derived from healthy volunteers were used as a positive control. We also investigated the changes of E2 and P in the PF according to revised ASRM staging and morphologic appearances of endometriosis.

We found the highest concentration of E2 and P in the PF, intermediate levels in the menstrual fluid and the lowest levels in the serum (Fig. 8). Both E2 and P levels in these body fluids were higher in women with endometriosis than that in control women, irrespective of menstrual cycle, revised-ASRM staging and color appearance. Although, no significant difference was observed in E2 and P levels between these two groups of women, when we distributed the patients in a different revised-ASRM staging, PF levels of E2 in women with stage I–II endometriosis were found to be significantly higher than in women with stage III–IV endometriosis or without endometriosis.[14] An increased trend in P concentration was observed between them. When we distributed the patients into different morphologic appearances of endometriosis, PF levels of E2 and P were found to be significantly higher in women containing dominant red peritoneal lesions when compared with women containing other peritoneal lesions or women without endometriosis.[14] Estradiol (E2) level in the PF of women with endometriosis in the proliferative phase was higher than that of women without endometriosis. Women with endometriosis also displayed higher P levels in the secretory phase than those in the proliferative phase. No difference was observed in serum levels E2 or P according to revised-ASRM staging or color appearances of endometriosis.

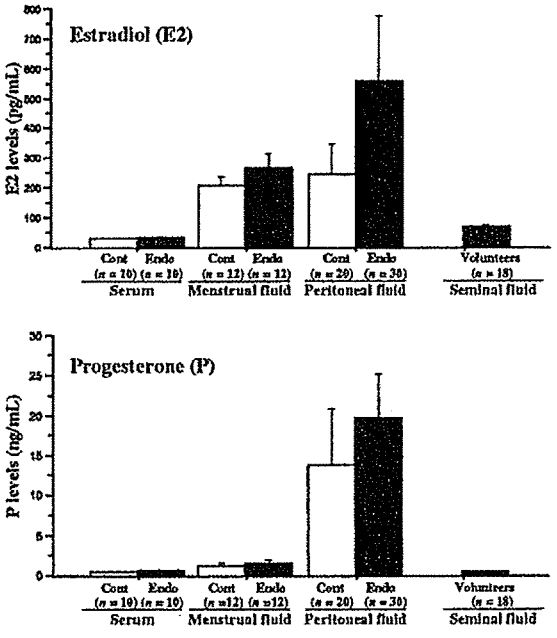

Fig. 8 Shows levels of estradiol (E2) and progesterone (P) in different body fluids collected from women with and without endometriosis. The level of ovarian steroids in seminal fluid was used as a positive control. A higher increase of E2 and P in the peritoneal fluid and menstrual fluid derived from women with endometriosis was observed when compared with corresponding levels in control women. cont, control women; endo, women with endometriosis.

Expression of E2 Receptor (ER) and P Receptor (PR) in Endometriosis

The expressions of ER and PR in the eutopic and ectopic endometria are well described.[85–94] Aside from typical endometriosis, there are atypical varieties of endometriosis characterized by a lack of glandular epithelial structures or stromal cells in non-pigmented lesions,[123,124] inclusion cyst, endosalpingiosis, reactive mesothelium, and columnar epithelial cells in the pelvic peritonea. Reports on the expression of ERs and PRs in these other peritoneal lesions are scarce, and the effect of ovarian steroids on them is not well understood.

We re-evaluated ER and PR expression in endometrium and typical endometriosis and extended our study to include other peritoneal lesions. The highest score of ERs and PRs was observed in the epithelial and stromal cells of the normal uterine endometrium at the early proliferative phase of the menstrual cycle. The ER and PR scores declined throughout the secretory phase. In typical endometriotic lesions, the ER and PR scores were constantly high independent of the menstrual cycle. The expression pattern of ER mRNA was mostly in parallel with that of ERs. In typical endometriosis, ERs and PRs were found in both glandular epithelial cells and their surrounding stromal cells. Expression of ER mRNA was found in typical endometriotic peritonea and in pelvic peritoneum with columnar epithelial cells, but not in normal pelvic peritoneum (mesothelium). ER and PRs were negative in mesothelium, but were positive in the nuclei of fibroblasts in the connective tissue.

Our findings indicate that the columnar type cells in mesothelium are more similar to the epithelial cells in endometrium and endometriosis than to flat mesothelium. The relation of the columnar cells

with their adjacent mesothelium and endometriotic lesions appears to be a key point that will decide whether endometriosis is an implant of the endometrium or is a metaplasia of mesothelium. In our previous study, clusters of columnar cells were found in mesothelium in cases in which endometriosis was absent.[125] The study suggested that the columnar cells did not originate from endometriosis and that those found in the periphery of endometriotic lesions may manifest eventually as endometriosis. Although there is no unequivocal evidence for this path, there are some supporting data: Murphy et al.[126] found a glandular orifice adjacent to normal mesothelium, and Nakamura et al.[127] documented columnar and ciliated cells in many peritoneal infoldings that dip into sub-peritoneal stroma. We strongly presume that pelvic peritoneum may have the potentiality for metaplastic changes under the influence of steroid hormones and/or a stimulating substance such as growth factors. In fact, we already described that HGF, as a pleiotropic growth factor, has the parallel potentiality involving in cellular changes of peritoneal mesothelium to a columnar phenotype and consequent gland-like invagination mimicking endometriosis.[3]

Expression of ERs and PRs in Macrophages

As mesenchymal cells retain ER, production of different cytokines by endometrial stromal cells and its modulation by estrogen has been demonstrated.[16] Considering that infiltrated Mφ is one of the cell components of endometriotic lesion in pelvic environment, reports describing expression of steroid receptors by Mφ and the secretion of different macromolecules in response to steroid hormones are scanty.

