Estrogen and Progesterone Receptor Expression by Macrophages and Regulation of Hepatocyte Growth Factor by Ovarian Steroids in Women with Endometriosis

Khaleque Newaz Khan, Hideaki Masuzaki, Akira Fujishita, Michio Kitajima,
Ichiro Sekine*, Toshifumi Matsuyama [‡], Tadayuki Ishimaru

Department of Obstetrics and Gynecology, [‡]Division of Cytokine Signaling,
Department of Molecular Microbiology and Immunology
Graduate School of Biomedical Sciences, Nagasaki University,
*Department of Molecular Pathology, Atomic Bomb Disease Institute,
Nagasaki 852-8501, Japan.

Running title: Ovarian steroid-mediated regulation of HGF by M ϕ

Address for correspondence and reprint request:

Khaleque Newaz Khan, M.D, PhD
Department of Obstetrics and Gynecology
Graduate School of Biomedical Sciences, Nagasaki University
1-7-1 Sakamoto, Nagasaki city 852-8501, Japan.
Tel: +81-95-849 -7363
Fax: +81-95-849-7365
E-mail: nemokhan@net.nagasaki-u.ac.jp

Abstract

Background: The production of hepatocyte growth factor (HGF) by mesenchymal cells and macrophages (Mϕ) in response to different inflammatory mediators and its involvement in the growth of endometriosis has been described. However, information regarding Mϕ-mediated regulation of HGF by ovarian steroid hormones in women with endometriosis is limited. Therefore, we planned to investigate the regulation of HGF by steroid hormones in isolated Mϕ and stromal cells derived from women with or without endometriosis. **Methods**: We isolated CD68 immunoreactive adherent Mϕ in vitro from 46 women with endometriosis and 30 women without endometriosis. The expression of estrogen receptor (ER) and progesterone receptor (PR) by Mϕ was demonstrated by immunohistochemistry and RT-PCR. The production of HGF in the culture media of basal and ovarian steroid-stimulated Mϕ was examined by enzyme-linked immunosorbent assay. The expression of mRNA for HGF and its receptor, c-Met in the Mϕ and stromal cells in response to ovarian steroid was investigated by RT-PCR. The single and combined effect of HGF and estrogen on the growth of Mϕ and stromal cells was analyzed by bromodeoxyuridine (BrdU) incorporation. **Results**: ER

and PR were expressed in isolated M ϕ and intact tissue both at the protein and mRNA levels. Macrophages derived from women with endometriosis produced significantly higher concentration of HGF (352.2 $\pm$ 4.9 pg/mL) in the conditioned media after treatment with estradiol (10^{-8}M) than that of basal M ϕ (221.5$\pm$32.8 pg/mL, p<0.05) or women without endometriosis (170.6$\pm$2.6 pg/mL, p<0.05). These effects were less evident after treatment with progesterone. An anti-estrogenic treatment of these M ϕ with tamoxifen (10^{-6}M) reversed the production of HGF and other macromolecules towards non-treated M ϕ. It was interesting to observe that 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 were also detected in M ϕ and stroma in response to estrogen, suggesting an autocrine regulation. HGF mRNA expression was higher in these cells of women with endometriosis than that of non-endometriosis without displaying any phase difference of menstrual cycle. The bromodeoxyuridine (BrdU) incorporation study indicated that exogenous stimulation with HGF and estrogen, either alone or in combination, significantly increased the cell proliferation of both endometrial stroma and M ϕ comparing to that of non-endometriosis or non-treated cells.

Conclusion: These results suggested that besides other inflammatory mediators, ovarian steroids also participate in the production of HGF by peritoneal Mϕ which may be involved in the growth of endometriosis either alone or in combination with estrogen.

Key words: estrogen receptor / progesterone receptor / macrophage / peritoneal fluid / hepatocyte growth factor / ovarian steroids / lipopolysaccharide / endometriosis

Introduction

Endometriosis, the presence of functional endometrium outside of the uterine cavity, is a common disease, causing abdominal pain, dysmenorrhea, dyspareunia and infertility in about 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 (Sugawara et al., 1997; Ishimaru et al., 2004; Sampson J, 1927; Thomas et al., 1992). A number of literatures 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 pelvic environment, the growth or persistence of endometriosis can also be regulated by 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 et al., 1987).

As a cell component of innate immune system, peritoneal fluid (PF) 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 PF as produced by the stimulated-M ϕ in these patients (Halme et al., 1988, 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 M ϕ is one of the cell components of endometriotic lesion in pelvic environment, reports describing expression of steroid receptors by M ϕ 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 has already been described (Khan et al., 2005). HGF production by mesenchymal cells and M ϕ 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., 2003a, 2005). However, information regarding ovarian steroid-mediated production of HGF by peritoneal M ϕ is yet to be determined. Therefore, we report for the first time the production of HGF by the peritoneal M ϕ in response to ovarian steroids in women with or without endometriosis and examined 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):Hams F12 medium for stromal cells and were supplemented with 100 IU/mL of penicillin G, 50 mg/mL of streptromycin, 2.5 μ g/mL of amphotericin B (GIBCO, Grand Island, NY). Fetal bovine serum (FBS), 17 β -estradiol (E2), progesterone (P), hydroxytamoxifen (TMX), lipopolysaccharide (LPS, derived from *Escherichia coli*, serotype 0111:B4) were purchased from Sigma Chemical Co. (St. Louis, MO). Recombinant hepatocyte growth factor (HGF) was from R&D

system (Quantikine, R & D system, Minneapolis, MN).

Subjects. Women between 20 and 42 years old 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 harboring mixed black and white lesions. Eutopic endometrium from ten control women was also collected for parallel study by immunohistochemistry. Peritoneal fluid (PF) was obtained from 46 women with endometriosis and cycle matched 30 women with non-endometriosis. The control group, between 18 to 32 years old, consisted of fertile women without any evidence of endometriosis and operated for dermoid cyst 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 proliferative phase and three in secretory phase). The extent of the disease was staged according to the revised-classification of American Society of Reproductive Medicine (revised-ASRM,

1997). All biopsy specimens and PF were collected in accordance with the guidelines of the Declaration of Helsinki. This study protocol was approved by the Institutional Review Board (IBR) of Nagasaki University. An informed consent was obtained from all women.

The distribution of patients in different 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 cycles is 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 et al., 1996) and categorized as red,

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

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

according to color appearance of endometriosis for our current study was done as follows:

(1) women with red lesion (n=9), women containing blood-filled opaque red lesions or

non-opaque transparent and/or translucent red lesions; (2) women with combined black

and white lesions (n=22), women containing dominant distribution of black or white

lesions without existence of any opaque red lesion; (3) women with chocolate cysts

(n=15), women containing variable sizes of chocolate cysts with scanty distribution of

either black lesion or white lesion or both but without any evidence of red lesion.

Isolation of peritoneal fluid macrophages. Peritoneal fluid was aspirated

from the posterior cul-de-sac. Peritoneal fluid M ϕ were isolated by the method of Halme

et al. (1987), and this method has been extensively characterized before (Helme et al.,

1987; Khan et al., 2005). It results in an enriched population of M ϕ (>95%) which are

not activated during the isolation procedure and are capable of being maintained as viable

cultures for up to 72 hour. Isolated PF macrophages for immunohistochemistry were

spotted onto untreated four-well chamber slides (Nunc, Naperville, IL) and the purity of

the preparation was determined using immunohistochemical staining using CD68 (KP1), a mouse monoclonal antibody for Mϕ (1:50 dilution) from Dako, Denmark. The detail 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 counter staining of Mϕ was also performed and we did not find any contaminating cells in isolated Mϕ. The concentration of Mϕ in PF was counted by a hemocytometer counter.

