ORIGINAL

Lipopolysaccharide promotes early endometrial-peritoneal interactions in a mouse model of endometriosis

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Abstract : Purpose : The aims of this study were to clarify the effects of lipopolysaccharide (LPS) on the early development of endometriosis and on the production of cytokines and chemokines in the murine peritoneal cavity. Methods : Endometriotic lesions were induced in C57BL/6J adult female mice by intraperitoneal injection of endometrial fragments plus blood or endometrial fragments plus blood with LPS. On day 7, endometriotic lesions were assessed by gross and microscopic evaluations. Time-dependent changes in the secretion of TNF-α, IL-6, and CXCL2/MIP-2 in peritoneal lavage fluid after the intraperitoneal injection of LPS (50 μg/body) were measured by their respective enzyme-linked immunosorbent assays. Results : The areas of endometriotic lesions in the LPS group (10.8 ± 8.6 mm^2) were significantly larger than those in the control group (3.1 ± 3.7 mm^2). The levels of TNF-α and IL-6 peaked within 2 hours and the level of MIP-2 reached a maximum on day 1 after the injection of LPS. Conclusions : LPS promotes development of the early stages of murine endometriotic lesions. J. Med. Invest. 66 : 70-74, February, 2019

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INTRODUCTION

Endometriosis is a common gynecologic disorder associated with dysmenorrhea and infertility, which is characterized by the growth of endometrial tissues within the peritoneal cavity. The implantation theory of retrograde menstruation is widely accepted as the pathogenesis of peritoneal endometriosis (1). However, although retrograde menstruation occurs in 76–90% of women with patent tubes (2, 3), it remains to be elucidated why it progresses to the development of peritoneal endometriosis in only some women.

Reportedly, retrograde menstruation can induce the production of inflammatory cytokines, growth factors, and angiogenic factors, which may promote the adhesion of refluxed endometrial fragments (4, 5). Recently, Khan *et al.* reported that the menstrual blood of women with endometriosis was highly contaminated with *Escherichia coli* as compared to that of control women, and that the contamination corresponds to elevation in the concentrations of endotoxins in menstrual and peritoneal fluids (6). The number of colony forming units of *Gardnerella, α-Streptococcus, Enterococci*, and *E. coli* were significantly higher in endometrial samples obtained from women with endometriosis than in control women (7), suggesting that lipopolysaccharides (LPS) derived from gram-negative bacteria may play a role in the development of endometriosis. Furthermore, the prevalence of chronic endometritis was significantly higher in women with endometriosis as compared with women who did not have endometriosis, although the causal relationship between endometriosis and chronic endometritis remains unclear (8, 9).

Experimental mouse models have been developed to investigate the pathogenesis of endometriosis. Previously, we showed that the presence of blood accelerates the development of the early stage of endometriotic lesions when endometrial fragments plus blood are injected into the peritoneal cavity of 8- to 9-week-old adult mice (10). This syngeneic mouse model of endometriosis enabled us to assess the effect of LPS on the early endometrial-peritoneal interaction. The aim of the current study was to clarify the effect of inflammation on the early stage of development of endometriotic lesions by using a mouse model.

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MATERIALS AND METHODS

Animals

This study was approved by our Institutional Animal Care and Use Committee and carried out according to Tokushima University Animal Experimentation Regulations.

Mature (8- to 9-week-old) female C57BL/6J mice were purchased from CLEA Japan (Tokyo, Japan). The mice were fed a standard mouse diet and water ad libitum and kept at a light/dark cycle of 12/12 hours under controlled conditions. Prior to any invasive procedure, the mice were anesthetized with 4% sevoflurane.

All surgical procedures were performed under sterile conditions. This study was approved by the Committee of the Institute for Animal Experimentation of Tokushima University Graduate School.

Reagents

Estradiol and LPS from E. coli 0111 : B4 were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Induction of endometriotic lesions

Donor female C57BL/6J mice (n=10) were ovariectomized on day 0 under anesthesia using 4% sevoflurane and injected with depo-estrogen in peanut oil (100 μg/kg subcutaneously) for 7 days. On day 7, the mice were sacrificed and both uterine horns were removed and placed in a small sterile dish containing 0.5 mL of sterile normal saline. The endometrium was then gently peeled to separate it from the uterine muscle and cut into small pieces with small scissors.