Several reports demonstrated that these inflammatory cells retain the mRNA encoding both ER and PR.[71,128] Majority of CD68 immunoreactive Mφ as isolated from women with or without endometriosis showed stronger nuclear staining for ER but were less reactive to PR. In contrast, tissue localization of ER and PR was equally demonstrated in the same position of CD68 immunoreactive Mφ in the serial section of intact tissues derived from the eutopic endometrium of woman with endometriosis.[71] This indicates that besides glandular epithelium and stroma, ER and PR are also being synthesized and expressed by the infiltrated Mφ in intact tissue. This was further confirmed at gene level and revealed that basal Mφ isolated from the PF of women with or without endometriosis contained the mRNA encoding for ER and PR. No phase of the menstrual cycle-dependent variation in the expression of these receptor mRNAs was evident for either group.[71]

Production of Macromolecules by E2-, P-, and LPS-stimulated Mφ

Several reports have already demonstrated the potential role of ovarian steroid hormones in the regeneration of endometrium after menstruation and the growth of endometriosis.[6,7] However, information regarding inflammatory cell-mediated growth or persistence of endometriosis by ovarian steroids is limited.

We and others found that direct stimulation of Mφ in culture with E2 and P resulted in a variable increase in the secretion of HGF, VEGF, IL-6 and TNF-α by PF Mφ.[71,128] The production of HGF was significantly increased by E2 in women with endometriosis than that of control women or non-treated macrophages. A marked increase in the secretion of VEGF was observed by treatment with both E2 and P in women with endometriosis and also in women without endometriosis when compared with non-treated PF macrophages. No phase of cycle differences were seen. When we performed a blocking experiment on E2 by using ER antagonist, tamoxifen, we found that tamoxifen significantly reversed the secretion of and tended to reverse the secretion of HGF by the estrogen-treated PF macrophages towards the non-treated macrophages.[71] This indicates that it is the direct effect of estrogen on the PF macrophages that was able to produce significant amount of HGF and VEGF and is being mediated by ER as located on these inflammatory cells. We also found a substantial amount of increase in the levels of IL-6 and TNF-α in response to ovarian steroids without displaying any significant difference with non-treated cells.[71]

It has been reported that activation with LPS significantly increased the amount of a number of macromolecules secreted by inflammatory cells.[71,73] We observed that activation of basal macrophages further enhanced the response of these cells to ovarian steroids. In fact, exogenous treatment with E2 was able to further increase the amount of both HGF and VEGF secretion by PF macrophages when these cells were activated with LPS.[71] Although progesterone increased the secretion of VEGF by non-activated

Mφ, it was unable to further enhance the secretion of either HGF or VEGF by activated PF macrophages.[71] These results confirmed that irrespective of activation status, PF macrophages were independently stimulated to produce HGF and VEGF by E2. This also indicates that in addition to primary and secondary inflammatory mediators, ovarian steroids equally produce a pelvic inflammatory reaction mediated by macrophages. An inflammatory response and ovarian steroid hormones may function either alone or in combination to regulate the production of a variety of macromolecules by PF macrophages in pelvic microenvironment.

It is generally believed that ovarian steroid hormones are essential for the growth or persistence of ectopic endometrium and corresponding eutopic endometrium in women with endometriosis.[129] As the major cellular constituents of PF are macrophages, comprising between 82% and 99% of the total cell population,[130] it is quite reasonable to speculate that these cells may be responsive to ovarian steroids.

Synergistic Effect Between HGF and E2 in the Growth of Endometriosis

The presence of c-Met receptor and ER in endometrial and endometriotic cells and Mφ might be expected to enable these cells to respond to endogenous or exogenous HGF and E2.[71,73,131] When we examined the effect of HGF and E2 on the proliferation of endometrial gland cells, stromal cells and Mφ, we found that these cells significantly proliferated in response to HGF and E2 either alone or in combination when compared with non-treated cells.[71] A synergistic effect between HGF and E2 on cell proliferation was observed. A similar pattern of cell proliferation was also found in gland cells and stromal cells derived from ectopic endometrium.