Immunoexpressions of estrogen receptor (ER) and progesterone receptor (PR) by isolated macrophages and in intact tissue. In order to immunolocalize ER and PR in the CD68 immunoreactive isolated Mϕ and to demonstrate that ER and PR are being synthesized and expressed by Mϕ in intact tissue, we performed immunohistochemistry using respective antibody and using serial section of eutopic and ectopic endometrium derived from women with or without endometriosis. The details of ER and PR antibody and procedure of immunohistochemistry 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 $M\phi$ or other leukocytes, and experiments were performed at passage 1. The detail procedures for the isolation of endometrial stromal cells were described previously (Sugawara et al., 1997; Osteen et al., 1989, Khan et al., 2005). The specificity of the immunocytochemical staining was confirmed by the deletion of the first antibody. A counter-staining of stroma was also performed to exclude the contamination by epithelial cell or endothelial cells in isolated stromal cell culture (data not shown).

Activation and ovarian steroid stimulation of peritoneal fluid macrophages. Isolated peritoneal fluid $M\phi$ as derived from six women with

endometriosis and six women without endometriosis were plated in 96-well microplates

and allowed to attach for 24 hour in phenol red free media plus 5% FBS. After this time

the cells were washed with phosphate-buffered saline (PBS) and incubated in serum free

and phenol red free RPMI-1640 media containing either E2 (10^{-8}M), P (10^{-6}M) or a

combination of both, for another 24 hr. These doses of E2 and P were used according to a

previous study protocol (McLaren et al., 1996). Control cells were incubated in just

phenol red free RPMI-1640 media. In order to examine the blocking effect of estrogen

receptor on the secretion of different macromolecules in the Mϕ condition media, we

performed extended experiment with tamoxifen (10^{-6}M). 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 hr incubation with E2

and P. This dose of LPS was selected from a previous dose-dependent study from our

laboratory that showed a maximum activation of Mϕ (Khan et al., 2005). The condition

media were collected in triplicate, pooled, and frozen at -70℃ until testing.

Cytokine assays in the condition media. The concentrations of HGF and

VEGF in the culture media of treated- and non-treated-Mϕ were measured in duplicate

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

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

MN). The antibodies used in HGF 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.

Reverse transcription-polymerase chain reaction (RT-RCR). Ribonucleic

acid (RNA) was extracted from each of 10^6 M ϕ and stromal cells 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.

The presence of mRNA encoding the estrogen and progesterone receptors in

basal M ϕ 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 analyzed in isolated peritoneal fluid

M ϕ 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 which we used for our current study are shown in Table 1. 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.

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) 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 [^{3}H]-thymidine-based cell proliferation and carries equal sensitivity and specificity

(Takagi, 1993).

Briefly, desired cells (endometrial stroma cells and Mϕ) were cultured in 96 well microtitre plate (10^4 cell/well). The cells were phenol red starved with 5% FBS before HGF (50 ng/mL) and E2 (10^{-8}M) treatment for a period of 24 hour. After a 24hr pre-incubation period, respective cells were treated with HGF and E2 either alone or in combination in a serum and phenol red free media and incubated for an additional 24 hours. The BrdU incorporation was assayed according to the manufacturer's instruction.

In order to confirm that growth promoting factor in the Mϕ conditioned media is HGF, we used antibody to deplete HGF in the conditioned medium. The Mϕ conditioned media was added to the cultured endometrial stromal cells and macrophages, and then incubated for 24 hr. HGF inhibition studies using a specific anti-human HGF neutralizing antibody (10μg/mL, R & D system) were carried out in parallel, incubated for another 24 hr 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 $\pm$ SEM. The concentrations of the studied

cytokines were not distributed normally and the data were analyzed using non-parametric test. The differences between two groups were compared using Mann-Whitney U-test or Student's t test. For comparison among three or more groups, the Kruskal-Wallis test was used to determine the difference among the groups. Differences were considered as statistically significant for $p<0.05$.

Results

We measured CD68 immunoreactive Mϕ concentrations in the PF of women with endometriosis and they were distributed according to revised-ASRM staging, color appearances of endometriotic lesions and also by the different phases of menstrual cycle. Peritoneal fluid of women with early endometriosis (stage I-II) and those containing red lesions in pelvic cavity harbored abundant macrophages in PF 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 (<0.05) (Fig. 1A and 1B). Although no menstrual phase difference was observed in control women, a dominant infiltration of Mϕ in PF was found in the secretory or menstrual phase in women with endometriosis ($p<0.05$ and

p<0.01, respectively, Fig. 1C).

Expressions of estrogen and progesterone receptors by Mϕ. The majority

of CD68 immunoreactive Mϕ as isolated from women with or without endometriosis

showed strong nuclear staining for ER but were less reactive to PR (Fig. 2A). In contrast,

tissue localization of ER and PR was equally demonstrated in the same position of CD68

immunoreactive Mϕ (Fig. 2B) (shown by corresponding arrow heads) in the serial

section of intact tissues derived from the eutopic endometrium of woman with

endometriosis. This indicates that besides glandular epithelium and stroma, ER and PR

are also being synthesized and expressed by the infiltrated Mϕ in intact tissue.

This was further confirmed by RT-PCR and revealed that basal Mϕ isolated

from the PF of women with or without endometriosis contained the mRNA encoding for

ER and PR (Fig. 3A). No phase of the menstrual cycle dependent variation in the

expression of these receptor mRNAs was evident for either group.

Production of HGF by peritoneal fluid Mϕ in response to ovarian

steroids. Ovarian steroid concentrations are elevated in peritoneal fluid (Khan et al.,

2002b; DeLeon et al., 1986) 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; Frazier-Jassen et al., 1995; Chao et al., 1995). Therefore, we tried to investigate the secretion of HGF and other macromolecules by PF macrophages in response to ovarian steroids. We found that direct stimulation of M ϕ in culture with E2 and P resulted in a variable increase in the secretion of HGF and VEGF by peritoneal fluid M ϕ (Fig. 4). The production of HGF was significantly increased by E2 in women with endometriosis than that of control women (352.2 ± 4.9 pg/mL vs. 170.6 ± 2.6 pg/mL, respectively, $p<0.05$) 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 P ($p<0.01$ for both) in women with endometriosis and also in women without endometriosis ($p<0.05$ for both) when compared with non-treated PF macrophages (Fig. 4). The production of HGF was about one half than that of VEGF in women with or without endometriosis. There was no statistical difference in response between E2 and P or a combination of E2 and P. No phase of cycle differences were seen.

Since progesterone failed to show a significant increase in the secretion of HGF by PF macrophage, we performed a blocking experiment on E2 by using ER antagonist,

tamoxifen. It was interesting to observe that tamoxifen significantly reversed the secretion of VEGF ($p<0.05$) and tended to reverse the secretion of HGF ($p=0.07$) by the estrogen-treated PF macrophages towards the non-treated macrophages (Fig. 4). This indicates that it is the direct effect of estrogen on the PF macrophages that was able to produce significant amount of HGF and VEGF and is being mediated by ER as located on these inflammatory cells.

