Estrogen and progesterone receptor expression in macrophages and regulation of hepatocyte growth factor by ovarian steroids in women with endometriosis

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BACKGROUND: Information regarding macrophage-mediated regulation of hepatocyte growth factor (HGF) by ovarian steroid hormones in women with endometriosis is limited. Therefore, we investigated the regulation of HGF by steroid hormones in isolated macrophages and stromal cells derived from women with or without endometriosis. METHODS: We isolated CD68 immunoreactive adherent macrophages from 46 women with endometriosis and 30 women without endometriosis. Estrogen receptor (ER) and progesterone receptor (PR) expression in macrophages was demonstrated by immunohistochemistry and RT–PCR. Production of HGF in the culture media of basal and ovarian steroid-stimulated macrophages was examined by enzyme-linked immunosorbent assay. Expression of mRNA for HGF and its receptor, c-Met in macrophages and stroma in response to ovarian steroid was investigated by RT–PCR. The single and combined effect of HGF and estrogen on the growth of macrophages and stromal cells was analysed by bromodeoxyuridine (BrdU) incorporation. RESULTS: ER and PR were expressed in isolated macrophages and intact tissue at the protein and mRNA levels. Macrophages derived from women with endometriosis produced significantly higher concentration of HGF (352.2 ± 4.9 pg/ml) in conditioned media after treatment with estradiol (10^{-8} mol/l) than that of basal macrophages (221.5 ± 32.8 pg/ml, \( P < 0.05 \)) or women without endometriosis (170.6 ± 2.6 pg/ml, \( P < 0.05 \)). These effects were less evident after treatment with progesterone. Treatment with tamoxifen (10^{-6} mol/l) reversed the production of HGF and other macromolecules. Secretion of HGF in response to ovarian steroids was further enhanced after activation with lipopolysaccharide. The mRNA expressions of HGF and its receptor, c-Met in macrophages and stroma in response to estrogen, suggesting an autocrine regulation. HGF mRNA expression was higher in cells of women with endometriosis than non-endometriosis women. Bromodeoxyuridine incorporation indicated that exogenous stimulation with HGF and estrogen, either alone or in combination, significantly increased the cell proliferation of both endometrial stroma and macrophages compared to that of non-endometriosis or non-treated cells. CONCLUSION: These results suggest that besides other inflammatory mediators, ovarian steroids also participate in the production of HGF by peritoneal macrophages which may be involved in the growth of endometriosis either alone or in combination with estrogen.

Key words: estrogen receptor/endometriosis/hepatocyte growth factor/ovarian steroids/progesterone receptor

Introduction

Endometriosis, the presence of functional endometrium outside of the uterine cavity, is a common disease, causing abdominal pain, dysmenorrhea, dyspareunia and infertility in ~10% of the female population (Strathy et al., 1982). Besides metaplastic transformation of endometrial and peritoneal mesothelial cells, the transplantation, implantation and growth of exfoliated menstrual debris on the peritoneal and ovarian surfaces are the widely accepted mechanisms of endometriosis (Sampson, 1927; Thomas and Prentice, 1992; Sugawara et al., 1997; Ishimaru et al., 2004). A number of publications have already demonstrated the potential role of ovarian steroid hormones in the regeneration of endometrium after menstruation and the growth of endometriosis (Fujishita et al., 1997; Nisolle et al., 1997). However, as a non-self lesion in the pelvic environment, the growth or persistence of...
endometriosis can also be regulated by the innate immune system. The mitogenesis or angiogenesis of eutopic and ectopic endometrium possibly involves an extensive interplay between endometrial cells, inflammatory cells, ovarian hormones, soluble factors and the extracellular matrix (Folkman and Klagesbrun, 1987).

As a cell component of the innate immune system, peritoneal fluid and intact tissue derived from women with endometriosis have been shown to contain higher numbers of activated macrophages (Halme et al., 1987; Khan et al., 2002a, 2004a) than that found in women without endometriosis. This results in the secretion of higher concentrations of growth factors including hepatocyte growth factor (HGF) and other cytokines in peritoneal fluid as produced by the stimulated macrophages in these patients (Halme et al., 1988; Halme, 1989; Khan et al., 2002b). This indicates that the growth or persistence of endometriosis is a normal inflammatory response.