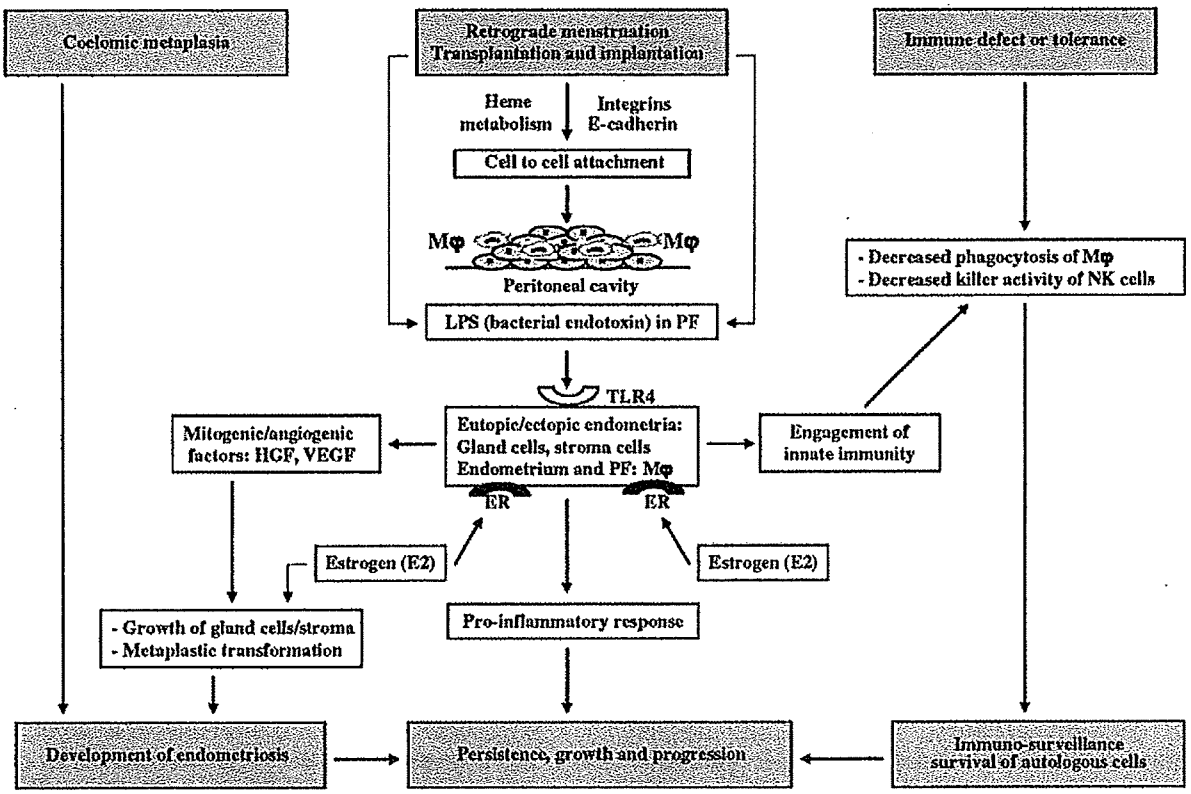

Fig. 9 Shows our proposed concepts on the immunopathogenesis of pelvic endometriosis by hepatocyte growth factor, bacterial endotoxin (LPS) via toll-like receptor 4, macrophages and ovarian steroid hormone (estrogen) and their cross-talk in the development, persistence and progression of endometriosis. The details of the development of early endometriosis by initial cell to cell contact and tissue invasion, the immuno-endocrine relationship and engagement of innate immune system in endometriosis are described in the text.

These results indicate that an innate immune system in pelvic environment and ovarian steroid hormones function in an orchestrated fashion and are involved in the growth of endometriosis. Our results are summarized in Fig. 9.

Conclusions

We now know that besides steroid hormones, innate immunity plays a pivotal role in the initiation of an array of inflammatory reactions against regurgitated endometrial cells and subsequent development of peritoneal endometriosis. Currently prevalent concepts on the genesis of pelvic endometriosis are retrograde dissemination of eutopic endometrial tissues during menstruation,[4] celomic metaplasia of the peritoneum as a secondary Mullerian system,[132,133] and compromised immuno-surveillance.[18–20] However, none of these theories can explain the pathogenesis of endometriosis consistently. Based on our serial studies on the etiological role of bacterial endotoxin (LPS), we would propose a novel concept for the genesis of pelvic endometriosis via LPS/TLR4-mediated engagement of innate immune response.

According to this concept, it would appear possible to integrate two conflicting thoughts of transplantation and metaplasia as reflecting the different phases of initiation and progression of pelvic endometriosis. Transplantation and consequent implantation of regurgitated endometrial cells during menstruation may trigger strong inflammatory reaction in early endometriosis. In addition, a variety of pro-inflammatory factors are also secreted from the infiltrated mononuclear cells of innate immune system. During progression of the affected lesion, cellular changes of juxtaposed mesothelium into endometrioid cells and gland-like structures subsequently ensue and was described as metaplasia of peritoneal mesothelium.[3] As a pleiotropic growth factor, HGF being produced by macrophages and stromal cells has been shown to serve this unique role. The multi-functional role of HGF can be performed with the aid of systemic or focal hormonal environment characterized by consistent estrogen synthesis. Our presenting findings demonstrate for the first time that besides other pro-inflammatory mediators, ovarian steroids also participate in the generation of a pelvic inflammatory response by producing different macromolecules including HGF by peritoneal Mφ. These pro-inflammatory mediators including HGF may be involved in the growth of endometriosis either alone or in combination with estrogen (Fig. 9).

A complete understanding of the mechanisms of endocrine-immune cross talk in the mammalian species and the function of innate immunity via toll-like receptor system will be helpful for the future development of innovative therapies for handling endometriosis.

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Toll-like receptor 4-mediated growth of endometriosis by human heat-shock protein 70

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BACKGROUND: We investigated the role of human heat-shock protein 70 (Hsp70) in Toll-like receptor 4 (TLR4)-mediated growth of endometriosis. METHODS: TLR4 expression was examined in macrophages (Mϕ) isolated in primary culture from the peritoneal fluid of women with and without endometriosis. The production of a number of macromolecules by non-treated Mϕ, Hsp70-treated Mϕ and after treatment with anti-TLR4 antibody was examined by enzyme linked immunosorbent assay (ELISA). The single and combined effects of Hsp70 and lipopolysaccharide (LPS) on the growth of endometrial stromal cells were analyzed by 5-bromo-2-deoxyuridine (BrdU) incorporation study. Hsp70 levels in eutopic and ectopic endometria were measured by ELISA. RESULTS: TLR4 was detected in isolated Mϕ at protein and gene level. Hsp70 (10 μg/ml) significantly stimulated the production of hepatocyte growth factor, vascular endothelial cell growth factor, interleukin-6 and tumor necrosis factor alpha by Mϕ derived from women with endometriosis compared with Mϕ derived from women with no endometriosis ($P < 0.05$ for each). This effect of Hsp70 was abrogated after pretreatment of Mϕ with anti-TLR4 antibody. BrdU incorporation indicated that Hsp70 significantly enhanced the growth of endometrial stromal cells (~50% increase) from women with endometriosis compared to non-treated cells. A synergistic effect on cell proliferation was observed between exogenous Hsp70 and LPS and this was significantly suppressed by 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 from other peritoneal lesions or from women with no endometriosis. CONCLUSIONS: A prominent stress reaction was observed in blood-filled opaque red peritoneal lesions. Human Hsp70 induces pelvic inflammation and may be involved in TLR4-mediated growth of endometrial cells derived from women with endometriosis.

