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Regulation of Hepatocyte Growth Factor by Basal and Stimulated-Macrophage in Women with Endometriosis

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Running Title: HGF production by macrophage of endometriosis.

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Abstract

Background: The different macromolecules as secreted by macrophages (Mφ) in pelvic environment are believed to enhance the growth of endometriosis. However, the possible mediator that stimulates Mφ for the production of different growth factors is not well described. Therefore, we investigated the possible production of hepatocyte growth factor (HGF) by the basal and lipopolysaccharide (LPS) stimulated-Mφ derived from women with or without endometriosis. Methods: Using primary culture and four-well chamber slides, adherent macrophages as immunoreactive to CD68 were isolated from the peritoneal fluid (PF) of 20 infertile women with endometriosis and 12 women without endometriosis. The proliferation of basal and LPS-treated Mφ was investigated by dimethylthiazole tetrazolioum bromide (MTT) assay. The production of HGF in the culture media of basal and LPS stimulated-Mφ was examined by ELISA. The mRNA expressions of HGF and its receptor, c-Met in the Mφ were investigated by reverse transcription-polymerase chain reaction (RT-PCR). The effect of HGF on the growth of endometrial cells and Mφ was analyzed by bromodeoxyuridine (BrdU) incorporation study. Results: A more than 100% increase in the proliferation of peritoneal Mφ derived from
women with endometriosis and particularly of those harboring dominant red lesions were observed after treatment with LPS than basal Mφ. A four-fold (243.6±45.8 pg/mL vs. 66±23.7 pg/mL) and three-fold (148±34.6 pg/mL vs. 49.6±17.3 pg/mL) increase in the production of HGF was observed by the LPS-treated Mφ derived from women with stage I-II endometriosis and stage III-IV endometriosis, respectively when compared to LPS-non-treated Mφ. At the transcriptional level, we found a five-fold increase in HGF mRNA expression by LPS-treated Mφ than basal Mφ in women with endometriosis. BrdU incorporation study indicates that the addition of 10-100ng/mL of HGF enhanced the growth of endometrial epithelial cells, stroma and Mφ (about 50% increase) derived from women with endometriosis, however, this cell proliferation was less observed in cells derived from women with non-endometriosis. **Conclusion:** Lipopolysaccharide could be an inflammatory mediator of macrophage stimulation in pelvic microenvironment. Besides mesenchymal cells, hepatocyte growth factor is also produced by the peritoneal macrophage and is possibly involved in the growth of endometriosis.

**Key Words:** cell growth / endometriosis / hepatocyte growth factor / lipopolysaccharide / macrophage.
Introduction

Peritoneal fluid (PF) from women with endometriosis has been shown to contain higher numbers of activated macrophages (Halme et al., 1987) than found in women without endometriosis. This results in higher concentrations of growth factors and cytokines released by activated Mϕ in these patients (Halme et al., 1988, 1989). This indicates that the growth or persistence of endometriosis is a normal inflammatory response. Activated Mϕ synthesize and secrete different cytokines including tumor necrosis factor α (TNFα), interleukin (IL)-1, IL-6 and IL-10 (Eisermann et al., 1988; Fakih et al., 1987; Ryan et al., 1995; Buyalos et al., 1992; Mosmann 1994). Several of these macromolecules are reported to be elevated in the PF of women with endometriosis (Eisermann et al., 1988; Fakih et al., 1987; Ryan et al., 1995). The increased levels of cytokines in the PF may reflect increased synthesis of cytokines by the peritoneal Mϕ, eutopic and ectopic endometrium, and/or mesothelial cells of the peritoneum, all of which have been shown to be capable of cytokine synthesis (Tabibzadesh et al., 1989; Betjes et al., 1993).

We and others have already demonstrated that hepatocyte growth factor (HGF) and vascular endothelial cell growth factor (VEGF) are also elevated in the PF of women with
endometriosis than healthy controls (Khan et al., 2002a; Fujishita et al., 1999; Osuga et al., 1999; Mahnke et al., 2000; Donnez et al., 1998). HGF was discovered as a mitogen for adult hepatocytes and is identical to scatter factor (Miyazawa et al., 1989; Nakamura et al., 1989; Weidner et al., 1991). Several lines of evidence have implied that HGF, produced by mesenchymal cells, exerts mitogenic, motogenic (migration), morphogenic and angiogenic activity after binding with its receptor, c-Met on various epithelial cells derived from rodents and humans (Nakamura et al., 1986; Tajima et al., 1992). The role of HGF in the proliferation, migration and metaplastic transformation of endometrial epithelial cells has been demonstrated in vitro and in vivo (Sugawara et al., 1997, Ishimaru et al., 2004).

Although production of HGF by hepatic kupffer cells and alveolar macrophages has been reported (Skrtic et al., 1999; Crestani et al., 2002; Morimoto et al., 2001), information regarding production of HGF by peritoneal Mϕ is unknown. Therefore, we report for the first time the production of HGF by the peritoneal fluid Mϕ derived from women with or without endometriosis and examined the ability of peritoneal fluid Mϕ for the synthesis of HGF in both basal condition without any prior treatment and after treatment with LPS.

Since our initial study (Khan et al., 2003a) demonstrated that peritoneal fluid of
women with endometriosis contains an increased concentration of LPS (endotoxin) than that in non-endometriosis, we speculated that LPS could be an inflammatory mediator of M\( \phi \) stimulation in pelvic microenvironment. In addition, we also demonstrated the metabolic activity of peritoneal M\( \phi \) in different staging and morphologic appearances of endometriosis. Finally, we examined the effect of HGF on the growth of bovine endometrial epithelial cells and isolated stroma or macrophage derived from women with or without endometriosis.