Since mesenchymal cells retain estrogen receptor, production of different cytokines by endometrial stromal cells and its modulation by estrogen has been demonstrated (Tabibzadeh et al., 1989). Considering that infiltrated macrophages are one of the cell components of endometriotic lesion in the pelvic environment, reports describing expression of steroid receptors by macrophages and the secretion of different macromolecules in response to steroid hormones are scanty.

The details of HGF and its receptor, c-Met, and the biological activity of this ligand-receptor have already been described (Khan et al., 2005). HGF production by mesenchymal cells and macrophages in response to different inflammatory mediators and its involvement in the growth of endometriosis has been reported (Sugawara et al., 1997; Jiang et al., 1999; Khan et al., 2003, 2005). However, information regarding ovarian steroid-mediated production of HGF by peritoneal macrophages is yet to be determined. Therefore, we investigated the production of HGF by the peritoneal macrophages in response to ovarian steroids in women with or without endometriosis and the effect of HGF on the growth of endometrial cells either alone or in combination with estrogen.

**Materials and methods**

**Reagents**

Culture media: RPMI-1640 medium for macrophage and Dulbecco’s modified essential medium (DMEM)–Ham’s F-12 medium for stromal cells were supplemented with 100U/ml of penicillin G, 50 mg/ml of streptomycin, 2.5 μg/ml of amphotericin B (Gibco, USA). Fetal bovine serum (FBS), 17β-estradiol (E2), progesterone, hydroxytamoxifen (TMX), lipopolysaccharide (LPS, derived from Escherichia coli, serotype 0111:B4) were purchased from Sigma Chemical Co. (USA). Recombinant hepatocyte growth factor (HGF; Quantikine) was from R&D Systems (USA).

**Subjects**

Women aged between 20 and 42 years who were undergoing either diagnostic laparoscopy for dysmenorrhea or elective laparoscopy for infertility were recruited for this study. Ectopic and corresponding eutopic endometrial tissue samples were collected from eight women containing blood-filled red lesions and two women harbouring mixed black-and-white lesions. Eutopic endometrium from 10 control women was also collected for parallel study by immunohistochemistry. Peritoneal fluid was obtained from 46 women with endometriosis and cycle matched to 30 women without endometriosis (non-endometriosis). The control group, between 18 and 32 years old, consisted of fertile women without any evidence of endometriosis and were operated on for dermoid cysts by laparoscopy.

The production of macromolecules in the culture media of basal and stimulated macrophages and cell proliferation assay were studied in six women with endometriosis and six women without endometriosis (three each in the proliferative phase and three in the secretory phase of the menstrual cycle). The extent of the disease was staged according to the revised classification of the American Society of Reproductive Medicine (ASRM, 1997). All biopsy specimens and peritoneal fluid were collected in accordance with the guidelines of the Declaration of Helsinki. This study protocol was approved by the Institutional Review Board of Nagasaki University and informed consent was obtained from all women.

The distribution of patients according to the revised ASRM staging of endometriosis was as follows: stage I–II (n = 24) and stage III–IV (n = 22). Neither the study group nor the endometriosis-free group had been on hormonal medication in the 3 months prior to the surgical procedure. All control women and women with endometriosis had regular menstrual cycles (28–32 days). The phase of the menstrual cycle was determined by histological dating of eutopic endometrium samples taken simultaneously with the peritoneal fluid samples. Menstrual dating was carried out by an independent pathologist. All induced menstrual cycles were excluded from the current study. The distribution of patients in different menstrual cycle phases was as follows: control women: proliferative phase, n = 10, secretory phase, n = 20; endometriosis women: proliferative phase, n = 20, secretory phase, n = 23 and menstrual phase, n = 3.

Peritoneal lesions of endometriosis were diagnosed by their macroscopic appearance according to published criteria (Jansen and Russel, 1996) and categorized as red, black and white lesions as proposed in the latest revision of the ASRM (1997) classification. As we described recently (Khan et al., 2004b), the grouping of patients according to colour appearance of endometriosis for our current study was done as follows: (i) women with red lesions (n = 9): women containing blood-filled opaque red lesions or non-opaque transparent and/or translucent red lesions; (ii) women with combined black and white lesions (n = 22): women containing dominant distribution of black or white lesions without existence of any opaque red lesions; (iii) women with chocolate cysts (n = 15): women containing variable sizes of chocolate cysts with scanty distribution of either black lesions or white lesions or both but without any evidence of red lesions.