Steroidal stimulations of HGF and VEGF production by LPS-activated peritoneal fluid Mϕ. To investigate the control of HGF and VEGF secretion by PF macrophages, we measured HGF and VEGF secretion by activated (LPS, 5 ng/mL) and non-activated PF 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 PF macrophages. However, activation with LPS significantly increased the amount of HGF ($p<0.05$) and VEGF ($p<0.01$) secreted by these cells (Fig. 5). We observed that activation of basal macrophages further enhanced the response of these cells to ovarian

steroids. In fact, exogenous treatment with E2 was able to further increase the amount of

both HGF (p<0.05) and VEGF (p<0.05) secretion by PF macrophages when these cells

were activated with LPS (Fig. 5). Although progesterone increased the secretion of

VEGF by non-activated Mϕ, it was unable to further enhance the secretion of either

HGF or VEGF by activated PF macrophages (Fig. 5). These results confirmed that

irrespective of activation status, PF 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 PF macrophages in pelvic microenvironment.

mRNA expression of HGF and c-Met in PF macrophages and endometrial

stroma. Since VEGF mRNA expression has already been demonstrated previously in

isolated PF macrophages (McLaren et al., 1996), we tried to examine the transcriptional

activity of HGF and its receptor, c-Met from the isolated PF macrophages and

endometrial stroma in response to ovarian steroids (Fig. 3B and 3C). In a time-dependent

study, HGF response to E2 (10^{-8}M) was delayed, with an increasing level of HGF mRNA

observed from 6 hr to 24 hr after the addition of E2 in both PF macrophages 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}M) displayed higher expression of HGF

mRNA (3.2 to 3.8 fold) than that of women without endometriosis (1.2 to 1.6 fold)

($p<0.05$). No difference in c-Met mRNA expression was observed in both Mϕ and

stromal cells between the normal and endometriosis group (Fig. 3B and 3C). Similar to

PF macrophages, an increase in the expression of HGF mRNA was observed in the

stromal cells derived from women with endometriosis than that of women without

endometriosis (Fig. 3C). Again no differences in the expression of HGF and c-Met

mRNA were seen between proliferative and secretory phases of menstrual cycle in these

two groups of women (Fig. 3B and 3C). This indicates an autocrine or paracrine

regulation of HGF by PF macrophages and endometrial or endometriotic stromal cells in

response to ovarian steroids 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 increases in stromal cell and macrophage proliferation.

The presence of c-Met receptor in endometrial stromal cells and M ϕ might be expected to enable these cells to respond to endogenous or exogenous HGF. The effect of media conditioned by peritoneal M ϕ on non-endometriotic stromal cell and M ϕ 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 PF macrophages of women with endometriosis resulted in a significantly greater ($p<0.05$ for both stroma and M ϕ) incorporation of BrdU than that of media conditioned by M ϕ derived from non-endometriosis women (Fig. 6A and 6B). This additional incorporation was significantly decreased by the inclusion of anti-HGF antibody to the culture medium ($p<0.05$ for both stroma and M ϕ) that is comparable to those seen with medium conditioned by M ϕ being collected from women without endometriosis (Fig. 6A and 6B). This indicates that the proliferation of stromal cells and macrophages induced by the conditioned media of endometriosis may be contributed by HGF.

We finally tried to examine the effect of exogenous HGF and E2 on the proliferation of endometrial stromal cells and M ϕ. 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}M) either alone or in combination when compared with BrdU incorporation of non-treated stromal cells (p<0.05 for all, Fig. 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 similar significantly greater BrdU incorporation was observed in PF macrophages derived from women with endometriosis than that of non-treated Mϕ (p<0.05 for all, Fig. 7B). Besides combined treatment of HGF and E2, macrophages were also responsive to significantly incorporate 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, therefore, we also examined the cell growth of stroma and Mϕ by cell number (initial plating 10^5 cells/well) under HGF and E2 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 M ϕ in response to ovarian steroids in women with or without endometriosis. Although macrophage infiltration in PF was dominant in the secretory or menstrual phase, we found a little relation of HGF production by E2 or ER and PR expression with the phase of menstrual cycle. Our results of HGF production in response to E2 indicate that HGF production is possibly related to the activation status of macrophages in peritoneal milieu. In fact, the activation status of macrophages retains increased potentiality to produce and secrete different macromolecules in women endometriosis than that of non-endometriosis. Our current findings are in consistent with the increased production of other cytokines and growth factors by the activated macrophages as described previously from our laboratory and by other literatures (Khan et al., 2002b, 2004b; Halme et al., 1988, 1989).

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 was assumed that this was 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

(PF) have generally been overlooked or scarcely described. Since the major cellular

constituents of PF are macrophages, comprising between 82 and 99% of the total cell

population (Eischen et al., 1994), it is quite reasonable to speculate that these cells may be

responsive to ovarian steroids. Our current study is a further piece of evidence to describe

this interaction between macrophages and ovarian steroid hormones.

We demonstrated that these inflammatory cells retain the mRNAs encoding

both ER and PR and showed nuclear staining for both ER and PR in isolated $M\phi$ 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 $M\phi$ in the

PF of women with early endometriosis and those containing active blood-filled red

lesions in pelvic cavity are in parallel with the increased tissue infiltrations of $M\phi$ in

women with endometriosis as we reported recently (Khan et al., 2004a).

In this study we have demonstrated that HGF can be produced by PF

macrophages derived from pelvic cavity in addition to its production by alveolar

macrophages or hepatic kupffer cells (Skrtic et al., 1999; Crestani et al., 2002; Morimoto

et al., 2001). We reported that PF macrophages could be a constant source of different macromolecules including HGF and VEGF in response to ovarian steroids. We found that PF 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 have been previously activated with LPS.

Besides ER and PR expression, PF 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 were more higher in women with endometriosis than that of non-endometriosis. This indicates that expressions of HGF ligand-receptor by PF macrophages may depend on the activation status of these inflammatory cells. Finally, we demonstrated that the enhanced proliferation of endometrial stromal cells and PF macrophages induced by conditioned media of 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-folds of biological implications, one, ovarian steroids

may influence the autocrine regulation of macrophages or stormal cell functions; two, an inflammatory response in 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 M ϕ also retain c-Met receptor and the proliferation of these cells in response to exogenous HGF strengthened the notion that besides paracrine mode of action, HGF may also play an autocrine mode of action in the growth of endometriosis.

Although PF macrophages are the principal source of VEGF production in 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 PF macrophages. In fact, we found that the ovarian steroid-stimulated production of VEGF by PF macrophages was two-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 PF 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 promoter region of HGF and VEGF. In fact, several half-palindromic consensus sequences for ERE and PRE were found on the promoter region of VEGF gene and claimed that VEGF 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 expressions of its ligand-receptor are up-regulated by E2-stimulated PF 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 PF 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 already demonstrated that the concentrations of HGF and other inflammatory mediators in PF were significantly elevated in women with early endometriosis and in those harboring blood-filled opaque peritoneal lesions comparing 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 PF macrophages further confirmed that the growth of endometriosis possibly depends on a mutual interaction between innate immune system and ovarian steroid hormones in pelvic microenvironment. The current therapeutic strategy of hypo-estrogenic medication in women with endometriosis can also be explained by its

effect on 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

American Society for Reproductive Medicine (1997) Revised American Society for Reproductive Medicine classification of endometriosis: 1996. Fertil. Steril. 67,817-821.

Bergqvist A (1992) Steroid receptors in endometriosis. *In* Modern Approaches to Endometriosis. E. Thomas and J. Rock, editors. Kluwer Acedmic Publishers Group, Dordrecht, The Netherlands. 257-274.

Chao TC, Van Alten PJ, Greager JA and Walter RJ (1995) Steroid sex hormones regulate the release of tumor necrosis factor by macrophages. Cell. Immunol 160,43-49.

Crestani B, Dehoux M, Hayem G, Lecon V, Hochedez F, Marchal J, Jaffre S, Stern JB, Durand G, Valeyre D, Fournier M, Aubier M (2002) Differential role of neutrophils and alveolar macrophages in hepatocyte growth factor production in pulmonary fibrosis. *Lab*

Invest., 82(8),1015-1022.

DeLeon FD, Vijoykumar R, Brown M, Rao CHV, Yussman MA, Schultz G (1986) Peritoneal fluid volume, estrogen, progesterone, prostaglandin, and epidermal growth factor concentrations of patients with and without endometriosis. Obstet. Gynecol. 68,189-194.

Eischen A, Duclos B, Schmitt-Gognel M, Rouyer N, Bergerat JP, Hummel M, Oskam R, and Oberling Fr (1994) Human resident peritoneal macrophages: phenotype and histology. Br. J. Haematol 88,712-722.

