Additive effects of inflammation and stress reaction on Toll-like receptor 4-mediated growth of endometriotic stromal cells

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STUDY QUESTION: Is there any combined effect between inflammation and stress reaction on Toll-like receptor 4 (TLR4)-mediated growth of endometriotic cells?

SUMMARY ANSWER: A combined effect of local inflammation and stress reaction in the pelvic environment may be involved in TLR4-mediated growth of endometriotic stromal cells.

WHAT IS KNOWN ALREADY: In endometriosis, higher endotoxin levels in the menstrual fluid (MF) and peritoneal fluid (PF) and higher tissue concentrations of human heat shock protein 70 (HSP70) in the eutopic and ectopic endometria promote TLR4-mediated growth of endometriotic cells.

STUDY DESIGN, SIZE AND DURATION: This is a case-controlled research study with prospective collection and retrospective evaluation of sera, MF, PF and endometrial tissues from 43 women with and 20 women without endometriosis.

PARTICIPANTS/MATERIALS, SETTING, METHODS: PF was collected from 43 women with endometriosis and 20 control women during laparoscopy. Sera and endometrial biopsy specimens were collected from a proportion of these women. MF was collected from a separate population of 20 women with endometriosis and 15 control women. HSP70 concentrations in sera, MF, PF and in culture media were measured by ELISA. Gene expression of HSP70 by endometrial cells in response to lipopolysaccharide (LPS) was examined by qRT–PCR. The individual and combined effects of LPS and HSP70 on the secretion of interleukin-6 (IL-6) and tumor necrosis factor α (TNFα) by PF-derived macrophages (Mφ) were examined by ELISA, while their effects on endometrial cell proliferation were examined by bromodeoxyuridine and [³H]-thymidine incorporation assay.

MAIN RESULTS AND THE ROLE OF CHANCE: Concentrations of HSP70 were maximal in MF, intermediate in PF and the lowest in sera. In MF and PF, HSP70 levels were higher in women with endometriosis than in controls. LPS stimulated gene expression and secretion of HSP70 by eutopic endometrial stromal cells (ESCs) and this effect was abrogated after pretreatment of cells with an anti-TLR4 antibody. This effect was significantly higher for ESCs derived from women with endometriosis than for ESCs from control women. Exogenous treatment with either HSP70 or LPS significantly stimulated the production of IL-6 and TNFα by Mφ and promoted the proliferation of ESCs, and a significant additive effect between LPS and HSP70 was observed. While individual treatment with either polymyxin B, an LPS antagonist, or anti-HSP70 antibody was unable to suppress the combined effects of LPS and HSP70 on cytokine secretion or ESC proliferation, pretreatment of cells with the anti-TLR4 antibody was able to significantly suppress their combined effects.

LIMITATIONS, REASONS FOR CAUTIONS: Further studies are needed to examine the mutual role between other secondary inflammatory mediators and endogenous stress proteins in promoting pelvic inflammation and growth of endometriotic stromal cells.
**WIDER IMPLICATIONS OF THE FINDINGS:** Our findings suggest that endotoxin and HSP70 are mutually involved in a stress reaction and inflammation. A combined effect between local inflammation and a stress reaction in pelvic environment may be involved in TLR4-mediated growth of endometriotic cells.

Since endometriosis is a multi-factorial disease, it is difficult to explain uniformly its growth regulation by a single factor. Our findings may provide some new insights in understanding the physiopathology or pathogenesis of endometriosis and may hold new therapeutic potential.

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**Introduction**

Endometriosis is an estrogen-dependent chronic inflammatory disease which affects 8–10% of the total women population (Strathy et al., 1982). The main clinical problems of endometriosis are a variable severity of pain, difficulty in achieving pregnancy and recurrence after medical or surgical treatment (Strathy et al., 1982; Khan et al., 2008a; Stratton and Berkley, 2011). The exact pathogenesis of endometriosis is still unclear. There are some established hypotheses and regulatory factors supporting the development or maintenance of this disease (Khan et al., 2008a; Burney and Giudice, 2012). However, it is difficult to explain uniformly the pathogenesis of endometriosis by a single factor. Hormonal factors and inflammation are commonly involved in the growth regulation of endometriosis (Khan et al., 2005a, 2008a; Stratton and Berkley, 2011).

