressed MF-stimulated replication of *E.coli* in a dilution-dependent fashion. The growth promoting effect of MF on *E.coli* was more prominent in women with endometriosis than in control women. This indicates that among different macromolecules in menstrual blood (Zhou et al., 1989; Badaway et al., 1985), PGE2 is one of the components of MF that may be involved in bacterial growth. The indirect effect of PGE2 in the growth of *E.coli* can be explained by the significant suppression of PHA-stimulated growth of PBL in response to PGE2. This immunosuppression effect of PGE2 was observed for PBL derived from women with both endometriosis and non-endometriosis. An unexpected observation was that we did not find any significant difference in lymphocyte growth in response to PHA between women with and without endometriosis. This can be explained by the fact that we collected total population of

peripheral blood lymphocytes (CD3+) from these two groups of women and not individual population of lymphocytes such as CD4+ or CD8+ lymphocytes. Further study may clarify this issue using individual phenotype of lymphocytes.

We recently demonstrated that menstrual blood of women with endometriosis was highly contaminated with non-pathogenic strain of *E.coli* with consequent significantly higher levels of endotoxin (LPS) in both MF and PF derived from women with endometriosis than in non-endometriosis (Khan et al., 2010). The colony formation of *E.coli* and endotoxin levels in MF was markedly higher in women with red lesion containing r-ASRM stage I-II endometriosis than in women with endometrioma containing stage III-IV endometriosis. We also reported that LPS was involved in Toll-like receptor 4-mediated pro-inflammatory response by macrophages and growth of both eutopic and ectopic endometrial cells (Khan et al., 2010). However, the mechanistic basis of bacterial growth in menstrual blood was unclear. Here we reported that PGE2-enriched MF as well as PGE2 itself might play a role in bacterial growth such as *E.coli*. Although we could not investigate the association between PGE2-stimulated growth of *E.coli* and endotoxin levels in bacteria culture media in our current study, we

can at least speculate that PGE2-enriched MF may be involved in *E.coli* replication by its direct growth promoting effect.

Macrophages and endometrial stromal cells are predominant cell types producing PGE2 (Herath et al., 2006). We detected higher levels of basal and LPS-treated PGE2 in the culture media of both Mϕ and endometrial stromal cells derived from women with endometriosis than in control women. We found that in addition to suppressing PHA-stimulated growth of lymphocytes by PGE2, application of PGE2 containing culture media derived from both of these cells was able to significantly suppress PHA-promoted growth of lymphocytes. This growth inhibiting effect of culture media on lymphocytes was lost after pre-treatment of cells with anti-PGE2 antibody. Information regarding PGE2-mediated suppression of T-cell function has been previously reported by other investigators (Benedetto, 1989; Koike et al., 1992). Here we reported that PGE2 has the similar capacity to suppress immune cell function in women with endometriosis. This finding is biologically important. First, during the menstrual period, higher levels of PGE2 in menstrual blood being produced and released by both of these cells in intrauterine environment may directly or indirectly enhance *E.coli* growth.

Second, as an anti-apoptotic agent, higher levels of PGE2 in MF and PF may facilitate survival of endometrial cells in pelvic environment. Third, prostaglandin suppressing agents, such as current hormonal therapies for endometriosis including NSAIDS, may be clinically useful for not only suppressing the growth of endometriosis but also for protecting bacterial growth in women with endometriosis. It is unknown regarding the relationship between PGE2 levels and numbers of immune cells in menstrual blood. Further studies are needed to clarify our growth suppressing effect of PGE2 on lymphocytes and other immune cells.

Our findings provide some evidence that in addition to viral replication, PGE2 has the capacity to stimulate bacterial growth. The fluctuating pattern in the growth of *E.coli* in response to PGE2 may be due to polyclonal nature of *E.coli*. It means that some clones of *E.coli* are responsive to grow at lower doses and some clones at higher doses of PGE2. This can also be explained by differential binding affinity between PGE2 and its receptors, EP2/EP4 as reported to be expressed by different epithelial cells and microbial pathogens (George et al., 2010; Goldmann et al., 2010; Wu el al., 2010). A strong receptor-ligand binding affinity by lower and higher doses of PGE2 and decreased

functional affinity by the intermediate dose of PGE2 may explain our current findings. We need to clarify this variation in the growth pattern of *E.coli* in response to variable doses of PGE2 by further experiments.

The optimal concentrations of PGE2 and PGF2α in the menstrual blood and peritoneal fluid corresponds to 1pg/ml to 100pg/ml for women who complain of mild to moderate pelvic pain and 10ng/ml to 100ng/ml for women with severe pelvic pain associated with endometriosis (unpublished data). In fact, during the menstrual period, higher levels of PGE2, in association with PGF2α, may be involved in uterine contraction with consequent pain symptoms (Benedetto, 1989; Koike et al., 1992; Wu et al., 2007). Although we did not investigate in our current study, endotoxin (LPS) itself may also have an effect on uterine contraction in women with endometriosis via COX2 up-regulation and production of prostaglandins (Takenaka et al., 2010). Therefore, women with endometriosis who complain a variable severity of pelvic pain or menstrual pain might be susceptible to ongoing growth of migrating *E.coli* in their menstrual blood.