Frazier-Jassen MR, and Kovacs EJ (1995) Estrogen modulation of JE/monocyte chemoattracttant protein-1 mRNA expression in murine macrophages. Cell. Immunol. 160,1838-1845.

Folkman J and Klagsbrun M (1987) Angiogenic factors. Science (Wash. DC). 235,442-446.

Fujishita A, Nakane PK, Koji T, Masuzaki H. Chavez RO, Yamabe T, Ishimaru T (1997) Expression of estrogen and progesterone in endometrium and peritoneal endometriosis: an immunohistochemical and in situ hybridization study. Fertil Steril 67,856-864.

Gockerman JP, Spremulli EN, Raney M, Logan T (1986) Randomized comparison of tamoxifen versus diethylstilbestrol in estrogen receptor-positive or unknown metastatic breast cancer: a Southeastern cancer Study Group trial. Cancer Treat Rep 70,1199-1203.

Halme J, Becker S, Haskill S (1987) Altered maturation and function of peritoneal macrophages: possible role in pathogenesis endometriosis. Am J Obstet Gynecol, 156, 783-789.

Halme J, White C, Kauma S, Estes J, Haskill S (1988) Peritoneal macrophages from patients with endometriosis release growth factor activity in vitro. J Clin Endocrinol Metab 66,1044-1049.

Halme J (1989) Release of tumor necrosis factor-alpha by human peritoneal macrophages in vivo and in vitro. Am J Obstet Gynecol 161,1718-1725.

Hu SK, Mitcho YL, and Rath NC (1988) Effect of estradiol on interleukin 1 synthesis by macrophages. Int. J. Immunopharmacol 10,247-252.

Ishimaru T, Khan KN, Fujishita A, Kitajima M, Masuzaki H (2004) Hepatocyte growth factor may be involved in cellular changes to the peritoneal mesothelium adjacent to pelvic endometriosis. Fertil Steril, 81(suppl 1), 810-818.

Jiang W, Hiscox S, Matsumoto K, Nakamura T (1999) Hepatocyte growth factor/scatter factor, its molecular, cellular and clinical implications in cancer. Crit Rev Oncol Hematol 29,209-248.

Jansen, RPS and Russel P (1996) Non-pigmented endometriosis: clinical, laparoscopic and pathologic definition. Am J. Obstet Gynecol 155,1154-1159.

Khan KN, Fujishita A, Kitajima M, Masuzaki H, Sekine I, Ishimaru T (2002a) Infiltrated macrophage activity in intact tissue of endometriosis. Proceedings of Endometriosis, 23,137-142 (in Japanese).

Khan KN, Masuzaki H, Fujishita A, Hamasaki T, Kitajima M, Hasuo A, Ishimaru T (2002b) Association of interleukin-6 and estradiol with hepatocyte growth factor in peritoneal fluid of women with endometriosis. Acta Obstet Gynecol Scand 81,764-771.

Khan KN, Masuzaki H, Fujishita A, Kitajima M, Sekine I, Ishimaru T (2003) Immunoexpression of hepatocyte growth factor and c-Met receptor in the eutopic endometrium predicts the activity of ectopic endometrium. Fertil Steril 79,173-181.

Khan KN, Masuzaki H, Fujishita A, Kitajima M, Sekine I, Ishimaru T (2004a) Differential macrophage infiltration in early and advanced endometriosis and adjacent

peritoneum. Fertil Steril, 81,652-661.

Khan KN, Masuzaki H, Fujishita A, Kitajima M, Sekine I, Ishimaru T (2004b) Higher activity by opaque endometriotic lesions than non-opaque lesions in women with endometriosis. Acta Obstet Gynecol Scand 83,375-382.

Khan KN, Masuzaki H, Fujishita A, Kitajima M, Kohno T, Sekine I, Matsuyama T, Ishimaru T (2005). Regulation of hepatocyte growth factor by basal and stimulated macrophages in women with endometriosis. Hum Reprod 20,49-60.

McLaren J, Prentice A, Charnock-Jones DS, Millican SA, Muller KH, Sharkey AM, et al. (1996) Vascular endothelial growth factor is produced by peritoneal fluid macrophages in endometriosis and is regulated by ovarian steroids. J Clin Invest 98,482-489.

Morimoto K, Amano H, Sonoda F, Baba M, Senba M, Yoshimine H, Yamamoto H, Ii T, Oishi K, Nagatake T (2001) Alveolar macrophages that phagocytose apoptotic neutrophils produce hepatocyte growth factor during bacterial pneumonia in mice. Am J Respir Cell Mol Biol 24(5),608-615.

Nisolle M, Casanas-Rouz F, Donnez J (1997) Immunohistochemical analysis of proliferative activity and steroid receptor expression in peritoneal and ovarian

endometriosis. Fertil Steril 68,912-919.

Nakamura T, Teramoto H, Ichihara A (1986) Purification and characterization of a growth factor from rat platelets from mature parenchymal hepatocytes in primary cultures. Proc Natl Acad Sci USA, 83,6489-493.

Osteen KG, Hill GA, Hargrove JT, Gorstein F (1989) Development of a method to isolate and culture highly purified populations of stromal and epithelial cells from human endometrial biopsy specimens. Fertil Steril 52,965-972.

Sampson J (1927) Peritoneal endometriosis due to menstrual dissemination of endometrial tissue into the peritoneal cavity. Am J Obstet Gynecol 14,422-429.

Shifren JL, Tseng JF, Zaloudek CJ, Ryan IP, Meng YG, Ferrara N, Jaffe RG, and Taylor RN (1996) Ovarian steroid regulation of vascular endothelial growth factor in the human endometrium: implications for angiogenesis during menstrual cycle and in the pathogenesis of endometriosis. J Clin Endocrinol Metab 81,3112-3118.

Skrtic S, Wallenius V, Ekberg S, Brenzel A, Gressner AM, Jansson JO (1999) Hepatocyte-stimulated expression of hepatocyte growth factor (HGF) in cultured rat hepatic stellate cells. J Hepatol 30(1),115-124

Strathy JH, Molgaard CA, and Coulman CB (1982) Endometriosis and infertility:

a laparoscopic study of endometriosis among fertile and infertile women. Fertil Steril

38,667-672.

Sugawara J, Fukaya T, Murakami T, Yoshida H, Yajima A (1997) Hepatocyte growth

factor stimulates proliferation, migration, and lumen formation of human endometrial

epithelial cells in vitro. Biol Reprod 57,936-942.

Tabibzadeh SS, Santhanan V, Sehgal PB, May LT (1989) Cytokine induced production of

IFN-β2/IL-6 by freshly implanted human endometrial stromal cells: modulation by

estradiol 17β. J Immunol 42,3134-3139.

Tajima H, Matsumoto K, Nakamura T (1992) Regulation of cell growth and motility by

hepatocyte growth factor and receptor expression in various cell species. Exp Cell Res

202,423-431.

Takagi S (1993) Detection of 5-bromo-2-deoxyuridine (BrdU) incorporation with

monoclonal anti-BrdU antibody after deoxyribonuclease treatment. Cytometry

14,640-649.

Thomas EJ and Prentice A (1992) The etiology and pathogenesis of endometriosis.

Reprod. Med. Rev. 1,21-36.

Zarnegar R (1995) Regulation of HGF and HGFR gene expression.

Epithelial-Mesenchymal Interactions in Cancer, 33-49.

Figure Legends

Figure 1. Shows the peritoneal fluid concentrations of macrophages derived from women with or without endometriosis and are distributed according to the revised-ASRM staging (**A**), morphologic appearance with chocolate cysts (**B**) and different phases of menstrual cycle (**C**). The results are expression as mean $\pm$ SEM. **A**, *p<0.05, stage I-II vs. stage III-IV or without endometriosis. **B**, *p<0.05, women containing red lesions vs. women with other lesions/chocolate cysts or without endometriosis. **C**, *p<0.05, secretory phase vs. proliferative phase; **p<0.01, menstrual phase vs. secretory phase or proliferative phase.