Keywords: cell growth; endometriosis; TLR4; Hsp70; LPS; macrophages

Introduction

Endometriosis induces a variable amount of inflammatory reaction in the pelvic environment depending on the stage and morphology of the 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 and the presence of a number of primary and secondary inflammatory mediators in tissue and body fluids (Halme *et al.*, 1988, 1989; Keenan *et al.*, 1994, 1995; Osuga *et al.*, 1999; Mahnke *et al.*, 2000; Khan *et al.*, 2002a,b, 2004a, 2005a, 2007). The primary inflammatory mediators derived from Gram-negative and -positive microbes can elicit an immune response in the pelvic environment through pattern-recognition receptors, which belong to the family of Toll-like receptors (TLRs) (Akira and Takeda, 2004; Takeda and Akira, 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; McLaren *et al.*, 1996; Harada *et al.*, 2001; Khan *et al.*, 2005a,b,c). There is a possibility that, in addition to the pelvic inflammation, endometriosis may also produce a stress reaction and release endogenous heat-shock proteins (Hsps) in the 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 Hsp, their mechanism of action and levels in the 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 and Kaufmann, 1999; Asea *et al.*, 2000, 2002). Mammalian Hsp60, Hsp70 and Hsp90 have been 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 Hsps (Hsp60, Hsp70 and Hsp90) are reported to be produced by macrophages (Mϕ), 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 that of either Hsp60 or Hsp90 (Wallin *et al.*, 2002), we report here TLR4 expression in Mϕ derived from the peritoneal fluid (PF) 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 had endometriosis of Stages I–II and the remaining 13 women had endometriosis of Stages III–IV 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) (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 phase of the menstrual cycle was determined by histological dating of eutopic endometrium samples taken simultaneously with the PF 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 the published criteria (Jansen and Russel, 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 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 the PF were reported elsewhere (Khan *et al.*, 2002b, 2004a,b, 2006).

All biopsy specimens and the PF 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 the Mϕ from the PF

The PF was obtained from all women with or without endometriosis with the use of laparoscopy. The Mϕ were isolated in primary culture from the PF of six women with and without endometriosis. The detailed procedure of Mϕ isolation in primary culture was described previously (Khan *et al.*, 2005a,b). 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 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 of Mϕ was plated in four-well chamber slides (Nunc, Naperville, IL, USA) for immunostaining and the rest were used for culture. The detailed 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 and without endometriosis. The detailed 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 a four-chamber slide (Nunc) for immunostaining and the rest was used for culture. After 24 h, the slides were washed in PBS, fixed with 4% paraformaldehyde for 10 min and rinsed with PBS. The slides then were incubated in 0.1% Triton X-100 for 5 min and were incubated for 3 h at 37°C 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 Mϕ and stromal cells

The isolated peritoneal Mϕ were cultured in triplicate (10^5cells/well) for 24 h 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 h and then serum free Mϕ were cultured for another

24 h with different concentrations of highly purified recombinant human Hsp70 (1, 5, 10, 15 and 20 μg/ml) (low endotoxin, ESP-555, Stressgen, Victoria, Canada). A blocking experiment was performed with anti-TLR4 antibody (10 μg/ml) (HTA-125, HyCult Biotechnology) 20 min prior to the treatment with recombinant human Hsp70 (10 μg/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 h, the cultured media were collected in triplicate, pooled and frozen at −70°C 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 (1 μg/ml, Sigma), an LPS antagonist and by heat treatment (65°C) of Hsp70-treated cells.

Next, to examine the direct effects of recombinant Hsp70 (1, 5 and 10 μg/ml) and LPS (10 ng/ml) derived from *Escherichia coli* (serotype 0111:b4; Sigma, St Louis, MO, USA) on the proliferation of endometrial stromal cells, 10^4cells/ml were plated in 96-well microtitre plate and treated with various doses of recombinant Hsp70 either alone or in combination with LPS (10 ng/ml) in serum free RPMI medium and incubated for another 24 h. The neutralizing effects of polymyxin B (1 μg/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 Mϕ and cell proliferation assay were studied in six women with and without endometriosis (three each in proliferative phase and three in secretory phase).

Cytokine assays in the culture media of Mϕ

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, USA). The antibodies used in HGF, VEGF, IL-6 and TNFα determinations 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- and inter-assay coefficients of variation were <10% for all these assays.

Immunolocalization of TLR4 in Mϕ

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 detailed procedure of immunocytochemistry was described previously (Fujishita *et al.*, 1997; Nisolle *et al.*, 1997; Khan *et al.*, 2005a,b). Non-immune mouse Ig G1 antibody (1:50) was used as a negative control.

Western blotting

Cultured cells in six-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 the use of an enhanced chemiluminescence western blotting system (Amershan Pharmacia Biotech).

Gene expression of TLR4 and HGF in Mϕ

Ribonucleic acid (RNA) was extracted from the 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 analyzed in the Mϕ derived from women with or without endometriosis. The presence of mRNA encoding TLR4 in the 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 the sense and anti-sense 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,b,c). 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 quantify the differences in levels of transcripts between Hsp70-treated and non-treated samples derived from the control women and women with endometriosis. Values of each transcript after the 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°C for 10 s, 64°C for 10 s and 72°C for 12 s; for HGF, 40 cycles at 95°C for 10 s, 64°C for 10 s and 72°C for 12 s; for β-actin, 30 cycles at 95°C for 10 s, 60°C for 10 s and 72°C for 10 s. All PCR conditions were followed by the 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, USA).

Cell proliferation assays

5-Bromo-2-deoxyuridine (BrdU) can be used to measure cell proliferation by quantifying BrdU incorporated into the newly synthesized DNA of replicating cells (Takagi, 1993; Khan *et al.*, 2005a,b). 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 detailed procedure of BrdU incorporation assay was described previously (Khan *et al.*, 2005a,b,c). 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 with the amount of DNA synthesis and thereby with 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 min to obtain the supernatant and stored at −80°C 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™, EKS-700, Stressgen, Victoria, Canada) 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 200 pg/ml. Both the intra- and inter-assay coefficients of variation were <10% for this assay. The tissue concentration of Hsp70 was expressed as nanogram per microgram protein.

Statistical analysis

The clinical characteristics of the subjects were evaluated by one-way analysis of variance. The data are expressed as either mean ± SEM or mean ± 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 and without endometriosis (data not shown). As an initial study, we also examined five women with endometriosis but without infertility. We did not find any differences in cytokine profile or cell growth in response to Hsp70 in these five women compared with the women with endometriosis and infertility. Therefore, we represented our data only in women with infertility.