**Materials and Methods**

**Subjects.** The subjects in this study were women of reproductive age. The endometriosis group (n=20) included infertile women with 10 women belonging to stage I-II endometriosis and the remaining 10 women belonging to stage III-IV endometriosis at the time of diagnostic laparoscopy. The control group (n=12) consisted of fertile women 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). As we reported recently (Khan et al, 2002a, 2004a), the selection of patients
containing a peritoneal lesion was based on the dominant distribution (70-80%) of a particular lesion in one patient and was assessed by measuring the size and depth of each lesion. This was confirmed by a second blind observer from the photographic or video evidence of recorded files during laparoscopy. All laparoscopic procedures were performed during the luteal phase of the menstrual cycle in both control and study subjects. 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 and informed consent was obtained from all women.

**Isolation of Macrophage from the PF.** Peritoneal fluid was obtained from all women with or without endometriosis with the use of laparoscopy. Macrophages were isolated in primary culture. Peritoneal fluid samples were centrifuged at 400 x g for 10 minutes and the cellular pellet was underlayered with Lymphocyte Separation Medium (ICN, Aurora, OH) and centrifuged at 400 x g for 10 minutes. Macrophages were collected from the interface and cultured in RPMI-1640 medium (GIBCO, Grand Island, NY) supplemented with 100 IU/mL of penicillin G, 50 mg/mL of streptomycin, 2.5 μg/mL of amphotericin B and 10% fetal bovine serum at 37°C in 5% CO₂ in air.
The macrophages 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% macrophages as estimated by their morphology and by immunocytochemical staining using CD68 (KP1), a mouse monoclonal antibody from Dako, Denmark. The cells used for immunocytochemical staining were plated in four-well chamber slides (Nunc, Naperville, IL) and grown to near confluence. The detail procedures of immunocytochemical staining are described elsewhere (Rana et al., 1996; Tsudo et al., 2000). Nonimmune mouse immunoglobulin (Ig) G1 antibody in 1:50 dilution was used as a negative control. A counter staining of macrophages was also performed and we did not find any contaminating cells in isolated macrophage (data not shown).

The isolated peritoneal macrophages were cultured in triplicate \((10^5\text{ per well})\) for 24 hours to assess basal (constitutive) production of cytokines. To evaluate the stimulated (induced) secretion of cytokines, after initial culture with serum containing medium, macrophages were serum starved for 24 hours and then serum free macrophages were cultured for another 24 hours with lipopolysaccharide (LPS) derived from Escherichia coli
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(serotype 0111:B4; Sigma, St. Louis, MO). After 24 hours, the cultured supernatants were collected in triplicate, pooled, and frozen at -70°C until testing.

**Proliferation of macrophage by MTT assay.** The proliferative status of macrophage can be assessed by the metabolic activity of viable cells using MTT (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyl tetrazolioum bromide) assay. The principle of this assay is that the tetrazolium salt MTT is cleaved to formazan by the succinate-tetrazolium reductase which belongs to the mitochondrial respiratory chain and is active only in viable cells. A microtitre plate assay which uses the tetrazolium salt MTT is now widely used to quantitate cell proliferation and cytotoxicity (Sugawara et al., 1997; Mosmann, 1983).

Isolated Mφ from women with or without endometriosis were first placed in 96-well microtitre plate (Griener labortechnik, Germany) at a density of 10^4 cells per well and examined the time-dependent (6hr, 12hr, 24hr, 48hr and 72hr) proliferation of Mφ. For the dose-dependent study, after a 24hr pre-incubation period without serum, culture media were replaced for another 24 hr with serum-free media, containing 0, 1, 5, 10, 15 ng/mL of LPS (Sigma, MO). The dose–dependent study of LPS was applied in isolated Mφ derived from women with or without endometriosis and according to the dominant distribution of either
blood-filled red lesions or black lesions of pelvic endometriosis as we reported previously (Khan et al, 2002a, 2004a).

The MTT assay was then performed in triplicate samples by adding 10 $\mu$l of 5mg/mL MTT solution per well and incubated for 4 hours. After that, 100 $\mu$l of dimethyl sulfoxide (DMSO) was added, kept at room temperature for a few minutes to dissolve the dark blue crystals (formazan), and finally their absorbance was measured at 570 nm with a microplate reader. We found a direct relationship between cell number and the amount of MTT formazan generated. This indicates that the absorbance at MTT assay was directly proportional to the number of viable cells.

**Cytokine assays in the culture media of treated and non-treated Mφ.** The culture media of basal (non-treated) and stimulated (5ng/mL of LPS) Mφ were prospectively collected in triplicate and assays were performed retrospectively. The concentrations of HGF and VEGF 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 VEGF and HGF determination do not cross-react with other cytokines. The
limits of detection were 9.0 pg/ml for VEGF and 40.0 pg/ml for HGF. Both the intra-assay and inter-assay coefficients of variation were <10% for all these assays.

**Immunolocalization of HGF in Macrophage.** In order to immunolocalize HGF in CD68 immunoreactive Mφ, we performed immunohistochemistry using respective antibody and using serial section of eutopic endometrium derived from women with endometriosis. A 1:50 dilution of a rabbit polyclonal antibody against a recombinant protein of HGFα (H-145) (sc-7949; Santa Cruz Biotechnology, Santa Cruz, CA) of human origin was used.

Immunohistochemistry was performed in the 5 μm thick serial section of paraffin-embedded tissues and as described previously (Khan et al., 2003b). Non-immune mouse immunoglobulin (Ig) G1 antibody (1:50) was used as a negative control. Placental tissue, which is known to exhibit high levels of HGF, was used as a positive control.