Figure 2. Shows the immunohistochemical staining of estrogen receptor (ER) and progesterone receptor (PR) in isolated peritoneal fluid Mϕ (**A**) and in the serial sections of intact tissue (**B**). The isolated PF macrophages show strong nuclear staining for ER and weak staining for PR. The ER and PR expressions are shown in the same

position of CD68 immunoreactive M ϕ and were demonstrated in the stromal component

of eutopic endometrium derived from woman with endometriosis. The arrow heads show

the co-localization of ER and PR at the same position of CD68 stained M ϕ. Final

magnification was adjusted at x50 and using light microscope connected to a camera

(Olympus-VANOX, model-AHBS, Tokyo, Japan).

Figure 3. Expression of mRNA encoding for estrogen receptor, progesterone

receptor, hepatocyte growth factor (HGF) and its receptor, c-Met in isolated peritoneal

fluid (PF) macrophages and endometrial stromal cells as assayed by RT-PCR.

Ethidium-stained agarose gels show representative products amplified from cDNA

derived from PF macrophages or stromal cells. (**A**) Estrogen and progesterone receptors;

endo (+), women with endometriosis; endo (-), women without endometriosis; P,

proliferative phase; S, secretory phase; c-DNA (-), negative control with no cDNA, M,

marker for DNA. (**B and C**) Effect of estradiol (10^{-8}M) on the mRNA expression of HGF

and c-Met in isolated PF macrophages (**B**) and in endometrial stromal cells (**C**) derived

from women with or without endometriosis. The mRNA expression of HGF was found to

be significantly higher in women with endometriosis than that of non-endometriosis. No

differences in the expression of c-Met mRNA or in the phases of menstrual cycle were seen between these two groups of women. β-actin, internal control. Calculated molecular weights of product bands are indicated by arrows.

Figure 4. Shows the production of hepatocyte growth factor (HGF) and vascular endothelial cell growth factor (VEGF) in the conditioned media of peritoneal fluid macrophages (Mϕ) derived from women with or without endometriosis and in response to estradiol (E2), progesterone (P), a combination of E2 and P (E2+P), and an estrogen receptor (ER) antagonist, tamoxifen (TMX). The results are expressed as mean $\pm$ SEM of six patients from each group (three proliferative and three secretory phase samples). HGF, *p<0.05 vs. non-treated Mϕ or control women; VEGF, *p<0.05 vs. non-treated Mϕ, **p<0.01 vs. non-treated Mϕ or control women. The blocking of ER by tamoxifen tended to reverse the production of HGF and VEGF towards non-treated Mϕ. VEGF, #p<0.05 vs. E2-treated Mϕ.

Figure 5. Shows the production of hepatocyte growth factor (HGF) and vascular endothelial cell growth factor (VEGF) in the conditioned media of activated and/or estradiol (E2)- and progesterone (P)-treated macrophages (Mϕ) that were derived

from six women with non-endometriosis (three proliferative and three secretory phase samples). Isolated peritoneal fluid Mϕ, untreated or activated by lipopolysaccharide (LPS, 5 ng/mL), were incubated with E2 or P for 24 hr, and the resultant conditioned media were assayed for HGF and VEGF. The results are expressed as mean $\pm$ SEM of six patients. HGF, *p<0.05 vs. LPS non-treated Mϕ; ¶p<0.05 vs. LPS-treated Mϕ. VEGF, *p<0.01 vs. LPS non-treated Mϕ; ¶p<0.05 vs. LPS-treated Mϕ; #p<0.05 vs. LPS non-treated Mϕ.

Figure 6. Shows the effect of conditioned media of peritoneal fluid macrophages (Mϕ) of women with or without endometriosis on the proliferation of non-endometriotic stromal cells (**A**) and Mϕ (**B**) and was measured by bromodeoxyuridine (BrdU) incorporations in these cells. Neutralization studies were carried out using anti-human HGF neutralizing antibody. The results are expressed as percentage of control (mean $\pm$ SEM). Non-macrophage conditioned media equals 100%. Six patients were examined from each group (three proliferative and three secretory phase). *p<0.05, endometriosis vs. non-endometriosis for both stromal cells and Mϕ; #p<0.05, anti-HGF antibody vs. without anti-HGF antibody for both stromal cells and M

ϕ . Endo (-) means women without endometriosis, Endo (+) means women with endometriosis.

