Toll-like receptor 4 (TLR4)-mediated growth of endometriosis by human heat-shock protein 70 (Hsp70)

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Abstract

Background: We investigated the role of human heat shock protein 70 (Hsp70) in toll-like receptor 4 (TLR4)-mediated growth of endometriosis. **Methods:** Macrophages were isolated in primary culture from the peritoneal fluid of women with and without endometriosis. TLR4 expression was examined in Mϕ. The production of a number of macromolecules by non-treated Mϕ, Hsp70-treated Mϕ and after treatment with anti-TLR4 antibody was examined by ELISA. The single and combined effects of Hsp70 and lipopolysaccharide (LPS) on the growth of endometrial stromal cells were analyzed by bromodeoxyuridine (BrdU) incorporation study. Hsp70 level in eutopic and ectopic endometria was measured by ELISA. **Results:** Toll-like receptor 4 (TLR4) was detected in isolated Mϕ at protein and gene level. Hsp70 (10μg/mL) significantly stimulated the production of hepatocyte growth factor (HGF), vascular endothelial cell growth factor (VEGF), interleukin (IL)-6 and tumor necrosis factor alpha (TNFα) by Mϕ derived from women with endometriosis than that in non-endometriosis (p<0.05 for each). This effect of Hsp70 was abrogated after pretreatment of Mϕ with anti-TLR4 antibody. BrdU incorporation study indicated that Hsp70 significantly enhanced the growth of

endometrial stromal cells (about 50% increase) derived from women with endometriosis when compared to non-treated cells. A synergistic effect on cell proliferation was observed between exogenous Hsp70 and LPS and this growth promoting effect was significantly suppressed after pretreatment of cells with anti-TLR4 antibody ($p<0.05$). Tissue levels of Hsp70 were significantly higher in the eutopic endometria ($p<0.05$) and opaque red lesions ($p<0.01$) derived from women with endometriosis than that of non-endometriosis or other peritoneal lesions. **Conclusion:** A prominent stress reaction was observed in blood-filled opaque red peritoneal lesions. Human Hsp70 also induces pelvic inflammation and may be involved in TLR4-mediated growth of endometrial cells derived from women with endometriosis.

Introduction

Endometriosis induces a variable amount of inflammatory reaction in pelvic environment depending on the staging and morphologic appearance of disease (Halme et al., 1987; Harada et al., 2001, Lebovic et al., 2001; Khan et al., 2003; Wira et al., 2005). The inflammatory reaction associated with endometriosis was demonstrated both in vitro and in vivo by the infiltration of immune cells, presence of a number of primary and secondary inflammatory mediators in tissue and body fluids (Halme et al., 1988, 1989; Osuga et al., 1999; Mahnke et al., 2000; Keenan et al., 1994, 1995; Khan et al., 2002a, 2002b, 2004a, 2005a, 2007). The primary inflammatory mediators derived from Gram-negative and Gram-positive microbes can elicit immune response in pelvic environment through pattern-recognition receptors, which belong to the family of Toll-like receptors (TLRs) (Akira et al., 2004; Takeda et al., 2005; Khan et al., 2007). Other secondary inflammatory mediators that are produced in response to primary inflammatory mediators interact with their respective receptors in immune cells or endometrial cells and may be involved in the growth of endometriosis either alone or in combination (Halme et al., 1987; Harada et al., 2001, Khan et al., 2005a, 2005b, 2005c; McLaren et al., 1996). There is a possibility that in addition to pelvic inflammation,

endometriosis may equally produce a stress reaction and release endogenous heat-shock proteins in pelvic environment as a result of tissue damage, tissue invasion and by inflammatory reaction itself. However, studies are limited regarding immune cell-mediated regulation of pelvic endometriosis by human heat-shock protein, their mechanistic basis and levels of this stress protein in pelvic environment.

A wide variety of stressful stimuli, such as heat shock, ultraviolet radiation, viral or bacterial infections, internal physical stress, chemical stress and pelvic inflammation, induce an increase in the intracellular synthesis of Hsps (Zugel et al., 1999; Asea et al., 2000, 2002). Recognition of mammalian Hsp60, Hsp70 and Hsp90 was implicated in a variety of autoimmune and inflammatory conditions (Wallin et al., 2002). The so-called 'danger theory' states that antigen presenting cells can be activated by endogenous substances released by damaged or stressful tissues (Matzinger, 1998). Members of the Hsp family are candidate molecules that potentially signal tissue damage or cellular stress to the immune system.

Human heat-shock proteins (Hsp60, Hsp70 and Hsp90) are reported to be produced by macrophages, vascular endothelial cells, smooth muscle cells, endometrial

cells and other dendritic cells (Wallin et al., 2002). In a manner similar to the recognition

of lipopolysaccharide (LPS), recognition of Hsp60 and Hsp70 seems to be mediated by a

complex of TLR4 and MD-2 (Kol et al., 1999; Wallin et al., 2002). Since the biological

potentiality of human hsp70 is stronger than either Hsp60 or Hsp90 (Wallin et al., 2002),

we report here TLR4 expression in macrophages derived from the peritoneal fluid of

women with and without endometriosis and TLR4-mediated growth of pelvic

endometriosis in response to human Hsp70. We also examined the pattern of stress

reaction by measuring endogenous Hsp70 concentration in the eutopic endometria and

different peritoneal lesions of women with pelvic endometriosis.

