Regulation of hepatocyte growth factor by basal and stimulated macrophages in women with endometriosis

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BACKGROUND: The different macromolecules as secreted by macrophages (Mφ) in the 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 4-well chamber slides, adherent Mφ 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 the dimethylthiazole tetrazolioum bromide (MTT) assay. The production of HGF in the culture media of basal and LPS-stimulated Mφ was examined by enzyme-linked immunosorbent assay. The expression of mRNA for HGF and its receptor, c-Met, in the Mφ was investigated by RT–PCR. The effect of HGF on the growth of endometrial cells and Mφ was analysed by bromodeoxyuridine (BrdU) incorporation.

RESULTS: A >100% increase in the proliferation of peritoneal Mφ derived from women with endometriosis, and particularly of those harbouring dominant red lesions, was observed after treatment with LPS (P<0.05). A 4- and 3-fold 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 with non-LPS-treated Mφ (P<0.001). At the transcriptional level, we found a 5-fold increase in HGF mRNA expression in LPS-treated Mφ versus basal Mφ in women with endometriosis (P<0.001). The BrdU incorporation study indicates that 10–100 ng/ml of HGF enhanced the growth of endometrial epithelial cells, stroma and Mφ (~50% increase) derived from women with endometriosis (all P<0.05).

CONCLUSION: LPS could be an inflammatory mediator of macrophage stimulation in the pelvic microenvironment. Besides mesenchymal cells, HGF is also produced by peritoneal Mφ 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 (Mφ) (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; Halme, 1989). This indicates that the growth or persistence of endometriosis is a normal inflammatory response. Activated Mφ synthesize and secrete different cytokines including tumour necrosis factor-α (TNF-α), interleukin (IL)-1, IL-6 and IL-10 (Fakih et al., 1987; Eissmann et al., 1988; Buylatos et al., 1992; Mosmann, 1994; Ryan et al., 1995). Several of these macromolecules are reported to be elevated in the PF of women with endometriosis (Fakih et al., 1987; Eissmann et al., 1988; 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 higher in the PF of women with endometriosis than that of healthy controls (Donnez et al., 1998; Fujishita et al., 1999; Osuga et al., 1999; Mahnke et al., 2000; Khan et al., 2002a). HGF was discovered as a mitogen for adult hepatocytes and is identical to scatter factor...
with the use of laparoscopy. Mϕ was obtained from all women with or without endometriosis (Sugawara in vivo 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; Morimoto et al., 2001; Crestani et al., 2002), information regarding production of HGF by peritoneal Mϕ is unknown. Therefore, we report for the first time the production of HGF by the PF Mϕ derived from women with or without endometriosis and examined the ability of PF Mϕ to synthesize HGF in basal conditions and after treatment with lipopolysaccharide (LPS).

Since our initial study (Khan et al., 2003a) demonstrated that PF of women with endometriosis contains a higher concentration of LPS (endotoxin) than that of those without endometriosis, we speculated that LPS could be an inflammatory mediator of Mϕ stimulation in the pelvic microenvironment. In addition, we also demonstrated the metabolic activity of peritoneal Mϕ in different stages and morphological appearances of endometriosis. Finally, we examined the effect of HGF on the growth of bovine endometrial epithelial cells and isolated stroma or Mϕ 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 having stage I–II endometriosis and the remaining 10 women having 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 for Reproductive Medicine (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 blinded 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 PF were collected in accordance with the guidelines of the Declaration of Helsinki and with the approval of the Nagasaki University Institutional Review Board, and informed consent was obtained from all women.

Isolation of Mϕ from the PF

PF was obtained from all women with or without endometriosis with the use of laparoscopy. Mϕ were isolated in primary culture. PF samples were centrifuged at 400 g for 10 min and the cellular pellet was under-layered with lymphocyte separation medium (ICN, Aurora, OH) and centrifuged at 400 g for 10 min. Mϕ were collected from the interface and cultured in RPMI-1640 medium (GIBCO, Grand Island, NY) supplemented with 100 I.U/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 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 >95% Mϕ 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 4-well chamber slides (Nunc, Naperville, IL) and grown to near confluence. The detailed procedure of immunocytochemical staining is described elsewhere (Rana et al., 1996). Non-immune mouse immunoglobulin (Ig) G1 antibody at 1:50 dilution was used as a negative control. A counterstaining of Mϕ was also performed and we did not find any contaminating cells in isolated Mϕ (data not shown).