Figure 7. Shows the exogenous single or combined effect of hepatocyte growth factor (HGF) and estradiol (E2) 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) incorporations are expressed as percentage of control (mean $\pm$ SEM). BrdU incorporations of non-treated cells equals 100%. Stroma of endometriosis, **$p<0.05$ vs. non-treated cells; stroma of non-endometriosis, *$p<0.05$ vs. non-treated cells. M ϕ of endometriosis, **$p<0.05$ vs. non-treated M ϕ ; M ϕ of non-endometriosis, *$p<0.05$ vs. non-treated M ϕ .
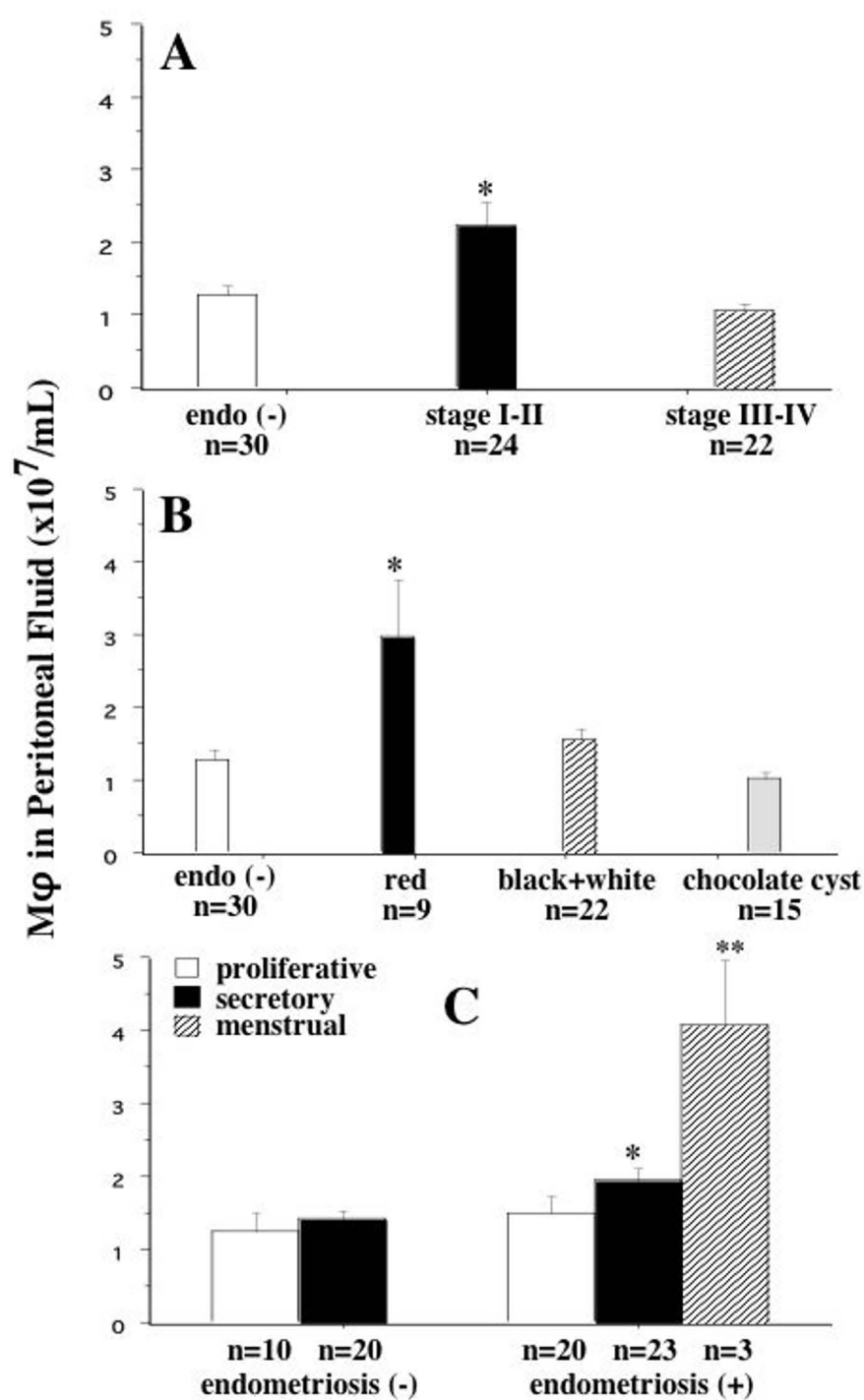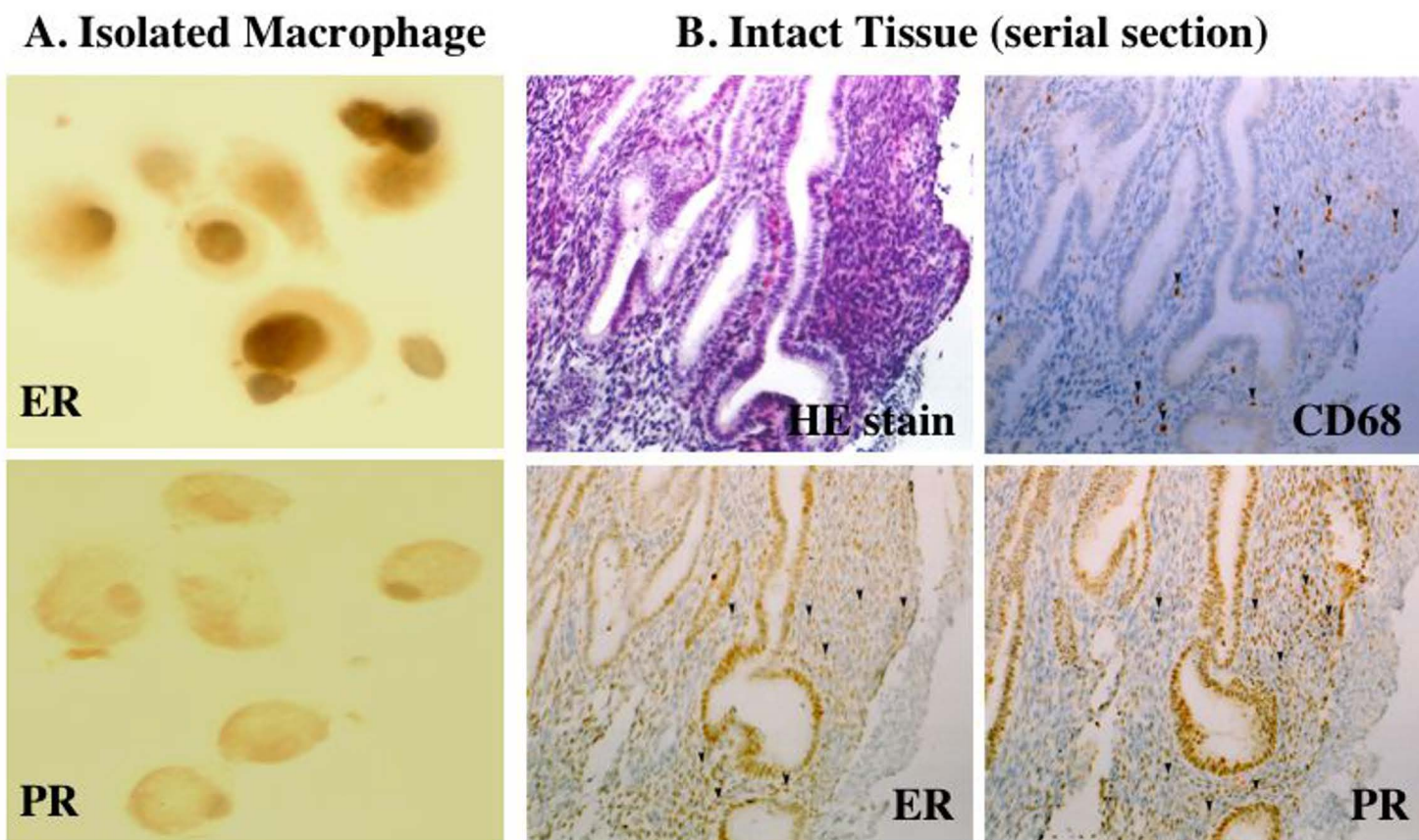
Figure 1