**Determination of HGF and c-Met mRNA by Reverse Transcription-Polymerase Chain Reaction (RT-RCR) in Macrophage.** We have used the RT-PCR technique to determine the mRNA levels of HGF and its receptor, c-Met in basal (non-treated) and stimulated Mφ (treated by 1, 5, 10ng/mL of LPS) derived from women.
with or without endometriosis. Ribonucleic acid (RNA) was isolated from each of $10^6$ M φ cultured 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.

RNA was treated with DNase 1 RNase-free 10mM Tris-Cl, pH 8.3, 50mM KCl and 1.5 mM MgCl$_2$ in the presence of RNasin ribonuclease inhibitor and incubated at 37°C for 30 minutes to remove DNA contamination. After extraction with phenol-choloform and ethanol precipitation, the RNA was re-dissolved in autoclaved ultrapure water (Milli-Q, MILLIPORE Inc. Corp., Yonezawa, Japan).

**cDNA synthesis.** The first-strand cDNA was synthesized using a RT-PCR kit (Stratagene, La Jolla, CA) with oligo-dT primers. Five micrograms of total RNA was heated to 65°C for five minutes in the presence of 3 μL of oligo-dT primer and then rapidly chilled on ice. A master mix containing 5 μL of 10 x first-strand buffer, 2 μL of 10 mM dNTP mix, 1 μL of MMLV-RT and (50 U/μL) and 1 μL of RNase inhibitor (40 U/μL) and each reaction was incubated at 37°C for 1 hour with further incubation at 90°C for 5 minutes.

**PCR amplification.** Aliquots from the cDNA reaction were PCR amplified in 20
μL reactions as follows: 10 x PCR reaction buffer [100 mM Tris-HCl (pH 9.0), 400 mM KCl, 15 mM MgCl₂, 10 mM from each of the deoxynucleotide triphosphate (dNTP mixture), 10 μM from each primer, and 1 U Taq DNA polymerase enzyme (Bioneer Corporation, Seoul). An amount of 1 μL cDNA was added for each PCR reaction. The reaction was initiated by heat denaturation at 94°C for 1 minute, annealing of the primers for 1 minute at 59°C, and then extension for 1 minute at 72°C. This was repeated for 32 cycles for each of HGF and c-Met using the PCR apparatus (Takara Biomedicals, Tokyo). The amplification protocol for β-actin used as an internal control was same as above except annealing condition of the primer (62°C for 1 minute) and the reaction was repeated for 23 cycles. After the final cycle, the temperature was maintained at 72°C for 10 minutes to allow completion of synthesis of amplification products. A control with no reverse transcription was run with each sample to confirm that PCR products were free of DNA contamination.

Analysis of PCR-amplified products was performed by fractionation over a 1.5% agarose gel followed by ethidium bromide staining of DNA bands. A scanner densitometer was used to determine the ratio of intensity of each band relative to β-actin and is represented as the relative expression of the target gene. Autoradiographs were analyzed to quantitate
differences in levels of transcripts between LPS non-treated samples and LPS-treated samples derived from control women and women with endometriosis. Values of each transcript after treatment with LPS were normalized to 1. Densitometric analysis of gel bands was performed using the National Institutes of Health image analysis program.

In order to confirm the PCR products, the corresponding PCR product was size fractionated and subcloned into the plasmid pCRII (In Vitrogen, San Diego, CA) for cDNA cloning. The PCR products were sequenced by the dideoxy chain termination method and the products for each primer pair was confirmed in both directions (sense and anti-sense). There was no difference between the sequence products after subcloning and the sequence of the target gene used for the study.

**Primer Design and Controls.** Optimal oligonucleotide primer pairs for RT-PCR amplification of oligo-dT-primed reverse-transcribed cDNA were selected with the aid of the computer program Oligo, version 4.0 (National Biosciences, Inc., Plymouth, MN). Human oligonucleotide primers of HGF, c-Met and β-actin as we used for our current study, their location on cDNA and corresponding GenBank accession numbers are shown in Table 1.

Because quantitative application of this method is contingent upon the analysis of the
PCR products during the amplification phase before the plateau, cycle relationships and dilution curves for cDNA of each target molecule and the housekeeping gene $\beta$-actin were determined.

**Isolation of stroma in primary culture.** Stroma was collected from the biopsy specimens of the eutopic and ectopic endometrium derived from the women with or without endometriosis. The detail procedure of the isolation of stroma is described previously (Osteen et al. 1989; Sugawara et al. 1997).

The characteristics of the cultured stromal cells were determined by morphological and immunocytochemical studies. The isolated cells were placed in four-chamber slide (Nunc, Naperville, IL). 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°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. A counter staining of stroma was also performed to exclude the contamination of epithelial cell or endothelial cell in isolated stromal cell culture (data not shown).

**DNA Synthesis Assay by Bromodeoxyuridine (BrdU) Incorporation Study.** Unlike MTT assay, the BrdU labeling and detection kit measures cell proliferation by quantitating BrdU incorporated into the newly synthesized DNA of replicating cells. 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 [³H]-thymidine-based cell proliferation and carries equal sensitivity and specificity (Takagi, 1993).

Besides isolated stroma and macrophage, we also used bovine endometrial epithelial cell line (BEND cells, ATCC, USA, CRL-2398) as a source of non-cancerous epithelial cells derived from bovine normal endometrium of the uterus. These bovine endometrial cells retain functional characteristics similar to that of human endometrial cells as
described previously (Austin et al., 1999; Parent et al., 2003). These normal endometrial epithelial cells were grown in a medium containing a 1:1 mixture of Ham’s F12 medium and Eagles Minimal Essential medium (EMEM) with Earle’s salt and 1.5 mM L-glutamine adjusted to contain 2.2 g/L sodium bicarbonate and supplemented with 0.126 g/L D-valine, 10% fetal bovine serum and 10% horse serum.