**Isolation of peritoneal fluid macrophages**

Peritoneal fluid was aspirated from the posterior cul-de-sac. Peritoneal fluid macrophages were isolated by the method of Halme et al. (1987), and this method has been extensively characterized before (Halme et al., 1987; Khan et al., 2005). It results in an enriched population of macrophages (>95%) which are not activated during the isolation procedure and are capable of being maintained as viable cultures for up to 72 h. Isolated peritoneal fluid macrophages for immunohistochemistry were spotted onto untreated 4-well chamber slides (Nunc, USA) and the purity of the preparation was determined using immunohistochemical staining using CD68 (KP1),
a mouse monoclonal antibody for macrophages (1:50 dilution) from Dako (Denmark). The detailed procedures of immunohistochemical staining are described elsewhere (Khan et al., 2004a, 2005). The adherent cells on the slides routinely contained >95% macrophages. Non-immune mouse immunoglobulin (Ig) G1 antibody in 1:50 dilution was used as a negative control. A counterstaining of macrophages was also performed and we did not find any contaminating cells in isolated macrophages. The concentration of macrophages in peritoneal fluid was counted by a haemocytometer counter.

**Immunoeexpression of estrogen receptor (ER) and progesterone receptor (PR) by isolated macrophage and in intact tissue**

In order to immunolocalize ER and PR in the CD68 immunoreactive isolated macrophages and to demonstrate that ER and PR are being synthesized and expressed by macrophages in intact tissue, we performed immunohistochemistry using the respective antibody and using serial sections of eutopic and ectopic endometrium derived from women with or without endometriosis. The details of ER and PR antibodies and the immunohistochemistry procedure were described previously (Fujishita et al., 1997; Nisolle et al., 1997). Non-immune mouse immunoglobulin (Ig) G1 antibody (1:50) was used as a negative control.

**Human endometrial cell cultures**

Primary endometrial cell cultures were prepared from the biopsy specimens of the eutopic and ectopic endometrium derived from six women with endometriosis and six women without endometriosis (three each in the proliferative phase and three in the secretory phase). Glandular epithelial cells were separated from stromal cells and debris by filtration through narrow gauge sieves. The characteristics of stromal cells were determined by morphological and vimentin-positive immunocytochemical studies. Stromal cells were sub-cultured to eliminate contamination by macrophages or other leukocytes, and experiments were performed at passage 1. Details of procedures for the isolation of endometrial stromal cells were described previously (Osteen et al., 1989; Sugawara et al., 1997; Khan et al., 2005). The specificity of the immunocytochemical staining was confirmed by the deletion of the first antibody. A counterstaining of stroma was also performed to exclude the contamination by epithelial cells or endothelial cells in isolated stromal cell culture (data not shown).

**Activation and ovarian steroid stimulation of peritoneal fluid macrophages**

Isolated peritoneal fluid macrophages as derived from six women with endometriosis and six women without endometriosis were plated in 96-well microplates and allowed to attach for 24 h in Phenol Red-free Roswell Park Memorial Institute (RPMI)-1640 medium containing either E_2_ (10^{-8} mol/l), progesterone (10^{-8} mol/l) or a combination of both, for another 24 h. These doses of E_2_ and progesterone were used according to a previous study protocol (McLaren et al., 1996). Control cells were incubated in Phenol Red-free RPMI-1640 medium only. In order to examine the blocking effect of estrogen receptor on the secretion of different macromolecules in the macrophage-conditioned media, we performed extended experiments with tamoxifen (10^{-6} mol/l). This dose was used as described in a previous report (Gockerman et al., 1986). Activation studies involved the addition of 5 ng/ml of LPS just before commencement of the 24 h incubation with E_2_ and progesterone. This dose of LPS was selected from a previous dose-dependence study from our laboratory that showed a maximum activation of macrophages (Khan et al., 2005). The conditioned media were collected in triplicate, pooled, and frozen at −70°C until testing.

**Cytokine assays in the conditioned media**

The concentrations of HGF and vascular endothelial growth factor (VEGF) in the supernatant of treated and non-treated macrophages were measured in duplicate using a commercially available sandwich enzyme-linked immunosorbant assay (ELISA; Quantikine; R&D Systems) in a blind fashion. The antibodies used in HGF and VEGF determination do not cross-react with other cytokines. The limits of detection were 40.0 pg/ml for HGF and 9.0 pg/ml for VEGF. Both the intra-assay and inter-assay coefficients of variation were <10% for all these assays.