Figure 2

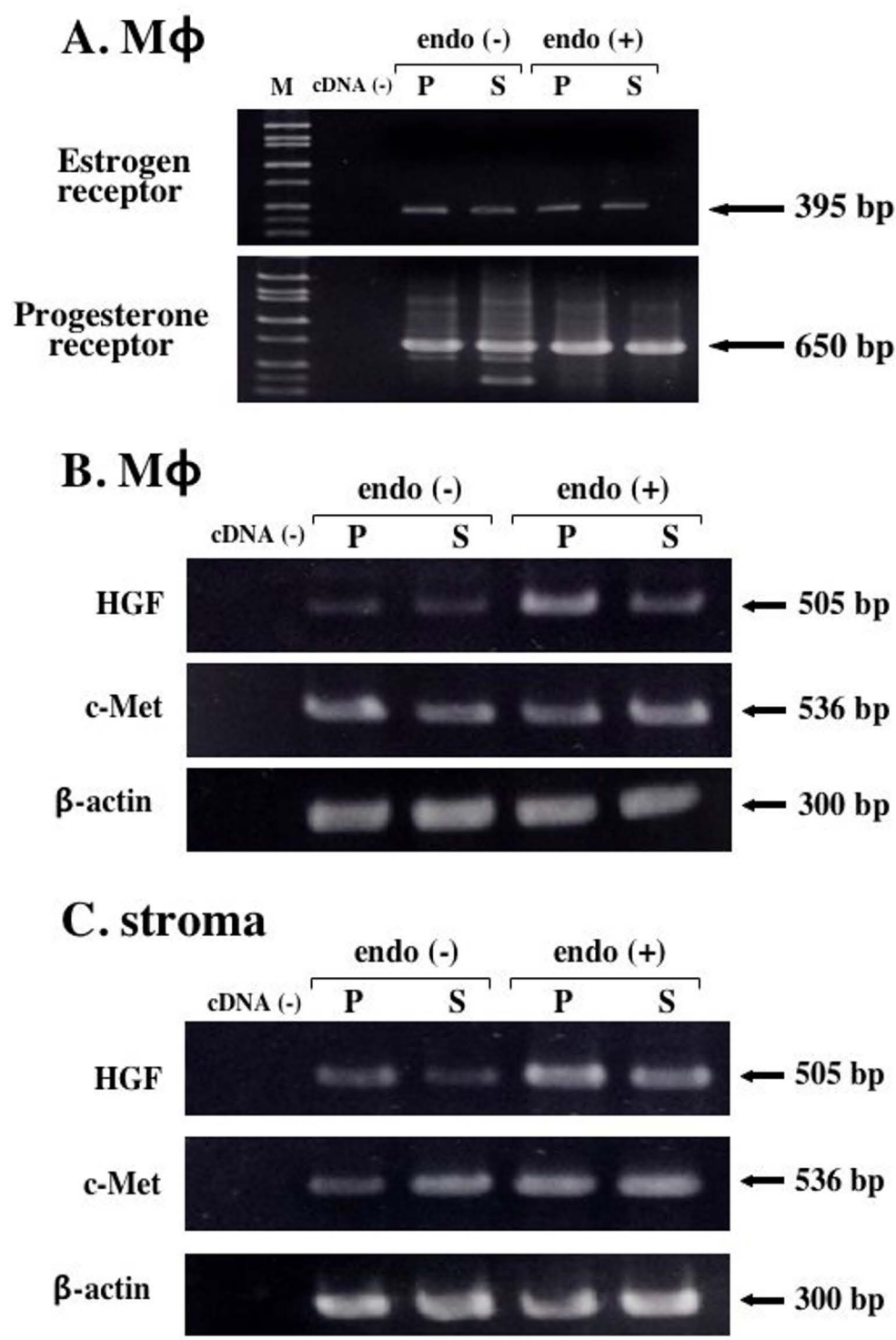## A. Isolated Macrophage

ER

PR

B. Intact Tissue (serial section)

HE stain

CD68

ER

PR
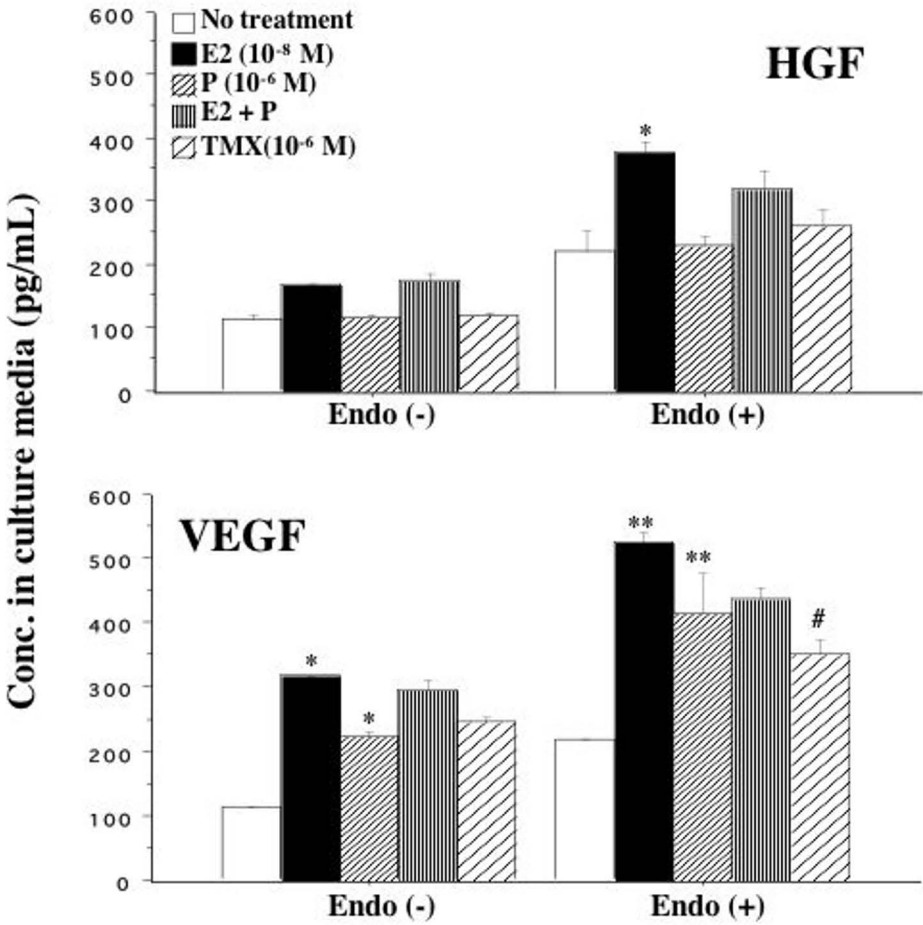
Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

A. stroma

BrdU incorporation (% of control)

□ endometriosis (-)
■ endometriosis (+)

HGF	0	50	0	50	(ng/mL)
E2	0	0	10^{-8}	10^{-8}	(M)

B. Mφ

□ endometriosis (-)
■ endometriosis (+)

