Vascular Endothelial Growth Factor Is Produced by Peritoneal Fluid Macrophages in Endometriosis and Is Regulated by Ovarian Steroids


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Abstract

Angiogenesis is important in the pathophysiology of endometriosis, a condition characterized by implantation of ectopic endometrium in the peritoneal cavity. Vascular endothelial growth factor (VEGF) is a potent angiogenic factor involved in physiological and pathological angiogenesis, and elevated levels of VEGF are found in peritoneal fluid of patients with endometriosis. Our aim was to investigate the site of expression and regulation of VEGF in endometriosis. VEGF immunoreactivity was found in tissue macrophages present in ectopic endometrium and in activated peritoneal fluid macrophages. Macrophage activation was highest in women with endometriosis, and media conditioned by peritoneal fluid macrophages from these women caused a VEGF-dependent increase in endothelial cell proliferation above that seen from normal women. Peritoneal fluid macrophages secreted VEGF in response to ovarian steroids, and this secretion was enhanced after activation with lipopolysaccharide. Peritoneal fluid macrophages expressed receptors for steroid hormones. VEGF receptors flt and KDR (kinase domain receptor) were also detected, suggesting autocrine regulation. During the menstrual cycle, expression of flt was constant but that of KDR was increased in the luteal phase, at which time the cells migrated in response to VEGF. KDR expression and the migratory response were significantly higher in patients with endometriosis. This study demonstrates that activated macrophages are a major source of VEGF in endometriosis and that this expression is regulated directly by ovarian steroids. (J. Clin. Invest. 1996. 98:482–489.) Key words: endometriosis • VEGF • angiogenesis • VEGF receptors (flt, KDR)

Introduction

Endometriosis, the presence of functional endometrium outside of the uterine cavity, is a common disease, causing abdominal pain, dysmenorrhoea, dyspareunia and infertility in about 10% of the female population (1). It is believed to arise from the implantation and growth of exfoliated menstrual en- dometrium on the peritoneal and ovarian surfaces (2). Active endometriotic explants have pronounced vascularization both within and around the tissue (3), achieved through the development of new blood vessels by the process of angiogenesis. Angiogenesis involves extensive interplay between cells, soluble factors, and the extracellular matrix (4). Physiologically it occurs during endometrial repair (5), the formation of the corpus luteum (6), and embryogenesis (7). Angiogenesis is also important in the pathogenesis of cancer (8–10), arthritis (11), and arteriosclerosis (12). The identification of the key mediators of angiogenesis and their sources in the peritoneal cavity of women with endometriosis would be important for the understanding of the pathogenesis of this disease.

An important mediator of angiogenesis is vascular endothelial growth factor (VEGF) (1). VEGF is a secreted, heparin-binding homodimeric glycoprotein of ~46 kD, with several protein variants resulting from alternative splicing of VEGF mRNA (5, 13). It is a potent endothelial cell mitogen, morphogen, and vascular permeability–inducing agent (14–16) and displays angiogenic activity in the rabbit cornea (17) and chick choioallantoic membranes (18). VEGF binds to either one of two tyrosine kinase receptors, the fms-like tyrosine kinase (flt) (19) and the kinase domain receptor (KDR) (20). These receptors are found predominately on endothelial cells (14, 15, 21), and activation leads not only to proliferation and increased vascular permeability but also to the expression of a number of proteolytic enzymes involved in the process of angiogenesis (22, 23).

Peritoneal fluid bathes the peritoneal and ovarian surfaces, on which most endometriotic lesions occur. The angiogenic activity of peritoneal fluid from women with endometriosis is elevated (24), and elevated levels of VEGF are found in the peritoneal fluid of women with endometriosis (25). VEGF is expressed by eutopic endometrium (5), but the major cellular constituents of peritoneal fluid are macrophages, comprising between 82 and 99% of the total cell population (26). These cells are involved in immune surveillance of the peritoneal cavity. They are also known to secrete a number of growth factors including TNF-α (27), IL-1β (28), IL-1 (29), IL-6 (30) and INF-γ (31), which may regulate the actions of leukocytes in the peritoneal fluid or which could act directly on ectopic endometrium. The number, size, and activation (32, 33) of these cells are increased in endometriosis, and the presence of mRNA encoding VEGF has been demonstrated in activated guinea pig peritoneal fluid macrophages (34). The elevated levels of angiogenic and other growth factors in the peritoneal fluid may contribute to the development of an adequate vascu-
lature and the subsequent maintenance of endometriotic ex-plants.

The aim of this study was to investigate the site of expres-
sion of VEGF in the peritoneal cavity, to determine the factors which regulate this expression, and to identify differences be-
tween women with and without endometriosis.

Methods

Patient details. Women between 24 and 44-yr-old who were undergo-
ing either diagnostic laparoscopy for dysmenorrhea or elective lap-
aroscopy for infertility were recruited for this study. Patients were di-
agnosed as normal or endometriotic after laparoscopic investigation. Ectopic endometrial tissue samples (n = 8) and peritoneal fluid were obtained from endometriotic (n = 28) and cycle matched nonen-
dometriotic patients (n = 34). The extent of endometriosis was deter-
mained according to the revised American Fertility Society scoring
system. All patients sampled had minimal to mild endometriosis
=scoring 1–12). This study was approved by the Cambridge Health
Authority Local Research Ethics Committee, and informed consent
was obtained from each patient.

Isolation of peritoneal fluid macrophages. Peritoneal fluid was as-
pirated from the posterior cul-de-sac. Peritoneal fluid macrophages
were isolated by the method of Halme et al. (32, 33), and this method
had been extensively characterized. It results in an enriched popula-
tion of peritoneal fluid 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 macro-
phages for immunohistochemistry were spotted onto untreated slides
and the purity of the preparation was determined using immunohis-
tochemical staining. Slides routinely contained > 95% macrophages.