Endometriosis induces a variable amount of inflammatory reaction in the pelvic environment depending on the staging and morphologic appearance of disease (Halme et al., 1987; Harada et al., 2001; Khan et al., 2004a). As an initial inflammatory mediator, bacterial endotoxin or lipopolysaccharide (LPS) has been recently reported to regulate Toll-like receptor 4 (TLR4)-mediated growth of endometriotic cells (Khan et al., 2010a, 2012). Endometriosis also induces a variable amount of stress reaction in pelvic environment during cyclic menstrual reflux, implantation and invasion of endometriotic cells into pelvis (Matzinger, 1998; Zugel and Kaufmann, 1999; Asea et al., 2000, 2002). In fact, tissue stress reaction in endometriosis may occur in response to several types of stress-related stimuli such as inflammation, physical stress (cell proliferation, invasion), chemical stress (receptor/ligand binding), neurogenic stress, pain sensation and oxidative stress (Khan et al., 2008b). As a marker of tissue stress reaction, we recently demonstrated that human heat shock protein-70 (HSP70) regulates TLR4-mediated growth of endometriotic cells (Khan et al., 2008b). However, information on the combined role of inflammation and stress reaction in women with endometriosis is lacking.

Therefore, in the first part of this study, we aimed to measure the HSP70 levels in sera, menstrual fluid (MF) and peritoneal fluid (PF) collected from women with and without endometriosis. Secondly, we examined the role of LPS in the production of HSP70 by eutopic endometrial stromal cells (ESCs) in primary culture. Thirdly, we investigated single and combined effects of LPS and HSP70 on the production of cytokines by peritoneal macrophages (Mφ) and in the proliferation of eutopic/ectopic ESCs derived from women with and without endometriosis.

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**Materials and Methods**

**Reagents**

Culture media: RPMI-1640 medium for Mφ and Dulbecco’s modified essential medium (DMEM); Ham’s F12 medium for stromal cells and were supplemented with 100 IU/ml of penicillin G, 50 mg/ml of streptomycin, 2.5 μg/ml of amphotericin B (GIBCO, Grand Island, NY, USA), Fetal bovine serum, LPS (derived from Escherichia coli, serotype 011:B4), polymyxin B (1 μg/ml) and anti-HSP70 neutralizing antibody (10 μg/ml) were all purchased from Sigma Chemical Co. (St. Louis, MO, USA). Anti-TLR4 neutralizing antibody (HTA-125, 10 μg/ml) was purchased from HyCult Biotechnology, PA, USA, and highly purified recombinant human HSP70 (10 μg/ml) (low endotoxin, ESP-55S) was purchased from Stressgen, Victoria, Canada. GnRH agonist (GnRHa, 10−8 M) (Leuplin: leuprolide acetate) was kindly provided by Takeda Pharmaceutical Co. (Tokyo, Japan). The concentrations of LPS, HSP70, polymyxin B, anti-HSP70 antibody, anti-TLR4 antibody and GnRHa were chosen based on previously published literature (Khan et al., 2008b, 2010a,b; Liu et al., 2010).

**Patient samples**

The subjects in this study were women of reproductive age. PF was collected from 43 women with endometriosis and cycle matched to 20 women without endometriosis. Women with endometriosis aged between 20 and 42 years were recruited by either elective laparoscopy for infertility or diagnostic laparoscopy for dysmenorrhea and were subsequently confirmed by histology. The control group, between 18 and 32 years old, consisted of fertile women who were operated on for dermoid cyst. All patients included had laparoscopies to confirm the presence or absence of endometriosis. The staging and the morphological distribution of peritoneal lesions were based on the revised classification of the American Society of Reproductive Medicine (r-ASRM, 1997). Neither the study group nor the endometriosis-free group had been on hormonal medication in 3 months prior to the surgical procedure. All control women and women with endometriosis had regular menstrual cycles (28–32 days). The phases of the menstrual cycle were determined by histological dating of eutopic endometrial samples taken simultaneously with pathological lesions derived from these women. The distribution of patients in different revised-ASRM staging of endometriosis and in different phases of the menstrual cycle is shown in Table I. Sera and biopsy specimens were collected from a proportion of these women before and during laparoscopy.

All body fluids and biopsy specimens were collected in accordance with the guidelines of the Declaration of Helsinki and were approved by the Institutional Review Board of Nagasaki University. An informed consent was obtained from all women.
Collection of menstrual blood

Under strict aseptic measure, we collected menstrual blood from a separate population of 20 women with endometriosis and 15 women without endometriosis on Day 1 to Day 3 of the menstrual cycle, as described previously (Kamijama et al., 2004; Khan et al., 2010a,b). The samples obtained were transferred into heparinized endotoxin-free plastic containers, processed, centrifuged and stored. All samples (sera, MF and PF) were stored at −80°C for subsequent analysis.