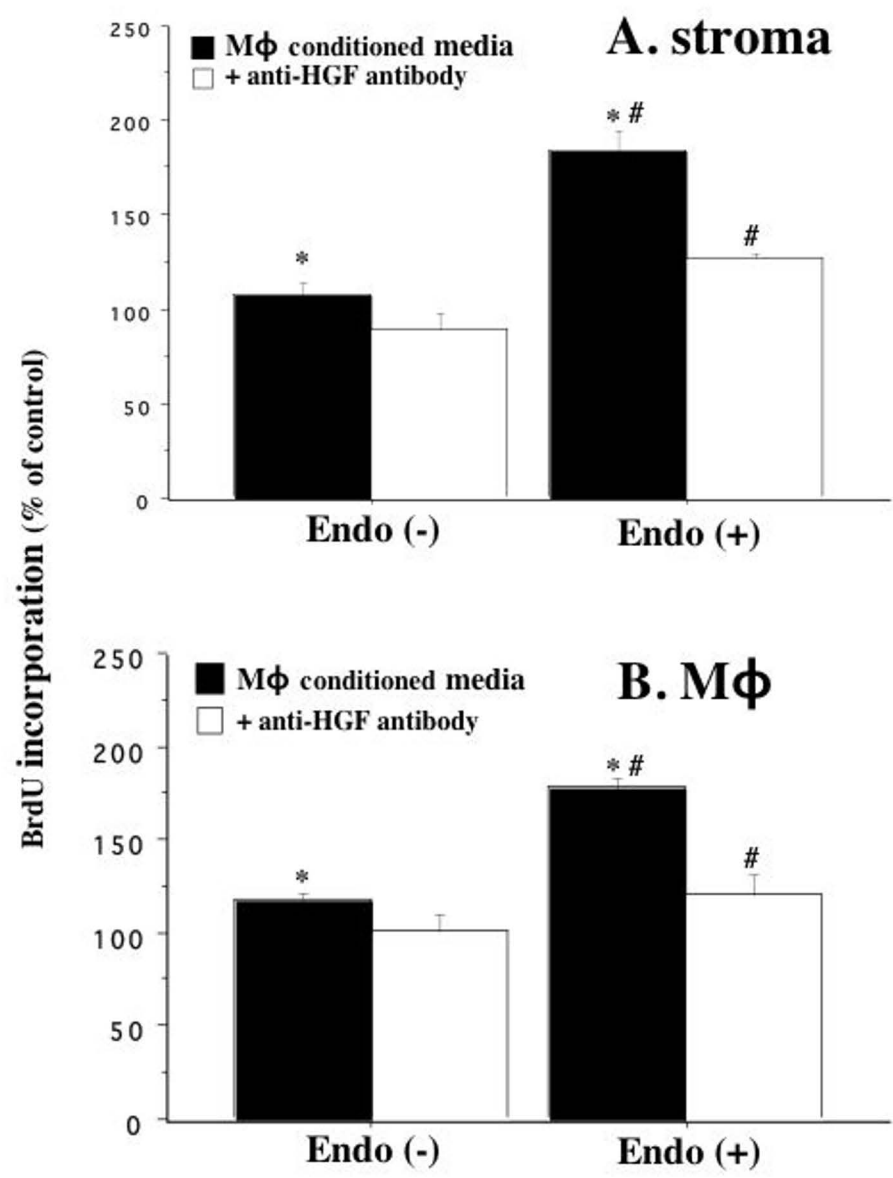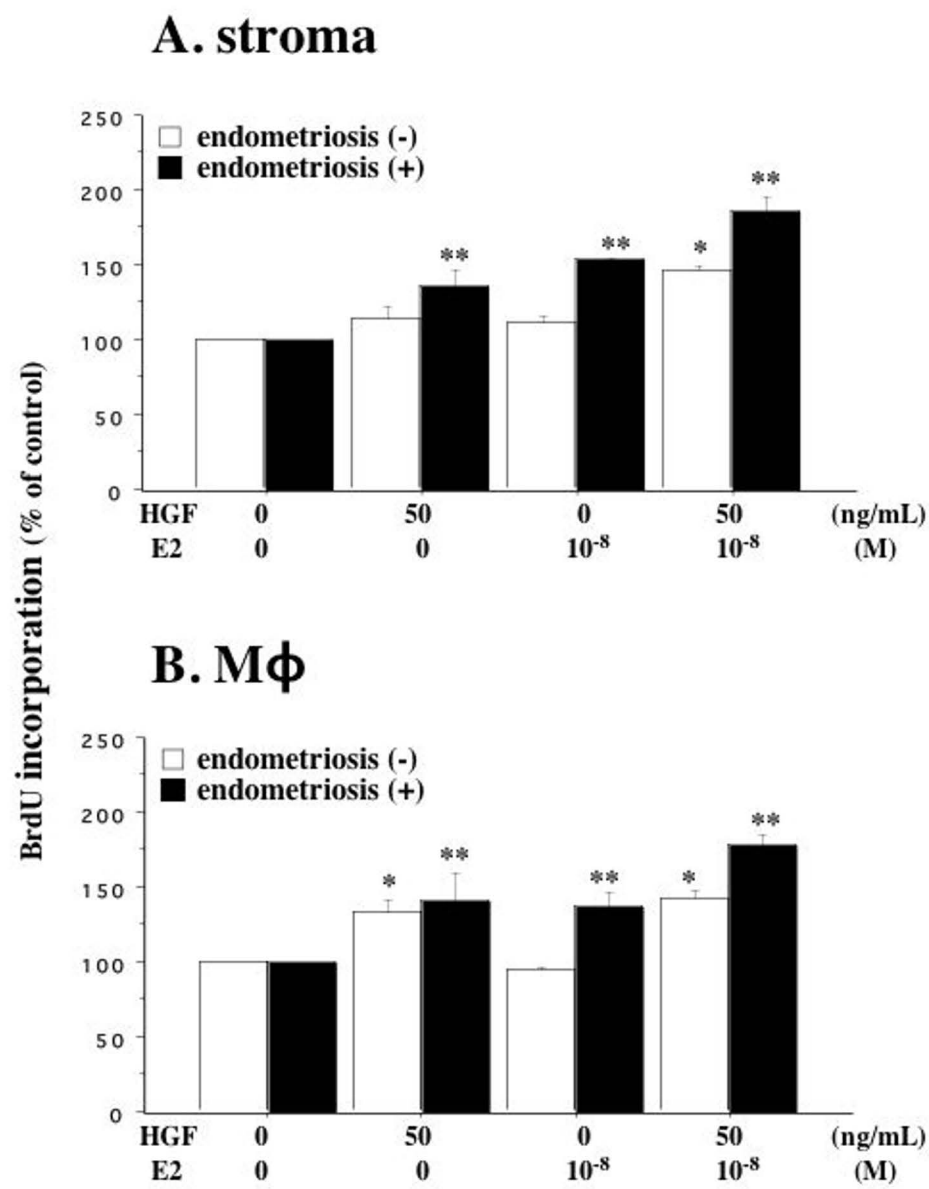|			
HGF	0	50	0	50	(ng/mL)
E2	0	0	10^{-8}	10^{-8}	(M)
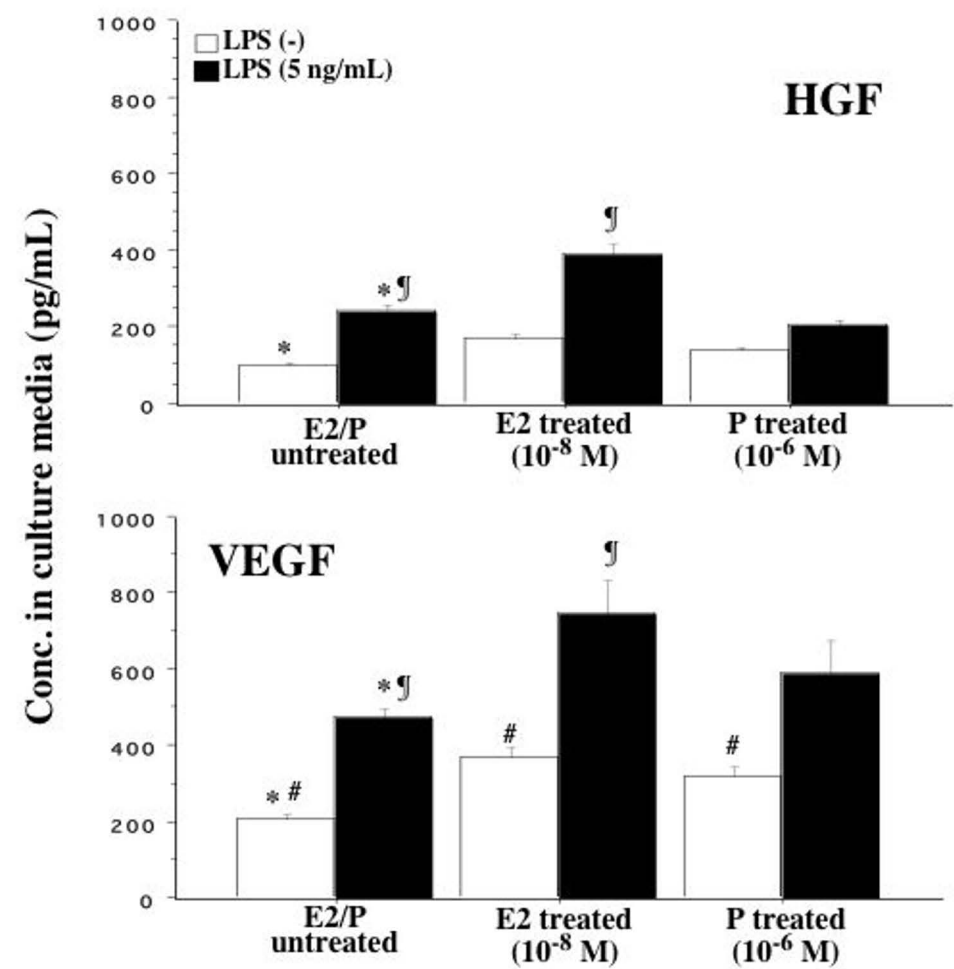
Table 1. Human oligonucleotide primers of ER, PR, HGF, c-Met and β-actin used.

Locus	Sense (5'-3')	Antisense (5'-3')	Size (bp)
ER	Primer 1: GGAGACATGAGAGCTGCCAA Primer 3: CTTTGGCCAAGCCCGCTC	Primer 2: CCAGCAGCATGTCGAAGATC Primer 4: TCATCATGCGGAACCGAGCT	395
PR	Primer 1: GGTCTTCCTTAAGAGGACA Primer 3: GGAAGGGCAGCACAACTA	Primer 2: GACTTCGTAGCCCTTCCA Primer 4: AAGGAATTGTATTAAGAAGTAA	650
HGF	ACTGGCTCTTTTAGGCACTGACTC	TGTTCCCTTGTAGCTGCGTCCTTT	505
c-Met	ACTCCCCCTGAAAACCAAAGCC	GGCTTACACTTCGGGCACTTAC	536
β-actin	ATGTGCTGTACCAAGAGTTTGC	CCAATTCCATTCCAGAAAAGCC	300

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