**RT-RCR**

Ribonucleic acid (RNA) was extracted from each of 10^6 macrophages and stromal cells cultured in a 60 mm Petri dish (Greiner) using the monophasic solution of 40% phenol and Isogen method (Molecular Research Center, Tokyo, Japan), according to the manufacturer’s protocol.

The presence of mRNA encoding the estrogen and progesterone receptors in basal macrophages was determined using forward and reverse primers synthesized to anneal with cDNA for respective receptors. Amplification of cDNA reaction mixture for ER and PR was done in two stages and as described previously (McLaren et al., 1996). The mRNA expressions of HGF and its receptor c-Met were also analysed in isolated peritoneal fluid macrophages and endometrial stromal cells by RT–PCR and using sense and antisense primers of HGF and c-Met as previously described (Jiang et al., 1999; Khan et al., 2005). PCR-generated bands were cloned and found to match the published sequences for the expected products. Human oligonucleotide primers of ER, PR, HGF, c-Met and β-actin used for our current study are shown in Table I. A scanner densitometer was used to determine the ratio of intensity of each band relative to β-actin which was used as an internal control.

**Table I. Human oligonucleotide primers used**

<table>
<thead>
<tr>
<th>Locus</th>
<th>Sense (5’–3’)</th>
<th>Antisense (5’–3’)</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ER</td>
<td>Primer 1: GGAGACATGAGAGCTGTGCCAA Primer 3: CTTGCGCAAGCCCGCTC</td>
<td>Primer 2: CCACGACATGAGAGCTGTGCCAA Primer 4: TCATCAGTAGAAATCG</td>
<td>395</td>
</tr>
<tr>
<td>PR</td>
<td>Primer 1: GGTCTTTTCAAGAGAGACA Primer 3: GAGAAAAGCATC</td>
<td>Primer 2: GCACTGACATC Primer 4: AAGAAATGTATTAGAAGG</td>
<td>650</td>
</tr>
<tr>
<td>HGF</td>
<td>Primer 3: CTTGCGCAAGCCCGCTC</td>
<td>Primer 2: CCACGACATGAGAGCTGTGCCAA Primer 4: TCATCAGTAGAAATCG</td>
<td>505</td>
</tr>
<tr>
<td>c-Met</td>
<td>ACTGCTCGTATTGAGCCGCACGACT</td>
<td>Primer 2: GCACTGACATC Primer 4: AAGAAATGTATTAGAAGG</td>
<td>536</td>
</tr>
<tr>
<td>β-Actin</td>
<td>Primer 1: GGAGACATGAGAGCTGTGCCAA Primer 3: CTTGCGCAAGCCCGCTC</td>
<td>Primer 2: CCACGACATGAGAGCTGTGCCAA Primer 4: TCATCAGTAGAAATCG</td>
<td>300</td>
</tr>
</tbody>
</table>

ER = estrogen receptor; PR = progesterone receptor; HGF = hepatocyte growth factor; c-Met = receptor for HGF; β-actin = internal control.

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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.

**Cell proliferation assay**

Bromodeoxyuridine (BrdU) labelling and detection kit measures cell proliferation by quantifying 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).

Briefly, desired cells (endometrial stroma cells and macrophages) were cultured in 96-well microtitre plates (10⁴ cell/well). The cells were Phenol Red-starved with 5% FBS before HGF (50 ng/ml) and E₂ (10⁻⁸ mol/l) treatment for a period of 24 h. After a 24 h preincubation period, respective cells were treated with HGF and E₂ either alone or in combination in serum- and Phenol Red-free media and incubated for an additional 24 h. The BrdU incorporation was assayed according to the manufacturer’s instructions.

In order to confirm that a growth-promoting factor in the macrophage-conditioned medium is HGF, we used antibody to deplete HGF in the conditioned medium. The macrophage-conditioned medium was added to the cultured endometrial stromal cells and macrophages, and then incubated for 24 h. HGF inhibition studies using a specific anti-human HGF neutralizing antibody (10 µg/ml; R&D systems) were carried out in parallel, the samples incubated for another 24 h and BrdU incorporation was measured to examine the change in cell proliferation.