Endometrial fragments from each uterine horn were suspended in 0.1 mL of sterile normal saline.

Injection of endometrial fragments with blood and LPS

Recipient mice (n=20) were anesthetized using 4% sevoflurane. After a 0.5 cm sub-abdominal midline incision was made, endometrial fragments from one of the uterine horns were mixed with 0.1 mL of blood immediately after its aspiration from the inferior vena cava without any anticoagulants (Control group, n=10) or mixed with 0.1 mL of blood and 50 μg of LPS (LPS group, n=10) and were injected into the peritoneal cavity. The peritoneum was then sutured.

Evaluation of transplanted tissues

The recipient mice were sacrificed on day 7 after the injection of endometrial fragments and the abdomen of each mouse was inspected and photographed. The endometrial lesions were measured with a scale and excised from the surrounding tissue. The maximal area of the lesion was calculated according to the formula for an ovoid : $D1 \times D2 \times \pi/4$.

Endometrial lesions were fixed in 10% formalin and embedded in paraffin. The tissue sections were cut into 5 μm sections and stained with hematoxylin and eosin. Sections were examined microscopically for the presence of histologic hallmarks of endometriosis.

Measurement of concentrations of cytokines

To investigate the effects of intra-peritoneal injection of LPS, 8- to 9-week-old female C57BL/6J mice (n=35) were treated with a single intraperitoneal injection of 50 μg LPS or 0.1 mL sterile saline under inhalation anesthesia. After 2 and 6 hours, and 1, 3, 5, 7, and 10 days of the injection, 1 mL of sterile saline was injected into the peritoneal cavity and peritoneal lavage fluids were aspirated with a heparin coated syringe by opening the abdomen surgically under anesthesia using 4% sevoflurane. Concentrations of interleukin (IL)-6, chemokine (C-X-C motif) ligand 2 (CXCL2)/macrophage inflammatory protein (MIP)-2, and tumor necrosis factor (TNF)-α in abdominal lavage fluid were measured by enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA). The sensitivities for IL-5, TNF-α, and CXCL2/MIP-2 were 1.6 pg/mL, 1.9 pg/mL, and 1.5 pg/mL, respectively. The intra-and interassay coefficients of variation for IL-6, TNF-α, and CXCL2/MIP-2 were 6.7% and 8.8%, 3.8% and 7.7%, and 2.8% and 5.9%, respectively.

Statistical analysis

Statistical analyses were performed by the Chi-square test and the non-parametric Mann-Whitney U test. Values of $p < 0.05$ were considered to be significant. Statistical analyses were carried out using SPSS 20.0 software (IBM, Armonk, NY, USA).

RESULTS

Endometrial lesions

Endometrial lesions were observed in both control and LPS groups. The lesions were red and cystic upon gross examination (Figure 1). Subsequently, the hematoxylin-eosin stained lesions were observed under a microscope, which demonstrated endometrial glands forming a duct-like configuration (Figure 2). The rate of development of endometriosis was 70% (7/10) in the control group and 90% (9/10) in the LPS group, indicating no significant differences between the two groups.

The mean area of endometriotic lesions was 3.1 ± 3.7 mm^2 in the control group (n=10), and 10.8 ± 8.6 mm^2 in the LPS group (n=10), indicating significantly larger lesions in the LPS group (Table 1).

Production of cytokines and chemokines in peritoneal lavage fluid in response to LPS

To investigate the effects of intraperitoneal injection of LPS, cytokines and chemokines in peritoneal lavage fluids were measured. The level of TNF-α in peritoneal lavage fluid peaked 2 hours after the injection of LPS, then declined to the basal level on day 3 (Figure 3). The level of IL-6 in peritoneal lavage fluid also peaked 2 hours after the injection of LPS, then declined to the basal level within a day (Figure 4). The level of CXCL2/MIP-2 in peritoneal lavage fluid peaked on day 1 after the injection of LPS, then declined to the basal level on day 3 (Figure 5).