The isolated peritoneal Mϕ were cultured in triplicate (105 per 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 medium, Mϕ were serum starved for 24 h and then serum-free Mϕ were cultured for another 24 h with LPS derived from Escherichia coli (serotype 0111:B4; Sigma, St Louis, MO). After 24 h, the cultured media were collected in triplicate, pooled, and frozen at −70°C until testing.

Proliferation of Mϕ by MTT assay

The proliferative status of Mϕ 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-tetrazolioum 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 (Mosmann, 1983; Sugawara et al., 1997).

Isolated Mϕ from women with or without endometriosis were first plated in a 96-well microtitre plate (Griener labotechnik, Germany) at a density of 104 cells per well and the time-dependent (6, 12, 24, 48 and 72 h) proliferation of Mϕ examined. For the dose-dependent study, after a 24 h pre-incubation period without serum, culture media were replaced for another 24 h with serum-free media, containing 0, 1, 5, 10 and 15 ng/ml of LPS (Sigma). 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 μl of 5 mg/ml MTT solution per well and incubated for 4 h. After that, 100 μl of dimethyl sulphoxide (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 (5 ng/ml of LPS) Mφ were collected in triplicate and assays were performed retrospectively. The concentrations of HGF and VEGF in the culture media were measured in duplicate in a blind fashion using a commercially available sandwich enzyme-linked immunosorbent assay (ELISA) developed by R&D Systems (Quantikine, R&D Systems, 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- and inter-assay coefficients of variation were <10% for all these assays.

Immunolocalization of HGF in Mφ
In order to immunolocalize HGF in CD68-immunoreactive Mφ, we performed immunohistochemistry using the respective antibody and using serial sections of eutopic endometrium derived from women with endometriosis. A 1:50 dilution of a rabbit polyclonal antibody against a recombinant protein of HGF (H-145) (sc-7949; Santa Cruz Biotechnology, Santa Cruz, CA) of human origin was used. Immunohistochemistry was performed in the 5 μm thick serial sections of paraffin-embedded tissues as described previously (Khan et al., 2003b). Non-immune mouse IgG1 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 RT–RCA in Mφ
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 and 10 ng/ml of LPS) derived from women with or without endometriosis. RNA was isolated from each of 106 Mφ cultured in a 60 mm Petri dish (Greiner) using a monophase solution of 40% phenol and the ISOGEN method (Molecular Research Center, Tokyo), according to the manufacturer’s protocol.

RNA was treated with RNase-free DNase I in 10 mmol/l Tris–HCl, pH 8.5, 50 mmol/l KCl and 1.5 mmol/l MgCl2 in the presence of RNasin ribonuclease inhibitor and incubated at 37°C for 30 min to remove DNA contamination. After extraction with phenol–chloroform 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 an RT–PCR kit (Stratagene, La Jolla, CA) with oligo(dT) primers. A cDNA synthesis master mix contained 5 mmol/l Tris–HCl (pH9.0), 400 mmol/l KCl, 15 mmol/l MgCl2, 10 mmol/l of each of the deoxynucleotide triphosphates (dNTP mixture), 10 μmol/l of each primer and 1 U of Taq DNA polymerase (Bioneer Corporation, Seoul). A 1 μl aliquot of cDNA was added for each PCR. The reaction was initiated by heat denaturation at 94°C for 1 min, annealing of the primers for 1 min at 59°C, and then extension for 1 min at 72°C. This was repeated for 32 cycles for HGF and c-Met using the PCR apparatus (Takara Biomedicals, Tokyo). The amplification protocol for β-actin used as an internal control was the same as above except for the annealing condition of the primer (62°C for 1 min), and the reaction was repeated for 23 cycles. After the final cycle, the temperature was maintained at 72°C for 10 min to allow completion of synthesis of the 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 analysed to quantitate differences in levels of transcripts between non-treated samples and LPS-treated samples derived from control women and women with endometriosis. The 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 plasmid pCRII (Invitrogen, San Diego, CA). The PCR products were sequenced by the dyeodeoxy chain termination method and the product of each primer pair was confirmed in both directions (sense and antisense). 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 which we used for our current study, their location on cDNA and corresponding GenBank accession numbers are shown in Table I.