Our findings have some clinical and biological implications. First, higher PGE2 levels in PF after menstrual reflux may be involved in the growth of endometriosis,

because PGE2 acts as a regulator of COX2/P450 aromatase activation resulting in tissue accumulation of estrogen (Attar and Bulun, 2006; Bulun, 2009). As a master molecule, PGE2 has multi-functional role such as cell proliferation, anti-apoptosis, immune suppression and angiogenesis during the development of endometriosis (Wu et al., 2010). Second, role of PGE2 in the bacterial growth such as *E.coli* by its direct and indirect effect may partly explain the mechanistic basis of *E.coli* contamination of menstrual blood in women with endometriosis as we reported recently (Khan et al., 2010).

In fact, we demonstrated that a higher colony formation of *E.coli* in menstrual blood with consequent higher endotoxin (LPS) levels in MF and PF of women with endometriosis significantly stimulated growth of eutopic and ectopic endometrial cells via LPS/TLR4 cascade (Khan et al., 2010). When we tried to link our current findings with our previous experiment on LPS/TLR4 system, we found parallel increase of both LPS and PGE2 in the menstrual blood collected from women with endometriosis. LPS is one of the mediators to stimulate overexpression of COX2 with consequent production of different of PGs including PGE2. LPS-mediated COX2 expression and PGE2 production have already been reported (Takenaka et al., 2010; Liu et al., 2011). If we consider the

inflammatory condition of intrauterine or pelvic environment, we can collectively link an association between LPS/TLR4/COX2 and PGE2 in the growth of endometrial cells as well as growth of bacteria.

After similar ascending migration of bacteria from vaginal cavity into uterine cavity for all women, differences in PGE2 levels of MF between women with and without endometriosis may be involved in higher colony formation of *E.coli* in the menstrual blood of women with endometriosis. Further studies are needed to investigate the sub-clinical infection within vaginal cavity and to clarify our current findings.

Acknowledgments: We thank Miss Kazumi Hayashida and Miss Kyoko Ishida, Department of Obstetrics and Gynecology, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan, for their excellent technical assistance.

Authors' roles: KNK was involved in concept, study design, experiments, data analysis and manuscript draft; MK and AF contributed equally to sample collection; NY was involved in experimental assistance; MN, TI and HM was equally involved in draft advice.

Funding: This work was supported by Grants-in-Aid for Scientific Research

(Grant No. 16591671 and 18591837) from the Ministry of Education, Sports, Culture, Science and Technology of Japan (to KN Khan).

Conflict of interest: The authors declare that there is no conflict of interest related to this article.

References

Attar E and Bulun SE: Aromatase and other steroidogenic genes in endometriosis: translational aspects. *Hum Reprod Update* 2006;**12(1):**49-56.

Badawy SZ, Marshall L, Cuenca V. Peritoneal fluid prostaglandins in various stages of the menstrual cycle: role in infertile patients with endometriosis. *Int J Feril* 1985;**30(2):**48-52.

Benedetto C. Eicosanoids in primary dysmenorrhea, endometriosis and menstrual migraine. *Gynecol Endocrinol* 1989;**3(1):**71-94.

Bulun SE. Endometriosis. *N Engl J Med* 2009;**360**:268-279.

Herath S, Fischer FD, Werling D, Williams EJ, Lilly ST, Dobson H, Bryant CE, Sheldon IM. Expression and function of Toll-like receptor 4 in the endometrial cells of the uterus. *Endocrinology* 2006;**147**:562-570.

George PA, Sharma-Walia N, Kerur N, White C, Cahndran B. Piracy of prostaglandin E2/EP receptor-mediated signaling by Kaposi's sarcoma-associated herpes virus (HHV-8) for latency gene expression: strategy of successful pathogen. *Cancer Res.* 2010;**70(9):**3697-3708.

Goldman O, Hertzen E, Hecht A, Schmidt H, Lehne S, Norrby-Teglund A, Medina E. Inducible cyclooxygenase released prostaglandin E2 modulates the severity of infection caused by Streptococcus pyrogens. *J Immunol* 2010;**185(4):**2372-2381.

Kuno S, Ueno R, Hayaishi O, Nakashima H, Harada S, Yamamoto N. Prostaglandin E2, a seminal constituent, facilitates the replication of acquired immune deficiency syndrome virus *in vitro*. *Proc. Natl. Acad. Sci. USA* 1986a;**83**:3487-3490.

Kuno S, Ueno R, Hayaishi O. Prostaglandin E2 administered via anus causes immunosuppression in male but not female rats: A possible pathogenesis of acquired

immune deficiency syndrome in homosexual males. *Proc. Natl. Acad. Sci. USA* 1986b;**83**:2682-2683.

Koike H, Egawa H, Ohtsuka T, Yamaguchi M, Ikenoue T, Mori N. Correlation between dysmenorrheic severity and prostaglandin production in women with endometriosis. *Prostaglandins Leukot Essent Fatty Acids* 1992;**46(2):**133-137.

Kojima M, Morisaki T, Uchiyama A, Doi F, Mibu R, Katano M, Tanaka M. Association of enhanced cyclooxygenase-2 expression with possible local immunosuppression in human colorectal carcinomas. *Ann. Surg. Oncol.* 2001;**8(5):**458-465.