Isolation of Mφ and eutopic/ectopic ESCs

Mφ from the PF and stromal cells from the eutopic and ectopic (peritoneal lesions) endometria were collected from six women each with and six women without endometriosis. The detailed procedures for the isolation of Mφ (Rana et al., 1996; Khan et al., 2005b) and stromal cells (Osteen et al. 1989; Sugawara et al., 1997) were described previously.

Briefly, PF samples were centrifuged at 400g for 10 min and the cellular pellet was underlayered with lymphocyte separation medium (Aurora, OH, USA) and centrifuged at 400g for 10 min. Mφ were collected from the interface and cultured in RPMI-1640 medium (GIBCO, Grand Island, NY). The Mφ were allowed to adhere to the culture plate for 2 h, after which the non-adherent cells were removed by washing the plates three times with RPMI medium. The adherent cells remaining on the plates were determined by their morphology and by immunocytochemical staining using CD68 (KP1, 1:50), a mouse monoclonal antibody from Dako, Denmark. The purity of Mφ was more than 95%, as judged by positive cellular staining for CD68.

Glandular epithelial cells were separated from stromal cells and debris by filtration through narrow gauge sieves. The characteristics of the cultured stromal cells were determined by morphological and immunocytochemical staining using CD10 (56C6, 1:40) and a mouse monoclonal antibody (Dako, Denmark). The purity of eutopic/ectopic ESCs preparation was more than 95%, as judged by positive cellular staining for CD10 and negative staining for CD45 (a pan-leukocyte marker), cytokeratin (an epithelial cell marker), von Willebrand factor (a micro-vessel marker) and alpha-smooth muscle actin (a marker of myofibroblasts).

Treatment of stromal cells and Mφ

ESCs (10⁴ cells/ml) derived from eutopic and ectopic endometria were plated in 96-well microtitre plates and culture media was collected in a time-dependent fashion (0, 6, 12, 24, 48, 72 h) to examine basal (non-treated) secretion of HSP70 in the culture media. At the pre-confluent stage, eutopic ESCs from women with and without endometriosis were treated with various doses of LPS (0, 5, 10, 100 ng/ml) in serum-free DMEM medium and incubated for another 24 h. Culture media and ESCs were collected for HSP70 assay and gene expression. A neutralizing experiment was performed with anti-TLR4 antibody (10 μg/ml) 20 min prior to treatment with LPS in order to examine any change in the secretion and gene expression of HSP70.

The isolated peritoneal Mφ derived from women with and without endometriosis were cultured in triplicate (10⁴ cells per well) for 24 h to assess basal production of IL-6 and TNFα. To evaluate the LPS/HSP70-stimulated secretion of IL-6/TNFα, after initial culture with serum containing RPMI medium, Mφ were serum starved for 24 h and then serum-free Mφ were cultured for another 24 h with highly purified recombinant human HSP70 (10 μg/ml) and/or LPS (10 ng/ml). A blocking experiment was performed with anti-HSP70 antibody (10 μg/ml), polymyxin B (1 μg/ml) and anti-TLR4 antibody (10 μg/ml) 20 min prior to LPS + HSP70 treatment in order to examine any change in the secretion of cytokines in culture media without washing the pre-incubated antibodies. After 24 h, the cultured media were collected in triplicate, pooled and frozen at −70°C until testing. Possible contamination of endotoxin with HSP70-treated cells was examined by measuring endotoxin levels in the culture media by the limulus amoebocyte lysate test (Endotoxin-Single Test; Wako-Jun-Yaku Co. Ltd., Tokyo, Japan), pre-treatment of cells with polymyxin B (1 μg/ml) and by heat treatment (65°C) of HSP70-treated cells.