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 β-actin were determined.

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primers</th>
<th>Location on cDNA</th>
<th>Size (bp)</th>
<th>GB accession no.</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>HGF</td>
<td>Sense (5’–3’): ACTGGCTTCCTTTAGGCACTGACTC</td>
<td>+47</td>
<td>505</td>
<td>D90325</td>
<td>Miyazawa et al. (1989)</td>
</tr>
<tr>
<td></td>
<td>Antisense (5’–3’): TGTTCCCTTGTAGTTGTTGCTTTC</td>
<td>+551</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c-Met</td>
<td>Sense (5’–3’): ACTCCCCCCTGAAAAACAAAGGC</td>
<td>2490</td>
<td>536</td>
<td>J02958</td>
<td>Park et al. (1987)</td>
</tr>
<tr>
<td></td>
<td>Antisense (5’–3’): GGCCTCACCTGGCCAGCCTAC</td>
<td>3025</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td>Sense (5’–3’): ATGTGCGCTGACCAAGGAGTTGCC</td>
<td>294–325</td>
<td>300</td>
<td>NM001101</td>
<td>Ponte et al. (1984)</td>
</tr>
<tr>
<td></td>
<td>Antisense (5’–3’): CCAAATCTCATTCCAGAAAGGCC</td>
<td>593–562</td>
<td></td>
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</tr>
</tbody>
</table>

HGF = hepatocyte growth factor; c-Met = met proto-oncogene, receptor for HGF; β-actin = internal control; GB = GenBank.
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 detailed procedure for the isolation of stroma has been 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 a four-chamber slide (Nunc, Naperville, IL). After 24 h, the slides were washed in phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde for 10 min, and rinsed with PBS. Slides were then incubated in 0.1% Triton X-100 for 5 min and incubated for 3 h at 37°C as follows: against human cytokeratin monoclonal antibodies (mAbs) (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 dilution (Dako). 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 by epithelial or endothelial cells in isolated stromal cell culture (data not shown).

DNA synthesis assay by bromodeoxyuridine (BrdU) incorporation

Unlike the MTT assay, the BrdU labelling 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 mAbs directed against BrdU. It offers a non-radioactive alternative to [³H]thymidine-based cell proliferation and carries equal sensitivity and specificity (Takagi, 1993).

Besides isolated stroma and M₇, we also used a bovine endometrial epithelial cell line (BEND cells, ATCC, USA, CRL-2398) as a source of non-cancerous epithelial cells derived from bovine uterus. These bovine endometrial cells retain functional characteristics similar to those 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 Eagle’s minimal essential medium (EMEM) with Earle’s salt and 1.5 mmol/l 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, the desired cells (bovine endometrial epithelial cells, human stroma and M₇) were cultured in 96-well microtitre plates (10⁴ cell/well). An average of 48 h were required to reach confluence for these various cells. After a 24 h pre-incubation period without serum, the respective cells were treated with or without HGF (recombinant HGF, R&D Systems) in a serum-free medium and incubated for an additional 24 h. After that, the cells were labelled with 10 μmol/l BrdU (100 μl/well) and incubated for 4 h at 37°C. The cells were fixed and genomic DNA was denatured by adding 200 μl/well of blocking reagent (1:10) for 30 min at room temperature. Peroxidase-labelled anti-BrdU antibody (1:100) was added (100 μl/well) and incubated for 90 min at room temperature. After washing three times, TMB (3,3',5,5'-tetramethylbenzidine) substrate solution was added.