Materials and Methods

Subjects. A total of 25 women between 20 and 38 years of age undergoing

laparoscopy for pelvic pain, dysmenorrhea and/or infertility were recruited in this study.

Among them, 12 women belonged to stage I-II endometriosis and the remaining 13

women belonged to stage III-IV endometriosis at the time of diagnostic laparoscopy. The

control group consisted of 12 fertile women between 21 and 36 years of age without any

evidence of pelvic or ovarian endometriosis and operated for dermoid cysts by

laparoscopy. 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 the 3 months prior to the surgical procedure. All control women

and women with endometriosis had regular menstrual cycles (28-32 days). The phase of

the menstrual cycle was determined by histological dating of eutopic endometrium

samples taken simultaneously with the peritoneal fluid samples. Menstrual dating was

carried out by an independent pathologist. All induced menstrual cycles were excluded

from the current study. The distribution of patients in different menstrual cycles is as

follows: control women, proliferative phase, n=4; secretory phase, n=8; endometriosis

women, proliferative phase, n=10; secretory phase, n=15.

Peritoneal lesions of endometriosis were diagnosed by their macroscopic

appearances according to published criteria (Jansen et al., 1996) and categorized as red,

black and white lesions as proposed in the latest revision of the ASRM classification

(ASRM, 1997). As we described recently (Khan et al., 2004b), the distribution of

peritoneal lesions according to color appearance of endometriosis for our current study

was done as follows: total red lesions (n=20) [blood-filled opaque red lesions (n=8) and

non-opaque transparent and/or translucent red lesions (n=12)]; black lesions (n=21), and white lesions (n=11). Biopsy specimens from each of these peritoneal lesions were collected for subsequent experimental analysis. The details of physical collection of tissue biopsies and peritoneal fluid (PF) were reported elsewhere (Khan et al., 2002b, 2004a, 2004b, 2006).

All biopsy specimens and peritoneal fluid were collected in accordance with the guidelines of the Declaration of Helsinki and with the approval by the Nagasaki University Institutional Review Board. An informed consent was obtained from all women.

Isolation of macrophages from the PF. Peritoneal fluid (PF) was obtained from all women with or without endometriosis with the use of laparoscopy. Macrophages (Mϕ) were isolated in primary culture from the PF of six women with endometriosis and six women without endometriosis. The detailed procedure of Mϕ isolation in primary culture was described previously (Khan et al., 2005a, 2005b). The Mϕ were allowed to adhere to the culture plate for 2 hours, 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 more than 95% Mϕ as estimated by their morphology and by

immunocytochemical staining using CD68 (KP1), a mouse monoclonal antibody from

Dako, Denmark. An aliquot Mϕ was plated in four-well chamber slides (Nunc,

Naperville, IL) for immunostaining and the rest were used for culture. The detail

procedure of immunocytochemical staining is described elsewhere (Rana et al., 1996;

Khan et al., 2003, 2004a). Non-immune mouse immunoglobulin (Ig) G1 antibody in 1:50

dilution was used as a negative control. A counter staining of Mϕ with hematoxylin-eosin

was also performed and we did not find any contaminating cells such as gland cells or

stromal cells in isolated Mϕ (data not shown).

Isolation of stromal cells in primary culture. Stromal cells were collected

from the biopsy specimens of the eutopic endometria derived from six women with

endometriosis and six women without endometriosis. The detail procedure of the

isolation of stroma is described previously (Osteen et al. 1989; Sugawara et al. 1997,

Khan et al. 2005c).

The characteristics of the cultured stromal cells were determined by

morphological and immunocytochemical studies. An aliquot of stromal cells was placed

in four-chamber slide (Nunc, Naperville, IL) for immunostain and the rest was used for culture. After 24 hours, the slides were washed in PBS, fixed with 4% paraformaldehyde for 10 minutes, and rinsed with PBS. Slides then were incubated in 0.1% Triton X-100 for 5 minutes and incubated for 3 hour in 37℃ as follows: against human cytokeratin monoclonal antibodies (mAb) (epithelial-cell specific) at a dilution of 1:50 (MNF 116;Dako, Denmark), against human vimentin mAb (stromal cell specific) at a dilution of 1:20 (V9; Dako), against human von Willebrand factor mAb (endothelial-cell specific) at a dilution of 1:50 (Dako), and against CD45 mAb (other leukocytes) at a 1:50 (Dako) dilution. The specificity of the immunocytochemical staining was confirmed by the deletion of the first antibody. Immunocytochemical staining was performed on at least three different isolated cells with similar results. The purity of stromal preparation was more than 95%, as judged by positive cellular staining for vimentin.

