Original article

High expression of high-mobility group box 1 in menstrual blood: Implications for endometriosis

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Abstract

Endometriosis is a benign gynecologic disease characterized by the presence of ectopic endometrium and associated with inflammation and immune abnormalities. However, the molecular basis for endometriosis is not well understood. To address this issue, the present study examined the expression of high-mobility group box (HMGB)1 in menstrual blood to investigate its role in the ectopic growth of human endometriotic stromal cells (ESCs). A total of 139 patients were enrolled in this study; 84 had endometriosis, and 55 were non-endometriotic gynecological patients (control). HMGB1 levels in various fluids were measured by enzyme-linked immunosorbent assay. Expression of receptor for advanced glycation end products (RAGE) in eutopic and ectopic endometrium was assessed by immunohistochemistry, and *RAGE* and *vascular endothelial growth factor* (*VEGF*) mRNA expression in HMGB1- and lipopolysaccharide (LPS)-treated endometriotic stromal cells (ESCs) was evaluated by real-time PCR. HMGB1 concentration was higher in menstrual blood than in serum or peritoneal fluid ($P < 0.001$ for both). RAGE was expressed in both normal and ectopic endometrium. Administration of 1000 ng/ml HMGB1 or co-administration of 100 ng/ml HMGB1 and 100 ng/ml LPS

induced VEGF production in ESCs relative to the control ($P < 0.05$). These results suggest that menstrual fluid has naturally high levels of HMGB1 and may promote endometriosis following retrograde menstruation when complexed with other factors such as LPS by inducing inflammation and angiogenesis.

Key words: endometriosis, high-mobility group box 1, lipopolysaccharide, receptor for advanced glycation end products, vascular endothelial cell growth factor

Introduction

Endometriosis is a common, benign gynecologic disease characterized by the presence of ectopic endometrium that causes dysmenorrhea, chronic pelvic pain, and infertility, and associated with inflammation and immune abnormalities as well as changes in ovarian steroid hormone output. The growth and maintenance of endometrial and endometriotic tissues is regulated by several cytokines and growth factors such as interleukin (IL)-6, IL-8, tumor necrosis factor (TNF)-α, and vascular endothelial growth factor (VEGF).[1,2] Retrograde menstruation into the peritoneal cavity via the fallopian tubes is thought to play an important role in the pathogenesis of endometriosis.[3] Menstrual fluid consists of blood cells, endometrial tissue, and debris, which are sources of endometrial cells. However, the profile of bioactive molecules in menstrual blood is unclear.

High-mobility group box (HMGB)1 is an abundant non-histone component of chromatin expressed in almost all nucleated mammalian cells. HMGB1 is mainly present in the nucleus and regulates DNA transcription, repair, replication, and remodeling.[4] However, it is also a late mediator of inflammation; in inflammatory events such as injury, infection, or cell necrosis, HMGB1 is passively released from necrotic cells or secreted by activated cells of the innate

immune system into the extracellular milieu. HMGB1 has little or no proinflammatory activity in itself but forms complexes with proinflammatory factors such as IL-1β or lipopolysaccharide (LPS) and thereby potentiates their biological activities. Thus, HMGB1 complexes stimulate the synthesis of proinflammatory cytokines and activate innate immunity and inflammation by binding to receptor for advanced glycation end products (RAGE) and Toll-like receptors (TLRs) 2, 4, and 9.[5–8] HMGB1 is also a potent proangiogenic factor that acts via RAGE and TLR4 signaling pathways; it stimulates endothelial cell proliferation and induces the release of proangiogenic cytokines such as VEGF, TNF-α, and IL-8 from endothelial cells and macrophages.[9,10] We speculate that HMGB1 or its complexes in menstrual blood induces inflammation and angiogenesis to promote the progression of endometriosis.

To investigate the role of HMGB1 in endometriosis, we measured HMGB1 concentrations in peripheral and menstrual blood and peritoneal fluid, as well as RAGE expression in normal endometrium and peritoneal endometriosis. We also evaluated the direct effect of HMGB1 and its complexes on human endometriotic stromal cells (ESCs) by measuring *VEGF* mRNA levels.

Materials and methods

Study population

A total of 139 patients who were not undergoing hormonal therapy were enrolled in this study, including 84 with endometriosis and 55 with benign gynecologic diseases other than endometriosis (control) (Table 1). Exclusion criteria were pregnancy, diabetes, cardiovascular disease, cancers, active pelvic inflammatory disease, or any other acute or chronic inflammatory disease.