**Statistical analysis**

The results were evaluated by one-way analysis of variance. The data are expressed as mean ± SEM. The concentrations of the studied cytokines were not distributed normally and the data were analysed using non-parametric test. The differences between two groups were compared using Mann–Whitney U-test or Student’s t-test. For comparison between three or more groups, the Kruskal–Wallis test was used to assess the differences. P < 0.05 was considered statistically significant.

**Results**

We measured CD68 immunoreactive macrophage concentrations in the peritoneal fluid of women with endometriosis and they were distributed according to revised ASRM staging, colour appearances of endometriotic lesions and also by the different phases of the menstrual cycle. Peritoneal fluid of women with early endometriosis (stage I–II) and those containing red lesions in the pelvic cavity harboured abundant macrophages in peritoneal fluid when compared with that of control women (P < 0.05), advanced endometriosis (stage III–IV, P < 0.5) or other pigmented lesions or chocolate cysts (P < 0.05) (Figure 1A and B). Although no menstrual phase difference was observed in control women, a dominant infiltration of macrophages in peritoneal fluid was found in the secretory or menstrual phase in women with endometriosis (P < 0.05 and P < 0.01 respectively, Figure 1C).

**Expression of estrogen and progesterone receptors in macrophages**

The majority of CD68 immunoreactive macrophages as isolated from women with or without endometriosis showed strong nuclear positivity for ER but were less reactive for PR (Figure 2A). In contrast, tissue localization of ER and PR was equally demonstrated in the same position of CD68 immunoreactive macrophages (Figure 2B) (shown by corresponding arrow heads) in the serial section of intact tissues derived from the eutopic endometrium of women with endometriosis. This indicates that besides glandular epithelium and stroma, ER and PR are also being synthesized and expressed by the infiltrated macrophages in intact tissue.

This was further confirmed by RT–PCR and revealed that basal macrophages isolated from the peritoneal fluid of women with early endometriosis (stage I–II) and those containing red lesions in the pelvic cavity harboured abundant macrophages in peritoneal fluid when compared with that of control women (P < 0.05), advanced endometriosis (stage III–IV, P < 0.5) or other pigmented lesions or chocolate cysts (P < 0.05) (Figure 1A and B). Although no menstrual phase difference was observed in control women, a dominant infiltration of macrophages in peritoneal fluid was found in the secretory or menstrual phase in women with endometriosis (P < 0.05 and P < 0.01 respectively, Figure 1C).

**Figure 1.** Peritoneal fluid concentrations of macrophages derived from women with or without endometriosis, distributed according to the revised ASRM staging (A), morphological appearance with chocolate cysts (B) and different phases of the menstrual cycle (C). The results are expressed as mean ± SEM. (A) *P < 0.05, stage I–II versus stage III–IV or without endometriosis. (B) *P < 0.05, women containing red lesions versus women with other lesions/chocolate cysts or without endometriosis. (C) *P < 0.05, secretory phase versus proliferative phase; **P < 0.01, menstrual phase versus secretory phase or proliferative phase.
women with or without endometriosis contained the mRNA encoding for ER and PR (Figure 3A). No phase of the menstrual cycle-dependent variation in the expression of these receptor mRNA was evident for either group.

Production of HGF by peritoneal fluid macrophages in response to ovarian steroids

Ovarian steroid concentrations are elevated in peritoneal fluid (DeLeon et al., 1986; Khan et al., 2002b) and data exist to show that these steroids can regulate the secretion of a number of factors from animal and human peritoneal fluid macrophages (Hu et al., 1988; Chao et al., 1995; Frazier-Jassen et al., 1995). Therefore, we tried to investigate the secretion of HGF and other macromolecules by peritoneal fluid macrophages in response to ovarian steroids. We found that direct stimulation of macrophages in culture with E2 and progesterone resulted in a variable increase in the secretion of HGF and VEGF by peritoneal fluid macrophages (Figure 4). The production of HGF was significantly increased by E2 in women with endometriosis (352.2 ± 4.9 pg/ml, P < 0.05) compared with that of control women (170.6 ± 2.6 pg/ml, P < 0.05) or non-treated macrophages (221.5 ± 32.8 pg/ml, P < 0.05). A marked increase in the secretion of VEGF was observed by treatment with E2 and progesterone (P < 0.01 for both) in women with endometriosis and also in women without endometriosis (P < 0.05 for both) when compared with non-treated peritoneal fluid macrophages (Figure 4). The production of HGF was about one-half that of VEGF in women with or without endometriosis. There was no significant difference in response between E2 and progesterone or a combination of E2 and progesterone. No phase-of-cycle differences were seen.