Treatment of macrophages and stromal cells. The isolated peritoneal Mϕ were cultured in triplicate (10^5 cells per well) for 24 hours to assess basal (constitutive) production of cytokines. To evaluate the stimulated (induced) secretion of cytokines, after initial culture with serum containing RPMI medium, Mϕ were serum starved for 24

hours and then serum free Mφ were cultured for another 24 hours with different

concentrations of highly purified recombinant human Hsp70 (1, 5, 10, 15, 20μg/mL)

(low endotoxin, ESP-555, Stressgen, Victoria, Canada). A blocking experiment was

performed with anti-TLR4 antibody (10μg/mL) (HTA-125, HyCult Biotechnology) 20

minutes prior to treatment with recombinant human Hsp70 (10μg/mL) in order to

examine any change in the secretion of cytokines and growth factors in culture media

without washing the pre-incubated antibodies. After 24 hours, the cultured media were

collected in triplicate, pooled, and frozen at -70℃ until testing. Possible contamination of

endotoxin with Hsp70 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 (1μg/mL, Sigma), an LPS

antagonist, and by heat treatment (65℃) of Hsp70-treated cells.

Next, to examine the direct effects of recombinant Hsp70 (1, 5, 10μg/mL) and

lipopolysaccharide (LPS, 10ng/mL) derived from *Escherichia coli* (serotype 0111:B4;

Sigma, St. Louis, MO) on the proliferation of endometrial stromal cells, 10^4 cells/ml were

plated in 96 well microtitre plate and treated with various doses of recombinant Hsp70

either alone or in combination with LPS (10ng/mL) in serum free RPMI medium and

incubated for another 24 hour. The neutralizing effects of polymyxin B (1μg/mL) and

anti-TLR4 antibody (10 μg/mL) on stromal cell growth were examined.

The production of macromolecules in the culture media of basal and stimulated

macrophages and cell proliferation assay were studied in six women with endometriosis

and six women without endometriosis (three each in proliferative phase and three in

secretory phase).

Cytokine Assays in the culture media of macrophages. The culture media of

basal (non-treated) and stimulated (treated with Hsp70) Mϕ were prospectively collected

in triplicate and assays were performed retrospectively. The concentrations of hepatocyte

growth factor (HGF), vascular endothelial cell growth factor (VEGF), interleukin (IL)-6,

and tumor necrosis factor alpha (TNFα) in the culture media were measured in duplicate

using a commercially available sandwich enzyme linked immunosorbent assay (ELISA)

developed by R & D system in a blind fashion (Quantikine, R & D system, Minneapolis,

MN). The antibodies used in HGF, VEGF, IL-6, and TNFα determination do not

cross-react with other cytokines. The limits of detection were 40.0 pg/ml for HGF, 9.0

pg/ml for VEGF, 0.70 pg/ml for IL-6, and 4.4 pg/ml for TNFα. Both the intra-assay and inter-assay coefficients of variation were <10% for all these assays.

Immunolocalization of TLR4 in macrophages. In order to immunolocalize TLR4 in the CD68-immunoreactive isolated Mϕ, we performed immunocytochemical staining of TLR4 using corresponding antibody (HTA-125, 1:50, Santa Cruz). The immunoreaction of TLR4 was examined in Mϕ derived from women with or without endometriosis. The detail procedure of immunocytochemistry was described previously (Fujishita et al., 1997; Nisolle et al., 1997, Khan et al., 2005a, 2005b). Non-immune mouse immunoglobulin G1 antibody (1:50) was used as a negative control.

Western blotting. Cultured cells in 6-well plates were homogenized in the lysis buffer containing 50 mM Tris HCl (pH 6.8), 2% sodium dodecyl sulfate, 10% glycerol, 50 mM dithiothreitol, and 0.1% bromophenol blue and were diluted to 1 mg total protein per milliliter. Plasma lysates and total cell lysates of Mϕ and Ramos, a B-lymphoma cell line were resolved in 8% SDS-PAGE. The procedure in the preparation of plasma lysates and total cell lysates was described elsewhere (Crawford et al., 1982). Proteins were blotted onto a nitrocellulose membrane and incubated with a rabbit antibody to TLR4

(1:300) as a primary antibody and an anti-rabbit antibody (1:1000; Amersham Pharmacia

Biotech, UK) as a secondary antibody. Immune complexes were visualized by use of an

enhanced chemiluminescence Western blotting system (Amershan Pharmacia Biotech).

Gene expression of TLR4 and HGF in macrophages. Ribonucleic acid (RNA)

was extracted from cultured Mϕ in 60 mm petridish (Greiner) using the monophasic

solution of 40% phenol and ISOGEN method (Molecular Research Center, Tokyo),

according to the manufacturer's protocol.