Briefly, desired cells (bovine endometrial epithelial cell, human stroma and Mφ) were cultured in 96 well microtitre plate (10^4 cell/well). An average of 48 hrs were required to reach confluence for these various cells. The cells were serum starved before HGF treatment for a period of 24 hour. After a 24hr pre-incubation period without serum, respective cells were treated with or without HGF (recombinant HGF, R & D system) in a serum free medium and incubated for an additional 24 hours. After that, the cells were labeled with 10 μM of BrdU (100 μ L/well) and incubated for 4 hours at 37°C. The cells were fixed and genomic DNA was denatured by adding 200 μL/well of blocking reagent (1:10) for 30 minutes at room temperature (RT). Peroxidase-labeled anti-BrdU antibody (1:100) was added (100 μL/well) and incubated for 90 minutes at RT. After washing three times, TMB (3,3’5,5’-tetramethylbenzidine) substrate solution was added (100 μL/well) and incubated for
15 minutes at RT for color appearance and finally optical density was measured using a microplate reader at an absorbance of 450 nm. The absorbance values correlated directly to the amount of DNA synthesis and thereby to the number of proliferating cells in culture.

In order to confirm that growth promoting factor in the LPS-treated culture medium is HGF, we used antibody to deplete HGF in the conditioned medium. Mφ were pre-treated with anti-HGF antibody (10 μ g/mL, R & D system), incubated for 4 hr and then treated with 5ng/mL of LPS for another 24 hrs and examined the change in cell growth.

**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 black lesions, LPS or HGF-treated and non-treated groups were compared using Mann-Whitney U-test or Student’s t test. The Tukey test of significant differences was used post hoc for multiple comparisons. Differences were considered as statistically significant for p<0.05.
Results

Although data not shown, we also measured PF concentrations of Mφ in a separate population of control women and women with endometriosis. In a parallel fashion of macrophage infiltration in intact tissue as we recently reported (Khan et al. 2004b), we also observed that PF concentrations of Mφ was significantly higher in women with endometriosis than that in non-endometriosis (mean, 1.8x10^7/mL vs. 1.3x10^7/mL, p<0.05).

**Proliferation of Basal and LPS-stimulated Mφ.** The isolated peritoneal fluid macrophages placed into primary culture, which were immunoreactive to CD68 and their negative control are shown in Figure 1A and B, respectively. The co-existing contamination by other leukocytes was excluded by their negative immunostaining to CD45 (data not shown).

We examined the metabolic activity of isolated Mφ as a measure of cell proliferation from women with or without endometriosis and in a time- and dose-dependent fashion (Figure 2) and these experiments were done in triplicate from three different patients. Cell proliferation was expressed as the percentage of control (without treatment with LPS). The proliferation of basal Mφ isolated from the peritoneal fluid of women with
endometriosis appeared to increase from 6 hour of incubation with a peak activity attained at 24 hour (>100% of control) and thereafter decreased (Fig. 2-A) (6 hr. vs. 12 hr., 24hr. and 48 hr., p<0.05). In contrast, the basal Mφ of women without endometriosis did not show any significant difference in their proliferation at any time point of incubation period.

Since the highest activity of Mφ was observed at 24 hour in the time-dependent study, all following dose-dependent study or the stimulation study was performed with an incubation period of 24 hour. LPS treatment of Mφ isolated from women with endometriosis induces their proliferation at 1ng/mL with a peak proliferation at 5ng/mL (>100% of control), thereafter decreased to >50% of control at 15ng/mL (Fig. 2-B). This enhanced proliferation of macrophage derived from women with endometriosis was significantly higher than that in women without endometriosis (maximum >50% of control) (p<0.05, at 1ng/mL, 5ng/mL and 10ng/mL) (Fig. 2-B). We further examined the proliferation of Mφ according to the dominant color appearance of endometriotic lesions, because red morphologic lesions of pelvic endometriosis displayed significantly higher biological activity than that in black lesions or white lesions (Khan et al, 2004a). We treated the Mφ with LPS after their isolation from three women containing dominant red lesions and three women containing
dominant black lesions. We found that Mϕ isolated from women containing red lesions displayed higher proliferative activity at all doses of LPS (>100% of control for red lesions and <50% of control for black lesions (Fig. 2-C) (p<0.05, at LPS 1ng/mL to LPS 15ng/mL, red lesion vs. black lesion).

**HGF Production by the Basal and LPS-Stimulated Mϕ**. The production of HGF by the LPS-treated (5 ng/mL) and non-treated Mϕ derived from the peritoneal fluid of control women, women with stage I-II endometriosis and stage III-IV endometriosis are shown in Figure 3. We compared the secretion of HGF (pg/mL) in the culture media between LPS-treated and non-treated Mϕ.

We found that a substantial amount of HGF was also produced by LPS-treated Mϕ in these three groups of women. There was no difference in the secretion of HGF by basal Mϕ in any of these three groups of women. The production of HGF by the LPS-treated Mϕ was more remarkable in women with stage I-II endometriosis than those with stage III-IV endometriosis or control women (Figure 3). These results indicate that the production of HGF is sensitive to LPS treatment than no treatment and Mϕ derived from women with stage I-II endometriosis are more responsive to produce significant amount of HGF than that of stage
III-IV endometriosis or control women.