Kamiyama S, Teruya Y, Nohara M, Kanazawa K. Impact of detection of bacterial endotoxin in menstrual effluent on the pregnancy rate in in vitro fertilization and embryo transfer. *Fertil Steril* 2004;**82**:788-792.

Khan KN, Masuzaki H, Fujishita A, Kitajima M, Kohno T, Sekine I, Matsuyama T, Ishimaru T. Regulation of hepatocyte growth factor by basal and stimulated-macrophages in women with endometriosis. *Hum Reprod* 2005a;**20**:49-60.

Khan KN, Masuzaki H, Fujishita A, Kitajima M, Hiraki K, Sekine I, Ishimaru T. Interleukin-6- and tumor necrosis factor α-mediated expression of hepatocyte growth

factor by stromal cells and its involvement in the growth of endometriosis. *Hum Reprod* 2005b;**20**:2715-23.

Khan KN, Kitajima M, Hiraki H, Fujishita A, Sekine I, Ishimaru T, Masuzaki H. Immunopathogenesis of pelvic endometriosis: role of hepatocyte growth factor, macrophages and ovarian steroids. *Am J Reprod Immunol (review*), 2008;**60**:383-404.

Khan KN, Kitajima M, Hiraki H, Fujishita A, Sekine I, Ishimaru T, Masuzaki H. Toll-like receptors in innate immunity: role of bacterial endotoxin and toll-like receptor 4 (TLR4) in endometrium and endometriosis. *Gynecol Obstet Invest (review)* 2009;**68**:40-52.

Khan KN, Kitajima M, Hiraki K, Yamaguchi N, Katamine S, Matsuyama T, Fujishita A, Nakashima M, Ishimaru T, Masuzaki H. Escherichia coli contamination of menstrual blood and effect of bacterial endotoxin on endometriosis. *Fertil Steril* 2010; **94(7):**2860-2863.

Liu Y, Hu J, Shen W, Wang J, Chen C, Han J, Zai D, Cai Z, Yu C. Peritoneal fluid of patients with endometriosis peomotes the proliferation of endometrial stromal cells and induces COX-2 expression. *Fertil Steril* 2011;**95(5):**1836-1838.

Osteen KG, Hill GA, Hargrove JT, Gorstein F. Development of a method to isolate and

culture highly purified populations of stromal and epithelial cells from human endometrial biopsy specimens. *Fertil Steril* 1989;**52**:965-72.

Osuga Y, Koga K, Hirota Y, Hirata T, Yoshino O, Taketani Y. Lymphocytes in endometriosis. *Am J Reprod Immunol* 2011;**65**:1-10.

Rana N, Gebel H, Braun DP, Rotman C, House R, Dmowski WP. Basal and stimulated secretion of cytokines by peritoneal macrophages in women with endometriosis. *Fertil Steril* 1996;**65**:925-30.

Takenaka Y, Taniguchi F, Miyakoda H, Takai E, Terakawa N, Harada T. Lipopolysaccharide promoted proliferation and invasion of endometriotic stromal cells via induction of cyclooxygenase-2 expression. *Fertil Steril* 2010;**93(1)**:326-327.

Wu MH, Shoji Y, Chuang PC, Tsai SJ. Endometriosis: disease pathophysiology and the role of prostaglandins. *Expert Rev Mol Med* 2007;**9(2)**:1-20.

Wu MH, Lu CW, Chuang PC, Tsai SJ. Prostaglandin E2: the master of endometriosis? *Exp Biol Med (Maywood)* 2010;**235(6):**668-677.

Zhou JP, Fraser IS, Caterson I, Grivas A, McCarron G, Norman T, Tan K. Reproductive hormones in menstrual blood. *J Clin Endocrinol Metab.* 1989;**69(2):**338-342.

Figure Legends

Figure 1. Shows PGE2 levels in the sera, peritoneal fluid (PF) and menstrual fluid (MF) derived from women with (hatched box) and without (white box) endometriosis. PGE2 level was found to be the highest in the MF, intermediate in the PF and the lowest in the sera derived from women with and without endometriosis. Although no group difference was observed in serum, PGE2 level was significantly higher in the PF and MF of women with endometriosis than in control women ($p<0.05$ for each). Boxes represent the distance (interquartile range) between the first (25%) and third (75%) quartiles, and horizontal lines in the boxes represent median values.

Figure 2. Shows the colony forming unit (CFU/mL) of *Escherichia coli (E.coli)* in response to variable doses of PGE2 (**A**). A fluctuating growth pattern of *E.coli* was observed in response to a lower dose to higher dose of PGE2. Multiple comparison analysis revealed that from a lower dose to higher dose of PGE2 was able to significantly stimulate the growth of *E.coli* (*$p<0.05$ for each indicated dose) when compared with non-treated (DMSO only) bacteria.

The colony formation (CFU/mL) of *E.coli* was significantly increased in response to PGE2-enriched menstrual fluid (MF) derived from women with endometriosis in a dilution-dependent fashion comparing to DMSO-treated *E.coli* (white bar, **$p<0.05$ for each indicated dilution, **B**). The MF-stimulated growth of *E.coli* was significantly suppressed after pre-treatment of *E.coli* with anti-PGE2 antibody (black bar, **B**) (*$p<0.05$ vs. anti-PGE2 antibody-non-treated bacteria for each dilution).