The expression of TLR4 was performed in Mϕ derived from women with or

without endometriosis. The presence of mRNA encoding TLR4 in basal Mϕ was

determined using forward and reverse primers synthesized to anneal with cDNA for

TLR4. Amplification of cDNA reaction mixture for TLR4 was done. The mRNA

expressions of TLR4 were analyzed by RT-PCR and using sense and antisense primers of

TLR4 as described previously (Hirata et al., 2005). PCR generated bands were cloned and

found to match the published sequences for the expected products.

The mRNA expression of HGF in response to Hsp70 and anti-TLR4 antibody

was also examined by standard RT-PCR and using sense and anti-sense primers as

described previously (Khan et al., 2005a, 2005b, 2005c). A scanner densitometer was used to determine the ratio of intensity of each band relative to β-actin that was used as an internal control. Autoradiographs were analyzed to quantitate differences in levels of transcripts between Hsp70- non-treated samples and Hsp70-treated samples derived from control women and women with endometriosis. Values of each transcript after treatment with Hsp70 were normalized to 1. Densitometric analysis of gel bands was performed using the National Institutes of Health image analysis program.

Real-time quantitative PCR was performed as reported previously (Koga et al., 2000). To assess TLR4 and HGF mRNA expression, real-time quantitative PCR and data analysis were performed using Light Cycler (Roche Diagnostics GmbH, Mannheim, Germany). Expression of TLR4 and HGF mRNA was normalized to RNA loading for each sample using β-actin mRNA as an internal control. The primers for TLR4, HGF and β-actin were the same as those used for standard PCR. PCR conditions were as follows: For TLR4, 40 cycles at 95℃ for 10 sec, 64℃ for 10 sec, and 72℃ for 12 sec; for HGF, 40 cycles at 95℃ for 10 sec, 64℃ for 10 sec, and 72℃ for 12 sec; for β-actin, 30 cycles at 95℃ for 10 sec, 60℃ for 10 sec, and 72℃ for 10 sec. 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 PRISMTM 310 genetic analyzer (Applied Biosystems, Foster city, CA).

Cell proliferation assays. 5-Bromo-2-deoxyuridine (BrdU) labeling and detection kit measures cell proliferation by quantitating BrdU incorporated into the newly synthesized DNA of replicating cells (Takagi, 1993; Khan et al., 2005a, 2005b). The incorporated BrdU can be detected by a quantitative cellular enzyme immunoassay (Biotrak, Amersham Pharmacia Biotech Ltd., UK) using monoclonal antibodies directed against BrdU. It offers a non-radioactive alternative to the [^{3}H]-thymidine-based cell proliferation and carries equal sensitivity and specificity (Takagi, 1993). The detail procedure of BrdU incorporation assay was described previously (Khan et al., 2005a, 2005b, 2005c). We examined the proliferation of endometrial stromal cells in response to Hsp70, LPS, polymyxin B and anti-TLR4 antibody and the differences in cell proliferation were expressed as the percentage of controls. The absorbance values correlated directly to the amount of DNA synthesis and thereby to the number of

proliferating cells in culture.

Endogenous Hsp70 assays in tissue extracts. A fraction of biopsy specimen from eutopic endometria of women with and without endometriosis and from different peritoneal lesions of women with endometriosis was homogenized in homogenizing buffer using a Polytron homogenizer (Kinematics, Luzern, Switzerland) and according to the procedure described previously (Miura et al., 2006). The respective tissue suspension was centrifuged at 1500 rpm for 5 minutes to obtain the supernatant and stored at -80℃ for the subsequent measurement of endogenous human Hsp70.

The tissue concentrations of human Hsp70 in the homogenized supernatant were measured in duplicate using a commercially available sandwich enzyme-linked immunosorbent assay (StressXpress[TM], EKS-700, Stressgen, Victoria, Canada) and according to the manufacturer's instructions. The protein concentration of samples was measured by the method of Bradford (1976) to standardize Hsp70 levels.

The antibodies used in Hsp70 determination do not cross-react with other cytokines. The sensitivity of this assay kit has been determined to be 200pg/mL. Both the intra-assay and inter-assay coefficients of variation were <10% for this assay. The tissue

concentration of Hsp70 was expressed as ng/μg protein.

Statistical Analysis. The clinical characteristics of the subjects were evaluated by one-way analysis of variance. The data are expressed as either mean $\pm$ SEM or mean $\pm$ SD. The concentrations of the studied cytokines were not distributed normally and the data were analyzed using non-parametric test. The differences between endometriosis and non-endometriosis, red lesions and other peritoneal lesions, Hsp70- or LPS-treated and non-treated groups were compared using Mann-Whitney U-test or Student's t test. For comparison among three or more groups, the Kruskal-Wallis test was used to determine the difference among the groups. P<0.05 was considered statistically significant.

Results

There were no significant differences in clinical characteristics between women with or without endometriosis (data not shown). As an initial study, we also examined five women with endometriosis but without infertility. We did not find any

difference in cytokine profile or cell growth in response to Hsp70 in these two groups of women with endometriosis with and without infertility. Therefore, we represented our data only in women with infertility.