Since progesterone failed to show a significant increase in secretion of HGF by peritoneal fluid macrophages, we performed a blocking experiment on E2 by using the ER antagonist, tamoxifen: this significantly reversed the secretion of VEGF (P < 0.05) and tended to reverse the secretion of HGF (P = 0.07) by the estrogen-treated peritoneal fluid macrophage towards levels seen in the non-treated macrophages (Figure 4). This indicates that it is the direct effect of estrogen on the peritoneal fluid macrophage that is able to produce significant amounts of HGF and VEGF and is being mediated by ER as located on these inflammatory cells.

Steroidal stimulation of HGF and VEGF production by LPS-activated peritoneal fluid macrophages

To investigate the control of HGF and VEGF secretion by peritoneal fluid macrophages, we measured HGF and VEGF secretion by activated (LPS, 5 ng/ml) and non-activated peritoneal fluid macrophages, isolated from women without endometriosis. Macrophages from women with endometriosis were not used in order to eliminate any bias in the basal activation status of these cells as already observed by our previous study (Khan et al., 2005). HGF and VEGF were secreted from both activated and non-activated peritoneal fluid macrophages. However, activation with LPS significantly increased the amount of HGF (P < 0.05) and VEGF (P < 0.01) secreted by these cells (Figure 5). We observed that activation of basal macrophages further enhanced the response of these cells to ovarian steroids. Exogenous treatment with E2 was able to further increase the amount of both HGF (P < 0.05) and VEGF (P < 0.05) secretion by peritoneal fluid macrophages when these cells were activated with LPS (Figure 5). Although progesterone increased the secretion of VEGF by non-activated macrophages, it was unable to further enhance the secretion of either HGF or VEGF by activated peritoneal fluid macrophages (Figure 5). These results confirmed that irrespective of activation status, peritoneal fluid macrophages were independently stimulated to produce HGF and VEGF by E2. This also indicates that...
an inflammatory response and ovarian steroid hormones may function either alone or in combination to regulate the production of HGF and VEGF by peritoneal fluid macrophages in the pelvic microenvironment.

mRNA expression of HGF and c-Met in peritoneal fluid macrophage and endometrial stroma

Since VEGF mRNA expression has already been demonstrated previously in isolated peritoneal fluid macrophages (McLaren et al., 1996), we tried to examine the transcriptional activity of HGF and its receptor, c-Met from the isolated peritoneal fluid macrophage and endometrial stromal cells in response to ovarian steroids (Figure 3B and C). In a time-dependent study, HGF response to E2 (10^{-8} mol/l) was delayed, with an increasing level of HGF mRNA observed from 6 to 24 h after the addition of E2 in both peritoneal fluid macrophage and stroma (data not shown).

As shown in Figure 3B, peritoneal fluid macrophages derived from women with endometriosis and in response to E2 (10^{-8} mol/l) displayed higher expression of HGF mRNA (3.2–3.8-fold) than that of women without endometriosis (1.2–1.6-fold) (P < 0.05). No difference in the expression of c-Met mRNA nor phases of menstrual cycle was seen between these two groups of women. β-Actin, internal control. Calculated molecular weights of product bands are indicated by arrows.
which may result in the elevation of HGF in the peritoneal fluid of women with endometriosis as we reported recently (Khan et al., 2002b, 2004b).

HGF-dependent increase in stromal cell and macrophage proliferation

The presence of c-Met receptor in endometrial stromal cells and macrophages might be expected to enable these cells to respond to endogenous or exogenous HGF. The effect of media conditioned by peritoneal macrophages on non-endometriotic stromal cell and macrophages proliferation was determined by BrdU incorporation assay. Neutralizing experiments using a specific anti-HGF antibody were carried out in parallel.

The culture media as conditioned by the peritoneal fluid macrophages of women with endometriosis resulted in a significantly greater (P < 0.05 for both stroma and macrophages) incorporation of BrdU than that of media conditioned by macrophages derived from non-endometriosis women (Figure 6A and B). This indicates that proliferation of stromal cells and macrophages by conditioned media from endometriosis may be contributed to by HGF.