As shown in Figure 3, a four-fold (243.6 ± 45.8 pg/mL vs. 66 ± 23.7 pg/mL, p<0.001) and three-fold (148 ± 34.6 pg/mL vs. 49.6 ± 17.3 pg/mL, p<0.001) increase in the production of HGF was observed by the LPS-treated Mφ derived from women with stage I-II endometriosis and stage III-IV endometriosis, respectively when compared to LPS-non-treated Mφ. However, only a 1.5 fold increase in the production of HGF was observed in the control women after LPS treatment than without treatment (74.2 ± 20.6 pg/mL vs. 51.6 ± 19.6 pg/mL, p<0.05). In contrast to HGF, the production of VEGF was also increased by both basal and LPS-stimulated Mφ in all three groups of women (data not shown). Although the production of VEGF in response to LPS was higher than that of HGF, we did not find any significant difference between the production of HGF and VEGF in these women (data not shown).

**Production of HGF by Mφ in intact tissue.** As shown in Figure 4, tissue localization of HGF (Fig. 4-C) was demonstrated in the same position of CD68 immunoreactive Mφ (Fig. 4-B) (shown by corresponding arrow heads) in the serial section of intact tissues derived from the eutopic endometrium of woman with endometriosis. This
indicates that HGF is also being synthesized and secreted by the infiltrated Mφ of intact tissue in addition to in-vitro production of HGF by the basal or LPS-stimulated Mφ.

**mRNA Expression of HGF and c-Met by the Basal and LPS-stimulated Macrophage.** In order to determine if the regulation of HGF and its receptor, c-Met also occurs at the transcriptional level, we examined the mRNA expression of HGF and c-Met by the treated (LPS, 1ng/mL, 5ng/mL and 10ng/mL) and non-treated Mφ using RT-PCR (Fig. 5-A). A control with no reverse transcription confirmed that PCR products were free of DNA contamination.

The RT-PCR reaction of HGF, c-Met and β-actin mRNAs gave rise to a band with sizes of 505 bp, 536 bp, and 300 bp, respectively (Fig. 5-A). In women without endometriosis, the mRNA expression of HGF was weaker than that in endometriosis after LPS-treatment. The mRNA expression of HGF appeared to be stronger in women with endometriosis and at a treatment dose of 5ng/mL. However, the mRNA expression of c-Met receptor was found to increase in a dose-dependent manner of LPS treatment and this was equally observed for women with and without endometriosis (Fig. 5-A).

A 3, 5 and 4-fold increase in the expression of HGF mRNA in Mφ was found in
women with endometriosis at 1ng/mL (p<0.01), 5ng/mL (p<0.001) and 10ng/mL (p<0.01) of LPS-treatment, respectively (Fig. 5-B). Although a very low expression of HGF mRNA was observed by gel band, a 1.5 to 2-fold increase in the relative expression of HGF mRNA was found in women without endometriosis (Fig. 5-B).

Similar to dose dependent increase in c-Met mRNA expression by gel band, a two to 4-fold increase in the relative expression of c-Met was observed in the Mφ of women with or without endometriosis and after LPS treatment (Fig. 5-C). This indicates that Mφ derived from women with or without endometriosis equally carries the receptor for HGF but the mRNA expression of HGF varies resulting in the differential production of HGF at the protein levels between these two groups of women.

**Effect of Culture Media from the Basal and LPS-treated Mφ on Cell Growth.**

According to the above results, the culture media of the basal and LPS-treated Mφ contained a variable concentrations of cytokines and growth factors including HGF. Therefore, we tried to examine the effect of 10% culture media derived from the basal and LPS (5ng/mL)-treated Mφ of women with endometriosis on the growth of normal epithelial cells derived from bovine endometrium and stroma which was isolated from the eutopic endometrium of women.
with endometriosis.

We confirmed the purity of isolated stroma by their positive immunoreaction to vimentin (Fig. 1-C and D) and negative immunoreaction to cytokeratin, von-Willebrand factor and CD45 (data not shown). This indicates that our isolated stroma were free of contamination with epithelial cells, endothelial cells or other leukocytes.

The cell growth was analyzed by counting the total cell number (initial plating $10^5$ per well) as shown in Figure 6. We found that the application of 10% culture medium from the treated M$\phi$ (LPS, 5ng/mL and 10 ng/mL) on bovine endometrial epithelial cells and isolated human endometrial stroma for an incubation period of 24 hour significantly increased the growth of these cells (>50% of control) when we compared them with basal macrophage M$\phi$ or low dose treatment (LPS 1ng/mL) of M$\phi$ ($p<0.05$ for both epithelial cell and stroma) (Fig. 6-A and 6-B).

Since the culture medium of M$\phi$ also retains LPS, we examined the cytotoxic effect of LPS on epithelial cell and stroma by both trypan blue exclusion test and also by BrdU incorporation study in separate experiments. We did not find any toxic effect or stimulated effect of the direct application of LPS on the growth of these cells (data not
shown).

In order to prove that the growth promoting factor in the LPS-treated condition medium may be HGF, we extended our experiment by using antibody to deplete HGF in the condition medium. We found that although not significant, blocking effect of HGF tended to reverse the growth of both epithelial cells and stroma towards the growth by non-treated condition medium (Fig. 6-A and 6-B, hatched bars). This further indicates that besides other growth factors, LPS-treated culture medium also contains HGF and this may promote the growth of endometrial cells.

**Effect of HGF on the Cell Proliferation by the BrdU incorporation Study.** The enhanced cell growth after application of culture medium derived from the LPS-treated Mϕ is the concerted effect of different cytokines and growth factors including HGF. We tried to investigate the direct effect of different concentrations of recombinant HGF on the proliferation of bovine endometrial epithelial cell and stroma or Mϕ of women with or without endometriosis by BrdU incorporation study.