Expression of TLR4 in Mϕ

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. TLR4 was immunolocalized in CD68-positive Mϕ (Fig. 1A), 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) (Fig. 1B). This was confirmed at the mRNA levels (406 bp) in basal Mϕ derived from the PF of women with and without endometriosis (Fig. 1C). The amount of TLR4 mRNA was dose-dependently increased in basal Mϕ with a maximum amount found at 24–48 h 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 and without endometriosis (Table I). 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,e, 2007).

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

In our initial time-dependent and dose-dependent study, we found a maximum increase in the levels of different

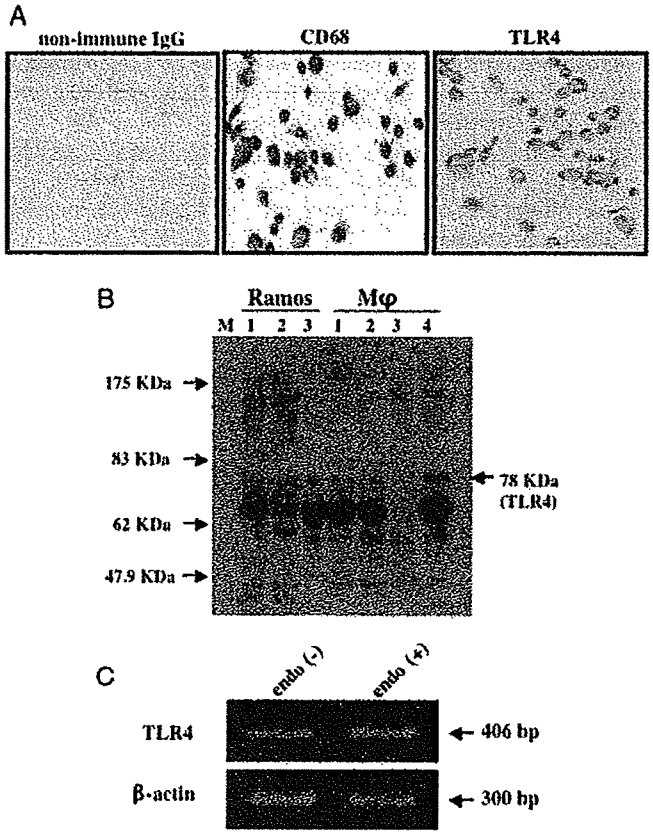

Figure 1: TLR4 protein and gene expression in macrophages. (**A**) The immunolocalization of Toll-like receptor 4 (TLR4) in CD68-positive macrophages (Mϕ) derived from the peritoneal fluid of women with or without endometriosis. (**B**) Western-blot analysis showing a band of TLR4 of 78 kDa molecular size. A lysate of Ramos, a B-lymphoma cell line was used to show a control band positive for TLR4. 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, Lanes 2 and 3 indicate 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. (**C**) TLR4 mRNA (406 bp) expression was detected by standard RT-PCR. Total RNA was extracted from cultured Mϕ derived from three women each with endometriosis (endo+) and without endometriosis (endo−).

Table I. Time-dependent study of TLR4 relative gene levels in basal peritoneal macrophages (Mϕ).

	Incubation time (h)				
	0	6	12	24	48
Endometriosis (−)	1	0.48 ± 0.12	0.55 ± 0.11	1.12 ± 0.13	1.42 ± 0.15
Endometriosis (+)	1	0.67 ± 0.15	0.87 ± 0.16	1.67 ± 0.22	1.75 ± 0.22

Basal (non-treated) peritoneal Mϕ derived from women with and without endometriosis were cultured for different incubation time. The expression of TLR4 mRNA was determined by real-time quantitative PCR using LightCycler and was expressed as fold changes in relative gene levels. The values shown are fold of control at initial incubation period (0 h). Normalization was performed by respective expression level of beta-actin gene. All values express mean ± SEM of three independent experiments. The relative gene level of TLR4 was time-dependently increased with a maximum level found at 24–48 h. No significant difference in TLR4 gene level was observed between women with and without endometriosis.

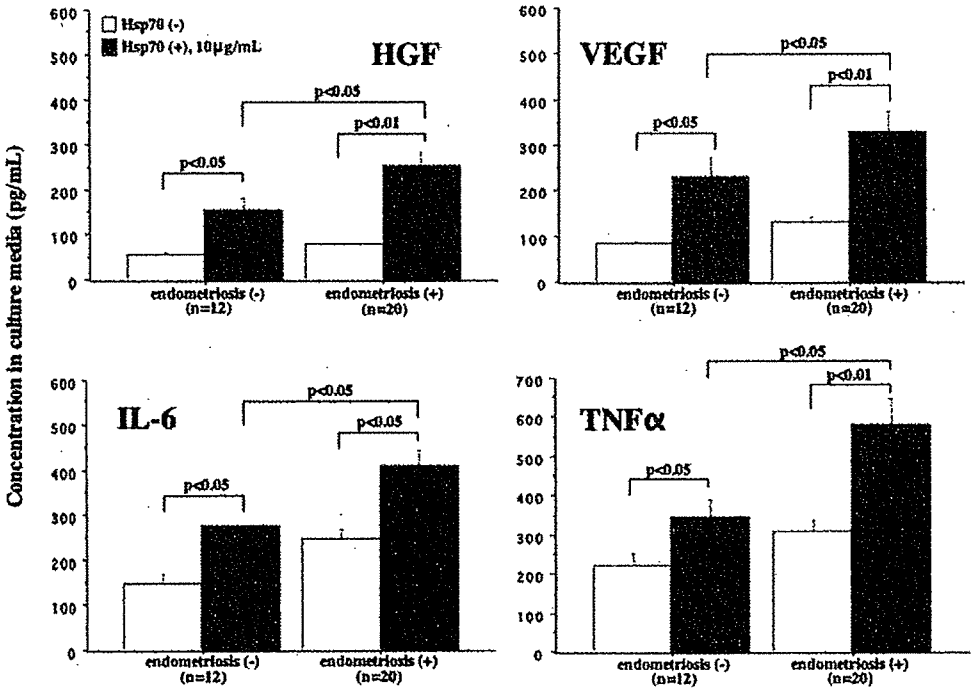

Figure 2: Production of different macromolecules by Hsp70-treated (black bar) and non-treated (white bar) Mϕ derived from the peritoneal fluid of women with and without endometriosis.
The levels of HGF, VEGF, IL-6 and TNFα in the culture media of Mϕ (10^5 cells /well) were significantly higher in the treated group comparing to non-treated group (either $P < 0.05$ or <0.01), and were also significantly higher in women with endometriosis than in women without endometriosis ($P < 0.05$ for each). All these data are expressed as mean ± SEM of three separate experiments for each group and are normalized for same number of cells.