The colony formation of *E.coli* was significantly higher in response to MF (1:100) collected from women with endometriosis than in control women ($p<0.05$, **C**). MF-stimulated *E.coli* growth was equally suppressed in both control women and in women with endometriosis after pre-treatment of bacteria with anti-PGE2 antibody

($p<0.05$ for each, **C**). DMSO, dimethylsulfoxide; anti-PGE2 (-) denotes colonies without pre-treatment with anti-PGE2 antibody; anti-PGE2 (+) denotes colonies with pre-treatment with anti-PGE2 antibody. The results are expressed as mean ± SD of triplicate experiments for each indicated dose of PGE2 or for each dilution of MF.

Figure 3. Shows PGE2 levels in the culture media of LPS-treated (black bar) and non-treated (white bar) Mϕ (**A**) and stromal cells (**B**) derived from the PF and eutopic endometria, respectively, of control women and women with endometriosis. Except stromal cells derived from control women, PGE2 levels in the culture media of LPS-treated Mϕ and stromal cells were significantly higher in women with and without endometriosis than in LPS-non-treated cells ($p<0.05$ for each cell) (**A, B**). PGE2 level in the culture media of LPS-non-treated cells was significantly higher in women with endometriosis than in control women ($p<0.05$ for each cell, **A, B**). LPS-stimulated PGE2 levels was also found to be significantly higher in the culture media of both Mϕ and stromal cells derived from women with endometriosis than in control women ($p<0.05$ for each cell, **A, B**). The results are expressed as mean ± SEM of six different experiments.

Figure 4. Shows the growth suppression effect of PGE2 on

phytohemagglutinin (PHA)-stimulated growth of lymphocytes derived from the peripheral blood of women with (black bar) and without (white bar) endometriosis (**A**). PHA stimulated the growth of lymphocytes in a dose-dependent manner. Co-treatment with a variable dose of PGE2 (10, 50, 100pg/mL) was able to significantly suppress PHA (50 μ g/mL)-stimulated growth of lymphocytes derived from women with endometriosis (**A**). This growth suppressing effect of PGE2 on lymphocytes was also observed in control women at a dose of 100pg/mL (**A**). The results are expressed as mean ± SEM of six different experiments.

Co-treatment with 10% culture media (10 μ L) of separately cultured M ϕ and stromal cells derived from women with endometriosis was also able to significantly suppress PHA-stimulated growth of lymphocytes (**B**). This effect of culture media on lymphocyte growth was abrogated after pretreatment of lymphocytes with anti-PGE2 antibody (10 μ g/mL) (**B**). The results are expressed as mean ± SEM of six different experiments.

Figure 1.