Finally, we investigated the effect of exogenous HGF and E2 on the proliferation of endometrial stromal cells and macrophages. We found that stromal cells derived from women with endometriosis significantly incorporated BrdU in response to recombinant HGF (50 ng/ml) and E2 (10^{-8} mol/l) either alone or in combination when compared with BrdU incorporation of non-treated stromal cells (P < 0.05 for all, Figure 7A). Only a combination of HGF and E2 treatment was able to significantly proliferate stromal cells of control women (P < 0.05). A similar pattern of BrdU incorporation was seen in stromal cells derived from ectopic endometrium (data not shown). A significantly greater BrdU incorporation was observed in peritoneal fluid macrophages derived from women with endometriosis, similar to that of non-treated macrophages (P < 0.05 for all, Figure 6A and B).
Discussion

Figure 7B). Macrophages also incorporated BrdU after single treatment of HGF in control women ($P < 0.05$, for both).

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, we also tried to examine the cell growth of stroma and macrophages by cell number (initial plating $10^5$ cells/well) under HGF and $E_2$ stimulation. We found a parallel and significantly increased cell growth under the similar stimulation for both stromal cells and macrophages (data not shown).

Discussion

We demonstrated for the first time the production of HGF by the peritoneal macrophages in response to ovarian steroids in women with or without endometriosis. Although macrophage infiltration in peritoneal fluid was not dominant in the secretory or menstrual phase, we found a weak association between the phase of menstrual cycle and HGF production by $E_2$ or ER and PR expression. Our results of HGF production in response to $E_2$ indicate that HGF production is possibly related to the activation status of macrophages in peritoneal milieu. The activation status of macrophages retains increased potential to produce and secrete different macromolecules in women with endometriosis compared to women without endometriosis. Our current findings are consistent with the increased production of other cytokines and growth factors by the activated macrophages as described previously by ourselves and others (Halme et al., 1988, 1989; Khan et al., 2002b, 2004b).

It is generally believed that ovarian steroid hormones are essential for the growth or persistence of ectopic endometrium and corresponding eutopic endometrium in women with endometriosis (Bergqvist, 1992). To date it has been assumed that this is a consequence of direct actions of the steroids on the endometrial or endometriotic tissues. Indirect actions of ovarian steroids on the inflammatory cells within the peritoneal fluid have generally been overlooked or scarcely described. Since the major cellular constituents of peritoneal fluid are macrophages, comprising between 82 and 99% of the total cell population (Eisen et al., 1994), it is quite reasonable to speculate that these cells may be responsive to ovarian steroids. Our current study provides further evidence to describe this interaction between macrophages and ovarian steroid hormones.

We demonstrated that these inflammatory cells retain the mRNA encoding both ER and PR and showed nuclear staining for both ER and PR in isolated macrophages and in intact tissue of endometriosis. These results are in accordance with the recently published results of McLaren et al. (1996). Our findings of increased concentrations of macromolecules in the peritoneal fluid of women with early endometriosis and those containing active blood-filled red lesions in the pelvic cavity are in parallel with the increased tissue infiltrations of macrophages in women with endometriosis as we reported recently (Khan et al., 2004a).

In this study we have demonstrated that HGF can be produced by peritoneal fluid macrophages derived from the pelvic cavity in addition to its production by alveolar macrophages or hepatic Kupffer cells (Skr tic et al., 1999; Morimoto et al., 2001; Crestani et al., 2002). We reported that peritoneal fluid macrophages could be a constant source of different macromolecules including HGF and VEGF in response to ovarian steroids. We found that peritoneal fluid macrophages were directly stimulated to secrete HGF and VEGF by ovarian steroids, a response which was blocked by ER receptor antagonist and which was enhanced if the cells were previously activated with LPS.

Besides ER and PR expression, peritoneal fluid macrophages as well as endometrial stromal cells expressed both HGF and its receptor, c-Met, transcripts which were independent of menstrual cycle, and the expression of HGF at both mRNA and protein levels was higher in women with endometriosis than without endometriosis. This indicates that expression of HGF ligand-receptor by peritoneal fluid macrophages may depend on the activation status of these inflammatory cells. Finally, we demonstrated that the enhanced proliferation of endometrial stromal cells and peritoneal fluid macrophages induced by conditioned media of macrophages