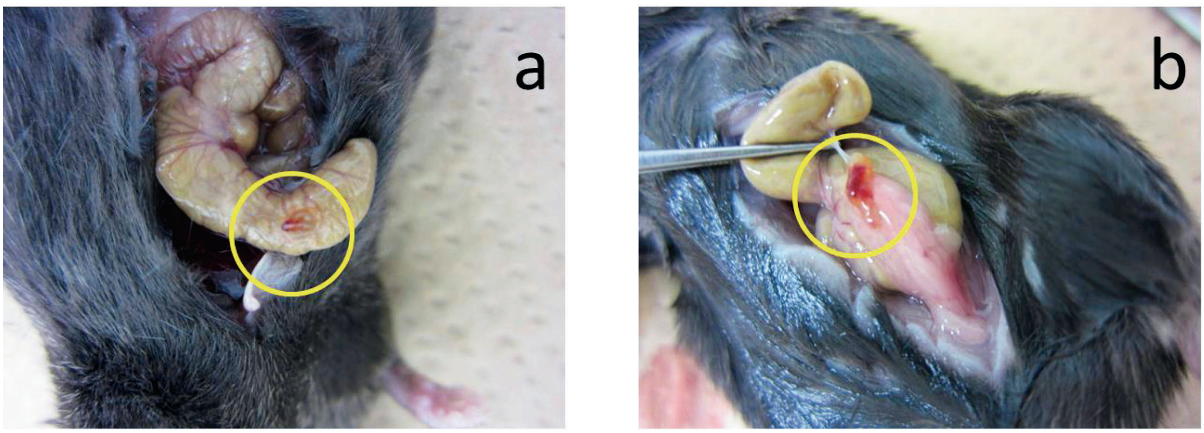

Figure 1 : Gross appearance of endometriotic lesions (*circle*) in the control (a) and LPS (b) groups.

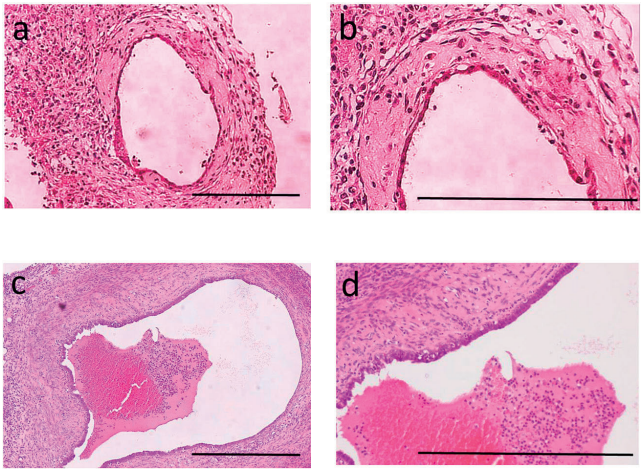

Figure 2 : Microscopic appearance (a, c = ×100 ; b, d = ×200) of endometriotic lesions in the control (a, b) and LPS (c, d) groups. Bar : 500 μm

Table 1. Morphological evaluation of endometriotic lesions in the control and LPS groups

	Control	LPS
No. of mice	10	10
No. of mice with lesions	7	9
Area of endometriotic lesions (mm^2)	3.1 ± 3.7	10.8 ± 8.6*

*p<0.05

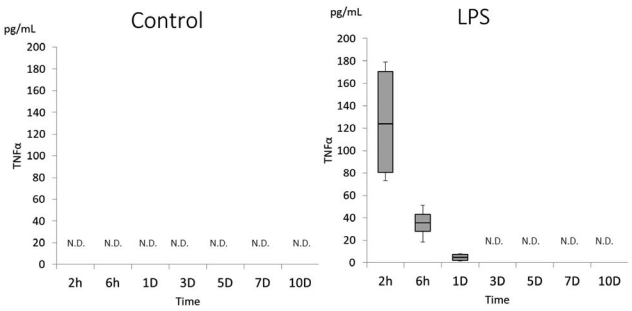

Figure 3 : Time-dependent changes in the production of TNF-α in the peritoneal lavage fluid of five mice after the injection of LPS (50 mg) or saline. The boxes represent the distance (interquartile range) between the first (25%) and third (75%) quartiles and horizontal lines in the boxes represent median values. N.D. : not detected