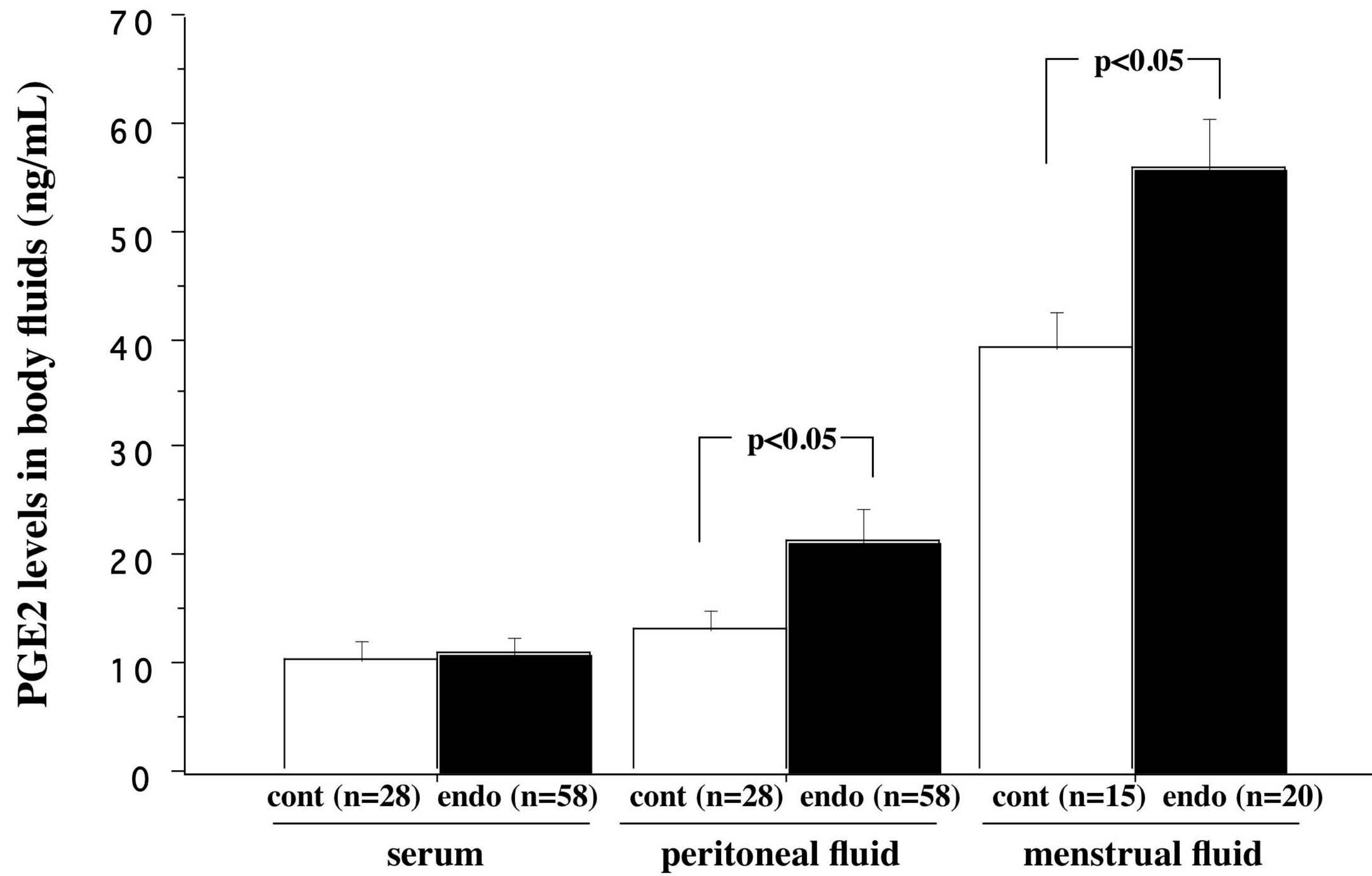
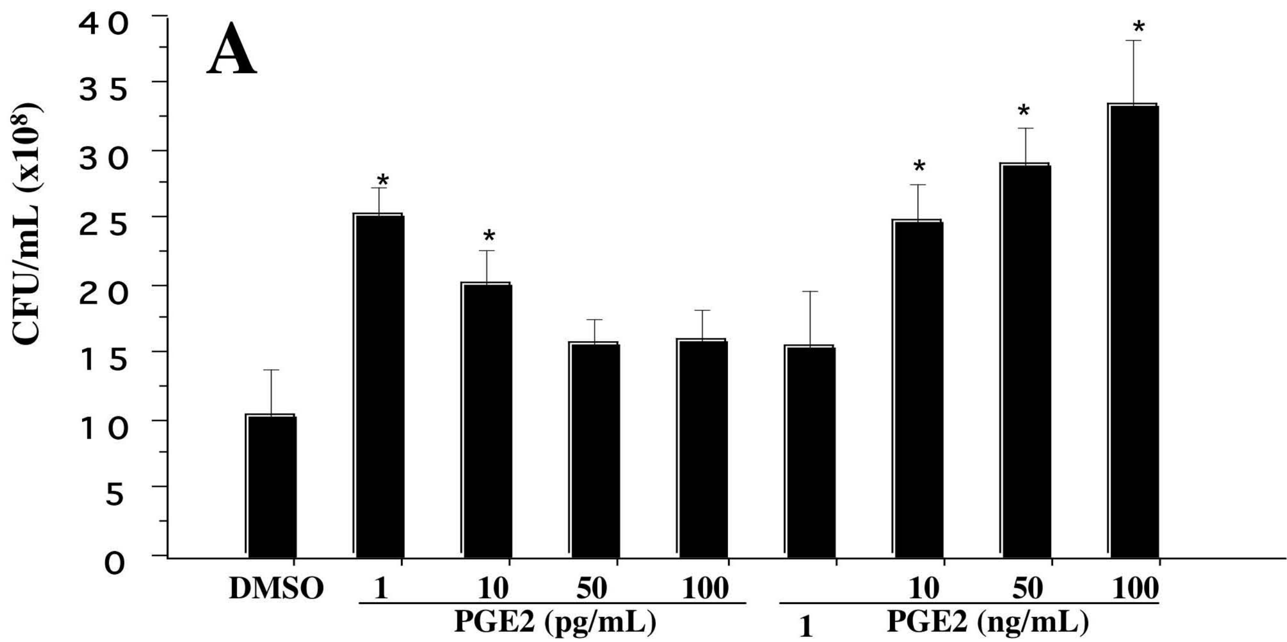