Gene expression of HSP70 in ESCs

Total RNA was isolated from LPS- or anti-TLR4 antibody-treated and -non-treated ESCs using an RNaseasy Mini Kit (Qiagen, Tokyo, Japan). RNA (1 μg) was added to reverse the transcription reaction (RT–PCR), and cDNA (1 μl) was subjected to real-time qPCR using an ABI 7900HT system (Applied Biosystems, Warrington, U.K). Primers used for HSP70 gene expression were as follows: forward, 5′-CGACCTGAAACAGAGCATCA-3′ and reverse, 5′-ATGACCTCCTGGAACGTC-3′. Gene expression of β-actin was used as an internal control. All primers and probes were designed as described previously (Khan et al., 2008b; Liu et al., 2010). The gene expression level of HSP70 were calculated and normalized by dividing the corresponding values of β-actin. PCR conditions were as follows: for HSP70, 40 cycles at 95°C for 10 s, 64°C for 10 s and 72°C for 1 s; for β-actin, 30 cycles at 95°C for 10 s, 60°C for 10 s and 72°C for 1 s. All PCR conditions were followed by melting curve analysis.

Each PCR product was purified with a QIAEX II gel extraction kit (QIAGEN), and their identities were confirmed using an ABI PRISM™ 310 genetic analyzer (Applied Biosystems, Foster city, CA, USA).

HSP70/cytokine assays in the body fluids/culture media

The culture media of basal (non-treated) and stimulated (treated) ESCs and Mφ were prospectively collected in triplicate and assays were performed retrospectively. The concentrations of HSP70 in sera/MF/PF and culture media were measured in duplicate by using a commercially available sandwich ELISA (StressXpress™, EKS-700; Stressgen Victoria, Canada) according to the manufacturer’s instructions. The concentrations of IL-6 and TNFα in culture media were measured in duplicate using ELISA developed by R&D system in a blind fashion (Quantikine; R&D system, Minneapolis, MN, USA). The antibodies used in HSP70, IL-6 and TNFα determination do not cross-react with other cytokines. The limits of detection were 200 pg/ml for HSP70, 0.70 pg/ml for IL-6 and 4.4 pg/ml for TNFα. Both the intra- and inter-assay coefficients of variation were <10% for all these assays.

Terminal deoxy-UTP-biotin nick end-labeling assay

The detailed procedure of terminal deoxy-UTP-biotin nick end-labeling (TUNEL) assay was described previously (Dmowski et al., 2001). The
isolated cells \((10^5 \text{ cells/ml})\) derived from the proliferative phase endometria of women with endometriosis were placed in four-chamber slide (Nunc, Naperville, IL). After 24 h, the slides were washed in PBS and after treating with 0.1% Triton X-100 for 5 min, the slides were co-treated with GnRHa, a pro-apoptotic marker \((10^{-4} \text{ M})\) as described previously (Khan et al., 2010b) and different doses of recombinant human HSP70 \((1, 5, 10, 50 \mu \text{g/ml})\) and further incubated for 3 h at 37°C. After that, slides were fixed with 4% paraformaldehyde for 10 min, rinsed with PBS, serially deparaffinized and the nuclei with fragmented DNA were detected using a TUNEL detection kit (Wako, Osaka, Japan). Cells were digested with protease for 5 min at 37°C. After washing with 0.01 M PBS for 15 min, the slides were incubated with the terminal deoxynucleotidyl transferase (TdT) reaction mixture in a humidified chamber at 37°C for 1 h. After reduction of endogenous peroxidase activity, slides were washed with 0.01 M PBS for 10 min. The slides were then treated with the peroxidase-conjugated antibody for 10 min at 37°C. After washing with 0.01 M PBS for 15 min, the immunoreaction was visualized with diaminobenzidine and H₂O₂. Counterstaining was done with methyl green dye.

Quantitative analysis of the apoptotic cells was performed with a cytometer under 400 × magnification using a Olympus (model DP20) microscope. The apoptotic index was defined as the number of apoptotic cells per 10 mm² unit area. A negative control was processed by omitting the TdT from TdT reaction mixture of the same TUNEL procedure.

**BrdU incorporation assay**

The detailed procedure of 5-Bromo-2-deoxyuridine (BrdU) incorporation assay was described previously (Takagi, 1993; Khan et al., 2005b). BrdU labeling and detection kit measure cell proliferation by quantitating BrdU incorporated into the newly synthesized DNA of replicating cells (Takagi, 1993; Khan et al., 2005c). The incorporated BrdU can be detected by a quantitative cellular enzyme immunoassay (Biotrak; Amersham Pharmacia Biotech., UK) using monoclonal antibodies directed against BrdU. It offers a non-radioactive alternative to the [³H]-thymidine-based cell proliferation and carries equal sensitivity and specificity (Takagi, 1993).