Expression of TLR4 in macrophages. We detected both protein and gene expression of TLR4 in Mϕ, endometrial stromal cells and epithelial cells and also in eutopic and ectopic endometria derived from women with and without endometriosis. The immunolocalization of TLR4 was found in CD68-positive Mϕ (Figure 1 A), vimentin-positive stromal cells and cytokeratin-positive epithelial cells. A parallel expression of TLR4 was also found in the glandular epithelial cells and stromal cells derived from both eutopic and ectopic endometria of women with and without endometriosis (data not shown). A 78 KDa molecular size of TLR4 was also visualized by western blot analysis in Mϕ and this was prominent in total cell lysates (Lane 3 for Ramos cells and Lane 4 for Mϕ) (Figure 1 B). This was confirmed at the mRNA levels (406bp) in basal Mϕ derived from the peritoneal fluid of women with and without endometriosis (Figure 1 C). The amount of TLR4 mRNA was dose-dependently increased in basal Mϕ with a maximum amount found at 24-48hr of incubated cells.

Although an apparent increase in the amount of TLR4 mRNA was found in basal Mϕ

derived from women with endometriosis, there was no significant difference in TLR4

expression between women with endometriosis and without endometriosis (Table 1).

The protein and gene expression of TLR4 in endometrial cells were reported elsewhere

(Young et al., 2004; Hirata et al., 2005; Khan et al., 2005d, 2005e, 2007).

TLR4-mediated production of different cytokines by human Hsp70-treated

macrophages. 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 to 48 hr and in response to 10-15μg/mL of Hsp70. Therefore, here we represented all

our experimental data in response to 10μg/mL of Hsp70 with a treatment duration of 24

hr. We found that the concentrations of HGF, VEGF, IL-6 and TNFα were significantly

higher in the culture media of Hsp70-treated Mϕ than that in non-treated Mϕ (p<0.01 or

p<0.05 for each, Figure 2). The levels of these cytokines and growth factors were also

markedly higher in Mϕ derived from women with endometriosis when compared to

non-endometriosis (p<0.05 for each, Figure 2). When we pre-treated Mϕ with antibody

against TLR4, then again treated them with Hsp70, the levels of all these cytokines and

growth factors were significantly decreased in comparison with cells without blocking

TLR4 (p<0.05, for each of HGF, VEGF, IL-6, and TNFα) (Figure 3). This effect was

observed in Mϕ derived from women with endometriosis but not from control women.

No difference in the production of these macromolecules was observed between Mϕ

collected during proliferative phase and secretory phase (data not shown).

TLR4-mediated gene expression of HGF by human Hsp70. We found that

gene expression of HGF in response to Hsp70 is stronger in Mϕ derived from women

with endometriosis than that from women without endometriosis (Figure 4A). Although a

dose-dependent increase in gene expression of HGF (505bp) was observed in these two

groups of women, a significant and a two-fold increase in mRNA expression of HGF was

found in women with endometriosis (p<0.05) comparing to women with

non-endometriosis in response to Hsp70 (Figure 4B). When we pre-treated Mϕ with

antibody against TLR4, then again treated them with Hsp70, HGF mRNA expression

level was significantly decreased in comparison with cells without blocking TLR4

(p<0.05, Figure 4B). When we examined the difference in the amount HGF mRNA in

response to Hsp70 and anti-TLR4 antibody by real-time PCR, we found almost parallel

findings in Mϕ similar to that when detected by standard RT-PCR (Table 2). This indicates that the stimulating effect of Hsp70 in the production of HGF and other macromolecules is mediated by TLR4.

Exclusion of endotoxin contamination with Hsp70-treated cells. In order to exclude the possible contamination of endotoxin with Hsp70-treated cells, both Mϕ and endometrial stroma, we repeatedly measured endotoxin level in the culture media. We could not detect any endotoxin in the culture media of Hsp70-treated Mϕ or Hsp70-treated stromal cells. Pre-treatment of Mϕ with polymyxin B (1μg/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 minutes. We could not detect any cytokine or growth factor in the culture media of Hsp70-treated cells, possibly due to degradation of Hsp70 after heat treatment.

Proliferation of stromal cells by Hsp70 and LPS. Bromodeoxyuridine (BrdU) incorporation study indicated that stromal cells derived from eutopic endometria of

women with endometriosis proliferated dose-dependently and significantly in response to

Hsp70 (p<0.05 at 5μg/ mL and 10μg/ mL vs. non-treated cells, Figure 5A). No

significant difference was observed between treated and non-treated cells derived from

women without endometriosis.