Figure 1. The immunocytochemical staining of CD68 in adherent M₇ which were collected from the peritoneal fluid of women with endometriosis (A) and the anti-mouse IgG-negative control (B). The purity of isolated M₇ 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 ×25 (A, B and D) and at ×50 (C) using a light microscope connected to a camera (Olympus-VANOX, model-AHBS, Tokyo, Japan).

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Differences were considered as statistically significant for \( P \) values. 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 the growth-promoting factor in the LPS-treated culture medium is HGF, we used antibody to deplete HGF in the conditioned medium. 

\( \text{M}_\text{w} \) were pre-treated with anti-HGF antibody (10 \( \mu \)g/ml, R&D Systems), incubated for 4 h and then treated with 5 ng/ml of LPS for another 24 h and examined for 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 \( \pm \) SEM or mean \( \pm \) SD. The concentrations of the studied cytokines were not distributed normally and the data were analysed using a 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 the 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 the data are not shown, we also measured PF concentrations of \( \text{M}_\text{w} \) in a separate population of control women and women with endometriosis. In a parallel fashion to \( \text{M}_\text{w} \) infiltration in intact tissue as we recently reported (Khan et al., 2004b), we also observed that PF concentrations of \( \text{M}_\text{w} \) were significantly higher in women with endometriosis than in those without endometriosis (mean, \( 1.8 \times 10^7 \) /ml versus \( 1.3 \times 10^7 / \) ml, \( P < 0.05 \)).

**Proliferation of basal and LPS-stimulated \( \text{M}_\text{w} \)**

The isolated PF \( \text{M}_\text{w} \) 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 for CD45 (data not shown).

We examined the metabolic activity of isolated \( \text{M}_\text{w} \) 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 \( \text{M}_\text{w} \) isolated from the PF of women with endometriosis appeared to increase from 6 h of incubation, with a peak activity attained at 24 h (\( > 100\% \) of control) and thereafter decreased (Figure 2A) (6 versus 12, 24 and 48 h, \( P < 0.05 \)). In contrast, the basal \( \text{M}_\text{w} \) of women without endometriosis did not show any significant difference in their proliferation at any time point of the incubation period.

Since the highest activity of \( \text{M}_\text{w} \) was observed at 24 h in the time-dependent study, all the following dose-dependent studies or the stimulation studies were performed with an incubation period of 24 h. LPS treatment of \( \text{M}_\text{w} \) isolated from women with endometriosis induces their proliferation at 1 ng/ml with a peak proliferation at 5 ng/ml (\( \approx 100\% \) of control), and thereafter decreased to \( > 50\% \) of control at 15 ng/ml (Figure 2B). This enhanced proliferation of \( \text{M}_\text{w} \) derived from women with endometriosis was significantly higher that in women without endometriosis (maximum \( > 50\% \) of control) (\( P < 0.05 \), at 1, 5 and 10 ng/ml) (Figure 2B). We further examined the proliferation of \( \text{M}_\text{w} \) according to the dominant colour appearance of endometriotic lesions, because red morphological lesions of pelvic endometriosis displayed significantly higher biological activity than did black lesions or white lesions (Khan et al., 2004a). We
HGF production by the basal and LPS-stimulated Mφ

The production of HGF in culture media by the LPS-treated (5 ng/ml) and non-treated Mφ derived from the PF of control women, women with stage I–II endometriosis (endo I-II) and 10 women with stage III–IV endometriosis (endo III-IV) is shown in Figure 3. 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 and that Mφ derived from women with stage I–II endometriosis are more responsive than those with stage III–IV endometriosis or control women.