Briefly, desired cells (eutopic/ectopic ESCs) were cultured in 96-well microtitre plates \((10^4 \text{ cell/well})\). The cells were serum starved before treatment for a period of 24 h. After a 24 h pre-incubation period without serum, respective cells were treated in a serum-free medium and incubated for an additional 24 h. After that, the cells were labeled with 10 μM of BrdU \((100 \mu \text{M/well})\) and incubated for 4 h at 37°C. The cells were fixed and genomic DNA was denatured by adding 200 μl/well of blocking reagent \((1:10)\) for 30 min at room temperature (RT). Peroxidase-labeled anti-BrdU antibody \((1:100)\) was added and incubated for 90 min at RT. After washing three times, TMB \((3,3',5',5'-\text{tetramethylbenzidine})\) substrate solution was added \((100 \mu \text{l/well})\) and incubated for 15 min at RT for color appearance and finally optical density was measured using a microplate reader at an absorbance of 450 nm.

We examined the proliferation of ESCs in response to HSP70, LPS and anti-HSP70 antibody, polyoxymixin B and anti-TLR4 antibody, and the differences in cell proliferation were expressed as the percentage of controls. The absorbance values correlated directly with the amount of DNA synthesis and thereby with the number of proliferating cells in culture.

**[³H]-thymidine incorporation assay**

To evaluate the effect of HSP70 and LPS on DNA synthesis in eutopic and ectopic ESCs, \(5 \times 10^5\) cells were plated into each well of 24-well multi-plates and were incubated for 48 h with fresh medium or fresh medium containing HSP70, LPS and anti-HSP70 antibody, polyoxymixin B and anti-TLR4 antibody. Eight hours before the desired incubation period, cells were treated with \(14.8 \text{ kBq/ml of [³H]-thymidine}\) and were incubated again at 37°C until cells were harvested. Radioactivity \((\text{cpm/well})\) was quantified in a Packard Tri-Carb 2100 liquid scintillation analyzer (Packard Instrument Co., Frankfurt, Germany).

**Statistical analysis**

The clinical characteristics of the subjects were evaluated by one-way analysis of variance. All results are expressed as either mean ± SD or mean ± SEM. The concentrations of HSP70 in different body fluids were not distributed normally and non-parametric tests such as Mann—Whitney U-test and Student’s t-test were used to analyze differences between groups. For comparison among groups, the Kruskal—Wallis test was used. A box plot analysis of HSP70 levels in different body fluids was performed using the medians and interquartile range (IQR). A P value < 0.05 was considered statistically significant.

**Results**

There were no significant differences in clinical characteristics between women with or without endometriosis (Table I). Six of the women with endometriosis were not infertile as they were included in an initial study. We did not find any difference in the cytokine profile or in cell growth in response to LPS/HSP70 in these two groups of women with endometriosis with and without infertility. Therefore, we represented our combined data below for women with and without infertility.

**HSP70 concentrations in body fluids**

We previously reported that endotoxin (LPS) levels in MF and PF are significantly higher in women with endometriosis than in women without endometriosis (Khan et al., 2010a,b, 2012). Here, we found that the concentrations of the potentially active stress protein, HSP70, were the highest in the MF, intermediate in the PF and the lowest in the sera. In fact, HSP70 levels in the MF and PF were significantly higher in women with endometriosis \((P < 0.05 \text{ for MF or PF})\) than in control women (Fig. 1A and B). No difference in HSP70 levels was observed in PF between r-ASRM stage I–II and stage III–IV endometriosis or in sera between women with and without endometriosis (Fig. 1A and B). Kruskal—Wallis test indicated that HSP70 levels in PF were the highest in the menstrual phase among all phases of menstrual cycle \((P < 0.05\) versus other phases, Fig. 1C).

**TLR4-mediated production of HSP70 by LPS in ESCs**

A substantial amount of HSP70 secretion in the culture media of basal eutopic and ectopic ESCs was observed. The basal secretion of HSP70 by ESCs was predominant at 12–48 h incubation and significantly higher for cells derived from ectopic endometria than from eutopic endometria in women with endometriosis at these incubation times \((P < 0.05\) for 12, 24, 48 h, Fig. 2A). Further incubation of ESCs showed a decline in basal HSP70 secretion. A significantly lower amount of basal HSP70 was secreted by eutopic ESCs derived from control women than by eutopic ESCs from women with endometriosis (data not shown).