We also found that individual treatment with Hsp70 (10μg/mL) or LPS

(10ng/mL) was able to significantly stimulate proliferation of stromal cells derived from

eutopic endometria of women with endometriosis (1.5-to 1.7-fold increase) comparing to

non-treated cells (p<0.05 for each, Figure 5B). A synergistic effect in cell proliferation

was observed between Hsp70 and LPS. In fact, combined treatment of stromal cells with

Hsp70 and LPS further increased BrdU incorporation when compared with only

Hsp70-treated cells (p<0.05, Figure 5B). In order to confirm the cellular specificity of

LPS, we treated cells with polymyxin B (1μg/mL), an LPS antagonist. We found that

polymyxin B significantly abrogated LPS-promoted cell proliferation (p<0.05) but failed

to decrease combined Hsp70- and LPS-promoted proliferation of stromal cells (Figure

5B). However, pre-treatment of stromal cells with anti-TLR4 antibody (10μg/mL) was

able to significantly decrease the combined Hsp70- and LPS-promoted proliferation of

stromal cells (p<0.05, Figure 5B). These results further indicate that both Hsp70 and LPS

have the capacity to directly stimulate stromal cell proliferation and this growth

promoting effect is mediated by TLR4.

Tissue levels of endogenous Hsp70 in eutopic and ectopic endometria. In

order to examine the in vivo variation of stress reaction at the tissue level, we measured

levels of endogenous Hsp70 in the eutopic endometria of women with endometriosis and

without endometriosis and in different peritoneal lesions as shown in Figure 6. The tissue

concentrations of Hsp70 were significantly higher in the homogenized samples of eutopic

endometria derived from women with endometriosis than that of similar tissues derived

from control women (p<0.05, Figure 6A). No significant difference was observed in

tissue levels of Hsp70 between stage I-II endometriosis and stage III-IV endometriosis

(data not shown). Although an apparent increase in tissue levels of Hsp70 was found in

the samples derived from women in the secretory phase, we did not find any significant

difference in Hsp70 levels when compared with Hsp70 levels in endometrial samples

derived from women in the proliferative phase (data not shown).

When we distributed endogenous Hsp70 levels in samples according to color

appearance of peritoneal lesions harbored by women with pelvic endometriosis, we found that tissue levels of Hsp70 was highest in red lesions, intermediate in black lesions and lowest in white peritoneal lesions (p<0.05 by Kruskal-Wallis test for red lesions, Figure 6B). No difference in tissue levels of Hsp70 was found in the samples between black lesions and white lesions. When total red lesions were subdivided into blood-filled opaque red lesions and non-opaque transparent or translucent red lesions as we described previously (Khan et al., 2004b), opaque red lesions showed significantly higher levels of Hsp70 at the tissue level when compared with either non-opaque red lesions, black lesions or white peritoneal lesions (p<0.01 for each, Figure 6B). No difference in tissue levels of Hsp70 was found among less active peritoneal lesions such as non-opaque red lesions, black lesions and white peritoneal lesions.

Discussion

We demonstrated in our current study that pelvic endometriosis induces stress reaction in pelvic environment in addition to inducing pelvic inflammation. This was confirmed by the release of a variable amount of endogenous heat shock protein 70 (Hsp70) by the different peritoneal lesions and eutopic endometria of women with

endometriosis. We also demonstrated that locally produced Hsp70 might be responsible

for TLR4-mediated induction of inflammatory reaction and direct promotion in the

growth of endometriosis. Although, polymyxin B, a potent LPS antagonist is able to

suppress LPS-mediated growth of endometrial cells derived from women with

endometriosis as reported previously (Hirata et al., 2005; Khan et al., 2007), in our

current study, polymyxin B was unable to suppress combined LPS- and Hsp70-mediated

growth of endometriosis. In contrast, the growth promoting effect of combined LPS and

Hsp70 was significantly suppressed when the biological function of TLR4 was blocked

with anti-TLR4 antibody. Our current findings indicated that LPS- and Hsp70-mediated

inflammatory reaction and growth of endometriosis may be mediated by TLR4 in pelvic

environment.

Toll-like receptor 4 (TLR4)-mediated production of different cytokines and

growth factors and endometrial cell proliferation in response to LPS has been

demonstrated previously from our laboratory and others (Hirata et al., 2005, Khan et al.,

2005d, 2005e, 2007). All of these studies were done in *in vitro* culture system. When we

consider the internal pelvic environment, besides LPS, there are a number of other

exogenous and endogenous ligands for TLR4 (Kiechl et al., 2002, Akira et al., 2004).

TLR4 is an essential receptor for bacterial endotoxin or LPS recognition. In addition to

LPS, as a potential endogenous ligand, Hsp70 can also transmit signal through TLR4

(Wallin et al., 2002; Triantafilou et al., 2004). Therefore, we presumed that growth of

endometriosis may be regulated by endogenous Hsp70 or LPS either alone or in

combination after their binding with TLR4. The expression level of TLR4 was reported to

be higher in the samples derived from the secretory phase of menstrual cycle (Fazeli et al.,

2005; Aflatoonian et al., 2007), however, we did not find any significant difference in

TLR4 expression levels in the samples between proliferative phase and secretory phase or

between women with and without endometriosis in our current study. This could be due

to small number of samples we used in the current study.