All patients underwent laparoscopic surgery or hysterectomy at the Department of Obstetrics and Gynecology, Okayama University Hospital. Written, informed consent was obtained from each patient before the start of the study, and the protocol was approved by the Institutional Review Board of Okayama University Hospital (no. d07001). The menstrual phase was determined based on basal body temperature and histological analysis of the endometrium. Most patients had regular cycles. Staging and morphological characterization of peritoneal endometriotic lesions were based on the revised classification of The American Society of Reproductive Medicine (Re-ASRM, 1996). Lesions were diagnosed by their macroscopic appearance and categorized as red or black according to the latest revision of the ASRM classification.[11]

Preparation of body fluid samples

Peripheral blood samples (endometriosis, n = 6 and control, n = 15) were obtained from patients on days 3–5 of the menstrual cycle and delivered to the laboratory within 30 min of collection. Peripheral blood was immediately centrifuged at 1500 × *g* for 10 min, and the supernatant was stored at −30°C until use.

Menstrual blood samples (endometriosis, n = 13 and control, n = 14) were obtained from patients on days 3–5 of the menstrual cycle. In the lithotomy position, an 18-gauge blunt-tip needle connected to a 1-ml plastic syringe was inserted into the uterine cervix to collect menstrual blood by manual suction. The blood was carefully diluted twice with phosphate-buffered saline (PBS) and then immediately centrifuged at 1000 × *g* for 10 min. The supernatant was stored at −30°C until use.

Peritoneal fluid samples were collected during laparoscopic surgery from 48 patients with endometriosis and nine control patients without endometriosis in the mid-proliferative phase of the menstrual cycle. Samples were centrifuged at 1000 × *g* for 10 min and the supernatant was stored at −30°C until use.

Preparation of tissue samples

Peritoneal endometriosis tissue samples were removed laparoscopically in the mid-proliferative phase of the menstrual cycle from 36 patients. Normal endometrial tissue samples were obtained from 23 hysterectomy patients with other benign gynecologic disease and without endometriosis. The walls of endometrial ovarian cysts were removed laparoscopically from four patients.

Enzyme-linked immunosorbent assay (ELISA)

HMGB1 levels in serum, menstrual blood, and peritoneal fluid were measured using ELISA kits (Shino-Test Corporation, Kanagawa, Japan) according to the manufacturer's protocols. The two-step sandwich ELISA employed 96-well microtiter assay plates where each well was pre-coated with a polyclonal antibody specific for HMGB1. Briefly, 10 μl of sample (i.e., standard, serum, peritoneal fluid, or 10 fold-diluted menstrual blood) and 100 μl of dilution buffer were added to each well; after mixing by shaking, the plate was incubated for 24 h at 37°C. Wells were then washed five times with wash solution. After incubation at room temperature for 2 h with 100 μl peroxidase-linked

anti-HMGB1 monoclonal antibody solution, the wells were washed five times with wash solution. A 100-µl mixture of equal volumes of tetramethyl benzidine and buffer containing 5 mM hydrogen peroxide was then added to each well at regular time intervals with incubation at room temperature for 30 min. The reaction was terminated by adding 100 µl sulfuric acid (0.35 mol/l), and absorbance at 450 nm was read on a Model 680 microplate reader (Bio-Rad, Osaka Japan).

Immunohistochemistry

Tissue samples (endometriosis, n = 36 and normal endometrium, n = 23) were fixed in 10% buffered formalin for 48 h. The tissue was embedded in paraffin, cut into 4-µm-thick sections, and mounted on silanized slides. Sections were stained with hematoxylin and eosin (Merck, Darmstadt, Germany) for histological analysis. For immunohistochemistry, slides were processed with the peroxidase-conjugated streptavidin-biotin system using diaminobenzidine (DAB) as a chromogen (labeled streptavidin-biotin kit; Dako, Carpinteria, CA, USA) according to the manufacturer's instructions. Briefly, sections were treated with 3% H_2O_2 for 5 min to quench endogenous peroxidase activity. After blocking