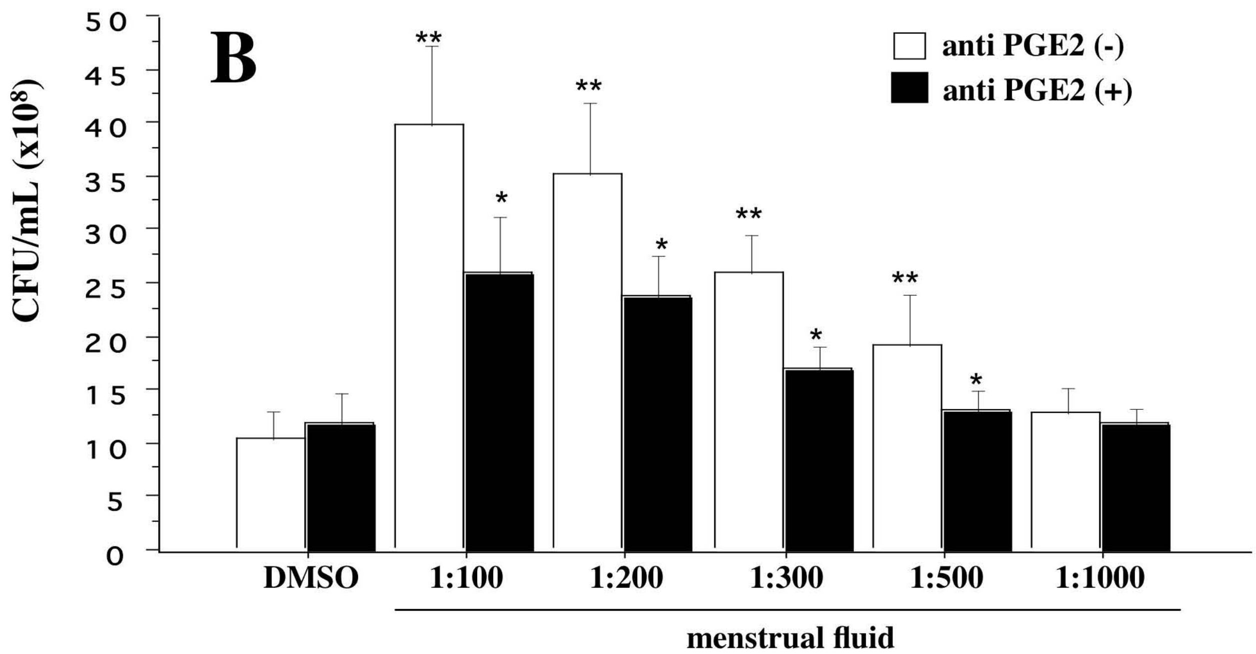