As a component of innate immune system, we found that macrophages (Mϕ)

derived from the peritoneal fluid of women with and without endometriosis equally

expressed TLR4 both at the protein and gene level. When we measured secretion levels of

HGF, VEGF, IL-6 and TNFα in the culture media of Hsp70-treated Mϕ, we found that

the production of all these macromolecules were significantly higher in treated cells when

compared to non-treated cells or in Mϕ derived from women with endometriosis than that of non-endometriosis. Again, direct stimulation with Hsp70 was able to significantly stimulate the proliferation of endometrial stromal cells derived from women with endometriosis comparing to similar cells derived from control women. All these pro-inflammatory response and cell promoting effects of Hsp70 were mediated by TLR4. In fact, we found a similar increase in the amount of HGF mRNA in Mϕ in response to Hsp70 and abrogation of cytokine secretion, HGF mRNA level and cell proliferation after pretreatment of cells with anti-TLR4 antibody. Our current findings are another piece of evidence that an internal stress reaction in pelvic environment could be responsible for pelvic inflammation and growth of endometriosis in addition to estrogen and other primary or secondary inflammatory mediators.

We learned from our present study that blocking of TLR4 could be more effective in reducing pro-inflammatory response and growth of pelvic endometriosis, because there are other endogenous and exogenous ligands for TLR4 in addition to LPS (Kiechl et al., 2002). The possible contamination of endotoxin in the study of Hsp70-treated cells during bio-culture procedure was a matter of concern in different

reports (Byrd et al., 1999; Triantafilaou et al., 2001; Wallin et al., 2002; Triantafilaou et al., 2004). However, we carefully excluded the possible contamination of Hsp70-treated cells with endotoxin by our serial exclusion experiments.

Endogenous heat shock proteins including Hsp70 can be produced in response to environmental stimuli (heat shock, ultraviolet radiation, heavy metals), pathological stimuli (viral, bacterial, parasitic infection, inflammation, malignancy or autoimmunity), and physiological stimuli (different physical or chemical stress) (Asea et al., 2000, 2002). Besides inflammation, pelvic endometriosis may induce a variable degree of physical stress (cell to cell contact, cell proliferation, cell differentiation or tissue invasion) or chemical stress (receptor-ligand interaction) in pelvic environment. In order to examine the degree of stress reaction in pelvic environment, we measured tissue levels of endogenous Hsp70 in the eutopic endometria and different peritoneal lesions of women with endometriosis. We found that endogenous stress reaction in eutopic endometria as measured by tissue levels of Hsp70 was significantly higher in women with endometriosis when compared with control women. Although a tendency of higher tissue levels of Hsp70 was observed in early endometriosis (stage I-II) and in the secretory

phase, there was no significant difference in Hsp70 levels between revised-ASRM stage

I-II and stage III-IV or between proliferative phase and secretory phase of the menstrual

cycle.

When we distributed tissue levels of Hsp70 according to color appearance of

endometriotic lesions, we found the highest levels in blood filled-opaque red lesions than

other peritoneal lesions. This could be due to higher stress reaction being contributed by

increased mitogenic, angiogenic and tissue invasion property of opaque red lesions as we

reported previously (Khan et al., 2003, 2004b). In fact, both opaque and non-opaque red

lesions are included in the same groups of red lesions according to the morphological

classification of revised-ASRM (1997). These results further indicated that this subgroup

of red lesions displayed strong stress reaction when compared with other peritoneal

lesions in pelvic environment.

Finally we conclude that women with endometriosis harboring different color

appearances of peritoneal lesions suffer different *in vivo* stress reaction. Among them, a

prominent stress reaction was observed in blood-filled opaque red peritoneal lesions. We

suggest that human Hsp70 also induces pelvic inflammation and may regulate

TLR4-mediated growth of endometriosis. A variation in pelvic inflammatory reaction and stress reaction may function together to regulate the growth of pelvic endometriosis. Our current findings may give us some clue in targeting TLR4 as a new therapeutic strategy in women with endometriosis. Current ongoing studies from our laboratory regarding relationships between stress reaction and inflammation or between stress reaction and angiogenic response in pelvic environment may support further evidence on the importance of stress protein in women with different endocrine diseases.

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Figure Legends

Figure 1. Toll-like receptor 4 (TLR4) protein localization (**A**) and content (**B**)

and gene levels (**C**) in macrophages derived from the peritoneal fluid of women with or

without endometriosis. The immunolocalization of TLR4 was found in CD68-positive

macrophages (Mϕ) (**A**). A 78kDa molecular size of TLR4 was visualized by western

blot analysis (**B**). The lysates of Ramos, a B-lymphoma cell line was used to show a

control band positive for TLR4 (**B**). For Ramos cells, lane 1 indicates plasma lysate, lane

2 indicates 50% diluted fraction of plasma lysate, lane 3 indicates expression in total cell

lysates (**B**). For Mϕ, Lane 1 indicates plasma lysate, lane 2 and 3 indicates 50% and

100% dilution of plasma lysate, and lane 4 indicates TLR4 expression in total cell lysates.