LPS dose dependently stimulated the production of HSP70 by eutopic ESCs derived from women with and without endometriosis (Fig. 2B). This effect of LPS was significantly higher in women with endometriosis than in control women \((P < 0.05\) for each of 5, 10 and 100 ng/ml). Pre-treatment of ESCs with anti-TLR4 antibody \((10 \mu \text{g/ml})\) significantly abrogated the LPS-promoted HSP70 secretion in the culture media.
The neutralizing experiment of anti-TLR4 antibody on HSP70 gene expression was performed and we found that, similar to protein secretion, blocking of TLR4 significantly suppressed LPS stimulated HSP70 gene expression (P < 0.05 versus anti-TLR4 antibody-non-treated cells). This effect was equally observed for ESCs derived from women with and without endometriosis (Fig. 2C).

(P < 0.05 versus anti-TLR4 antibody-non-treated cells, Fig. 2B). The neutralizing experiment of anti-TLR4 antibody on HSP70 gene expression was performed and we found that, similar to protein secretion, blocking of TLR4 significantly suppressed LPS stimulated HSP70 gene expression (P < 0.05 versus anti-TLR4 antibody-non-treated cells). This effect was equally observed for ESCs derived from women with and without endometriosis (Fig. 2C).
TLR4-mediated production of IL-6 and TNFα by HSP70- and LPS-treated Mφ

According to our initial time-dependent and dose-dependent study, we found a maximum increase in the levels of different macromolecules and cell growth at 24–48 h and in response to 10 μg/ml of HSP70 and 10–100 ng/ml of LPS. Therefore, here we and without endometriosis (Fig. 3). For women with endometriosis, IL-6/TNFα were each unable to suppress the combined LPS (1 μg/ml) was able to significantly decrease the LPS + HSP70-stimulated production of IL-6 and TNFα (Fig. 3). The neutralizing effect of anti-TLR4 antibody was observed on IL-6 secretion was observed for Mφ from women with and without endometriosis and on TNFα secretion in Mφ derived from women with endometriosis (Fig. 3). It was noted that only IL-6 secretion and not TNFα secretion by non-treated Mφ was significantly higher in women with endometriosis than in control women.

Effect of HSP70 on TUNEL-positive ESCs cells and apoptotic index

ESC derived from proliferative endometria of women with endometriosis showed increased numbers of TUNEL-positive cells (Fig. 4A, b, c, d) and an increased apoptotic index in response to GnRHa (10−8 M) (Fig. 4B). Exogenous treatment with HSP70 (10, 50 μg/ml) was found to significantly reduce the number of GnRHa-induced TUNEL-positive cells (Fig. 4A, e, f versus b, c) and the apoptotic index (Fig. 4B). This anti-apoptotic effect of HSP70 was not observed with lower doses (1, 5 μg/ml). GnRHa-treated ESCs showed minimal apoptotic cells (Fig. 4A, a).

Effect of HSP70 and LPS on the proliferation of ESCs

In vitro exposure of both eutopic and ectopic ESCs to HSP70 (10 μg/ml) allowed significant incorporation of BrdU into these cells, showing that ESCs proliferation in response to HSP70 was significantly higher when compared with HSP70-non-treated cells (P < 0.05 for both eutopic and ectopic ESCs). For ESCs derived from both eutopic and ectopic endometria of women with endometriosis, proliferation was significantly suppressed after pretreatment of cells with anti-TLR4 antibody (P < 0.05 for each of ESCs) (Fig. 5A). Although there was less BrdU incorporation, a similar pattern of suppression effect was observed for ESCs derived from eutopic endometria of control women (data not shown).

When we examined single and combined effects of HSP70 and LPS on BrdU incorporation into ESCs derived from ectopic endometria, we found that in addition to their individual significant effect on cell proliferation, an additive effect between HSP70 and LPS was observed in further promoting ESCs proliferation (P < 0.05 versus single treatment). While individual treatment with polymyxin B or anti-HSP70 antibody was unable to suppress the combined LPS + HSP70 promotion of cell proliferation, pretreatment of cells with the anti-TLR4 antibody was able to significantly suppress this combined effect on cell proliferation (P < 0.05 versus LPS + HSP70 treatment) (Fig. 5B).
In order to strengthen the results of BrdU incorporation assay in cell proliferation, we also performed [3H]-thymidine incorporation assay to examine DNA synthesis of eutopic/ectopic ESCs in response to HSP70, LPS and other neutralizing antibodies. We found a similar pattern of cell proliferation of eutopic and ectopic ESCs as shown in Fig. 6 (A and B).