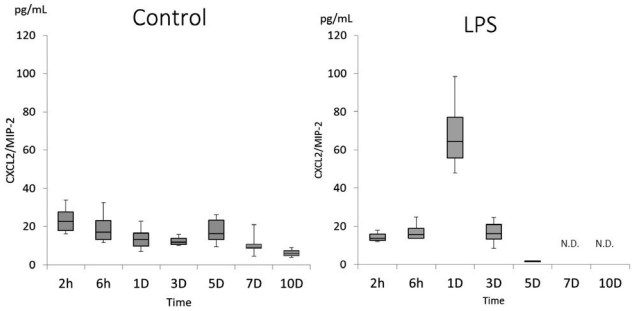

Figure 5 : Time-dependent changes in the production of CXCL2/MIP-2 in the peritoneal lavage fluid of five mice after the injection of LPS (50 mg) or saline. The boxes represent the distance (interquartile range) between the first (25%) and third (75%) quartiles, and horizontal lines in the boxes represent median values. N.D. : not detected

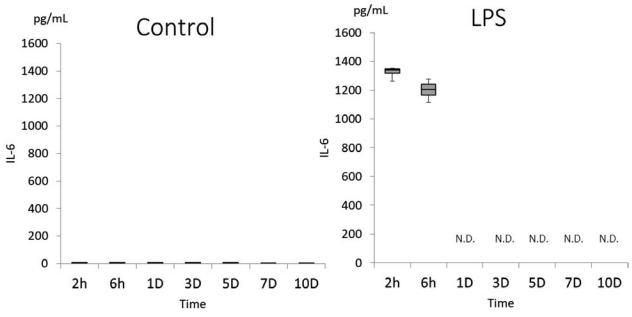

Figure 4 : Time-dependent changes in the production of IL-6 in the peritoneal lavage fluid of five mice after the injection of LPS (50 mg) or saline. The boxes represent the distance (interquartile range) between the first (25%) and third (75%) quartiles and horizontal lines in the boxes represent median values. N.D. : not detected

DISCUSSION

In this study, we demonstrated that injection of endometrial tissues together with LPS into the peritoneal cavity of female mice promoted the development of the early stage of murine endometriotic lesions. Furthermore, a single intraperitoneal injection of LPS stimulated the production of TNF-α, IL-6, and CXCL2/MIP-2 in peritoneal lavage fluid, suggesting that bacterial endotoxin has a stimulatory effect on the development of endometriosis.

Khan *et al.* demonstrated that menstrual blood of women with endometriosis was more contaminated with *E. coli* than that of control women, and that this elevation corresponds to elevation in the concentrations of endotoxin in menstrual and peritoneal fluids (6). Khan *et al.* also showed that intra-uterine microbial colonization that is considered as sub-clinical uterine infection was significantly higher in women with endometriosis than in control women

(7). These results suggest that the inflammation induced by LPS promotes the development of endometriosis.

Recently, Takebayashi *et al*. and Cicinelli *et al*. reported that a diagnosis of chronic endometritis was significantly more frequent in the endometriosis group than in the non-endometriosis group (38.5% vs. 14.1% and 52.9% vs. 27.0%, respectively) (8, 9). Although the prevalence was significantly higher in women with endometriosis as compared with women without endometriosis, the cause-effect relationship between endometriosis and chronic endometritis remains unclear (9). Furthermore, in a nationwide study of 79,512 patients, Lin *et al*. demonstrated that patients with inflammatory disease of the lower genital tract, such as the cervix, vagina, or vulva, had a 2.01-fold increased risk of endometriosis as compared to the general population (11), suggesting that chronic endometritis and lower genital tract infections are associated with a higher risk for the development of endometriosis.