nonspecific staining (Protein Block Serum-Free; Dako), sections were incubated with 2 μg/ml of anti-RAGE antibody (H-300 rabbit polyclonal IgG antibody; Santa Cruz Biotechnology, Dallas, Texas, USA) for 90 min at 37°C followed by biotinylated anti-rabbit immunoglobulin secondary antibody (Dako) for 20 min at room temperature. A cervical intraepithelial neoplasia sample was used to prepare a positive control slide for RAGE immunolabeling.[12] As a negative control, preimmune rabbit serum was used instead of the primary antibody. After staining with DAB, sections were counterstained with Mayer's hematoxylin solution (Merck). Immunoreactivity was scored as follows: 0, no staining; 1, weak; 2, same as positive control; 3, strong. Sections with a score of ≥ 2 were defined as positive. Scoring was independently performed by two researchers in at least three high-power (400×) fields.

ESC culture

ESCs were isolated from endometrial ovarian cyst biopsy specimens by selective enzyme digestion and filtration as previously described.[13] The cells were resuspended at a density of approximately 10^6 cells/ml of Dulbecco's modified Eagle's medium/F-12 containing 10% fetal bovine serum (Gibco BRL,

Grand Island, NY, USA) and 100 IU/ml penicillin-streptomycin (Invitrogen, Carlsbad, CA, USA), and grown to confluence for approximately 72 h at 37°C in a humidified atmosphere of 5% CO_2. ESCs were stimulated with 100 or 1000 ng/ml recombinant HMGB1 (Sigma-Aldrich, St. Louis, MO, USA) and then incubated with different doses (1–100 ng/ml) of LPS (Sigma-Aldrich) for 24 h.

Real-time PCR

VEGF and *RAGE* mRNA levels were evaluated 24 h after ESCs were exposed to HMGB1 and LPS. ESCs were harvested and rinsed twice with PBS, and total RNA was extracted using the RNeasy Mini kit (Qiagen, Hilden, Germany). Real-time PCR was carried out using the iTaq Universal SYBR Green One-Step kit and Mini Opticon reader (Bio-Rad); 1 μl of the product was used in a final volume of 20 μl according to the manufacturer's instructions. Human-specific sense and antisense primers used in for target gene amplification were as follows: VEGF, 5′ -GCAGAAGGAGGAGGGCAGAAT-3′ and 5′ -GTCTCGATTGGATGGCAGTAGC-3′ ; RAGE, 5′ -CAGGATGAGGGGATTTTC-3′ and 5′ -AGGAATCTGGTAGACACG-3′ ; and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), 5′

-GATGACATCAAGAAGGTGGTGAA-3′ and 5′ -GTCTTACTCCTTGGAGGCCATGT-3′ (all from Sigma-Aldrich).[14] *VEGF* and *RAGE* mRNA expression levels against the reference GAPDH were calculated with the $2^{-\Delta\Delta CT}$ method. Amplification was performed over 40 cycles under the following conditions: 50°C for 10 min, 95°C for 1 min, 95°C for 10 s, and 60°C for 20 s.

Statistical analysis

Differences between groups were evaluated with the Mann-Whitney U test and one-way analysis of variance. Data were analyzed with SPSS Statistics 20 software (IBM, Armonk, NY, USA). A P value < 0.05 was statistically significant.

Results

HMGB1 is highly expressed in menstrual blood

HMGB1 concentration was higher in menstrual blood than in serum or peritoneal fluid (P < 0.001 for both). However, there was no significant difference between endometriosis and control groups with respect to HMGB1 concentrations in serum, peritoneal fluid, and menstrual blood (serum: 29.3 ± 18.4 vs. 22.0 ± 9.4

ng/ml, n = 6 and 15, respectively; peritoneal fluid: 71.4 ± 68.1 vs. 68.7 ± 43.9 ng/ml, n = 48 and 9, respectively; menstrual blood: 667.1 ± 631.4 vs. 914.8 ± 844.0 ng/ml, n = 13 and 14, respectively) (Fig. 1A). When the endometriosis group was stratified according to disease severity, we found no differences among the ASRM I-II, ASRM III-IV, and control groups in terms of HMGB1 concentration in peritoneal fluid (ASRM I-II: 84.8 ± 60.6 ng/ml, n = 16; ASRM III-IV: 64.6 ± 71.5 ng/ml, n = 32; control: 68.7 ± 43.9 ng/ml, n = 9) (Fig. 1B).