Exclusion of endotoxin contamination with HSP70-treated cells

In order to exclude the possible contamination of HSP70-treated cells, both Mφ and endometrial stroma, with endotoxin (LPS), we repeatedly measured endotoxin levels in the culture media. We could not detect any endotoxin in the culture media of HSP70-treated Mφ or HSP70-treated stromal cells. Pretreatment of Mφ with polymyxin B (1 μg/ml) failed to decrease the levels of any of these macromolecules in the culture media of HSP70-treated cells (data not shown). Since LPS is heat stable and HSP70 is heat labile (Wallin et al., 2002), we further excluded endotoxin contamination by heat treatment (65°C) of HSP70-treated cells for 20 min. We could not detect either HSP70 or LPS in the culture media or cell proliferation of HSP70-treated cells due to degradation of HSP70 after heat treatment.

Discussion

We demonstrated here for the first time that a stress reaction occurs in the pelvic environment of women with endometriosis. When there is an inflammatory reaction in intrauterine environment or in pelvis as evidenced by the elevated endotoxin (LPS) levels in MF and PF (Khan et al., 2010a, 2012), LPS induces an internal tissue stress reaction and...
pro-inflammatory response as we confirmed here by the LPS stimulated cytokine production and increased HSP70 production in the culture media of basal and LPS stimulated ESCs. Again, as a marker of stress reaction, HSP70 also significantly stimulated the secretion of IL-6 and TNFα by Mφ and markedly promoted the proliferation of ESCs. An additive effect between LPS and HSP70 was observed in cytokine production and ESCs proliferation. These effects of LPS and HSP70 were more remarkable in cells derived from women with endometriosis than in cells from control women.

It can be noted that Mφ from control women were less responsive to single and combined treatment with HSP70 and LPS in the secretion of TNFα when compared with IL-6 secretion from similar control women. This could be due to the variations in the secretion ability or the receptor-ligand binding affinity in Mφ of control women for the production of IL-6 or TNFα. This also explains why there was no significant reduction in TNFα after pretreatment of Mφ from control women with the anti-TLR4 antibody.

We previously reported that LPS and HSP70 alone were able to exhibit TLR4-mediated increases in cytokine production and growth of endometriotic cells (Khan et al., 2008a, 2010a). In our current study, we demonstrated that pretreatment of Mφ and ESCs with either polymyxin B or neutralizing antibody against HSP70 was unable to abrogate the combined LPS + HSP70-promoted cytokine production and cell proliferation. In contrast, the cytokine production and growth-promoting effects of combined LPS + HSP70 were significantly suppressed when the biological function of TLR4 was blocked with anti-TLR4 antibody. This indicates that the LPS- and HSP70-mediated inflammatory reaction and growth stimulus of endometriotic cells may be mediated by TLR4 in the pelvic environment. Here, we confirmed the validity of the BrdU incorporation assay in ESCs proliferation by an additional [3H]-thymidine-based cell proliferation assay that showed a similar pattern of ESCs proliferation in response to LPS and HSP70.

TLR4 is an essential receptor for bacterial endotoxin or LPS recognition (Akira and Takeda, 2004; Takeda and Akira, 2005). In addition to E. coli-derived LPS, there are other exogenous (F protein from respiratory syncytial virus, chlamydial HSP60) and endogenous ligands (fibronectin, heparan sulfate, hyaluronic acid and HSP60) including HSP70 that can also transmit signals through TLR4 (Kiechle et al., 2002; Khan et al., 2009). Therefore, we presume that blocking of TLR4 may be more effective in reducing inflammatory responses and growth of endometriotic cells in the pelvis than blocking a single ligand of TLR4. The gene and protein expression levels of TLR4 in peritoneal Mφ and eutopic/ectopic endometrial cells are reported elsewhere (Fazeli et al., 2005; Hirata et al., 2005; Khan et al., 2008b, 2010a).