The application of recombinant HGF, at a concentration of 10ng/mL to 100ng/mL and with an incubation period of 24 hour, was able to significantly increase the proliferation
of epithelial cell than HGF non-treated cells or other low-dose treatment with HGF (p<0.05 for 10ng/mL, 50 ng/mL and 100 ng/mL) (Fig. 7-A). HGF was unable to induce the proliferation of stroma isolated from women with non-endometriosis. However, HGF at a concentration of 10ng/mL, 50ng/mL and 100ng/mL stimulated the proliferation of stroma (>50% of control) derived from women with endometriosis (p<0.05 vs. non-treated stroma) (Fig. 7-B). We also studied the effect of exogenous HGF on stromal cells of ectopic endometrium and we did not find any difference of cell proliferation between similar cells of eutopic and ectopic endometrium (data not shown).

It was interesting to observe that besides epithelial cell and stroma, HGF at a higher concentration (50ng/mL and 100ng/mL) was also able to stimulate the proliferation of Mϕ (>50% of control) which were derived from women with endometriosis (p<0.05 for both vs. non-treated Mϕ, Fig. 7-C). However, HGF was unable to do so in Mϕ derived from women with non-endometriosis (Figure 7-C).

Since BrdU incorporation study represents the simple incorporation of BrdU into the proliferated DNA of these cells and does not reflect the actual cell growth as accounted by increased cell number, therefore, we also tried to examine the cell growth of epithelial cell,
stroma and Mφ by cell number (initial plating $10^5$ cells/well) under HGF stimulation. We found a parallel and significantly increased cell growth under a stimulation dose of 10ng/mL, 50ng/mL and 100ng/mL of HGF for epithelial cell and stroma and at a dose of 50ng/mL and 100ng/mL of HGF for Mφ (data not shown).

**Discussion**

A line of evidence has already established that increased activity of endometriotic tissue and infiltrated Mφ has a crucial role in enhancing the growth of the disease or in causing infertility (Khan et al., 2004a and 2004b; Muscato et al., 1982). This can be achieved by the production of different macromolecules by the active tissues of stage I-II endometriosis and by the activated Mφ. In our current study we reported that infiltrations of Mφ in the peritoneal fluid of women with stage I-II endometriosis retain greater activity by their stimulated proliferation and by their ability to produce significantly greater concentrations of different glycoproteins. These findings were significantly higher than that of Mφ which were obtained from women with stage III-IV endometriosis or non-endometriosis.

The increased infiltration of Mφ in intact tissue and peritoneal fluid of women with
endometriosis has been reported (Khan et al., 2002b and 2004b; Halme et al., 1987). However, the metabolic activity of these Mφ in women with or without endometriosis and based on their morphologic appearance by laparoscopy is not well described. We found that proliferation of Mφ as a measure of their metabolic activity was significantly higher in women with endometriosis and also in those harboring dominant red peritoneal lesions than other pigments. This indicates that Mφ in the peritoneal fluid of women with active endometriosis equally retain higher metabolic activity for their consequent production of different macromolecules.

A number of growth factors, cytokines or chemokines are reported to be produced by the epithelial cells, endothelial cell, mesothelial cells or by the mesenchymal cells (Tabibzadeh et al., 1989; Betjes et al., 1993). In addition, the production of cytokines by LPS-stimulated Mφ from women with endometriosis has been reported (Wu et al., 1999). The growth or persistence of endometriosis has been considered as an inflammatory response as manifested by increased concentrations of IL-6, IL-8 and TNF-α in the peritoneal fluid of women with endometriosis (Khan et al, 2004a; Harada et al. 2001). As we reported recently (Khan et al., 2003a), concentration of LPS (endotoxin) in the PF of women endometriosis
appeared to be higher when comparing to that in non-endometriosis. Therefore, we presume that the peritoneal macrophages in women with endometriosis are at an activation status to produce more HGF after stimulation with LPS.

We demonstrated for the first time that beside alveolar Mφ and hepatic kupffer cell, a substantial amount of HGF is also produced by the peritoneal Mφ derived from women with endometriosis. This is demonstrated both at the protein level and also at the transcriptional level in response to LPS. The production of HGF and other macromolecules by the peritoneal Mφ in the pelvic microenvironment may be antigen-primed. Because peritoneal Mφ retains toll-like receptor 4 (TLR4) for LPS as demonstrated by a recent literature (Triantafilou et al., 2002). This is documented by the increased mRNA expression of HGF, the secretion of HGF and other macromolecules in the culture medium in response to LPS treatment than that by the basal Mφ. Comparing to transcriptional level, the decreased levels of HGF production at the protein level can be explained by the post-transcriptional or post-translational degradation of HGF. This dissociation in the regulation of HGF between the transcriptional level and the protein level requires further investigation.

Besides in vitro production of HGF by basal and LPS-stimulated Mφ, the
immunoreaction of HGF was also found to be co-localized in the same position of tissue Mφ as demonstrated in the serial section of intact endometrium. This result has shown for the first time that in addition to mesenchymal origin, HGF could also be synthesized and secreted by the infiltrated macrophage of the peritoneal fluid or the intact tissue derived from women with endometriosis. We already established that highly active blood-filled ectopic endometrium and corresponding eutopic endometrium equally harbor abundant Mφ (Khan et al., 2004b). Our current findings further strengthened the notion that the metabolic and biological activity of infiltrated Mφ in the eutopic endometrium of stage I-II endometriosis are higher than that of stage III-IV endometriosis or control women.