RAGE is highly expressed in normal endometrium and peritoneal endometriosis

RAGE was expressed throughout the menstrual cycle in normal endometrium. In the proliferative phase of the cycle, RAGE-positive cells were detected in the glandular epithelium and stromal cells in 90.9% (10/11) and 72.7% (8/11) of samples, respectively (Fig. 2A). In the secretory phase of the cycle, RAGE-positive cells were present in the glandular epithelium and stromal cells in 90.0% (9/10) and 80.0% (8/10) of samples, respectively (Fig. 2B). In red lesions of peritoneal endometriosis, RAGE was expressed in glandular and stromal cells in 100% (10/10) and 86.4% (19/22) of samples, respectively (Fig. 2C). In black lesions, RAGE expression was detected in glandular and stromal cells in 100%

(14/14) and 87.5% (14/16) of samples, respectively (Fig. 2D).

Co-administration of HMGB1 and LPS induces VEGF production

Treatment of ESCs with 1000 ng/ml HMGB1 induced VEGF production as compared to cells treated with 100 ng/ml HMGB1 or untreated cells ($P < 0.05$ and 0.05, respectively) (Fig. 3A). VEGF production was not induced by administration of 100 ng/ml HMGB1 alone but was observed upon co-administration of 100 ng/ml HMGB1 and 100 ng/ml LPS ($P < 0.05$) (Fig. 3B). *RAGE* mRNA level was unaltered in the presence of HMGB1 (Fig. 3C) and LPS (data not shown).

Discussion

This is the first report of elevated HMGB1 levels in menstrual blood as compared to the levels in peritoneal fluid or serum. We demonstrated that recombinant HMGB1 and LPS increased *VEGF* mRNA expression in ESCs. HMGB1 expression is clinically associated with the onset of sepsis, systemic inflammatory response syndrome, disseminated intravascular coagulation, cancer progression, arteriosclerosis, and ischemia-reperfusion injury. Serum

HMGB1 concentration is low in healthy individuals, but is upregulated in gastric,[15] colon,[16] and cervical[17] cancers and sepsis-induced organ dysfunction.[5] Hypoxia was shown to cause an increase in extracellular HMGB1 level in synovial fibroblast cells obtained from a patient with rheumatoid arthritis; additionally, the level was higher in the synovial fluid of patients with rheumatoid arthritis as compared to those with osteoarthritis (inflammatory vs. non-inflammatory arthropathy).[18]

A recent study reported HMGB1 secretion in human endometrial stromal cells following H_2O_2-induced cell necrosis. In addition, cell proliferation was increased in a concentration- and time-dependent manner by treatment with recombinant HMGB1.[19] Menstruation is a monthly hypoxic event in the endometrium caused by steroid hormone withdrawal; HMGB1 may consequently be released from necrotic endometrial cells. Indeed, our results demonstrate the extremely high concentration of HMGB1 in menstrual blood.

There was no difference in peritoneal fluid or menstrual blood HMGB1 concentrations between women with and without endometriosis. HMGB1 in peritoneal fluid with endometriosis may originate from ectopic endometrium, but an extremely high HMGB1 concentration in menstrual blood that refluxes into

the peritoneal cavity was presumed to influence HMGB1 level in peritoneal fluid in the mid-proliferative phase of the menstrual cycle, making it difficult to independently evaluate HMGB1 in peritoneal fluid.

HMGB1 stimulates proangiogenic cytokines such as VEGF via RAGE and TLR4 signaling pathways.[9,10] Endometriosis is a focal and chronic inflammatory disease; VEGF is also an angiogenic factor that likely modulates vascularization in endometriosis.[1] HMGB1 in menstrual blood that refluxes into the peritoneal cavity is thought to modulate angiogenesis in endometriosis via VEGF induction.

We found in this study that RAGE is expressed in the normal endometrium of the human uterus as well as the ectopic endometrium in peritoneal endometriosis. RAGE is a multi-ligand receptor for nonenzymatic glycation and oxidation products of proteins and lipids. RAGE activation by ligand binding stimulates diverse signaling cascades via transcription factors such as nuclear factor-κB.[20]The HMGB1/RAGE inflammatory pathway promotes pancreatic tumor growth by enhancing tumor cell mitochondrial complex I activity, ATP production, and tumor cell proliferation and migration;[21] moreover, alterations in human endothelial cell function in the pathogenesis of lupus vasculitis also involve signaling through this pathway.[22]

Retrograde menstruation has been implicated in the pathophysiology and pathogenesis of endometriosis.[3] We speculate that the extremely high concentration of HMGB1 that is expected in retrograde menstruation based on the current results promotes peritoneal seeding with viable endometrial cells, and subsequent development of endometriosis by induction of inflammation and angiogenesis via RAGE expressed in eutopic and ectopic endometrium.