As shown in Figure 3, a 4-fold (243.6 ± 45.8 versus 66 ± 23.7 pg/ml, \( P < 0.001 \)) and 3-fold (148 ± 34.6 versus 49.6 ± 17.3 pg/ml, \( P < 0.001 \)) increase in the production of HGF was observed in the LPS-treated Mφ derived from women with stage I–II and stage III–IV endometriosis, respectively, when compared with non-LPS-treated Mφ. However, only a 1.5-fold increase in the production of HGF was observed in the control women after LPS treatment compared with no treatment (74.2 ± 20.6 versus 51.6 ± 19.6 pg/ml, \( P < 0.05 \)). In contrast to HGF, the production of VEGF was 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 (Figure 4C) was demonstrated in the same position as CD68-immunoreactive Mφ (Figure 4B) (shown by the corresponding arrowheads) in the serial section of intact tissues derived from the eutopic endometrium of a 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 basal and LPS-stimulated Mφ

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

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

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

Similar to the dose-dependent increase in c-Met mRNA expression by gel band, a 2- 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 (Figure 5C). This indicates that Mφ derived from women with or without endometriosis carry the receptor for HGF but the HGF mRNA expression varies, resulting in the differential production of HGF at the protein level 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 concentration of cytokines and growth factors including HGF. Therefore, we tried to examine the effect of 10% culture medium derived...
from the basal and LPS (5 ng/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 (Figure 1C 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, as shown in Figure 6, was analysed by counting the total cell number (initial plating 10^5 per well). We found that the application of 10% culture medium from the treated Mφ (LPS, 5 and 10 ng/ml) on bovine endometrial epithelial cells and isolated human endometrial stroma for an incubation period of 24 h significantly increased the growth of these cells (50% of control) when we compared them with basal Mφ or low dose treatment (LPS 1 ng/ml) of Mφ (P < 0.05 for both epithelial cell and stroma) (Figure 6A and B).

Since the culture medium of Mφ also retains LPS, we examined the cytotoxic effect of LPS on epithelial cells and stroma by both the trypan blue exclusion test and BrdU incorporation 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 conditioned medium is HGF, we extended our experiment by using antibody to deplete HGF in the conditioned medium. We found that although not significant, the blocking effect of HGF tended to reverse the growth of both epithelial cells and stroma towards the growth by non-treated conditioned medium (Figure 6A and 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 cell proliferation by study of BrdU incorporation**

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 cells, epithelial cells and stroma or Mφ of women with or without endometriosis by BrdU incorporation.

The application of recombinant HGF, at a concentration of 10–100 ng/ml and with an incubation period of 24 h, was able to significantly increase the proliferation of epithelial cells compared with non-treated cells or other low dose treatment with HGF (P < 0.05 for 10, 50 and 100 ng/ml) (Figure 7A). HGF was unable to induce the proliferation of stroma isolated from women without endometriosis. However, HGF at a concentration of 10, 50 and 100 ng/ml stimulated the proliferation of stroma (>50% of control) derived from women with endometriosis (P < 0.05 versus non-treated stroma) (Figure 7B). We also studied the effect of exogenous HGF on stromal cells of ectopic endometrium and we did not find any difference in cell proliferation between similar cells of eutopic and ectopic endometrium (data not shown).
It was interesting to observe that besides epithelial cells and stroma, HGF at a higher concentration (50 and 100 ng/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 versus non-treated Mφ, Figure 7C). However, HGF was unable to do so in Mφ derived from women without endometriosis (Figure 7C).

Since the 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 for by increased cell number, we also tried to examine the cell growth of epithelial cells, stroma and Mφ by determining the 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 10, 50 and 100 ng/ml of HGF for epithelial cells and stroma and at a dose of 50 and 100 ng/ml of HGF for Mφ (data not shown).

Figure 5. The effect of variable concentrations of LPS (0–10 ng/ml) on mRNA expression at the transcriptional levels encoding HGF and its receptor, c-Met, in peritoneal Mφ derived from women with or without endometriosis as detected by RT–PCR (A). The individual mRNA bands of HGF (B) and c-Met (C) were normalized to the corresponding band of the internal control (β-actin) 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 asterisks denote 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.
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 (Muscato et al., 1982; Khan et al., 2004a, b). This can be achieved by the production of different macromolecules by the active tissues of stage I–II endometriosis and by activated Mφ. In our current study, we reported that infiltrations of Mφ in the PF 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 for Mφ which were obtained from women with stage III–IV endometriosis or without endometriosis.