Figure 2.





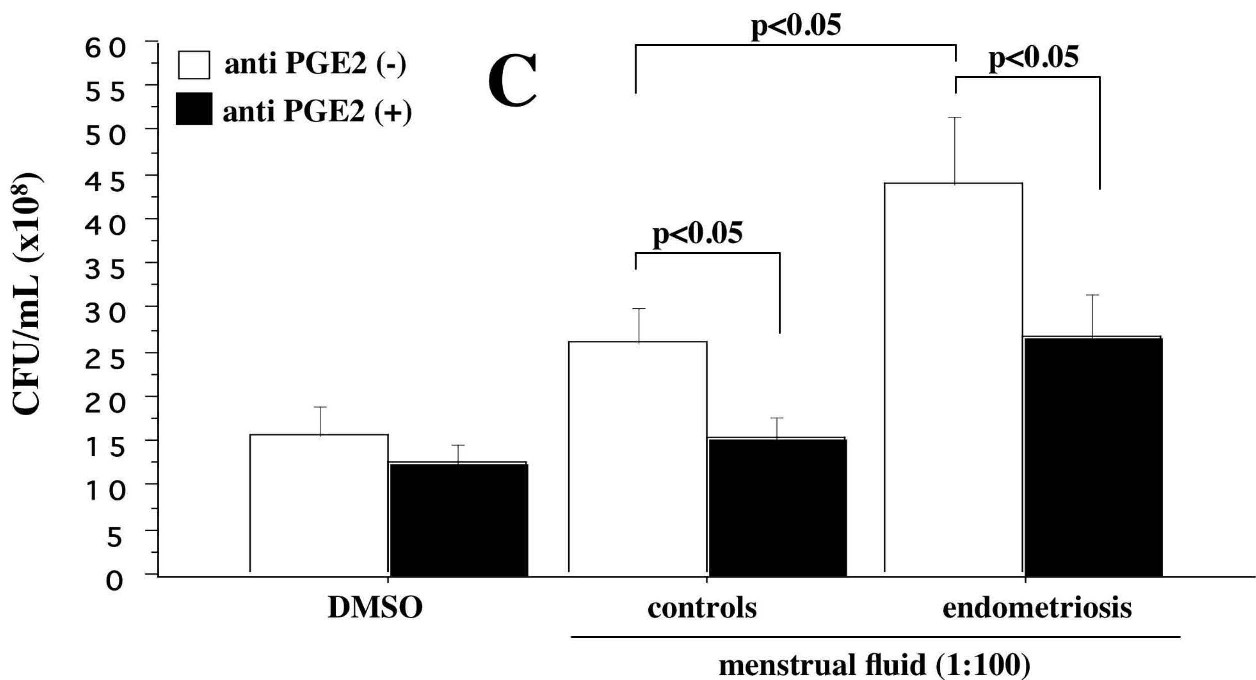
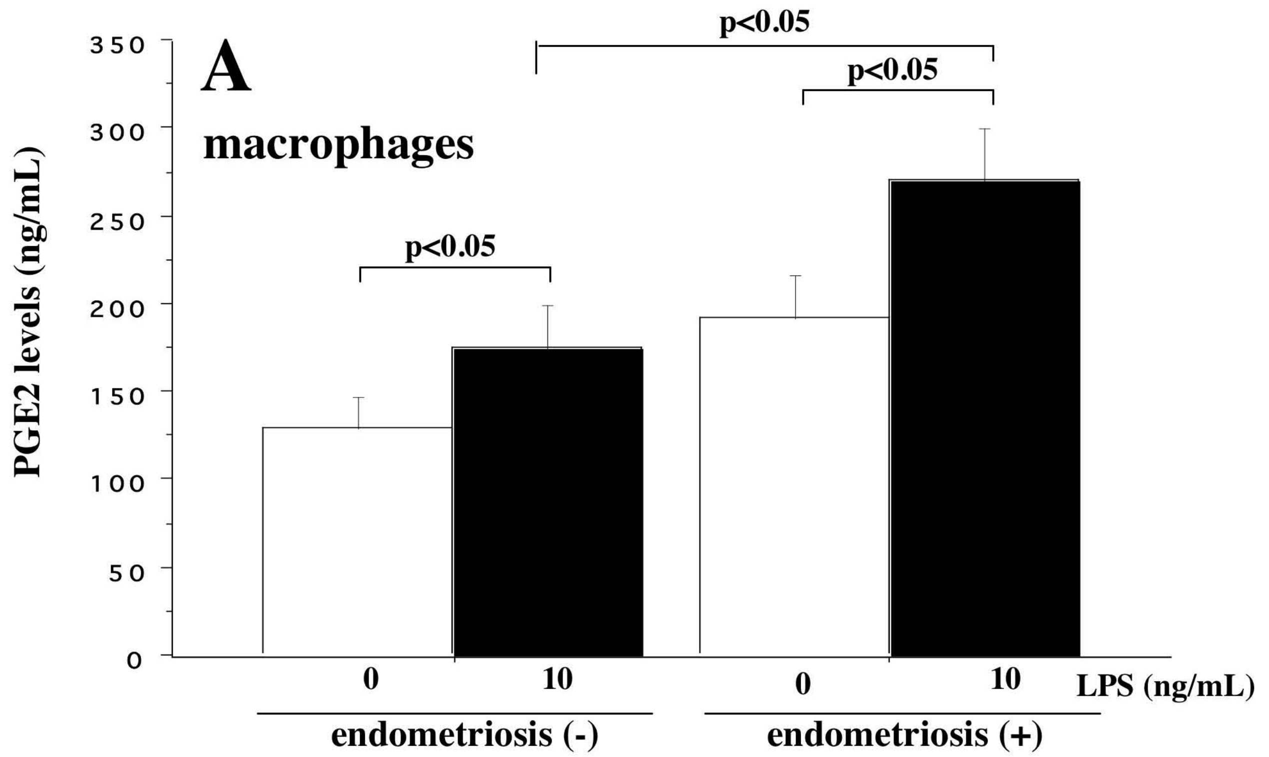