We demonstrated production of a small amount of HGF by the Mφ of control women in our current study and it is also reported that HGF can be produced by hepatic kupffer cells and alveolar macrophages (Skrtic et al., 1999; Crestani et al., 2002; Morimoto et al., 2001) in response to any stress, injury, apoptosis or reactive oxygen species. Therefore, it is quite reasonable to speculate that besides endometriosis, HGF may stimulate cells from normal subjects also. Further studies are required to approve this finding in normal subjects.

We demonstrated a three to five-fold increase in the expression of HGF mRNA in
M\(\phi\) derived from women with endometriosis. However, M\(\phi\) of women without endometriosis displayed 1.5-fold to two-fold increase in HGF mRNA. However, it was interesting to observe that these inflammatory cells of women with or without endometriosis displayed a dose-dependent and an almost equal expression of c-Met receptor. In fact, a two to four-fold increase in the expression of c-Met mRNA was observed in M\(\phi\) of these two groups of women.

The common belief until now is that the synthesis of HGF by mesenchymal cells and its interaction with c-Met as located on human endometrial epithelial and endothelial cells confer a paracrine mode of action (Sonnenberg et al., 1993). We recently reported a homogenous immunoexpressions of HGF and c-Met in the glandular epithelium and a heterogenous expression of this ligand-receptor in the stroma of women with endometriosis (Khan et al., 2003b).

If we consider that a typical lesion of pelvic endometriosis has three major cellular components such as glandular epithelial cell, stroma and infiltrated M\(\phi\), then we can postulate a possible inter-relationship between the co-expressions of HGF and its receptor, c-Met among these three types of cells. We believe that besides paracrine mode of action, the
ligand-receptor interaction of HGF in epithelial cell, stroma and in activated MØ may also exert an autocrine or intracrine mode of action in the regulation of the growth of endometriosis.

The autocrine and paracrine mode of actions of HGF among epithelial cell, stroma and MØ were further documented by the stimulated effect of HGF on the proliferation of endometrial epithelial cell, stroma and MØ. The proliferation of epithelial and mesothelial cells in response to HGF is well accepted (Sugawara et al., 1997; Yashiro et al., 1996). However, the proliferation of stroma and MØ in response to HGF treatment is completely new in our current study. The increased proliferation of stroma and MØ by HGF was only evident for the cells which were obtained from women with endometriosis and not from those without endometriosis. This can be explained by the increased co-expression of HGF and its receptor, c-Met in these cells comparing to those from non-endometriosis.

Our current findings of increased production of HGF and its receptor, c-Met by LPS-treated MØ and their exogenous response to cell proliferation by endometriotic cells are in parallel to some recently published reports (Khan et al., 2003b; Sugawara et al. 1997). The report by Khan et al. (2003b) demonstrated that the biological activity of eutopic
endometrium, as measured by the immunoreaction of HGF and c-Met in the glandular epithelium and stroma, was similar to highly active red peritoneal lesions and was significantly higher than that of controls and other lesions. The most recent publication by Khan et al (2004b) further demonstrated that peritoneal lesions of stage I-II endometriosis, their adjacent peritoneum and corresponding eutopic endometrium harbor abundant Mφ that could be involved in the growth of endometriosis. This indicates that a persistent inflammatory response in the peritoneal fluid as well as in intact tissue may be responsible for the growth and progression of endometriosis.

Finally we conclude that peritoneal Mφ of women with endometriosis can also produce a significant amount of HGF in response to LPS. These results further evaluated the crucial role of HGF in the histogenesis of endometriosis and its possible involvement in the growth or persistence of endometriosis. In fact, our current findings of HGF both at the transcriptional and protein levels indicate that besides other cytokines and growth factors, HGF may also play an important role in the pathogenesis of endometriosis.

Besides ovarian steroid hormones, the role of innate immune system in the regulation of endometriosis is also important. Since peritoneal Mφ retain the receptor
(TLR4) for LPS derived from gram-negative bacteria (Triantafilou et al., 2002), we can speculate that a sub-clinical concentrations of endotoxin could stimulate Mφ, produce different cytokines and growth factors and interacts with its neighboring cells in the pathology of endometriosis. Further studies are needed to find out the exact source of stimulation in regulating the Mφ activity and their possible association with infertility or the growth of endometriosis.

**Acknowledgment:** We are highly grateful to Dr. Kazuo Yamamoto, Division of Cytokine Signaling, Department of Molecular Microbiology and Immunology, Nagasaki University Graduate School of Medical Sciences, Nagasaki, Japan, for his excellent help and cooperation in the experimental use of epithelial cell line (BEND cells of ATCC) derived from bovine normal endometrium of the uetrus.
References


817-821.


Figure Legends

*Figure 1.* Shows the immunocytochemical staining of CD68 in adherent Mφ which were collected from the peritoneal fluid of women with endometriosis (A) and anti-mouse IgG positive negative control (B). The purity of isolated Mφ was confirmed by their negative immunoreaction to CD45 (other leukocytes). The vimentin-positive isolated stromal cells derived from the eutopic endometrium of women with endometriosis are shown in (C) and the specificity of the staining was confirmed by the deletion of the first antibody (D). The purity of isolated stroma was confirmed by their negative immunoreaction to cytokeratin (epithelial cell specific), von Willebrand factor (endothelial cell specific), and CD45 (other leukocytes). Final magnification was adjusted at x25 (A, B, D) and at x50 (C) using light microscope connected to a camera (Olympus-VANOX, model-AHBS, Tokyo, Japan).