The increased infiltration of Mφ in intact tissue and PF of women with endometriosis has been reported (Halme et al., 1987; Khan et al., 2002b, 2004b). However, the metabolic activity of these Mφ in women with or without endometriosis and based on their morphological 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 harbouring dominant red peritoneal lesions rather than other pigments. This indicates that Mφ in the PF 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 epithelial cells, endothelial cells,
mesothelial cells or 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 PF of women with endometriosis (Harada et al., 2001; Khan et al, 2004a). As we reported recently (Khan et al., 2003a), concentration of LPS (endotoxin) in the PF of women with endometriosis appeared to be higher when compared with that in those without endometriosis. Therefore, we presume that the peritoneal macrophages in women with endometriosis are at an activation state to produce more HGF after stimulation with LPS.

We demonstrated for the first time that beside alveolar Mϕ and hepatic kupffer cells, 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 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 retain Toll-like receptor 4 (TLR4) for LPS as demonstrated by a recent study (Triantafilou and Triantafilou, 2002). This is documented here by the increased mRNA expression of HGF, the secretion of HGF and other macromolecules in the culture medium in response to LPS treatment compared with that by the basal Mϕ. Comparing the 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 Mϕ of the PF or the intact tissue derived from women with endometriosis. We have already established that highly active blood-filled ectopic endometrium and corresponding eutopic endometrium equally harbour 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 is higher than that of stage III–IV endometriosis or in 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 (Skrice et al., 1999; Morimoto et al., 2001; Crestani et al., 2002) in response to any stress, injury, apoptosis or reactive oxygen species. Therefore, it is quite reasonable to speculate that besides endometriosis, HGF may also stimulate cells from normal subjects. Further studies are required to prove this finding in normal subjects.

We demonstrated a 3- to 5-fold increase in the expression of HGF mRNA in Mϕ derived from women with endometriosis. However, Mϕ of women without endometriosis displayed a 1.5- to 2-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. In fact, a 2- to 4-fold increase in the expression of c-Met mRNA was observed in Mϕ 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 (Sonnemberg et al., 1993). We recently reported a homogenous immunostainings of HGF and c-Met in the glandular epithelium and a heterogeneous 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, i.e. glandular epithelial cells, stroma and infiltrated Mϕ, 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 a paracrine mode of action, the ligand–receptor interaction of HGF in epithelial cells, stroma and activated Mϕ may also exert an autocrine or intracrine mode of action in the regulation of the growth of endometriosis.

The autocrine and paracrine modes of action of HGF among epithelial cells, stroma and Mϕ were documented further by the stimulated effect of HGF on the proliferation of endometrial epithelial cells, stroma and Mϕ. The proliferation of epithelial and mesothelial cells in response to HGF is well accepted (Yashiro et al., 1996; Sugawara et al., 1997). However, the proliferation of stroma and Mϕ in response to HGF treatment is completely new in our current study. The increased proliferation of stroma and Mϕ 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 compared with those from non-endometriosis.

Our current findings of increased production of HGF and its receptor c-Met by LPS-treated Mϕ and their exogenous response to cell proliferation by endometriotic cells are in agreement with some recently published reports (Sugawara et al. 1997; Khan et al., 2003b). 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 that of highly active red peritoneal lesions and was significantly higher than that of controls and other lesions. The most recent publication by Khan et al. (2004) further demonstrated that peritoneal lesions of stage I–II endometriosis, their adjacent peritoneum and corresponding eutopic endometrium harbour abundant Mϕ that could be involved in the growth of endometriosis. This indicates that a persistent inflammatory response in the PF 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 at both 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 the 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 and Triantafilou, 2002), we can speculate that a subclinical concentration of endotoxin could stimulate Mφ, produce different cytokines and growth factors and interact with its neighbouring cells in the pathology of endometriosis. Further studies are needed to find out the exact source of stimulation in regulating the activity of Mφ and their possible association with infertility or the growth of endometriosis.

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References


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