Figure 3.



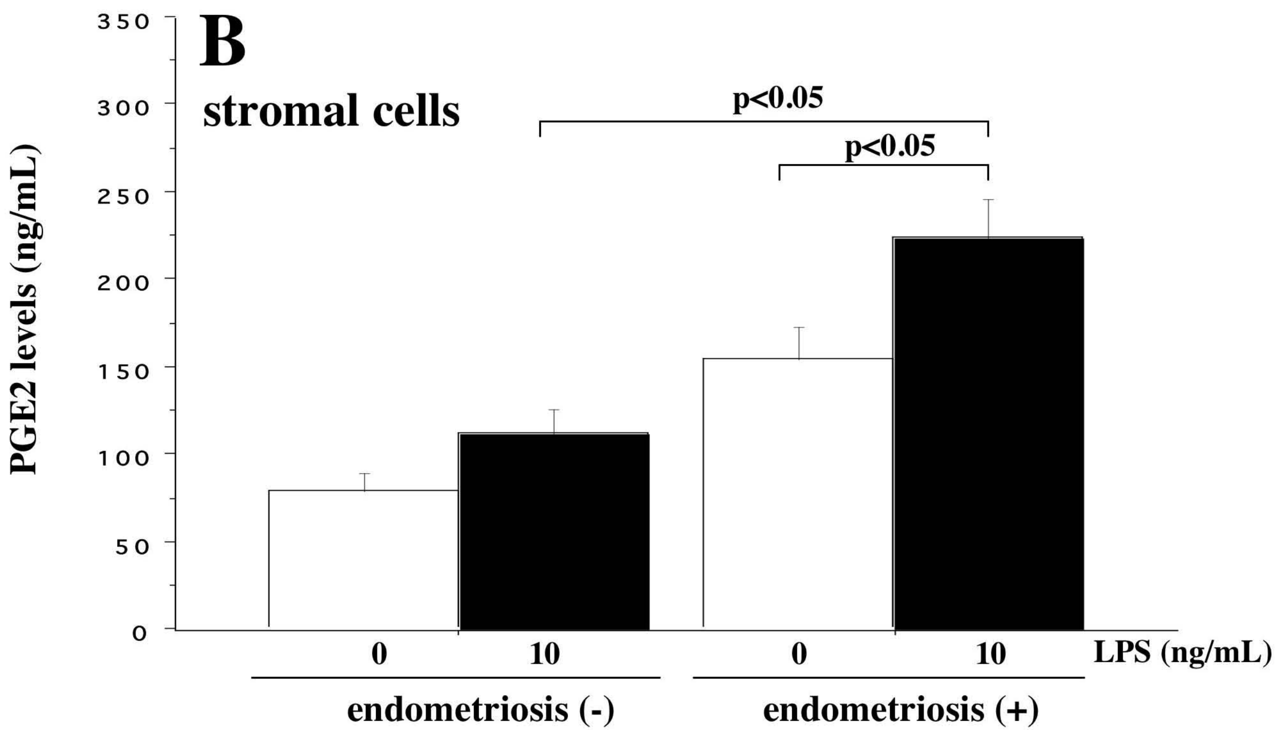
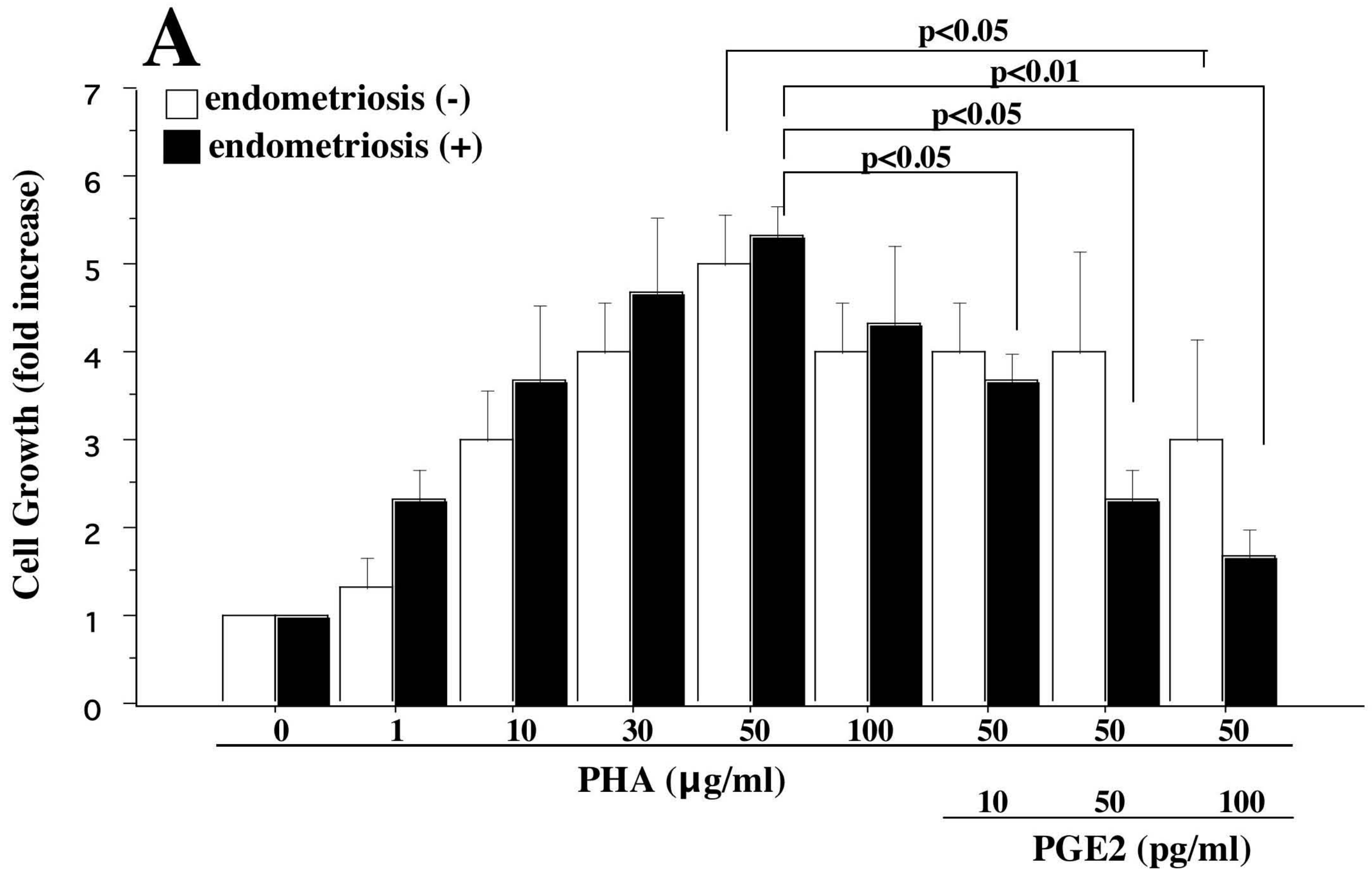


Figure 4.



B

p<0.05

p<0.05

Cell Growth (fold increase)

7
6
5
4
3
2
1
0

0
50
PHA (µg/ml)

0
10
anti-PGE2 (µg/ml)
Mφ culture media (10%)

0
10
anti-PGE2 (µg/ml)
stroma culture media (10%)

Table 1. Clinical profiles of patients with and without endometriosis.

	endometriosis (-) (n=28)	endometriosis (+) (n=58)
age in years (mean ± SD)	28.4 ± 3.9	30.2 ± 3.5
range of age in years	18-32	20-42
r-ASRM staging: I-II/III-IV		35/23
menstrual cycle: P/S/M/A	10/15/3/0	20/32/6/0

The results are expressed as mean ± SD. P, proliferative phase; S, secretory phase; M, menstrual phase; A, amenorrhea; r-ASRM, revised staging of American Society of Reproductive Medicine.