macromolecules and cell growth at 24–48 h and in response to 10–15 μg/ml of Hsp70. Therefore, we obtained all current experimental data in response to 10 μg/ml of Hsp70 with a treatment duration of 24 h. 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 < 0.05 for each, Fig. 2). The levels of these cytokines and growth factors were also markedly higher in Mϕ derived from women with endometriosis when compared with control women without endometriosis ($P < 0.05$ for each, Fig. 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 antibody ($P < 0.05$, for each of HGF, VEGF, IL-6 and TNFα) (Fig. 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 the proliferative phase and the 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 from women without endometriosis (Fig. 4A). Although a dose-dependent increase in gene expression of HGF (505 bp) was observed in both these two groups of women, a significant and a 2-fold increase in mRNA expression of HGF was found in women with endometriosis ($P < 0.05$) compared with women without endometriosis in response to Hsp70 (Fig. 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 antibody ($P < 0.05$, Fig. 4B). When we

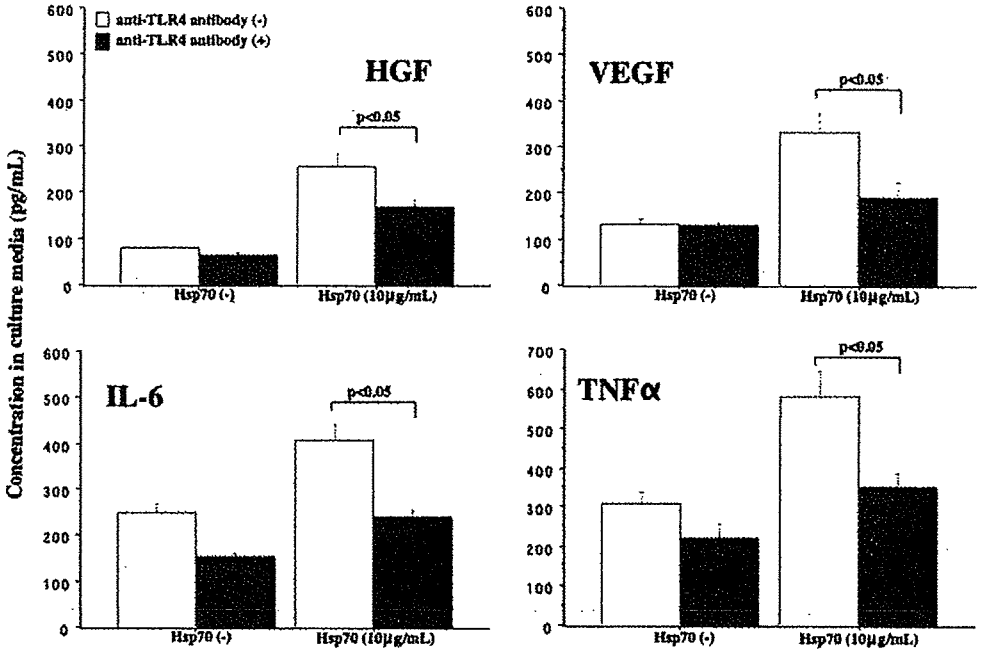

Figure 3: Neutralizing effect of anti-TLR4 antibody on the levels of HGF, VEGF, IL-6 and TNFα in the culture media of Mϕ (10^5 cells /well) derived from the peritoneal fluid of women with endometriosis.
Mϕ were pre-treated with anti-TLR4 antibody (10 μg/ml) (black bar) and without antibody (white bar) for 20 min and then further treated with and without Hsp70 for a period of 24 h. 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 ± SEM of three separate experiments for each group and were normalized with same number of cells.

examined the difference in the amount of HGF mRNA in response to Hsp70 and anti-TLR4 antibody by RT-PCR, results in Mϕ were almost parallel to those obtained by standard RT-PCR (Table II). This indicates that the stimulating effect of Hsp70 on the production of HGF and other macromolecules is mediated by TLR4.

Exclusion of endotoxin contamination of Hsp70-treated cells

In order to exclude the possible contamination by endotoxin of 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 (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 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

A 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 5 and 10 μg/ml versus non-treated cells, Fig. 5A). No significant difference was observed between treated and non-treated cells derived from women without endometriosis.

We also found that the individual treatment with Hsp70 (10 μg/ml) or LPS (10 ng/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) compared with non-treated cells ($P < 0.05$ for each, Fig. 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 cells treated with Hsp70 alone ($P < 0.05$, Fig. 5B). In order to confirm the cellular specificity of LPS, we treated cells with polymyxin B (1 μg/ml) and 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 (Fig. 5B). However, pre-treatment of stromal cells with anti-TLR4 antibody (10 μg/ml) was able to significantly decrease the combined Hsp70- and LPS-promoted proliferation of stromal cells ($P < 0.05$, Fig. 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 and without endometriosis and in different peritoneal lesions as shown in Fig. 6. The tissue concentrations of Hsp70 were significantly higher in the homogenized samples of eutopic endometria derived from