Figure 7. Exogenous single or combined effect of hepatocyte growth factor (HGF) and estradiol ($E_2$) on the proliferation of endometrial stromal cells (A) and peritoneal fluid macrophages (B) derived from six women with endometriosis and six women without endometriosis. Results of bromodeoxyuridine (BrdU) incorporation are expressed as percentage of control (mean ± SEM). BrdU incorporation of non-treated cells equals 100%. Endometriotic stroma, **$P < 0.05$ versus non-treated cells; non-endometriotic stroma, *$P < 0.05$ versus non-treated cells. Endometriotic macrophages, **$P < 0.05$ versus non-treated macrophages; non-endometriotic macrophages, *$P < 0.05$ versus non-treated macrophages.
was abolished by anti-HGF antibody. This mitogenic effect of endogenous HGF was in parallel with the exogenous stimulation of HGF on these cells either alone or synergistic with estrogen.

These results have two biological implications: (i) ovarian steroids may influence the autocrine regulation of macrophage or stromal cell functions; (ii) an inflammatory response in the pelvic environment and ovarian steroid hormones may function independently or in an orchestrated manner which may be involved in the growth or persistence of endometriosis. The production of HGF by the cells of mesenchymal origin and its interaction with c-Met receptor on epithelial cells, endothelial cells or mesothelial cells has been generally accepted (Nakamura et al., 1986; Tajima et al., 1992). We described here that endometrial stromal cells and infiltrated macrophages also retain c-Met receptor, and the proliferation of these cells in response to exogenous HGF strengthened the notion that besides a paracrine mode of action, HGF may also play an autocrine mode of action in the growth of endometriosis.

Although peritoneal fluid macrophages are the principal sources of VEGF production in the pelvic microenvironment (McLaren et al., 1996), it could also be produced by isolated endometrial cells (Shifren et al., 1996). These results are in agreement with our current results of VEGF production by peritoneal fluid macrophages. We found that the ovarian steroid-stimulated production of VEGF by peritoneal fluid macrophages was 2-fold higher than that of HGF production by these cells. In addition to the main source of product by mesenchymal cells, HGF may also be produced by peritoneal macrophages.

Besides a significant production of VEGF by both estrogen and progesterone, a higher production of HGF was demonstrated by estrogen only both at the transcriptional and protein levels. This indicates a variable response of peritoneal fluid macrophages to ovarian steroids in the production of HGF and VEGF. This can be explained by a differential interaction of ovarian steroid hormones with the estrogen (ERE) and progesterone response elements (PRE) located on the estrogen and progesterone receptor cistrome. Therefore, the regulation of HGF gene by macrophages may be the primary response gene for reproductive steroids in the endometrium (Shifren et al., 1996). In contrast, HGF gene retains only two putative ERE in its 3 kb 5’-promoter region in addition to having response elements for other cytokines and growth factors (Zarnegar, 1995). The existence of PRE on the promoter of HGF gene is now unknown. Therefore, the regulation of HGF gene by estrogen may be mediated by a direct interaction of the estrogen receptor complex with cis-acting ERE elements.

We previously demonstrated that, like VEGF, HGF also carries angiogenic and mitogenic activity in endometrial tissues (Khan et al., 2003). Since HGF production and mRNA expression of its ligand-receptor are up-regulated by E2-stimulated peritoneal fluid macrophages, when we extended our experiment to investigate the role of HGF in the pathogenesis of endometriosis, we found that the proliferation of both isolated stromal cells and peritoneal fluid macrophages derived from women with endometriosis were more enhanced by HGF either alone or in combination with estrogen.

Although not measured in our current study, we have already demonstrated that the concentrations of HGF and other inflammatory mediators in peritoneal fluid are significantly elevated in women with early endometriosis and in those harbouring blood-filled opaque peritoneal lesions compared to that of women without endometriosis or non-opaque lesions (Khan et al., 2002b, 2004b). These results of a persistent inflammatory response in women with endometriosis and estrogen-regulated production of HGF by activated and non-activated peritoneal fluid macrophages further confirmed that the growth of endometriosis possibly depends on a mutual interaction between the innate immune system and ovarian steroid hormones in the pelvic microenvironment. The current therapeutic strategy of hypo-estrogenic medication in women with endometriosis can also be explained by its effect on the innate immune system, which may suppress different cytokines and growth factors and thereby improve the growth of endometriosis or other endocrine diseases. Further studies are required to evaluate the effect of hypo-estrogenic medication on immune cells.

References


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Ovarian steroid-mediated regulation of hepatocyte growth factor by Me