HMGB1 has little or no proinflammatory activity on its own, but forms complexes with and increases the activity of exogenous or endogenous proinflammatory molecules.[6,7] Co-treatment of endometrial epithelial cells with LPS and HMGB1 was shown to increase TLR4 gene expression and consequently, IL-8 production.[23] In synovial fibroblasts co-cultured with the HMGB1-LPS complex, RAGE, TLR2, and TLR4 expression levels were upregulated relative to untreated cells.[24] We also demonstrated that VEGF mRNA expression was not induced by administration of 100 ng/ml HMGB1 alone but was observed upon co-administration of 100 ng/ml HMGB1 and 100ng/ml LPS.

It is well known that retrograde menstruation frequently occurs in women, but does not always lead to endometriosis. Furthermore, we did not find any

difference in menstrual blood HMGB1 concentrations between women with and without endometriosis. On the other hand, the menstrual blood of the former group shows greater contamination with *E. coli* and correspondingly higher levels of endotoxin than that of control women. It has been suggested that bacterial contamination promotes LPS/TLR4-mediated growth of endometriosis.[25] We therefore suggest that there are differences in HMGB1 complex formation with LPS or other molecules or in their affinity for receptors between women with and without endometriosis.

In conclusion, we report high levels of HMGB1 in menstrual blood and of RAGE in both eutopic and ectopic endometrium. We also confirmed that combined application of HMGB1 and LPS induced *VEGF* mRNA expression in human ESCs. These findings support our hypothesis that HMGB1 or its complexes promote the development of endometriosis following retrograde menstruation by inducing inflammation and angiogenesis, although further research is needed to clarify the molecular mechanisms involved.

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Author contributions

KS and YK drafted the manuscript. All authors contributed to the design, planning, and execution of experiments; data analysis; and writing of the manuscript.

Conflicts of interest

The authors declare no potential conflicts of interest.

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Table 1. Patient characteristics

	n	Age mean ± SD (range)	BMI mean ± SD (range)
Endometriosis	84	34.4 ± 5.4 (21–46)	21.1 ± 3.0 (15.9–35.7)
Re-ASRM I-II	33		
Re-ASRM III-IV	51		
Control	55	32.0 ± 6.5 (19–44)	21.2 ± 3.5 (14.9–32.0)

BMI = body mass index; Re-ASRM = revised classification of peritoneal endometriotic lesions of The American Society of Reproductive Medicine.

Figure legends

Figure 1. HMGB1 concentrations in body fluids.

HMGB1, a nuclear protein released to the extracellular compartment by necrotic cells. (A) HMGB1 concentrations in serum, menstrual blood, and peritoneal fluid. Bars show the group means. HMGB1 level was higher in menstrual blood than in the other fluids. (B) Comparison of HMGB1 concentration in peritoneal fluid among patients with two grades of endometriosis and control subjects. Bars show the group means. There was no difference in HMGB1 concentration among groups. Re-ASRM = revised classification of peritoneal endometriotic lesions of The American Society of Reproductive Medicine.

Figure 2. RAGE expression in normal endometrium and peritoneal endometriosis.

(A, B) RAGE-positive cells (brown) were detected in glandular epithelium and stromal cells of the normal endometrium in proliferative (A) and secretory (B) phases of the menstrual cycle. Hematoxylin-stained nuclei are blue. (C, D) RAGE expression was also detected in glandular and stromal cells in red (C) and black (D) lesions of peritoneal endometriosis.

Figure 3. Effect of HMGB1 on *VEGF* and *RAGE* mRNA expression in endometriotic stromal cells.

(A) Administration of 1000 ng/ml HMGB1 induced VEGF production in ESCs relative to cells treated with 100 ng/ml HMGB1 and untreated control cells. *$P < 0.05$. (B) Co-administration of HMGB1 (100 ng/ml) and LPS (100 ng/ml) induced

VEGF production. (C) *RAGE* mRNA level was unaltered by HMGB1 administration. Data are expressed as mean ± SD.