Experimental mouse models have been developed to investigate the pathogenesis of endometriosis. Previously, we developed a syngeneic mouse model of endometriosis by intraperitoneal injection of endometrial fragments with blood, to clarify the role of blood in the early stages of development of endometriosis (10). The areas of endometriotic lesions in the blood group were significantly larger than those in the saline group, suggesting that blood accelerates the development of endometriosis. This mouse model made it possible to provide insight into the early stages of development of endometriosis. In our model, 8- to 9-week-old adult female mice were used because their estrous cycles had become stable by this time. In_this study, one-time injection of LPS (about 2.5 mg/kg) with endometrial fragments accelerated the development of endometriosis. Macroscopic evaluation of the endometriotic lesions showed that they were red in appearance, as described previously (10), similar to the red lesions usually representing the early stage of endometriosis in women (12). These results suggest that acute pelvic inflammation as well as chronic inflammatory conditions accelerate the progression of endometriosis. Azuma *et al*. reported that LPS promoted the development of murine endometriosis-like lesions *via* the nuclear factor-kappa B pathway (13). In their study, 4-week-old juvenile female recipient and donor mice were used to avoid an environment of estrogen excess. LPS (2 mg/kg) was injected twice weekly for 4 weeks into the peritoneal cavity to mimic the chronic inflammatory condition, indicating that chronic pelvic inflammation promotes the progression of endometriosis. In our study, blood was injected into the peritoneal cavity of the mouse model to reproduce the effect of reflux of menstrual blood. Simultaneously injecting LPS and blood faithfully reproduces the effect of reflux of menstrual blood contaminated with *E. coli*. Therefore, the findings observed in our study are representative of the very early stages of endometriosis where menstrual blood contaminated with *E. coli* flows back into the peritoneal cavity.

Hormonal factors and inflammation are thought to be commonly involved in the regulation of endometriosis (14). Khan *et al*. demonstrated that individual treatment with estradiol (E_2) and LPS significantly increased the secretion of IL-6 and TNF-α in the culture media of macrophages derived from women with endometriosis than from control women, and the effects of LPS and E_2 on 5-Bromo-2-deoxyuridine (BrdU) incorporation into eutopic and ectopic endometrial stromal cells were significantly higher in women with endometriosis than in control women or untreated cells. These results suggest that E_2 and LPS might be involved in further worsening of pelvic inflammation and growth of endometriosis (15).

In this study, LPS was administered intraperitoneally to 8- and 9-week-old female mice, resulting in an early rise of TNF-α and IL-6 levels in abdominal lavage fluid and a sharp decline in the levels within a day after the injection. TNF-α and IL-6 are pleiotropic cytokines that are produced by macrophages and monocytes. Cao *et al*. showed that intraperitoneal administration of endometrial cells from 8- to 10-week-old Swiss Webster mice increased the number of peritoneal macrophages, production of IL-6, IL-1α, and monocyte chemoattractant protein-1. In their study, the levels of IL-1 and IL-6 in peritoneal lavage fluid were increased at 4 hours, decreasing thereafter at 24 hours (4). These results suggest that both endometrial cells and LPS induce an immediate inflammatory response. On the other hand, the level of CXCL2/MIP-2 in the current study reached a maximum on day 1 after the injection of LPS. CXCL2/MIP-2 is mainly secreted by macrophages and monocytes and is a neutrophil chemoattractant which induces marked recruitment of neutrophils to inflammatory sites. Lin *et al*. demonstrated that neutrophils peaked on day 2 to 4 after the transplantation of uterine tissue that was sutured to the peritoneum, although the number of macrophages did not change much, and that the levels of MIP-2 and MIP-1α in peritoneal fluid peaked on day 2 to 4 after tissue transplantation and then declined, which was parallel to the changes in neutrophils (16). Scapini *et al*. demonstrated that MIP-2 induces the recruitment of neutrophils that, in turn, release biologically active vascular endothelium growth factor-A, resulting in angiogenesis (17), suggesting that inflammatory angiogenesis mediated by neutrophils is important in the early stage of endometriosis.

In conclusion, we showed that one-time injection of blood with LPS accelerates the early stage of development of murine endometriotic lesions and the production of TNF-α, IL-6, and CXCL2/MIP-2 in peritoneal lavage fluid, suggesting that LPS promotes the early stages of development of murine endometriotic lesions.

CONFLICT OF INTERESTS

The authors have no conflicts of interest directly relevant to the content of this article.

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