*Figure 2.* Shows the time-dependent and dose-dependent proliferation of Mφ as measured by MTT assay. **A.** The time-dependent study of basal Mφ proliferation derived from women with or without endometriosis. Mφ proliferation was expressed as the percentage of control (OD with same no. of Mφ at time zero). The asterisk (*) denotes the significantly different from 6hr incubation (p<0.05). **B.** The dose-dependent effect of lipopolysaccharide (LPS) on
Mφ proliferation. The asterisk (*) denotes the significantly different between endometriosis and non-endometriosis (p<0.05). C. The dose-dependent effect of LPS on Mφ proliferation derived from women having dominant distribution of red lesions and black lesions. The asterisk (*) denotes the significantly different between red lesion and black lesion (p<0.05).

Mφ proliferation was expressed as the percentage of control (without treatment with LPS). The results are expressed as mean ± SEM of duplicate determinations of three different patients.

**Figure 3.** Shows the concentrations of HGF (pg/mL) in the culture media of basal Mφ (LPS non-treated) and LPS-treated (5ng/mL) Mφ which were derived from 12 control women (A), 10 women with stage I-II endometriosis (endo I-II, B) and 10 women with stage III-IV endometriosis (endo III-IV, C). The asterisk (*, **) denotes the significantly different between treated and non-treated Mφ. *p<0.05, **p<0.001. The results are expressed as mean ± SD of duplicate determinations of these three groups of women.

**Figure 4.** Shows the immunohistochemical analysis of HGF localization in the same position of CD68 immunoreactive Mφ and was demonstrated in the serial section of intact tissue derived from the eutopic endometrium of woman with endometriosis. A. Hematoxylin and
eosin stain of the same serial section of B and C. Arrow heads show the co-localization of HGF (C) at the same position of CD68 stained Mφ (B) in the stroma of eutopic endometrium. Final magnification was adjusted at x50 and using light microscope connected to a camera (Olympus-VANOX, model-AHBS, Tokyo, Japan).

**Figure 5.** Shows the effect of a variable concentrations of LPS (0-10ng/mL) on mRNA expression at the transcriptional levels encoding for HGF and its receptor, c-Met in peritoneal Mφ derived from women with or without endometriosis (A) and as detected by RT-PCR. The individual mRNA band of HGF (B) and c-Met (C) were normalized with the corresponding band of internal control (β-actin) and is represented by the fold increase of their corresponding control (without treatment with LPS). Values of each transcript after treatment with various doses of LPS were normalized to 1 (LPS0). The asterisk (*, **) denotes the significantly different from corresponding control. For HGF (B), *p<0.01, **p<0.001; for c-Met (C), *p<0.01 by the Tukey test of significant difference. RT (-), a control with no reverse transcription. The results are expressed as mean ± SEM of three different experiments derived from three separate patients.

**Figure 6.** Shows the effect of 10% culture media conditioned by the basal and LPS-treated M
\( \phi \) on the growth of epithelial cells derived from bovine normal endometrium of the uterus (A) and stroma of endometriosis (B). The hatched bars represent the effect on cell growth after depletion of HGF by a 4 hr pre-treatment with anti-HGF antibody (10 \( \mu \) g/mL) and then 24 hr treatment with 5ng/mL of LPS. The cell growth is represented by the percentage of control (without treatment with LPS). *p<0.05 vs. 0ng/mL and 1ng/mL of LPS treatment for both epithelial cell and stroma. The results are expressed as mean ± SEM of three different experiments derived from three separate patients.

**Figure 7.** Shows the effect of different concentrations of recombinant HGF (0-100ng/mL) on the proliferation of epithelial cells derived from bovine normal endometrium of the uterus (A), stroma of women with or without endometriosis (B), and M \( \phi \) of women with or without endometriosis (C) and as measured by the bromodeoxyuridine (BrdU) incorporation study. The results are represented as percentage of control (without treatment with recombinant HGF). The results are expressed as mean ± SEM of three different experiments derived from three separate patients. A. epithelial cell, *p<0.05 vs. non-treated cell or other low-dose treatment with HGF; B and C. stroma and M \( \phi \), *p<0.05 vs. non-treated cell or other low-dose treatment with HGF.
Figure 1
Figure 2

A

B

C

MΦ Proliferation by MTT assay (% of control)

Endo (-)  Endo (+)

6 hr  12 hr  24 hr  48 hr  72 hr

LPS 0  LPS 1  LPS 5  LPS 10  LPS 15 (ng/ml)

Black lesion  Red lesion

LPS 0  LPS 1  LPS 5  LPS 10  LPS 15 (ng/ml)
Figure 3

The graph shows the levels of HGF in Mφ supernatant (pg/mL) under different conditions. The x-axis represents LPS treatment status (- or 5 ng/mL), while the y-axis represents HGF levels.

- Control (n=12)
- Endo I-II (n=10)
- Endo III-IV (n=10)

Significance levels indicated with asterisks: *

The graph indicates a significant increase in HGF levels with LPS treatment (5 ng/mL) compared to the control group.
Figure 5

A. LPS (ng/ml)

<table>
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HGF: 505 bp

c-Met: 536 bp

β-actin: 300 bp

Endometriosis (-)  Endometriosis (+)

B. HGF

Relative Expression (fold increase)

Endo (-)  Endo (+)

LPS 0  LPS 1  LPS 5  LPS 10 (ng/ml)

C. c-Met

Relative Expression (fold increase)

Endo (-)  Endo (+)

LPS 0  LPS 1  LPS 5  LPS 10 (ng/ml)
Figure 7

A. Epithelial cell

B. Stroma

C. Macrophage
Table 1. Human oligonucleotide primers of HGF, c-Met and \( \beta \)-actin used in current study.

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<th>Primers</th>
<th>Location on cDNA</th>
<th>Size (bp)</th>
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HGF, hepatocyte growth factor; c-Met, met protooncogene, receptor for HGF; \( \beta \)-actin, internal control; cDNA, complimentary deoxyribonucleic acid; GB, GenBank.