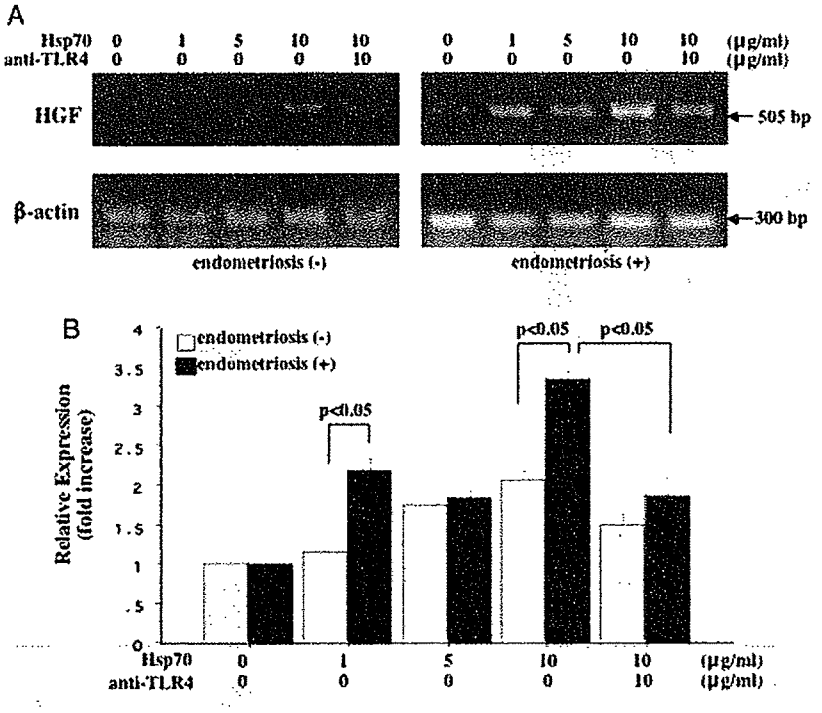

Figure 4: HGF gene expression in macrophages by human Hsp70.
(**A**) The effect of a variable concentration of Hsp70 (0–10 μg/ml) and anti-TLR4 antibody (10 μg/ml) on the mRNA expression of HGF in peritoneal Mϕ derived from women with or without endometriosis was studied by standard RT-PCR. (**B**) The individual mRNA band (505 bp) of HGF 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 (10 μg/ml) were normalized to 1 (dose 0). For HGF, a significance of $P < 0.05$ was found at the dose of 1 and 10 μg/ml of Hsp70 (endometriosis versus non-endometriosis) and $P < 0.05$ was found when compared with anti-TLR4 antibody non-treated Mϕ. The results are expressed as mean ± SEM of three different experiments derived from three separate patients.

Table II. Effects of Hsp70 and anti-TLR4 antibody on relative gene level of HGF in peritoneal macrophages (Mϕ).

Hsp70 (μg/ml) anti-TLR4 antibody (μg/ml)	0 0	1 0		5 0		10 0		10 10
Endometriosis (−)	1	1.26 ± 0.19	$P < 0.05$	1.57 ± 0.31	$P < 0.05$	2.24 ± 0.28	$P < 0.05$	1.81 ± 0.18
Endometriosis (+)	1	2.38 ± 0.37		2.89 ± 0.46		3.91 ± 0.21*		1.96 ± 0.34*

Peritoneal Mϕ were treated with or without Hsp70 for a period of 24 h. Mϕ were pretreated with anti-TLR4 antibody and incubated for another 24 h. The expression of HGF mRNA was determined by real-time quantitative PCR using LightCycler and was expressed as fold changes in relative gene levels. The values shown are fold of control without any treatment. Normalization was performed by respective expression level of beta-actin gene. All values express mean ± SEM of three independent experiments.
The relative gene level of HGF was dose-dependently increased and this was significantly higher in Mϕ derived from women with endometriosis than that in non-endometriosis. *$P < 0.05$ versus without anti-TLR4 antibody.

women with endometriosis than in similar tissues derived from control women ($P < 0.05$, Fig. 6A). No significant difference was observed in tissue levels of Hsp70 between endometriosis of Stages I–II and III–IV (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 in endometrial samples between the secretory phase and the proliferative phase (data not shown).

When we analyzed endogenous Hsp70 levels in samples according to color appearance of peritoneal lesions in women with pelvic endometriosis, we found that the tissue levels were highest in red lesions, intermediate in black lesions and lowest in white peritoneal lesions ($P < 0.05$ by Kruskal–Wallis test for red lesions, Fig. 6B). No difference in tissue levels of Hsp70 was found between the samples of 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, Fig. 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

In our current study, we demonstrated that pelvic endometriosis induces stress reaction in the pelvic environment in addition to inducing pelvic inflammation. This was confirmed by the