We found more expression of TLR4 in total cell lysates and minimal expression in diluted

fraction of plasma lysate (**B**). The expression of TLR4 mRNA (406bp) was detected by

standard RT-PCR (**C**). Total RNA was extracted from cultured Mϕ derived from three

women each with endometriosis (endo +) and without endometriosis (endo-).

Figure 2. Production of different macromolecules by Hsp70-treated (black bar)

and non-treated (white bar) Mϕ derived from the peritoneal fluid of women with and

without endometriosis. The levels of HGF, VEGF, IL-6 and TNFα in the culture media of

Mϕ (10^5 cells /well) were significantly higher in the treated group comparing to

non-treated group (either $p<0.05$ or $p<0.01$). The levels of all these macromolecules were

also found to be significantly higher in women with endometriosis than that in

non-endometriosis ($p<0.05$ for each). All these data are expressed as mean $\pm$ SEM of

three separate experiments for each group and were normalized with same number of

cells.

Figure 3. Neutralizing effect of anti-TLR4 antibody on the levels of HGF,

VEGF, IL-6 and TNFα in the culture media of macrophages (10^5 cells /well) derived

from the peritoneal fluid of women with endometriosis. Macrophages were pre-treated

with anti-TLR4 antibody (10μg/mL) (black bar) and without antibody (white bar) for 20

min and then further treated with and without Hsp70 for a period of 24 hr. Pre-treatment

of cells with anti-TLR4 antibody was able to significantly decrease all these

macromolecules when compared with non-pre-treatment group ($p<0.05$ for each). All

these data are expressed as mean $\pm$ SEM of three separate experiments for each group and

were normalized with same number of cells.

Figure 4. Effect of Hsp70 and anti-TLR4 antibody on the mRNA expression of

HGF and was detected by standard RT-PCR. A variable concentration of Hsp70 (0-10μ

g/mL) and anti-TLR4 antibody (10μg/mL) was applied and mRNA expression encoding

for HGF in peritoneal Mϕ derived from women with or without endometriosis (**A**) was

examined. The individual mRNA band (505bp) of HGF (**B**) was normalized with the

corresponding band of internal control (β-actin) and is represented by the fold increase of

their corresponding control (without treatment with Hsp70). Values of each transcript

after single treatment with Hsp70 or pre-treatment with anti-TLR4 antibody (10μg/mL)

were nomalized to 1 (dose 0). For HGF (**B**), p<0.05 was found at the dose of 1μg/mL and 10μg/mL of Hsp70 (endometriosis vs. non-endometriosis) and p<0.05 was found when compared with anti-TLR4 antibody non-treated Mϕ. The results are expressed as mean $\pm$ SEM of three different experiments derived from three separate patients.

Figure 5. Single and combined effect of exogenous Hsp70 and LPS on the proliferation of stromal cells derived from the eutopic endometria of women with endometriosis (black bar) and without endometriosis (white bar) and was measured by the bromodeoxyuridine (BrdU) incorporation study. Different concentration of recombinant human Hsp70 (0, 1, 5, 10μg/mL) was applied to stromal cells and is shown in upper panel (**A**). The single and combined treatment of stromal cells with Hsp70 (10μg/mL), LPS (10ng/mL) and pre-treatment of these cells with either polymyxin B (1μg/mL) or anti-TLR4 antibody (10μg/mL) are shown in lower panel (**B**). The results are represented as percentage of control (without any treatment). The results are expressed as mean $\pm$ SEM of three different experiments derived from three separate patients. **A.** p<0.05 vs. non-treated cells; **B**. p<0.05 (Hsp70 vs. control), p<0.05 (LPS vs. control),

p<0.05 (LPS alone vs. LPS+polymyxin B); p<0.05 (combined Hsp70+LPS vs. control);

p<0.05 (anti-TLR4 pre-treated cells vs. without anti-TLR4 pre-treated cells).

Figure 6. Tissue levels of Hsp70 in the eutopic and ectopic endometria derived from women with and without endometriosis. The concentration of Hsp70 was measured in the supernatant of tissue homogenates derived from the eutopic endometria (**A**) and different peritoneal lesions (**B**). The results are expressed as mean ± SEM. The tissue levels of Hsp70 were significantly higher in the eutopic endometria of women with endometriosis when compared with that in the similar tissues derived from control women (p<0.05, **A**). When we examined tissue levels of Hsp70 in different peritoneal lesions of women with endometriosis (**B**), we found that blood-filled opaque red lesions contained significantly higher amount of Hsp70 than that of either non-opaque red lesions (p<0.01) or black lesions (p<0.01) or white lesions (p<0.01). The tissue level of Hsp70 was the highest (p<0.05) in total red lesions comparing to black or white lesions.