As a source of endotoxin (LPS) in the pelvis, we recently reported significantly higher colony formation of E. coli in menstrual blood derived from women with endometriosis than in blood from control women (Khan et al., 2010a). We further established that higher prostaglandin E2 in MF was responsible for E. coli contamination of menstrual blood and this regulates down-stream cascade of LPS/TLR4/NF-κB in the development or maintenance of endometriosis (Khan et al., 2012). In a recent study, Hayashi et al. (2013) demonstrated a positive correlation between TLR4 and microsomal prostaglandin E synthase - 1 (mPGES-1) gene expression in endometriotic lesions. As a rate-limiting metabolizing enzyme, mPGES-1 may participate in the production of PGE2 in MF and PF in response to LPS or other inflammatory mediators in women with endometriosis.

Different types of physical stress, chemical stress, neurogenic stress, painful stimuli and oxidative stress may trigger variable degrees of tissue stress reaction in the pelvis of women with endometriosis (Khan et al., 2008b, 2010a). In a previous study (Khan et al., 2004b, 2008b), we observed more remarkable endogenous tissue stress reactions in active blood-filled opaque red lesions than in transparent/translucent lesions or other less active peritoneal lesions. LPS itself may stimulate Mφ for the production of TLR4-mediated reactive oxygen species (Khan et al., 2009). Therefore, we presume that there is a close relationship among LPS, HSP70 and oxidative stress. The increased expression of HSP70 or inflammatory responses in the pelvis might be related to oxidative stress induced by TLR4 stimulation (Gill et al., 2010).

Here we further demonstrated that as a potential marker of tissue stress reaction, soluble HSP70 level was significantly higher in the MF and PF of women with endometriosis than in MF and PF of control women. Persistent endogenous stimulation in the pelvis with LPS or HSP70 may change the cell membrane permeability causing the efflux of different cytokines out of immune cells or may cause the shifting of resting cells (S0) to proliferative phenotype (S2) in the cell cycle. This may also result in increases in cytokine levels by Mφ or increases in ESCs proliferation after exogenous exposure to LPS or HSP70. The possible contamination of endotoxin in the study of HSP70-treated cells

Figure 6 Single and combined effects of HSP70 and LPS on stromal cell proliferation derived from the eutopic and ectopic endometria of women with endometriosis were measured by [3H]-Thymidine incorporation assay. Radioactivity was expressed as count per minute per well (cpm/well). We found a similar pattern of growth-promoting effects of HSP70 and LPS in eutopic and ectopic ESCs in response to HSP70 and LPS (A and B) and a similar fashion of anti-proliferative effects in response to anti-TLR4 antibody (A and B) as shown in Fig. 5. The results are expressed as mean ± SEM of triplicate experiments on cells from six different patients.
during the bio-culture procedure has been a matter of concern in different reports (Byrd et al., 1999; Triantafilou et al., 2001; Wallin et al., 2002; Triantafilou and Triantafilou, 2004). We carefully excluded the possible contamination of HSP70-treated cells with endotoxin by serial exclusion experiments.

Increased HSP70 gene and protein expression in the eutopic/ectopic endometria of women with endometriosis carries some biological significance. As a molecular chaperon, HSP70 inhibits apoptosis of host cells by preventing recruitment of caspases to the apoptosome complex (Beere et al., 2000). Therefore, increased production of HSP70 by peritoneal Mφ and ESCs in pelvis may contribute to increased survival of endometrioid cells in women with endometriosis. HSP70 promoted survival of endometrial cells during the menstrual period and its retrograde entry into pelvis may facilitate their ectopic survival and implantation. This was further supported by the anti-apoptotic effect of HSP70 in our current study.

Our current findings provide us with new information that a vicious cycle between inflammatory reaction and stress reaction is constantly occurring in the pelvis of women with endometriosis. Endotoxin (LPS) and HSP70 are mutually involved in inducing stress reactions and inflammation in the pelvis. We conclude that a crosstalk between local inflammatory reaction and stress reaction is constantly involved in TLR4-mediated growth of endometriotic cells. Targeting TLR4 or the source of initial inflammatory mediator either in intrauterine environment or within vaginal cavity could be a potential therapeutic approach to effectively reduce pelvic inflammation and growth of endometriotic cells. Future studies investigating the relationship between secondary inflammatory mediators and other endogenous stress proteins or between ovarian steroids and inflammation in the pelvis of women with endometriosis may strengthen our current findings.

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Authors’ roles

K.N.K. was involved in original concept, study design, experiments, data analysis and manuscript drafting; M.K., T.I., A.F. and S.T. contributed equally to the operative procedure and sample collection; M.N. and H.M. were equally involved in revising the draft manuscript.

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Conflict of interest

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

References