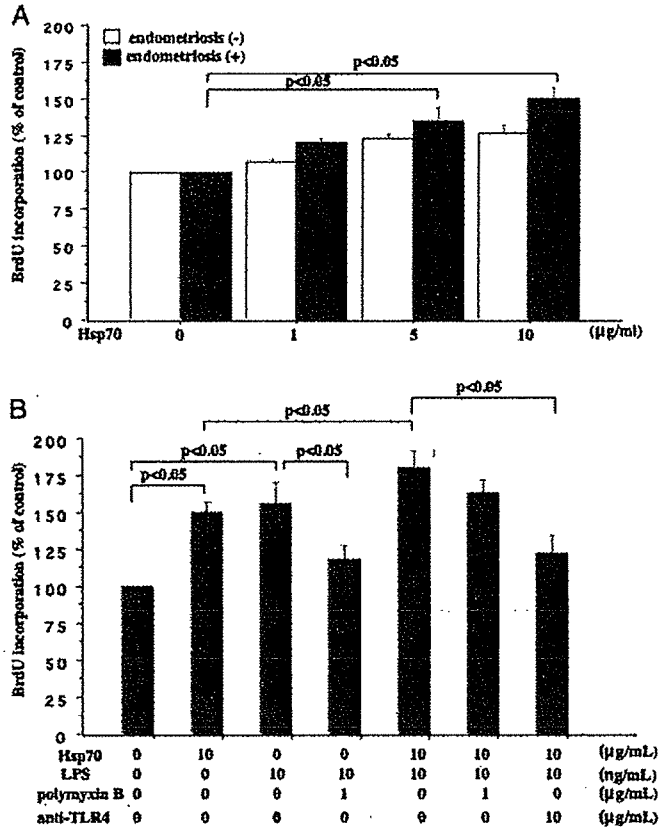

Figure 5: Single and combined effect of exogenous Hsp70 and LPS on the proliferation of stromal cells derived from the eutopic endometria of women with endometriosis (black bar) and without endometriosis (white bar) as measured by 5-bromo-2-deoxyuridine incorporation. Different concentrations of recombinant human Hsp70 (0, 1, 5, 10 μg/ml) were applied to stromal cells as shown in (**A**). The single and combined treatment of stromal cells with Hsp70 (10 μg/ml), LPS (10 ng/ml) and pre-treatment of these cells with either polymyxin B (1 μg/ml) or anti-TLR4 antibody (10 μg/ml) are shown in (**B**). The results are represented as percentage of control (without any treatment) expressed as mean ± SEM of three different experiments derived from three separate patients. **A.** $P < 0.05$ versus non-treated cells; **B.** $P < 0.05$ (Hsp70 versus control), $P < 0.05$ (LPS versus control), $P < 0.05$ (LPS alone versus LPS + polymyxin B); $P < 0.05$ (combined Hsp70 + LPS versus control); $P < 0.05$ (anti-TLR4 pre-treated cells versus without anti-TLR4 pre-treated cells).

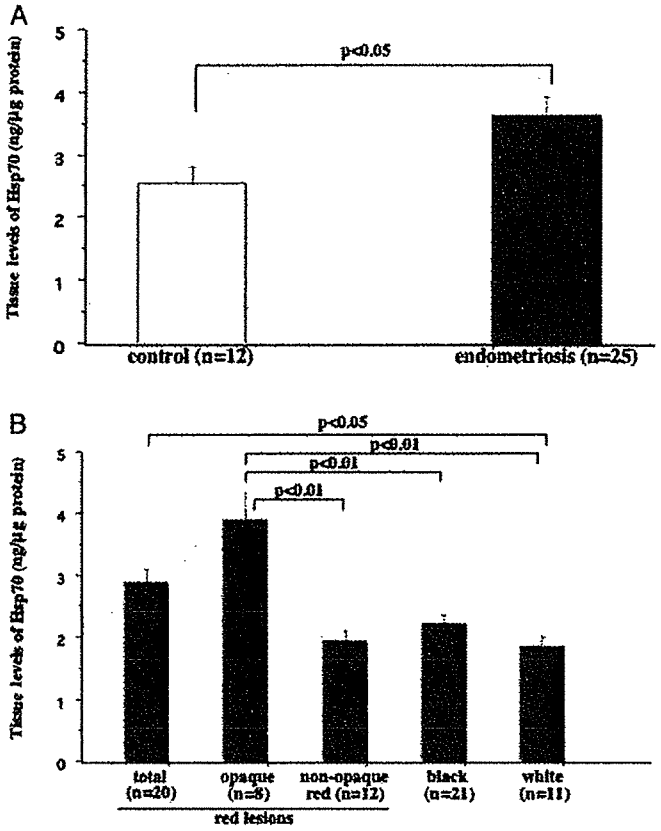

Figure 6: Tissue levels of Hsp70 in the eutopic and ectopic endometria derived from women with and without endometriosis. The concentration of Hsp70 was measured in the supernatant of tissue homogenates derived from the eutopic endometria (**A**) and different peritoneal lesions (**B**). The results 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 compared to black or white lesions.

release of a variable amount of endogenous 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 the pelvic environment.

TLR4-mediated production of different cytokines and growth factors and endometrial cell proliferation in response to LPS has been demonstrated previously (Hirata *et al.*, 2005; Khan *et al.*, 2005d,e, 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 and Takeda, 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 and Triantafilou, 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 between proliferative phase and secretory phase samples or between women with and without endometriosis in our current study. This could be due to the small number of samples we used in the current study.

As a component of the innate immune system, we found that Mϕ derived from the PF of women with and without endometriosis equally expressed TLR4 both at the protein and gene level. When we measured the 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 was significantly higher in treated cells when compared with non-treated cells or in Mϕ derived from women with endometriosis when compared with women without endometriosis. Again, direct stimulation with Hsp70 was able to significantly enhance the proliferation of endometrial stromal cells derived from women with endometriosis compared with 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 levels and cell proliferation after pretreatment of cells with anti-TLR4 antibody. Our current findings provide further evidence that an internal stress reaction in the 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 by endotoxin in the study of Hsp70-treated cells during bio-culture procedure was a matter of concern in different reports (Byrd *et al.*, 1999; Triantafilou *et al.*, 2001; Wallin *et al.*, 2002; Triantafilou and Triantafilou, 2004). However, we carefully excluded the possible contamination of Hsp70-treated cells with endotoxin by our serial exclusion experiments.

Endogenous Hsps including Hsp70 can be produced in response to environmental stimuli (heat shock, ultraviolet radiation and 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 the pelvic environment. In order to examine the degree of stress reaction in the 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 (Stages I–II) and in the secretory phase, there was no significant difference in Hsp70 levels between r-ASRM Stages I–II and III–IV or between proliferative and secretory phases of the menstrual cycle.

When we analyzed tissue levels of Hsp70 according to color appearance of endometriotic lesions, we found the highest levels in blood-filled opaque red lesions compared with other peritoneal lesions. This could be due to a higher stress reaction caused by increased mitogenic, angiogenic and tissue invasion properties 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 r-ASRM (ASRM, 1997). These results further indicated that this subgroup of red lesions displayed strong stress reaction when compared with other peritoneal lesions in the pelvic environment.

Finally, we conclude that women with endometriosis harboring peritoneal lesions of different color appearances 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 provide a 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 the pelvic environment may provide further evidence for the importance of stress protein in women with different endocrine diseases.

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