Immunohistochemical staining. Acetone-fixed cryostat sections
were used. Slides were incubated for 20 min in 10% goat serum to
block nonspecific staining, before inactivating endogenous peroxi-
dase activity with 1% H2O2 in PBS. Sections were then incubated for
1 h with either VEGF polyclonal rabbit antibody, raised against a peptide corresponding to the carboxy-terminal 20 amino acids of
VEGF (Santa Cruz Biotechnology, Hatfield, UK) (1:300) or VEGF
antibody that had been preabsorbed for 24 h with a 10-fold excess of
VEGF peptide (Santa Cruz Biotechnology). This latter treatment
served as negative control along with normal rabbit Igs (Dako, Cam-
bridge, UK). Binding was visualized after incubation with a biotiny-
lated goat anti–rabbit antibody (1:200) for 1 h, and subsequent com-
bination of both, for 24 h. Control cells were incubated in just
peritoneal fluid macrophages from both patient groups were
incubated with a 1:50 dilution for 30 min at 4
°C. VEGF inhibition
was performed with a chemotaxis chamber using
a combination of both, for 24 h. Control cells were incubated in just
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°C. VEGF inhibition
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Endothelial cell proliferation assay. Endothelial cell proliferation
assays were performed using human umbilical vein endothelial cells
(HUVEC) isolated according to the method of Jaffe (36). These cells
were grown for 48 h in Dulbecco’s Modified Essential Medium
(DMEM); Hams F12 (Gibco, Paisley, UK), without t-glutamine, con-
taining 15% FCS after which the FCS was reduced to 5% (vol/vol).
Isolated peritoneal fluid macrophages from both patient groups were
cultured in FCS-free M199 modified media for 48 h. This conditioned
media was then added to the cultured HUVECs, which had previ-
ously been washed twice with sterile PBS, pH 7.4, and then left for
24 h in a (5%) CO2 (95%) air incubator at 37°C. VEGF inhibition
studies were performed using a specific anti-human VEGF neutralizing antibody
(R&D Systems, Abingdon, UK) were carried out in parallel at a con-
centration of 800 ng/ml. After 24 h, [3H]thymidine (1 µCi/ml) (Amer-
sham International, Little Chalfont, UK) was added and incorpora-
tion was measured after a further 24 h. After incubation and TCA extraction,
[3H]thymidine uptake was determined by liquid scintillation counting.

Reverse transcription (RT) and PCR. RNA from isolated perito-
neal fluid macrophages was extracted and analyzed for mRNA en-
coding theflt and KDR receptors by RT-PCR as previously de-
scribed (37). The presence of mRNA encoding the estrogen and
progesterone receptors in peritoneal fluid macrophages was deter-
mined using forward and reverse primers synthesized to anneal with
cDNA for estrogen (38) and progestrone (39) receptors. Amplifica-
tion was in two stages: Estrogen receptor primers 1 (GGGAGCAT-
GAGGCTGCGCA) and 2 (CCACGCAGCTGCGAACTC) for
95°C, 90 s (95°C, 30 s; 95°C, 30 s; 72°C, 30 s) ×30 cycles. A 2-µl
aliquot of this was transferred to a new reaction mix containing primers 3
(CTTGGGGAAGCCTGCT) and 4 (TCATATCGCAGCAGAT) and reamplified using the above conditions but at a lower
annealing temperature (58°C). Progesterone receptor primers 1
(GGTTCCTCTAAAGGACA) and 2 (GACTCTGATCCCTC
TCA) for 95°C, 90 s (95°C, 30 s; 52°C, 30 s; 72°C, 30 s) ×30 cycles. A 2-µl aliquot of this was transferred to a new reaction mix containing primers 3
(GGAAGGGCCAGCAACTA) and 4 (AAGGAAAT-
TGATATTAGAAATGA) and reamplified using the above condi-
tions but at a lower annealing temperature (52°C). PCR generated
bands were cloned and found to match the published sequences for
the expected products.

Activation and ovarian steroid stimulation of peritoneal fluid mac-
rophages. Isolated peritoneal fluid macrophages from women with-
out endometriosis were plated in 96-well plates and allowed to attach
for 24 h in phenol red free M199 + 5% FCS. After this time the cells
were washed with PBS and incubated in phenol red free M199 media
containing either estradiol-17β (10-8 M), progesterone (10-8 M) or a
combination of both, for 24 h. Control cells were incubated in just
phenol red free M199 media. Activation studies involved the addition
of 10 ng/ml of lipopolysaccharide (LPS) (Sigma, Poole, UK) just be-
fore commencement of the 24-h incubation. The conditioned media
were removed and VEGF determined using a commercially available
ELISA kit (Peninsula Laboratories, Belmont, CA).

Flow cytometry forflt and KDR. Isolated peritoneal fluid mac-
rophages from both patient groups were incubated with a 1:50 dilu-
tion offlt or KDR rabbit polyclonal (Santa Cruz Biotechnology, Hat-
field, UK) for 30 min at 4°C. The cells were then washed and incubated in a 1:50 dilution of swine anti-rabbit FITC labeled F(ab)2,
fragment for 30 min at 4°C. After further washing, directly labelled
CD14 Phycoerythrin (PE) (Becton Dickinson, Cowley, UK), at a
concentration of 1:10, was added and again incubated at 4°C for 30
min before a final wash and fixing in 1% paraformaldehyde before fluo-
rescence activated cell sorting (FACS®) analysis. Controls comprised
of the same polyclonal antibodies which had previously been preab-
sorbed a relevant control peptide. In addition, tubes containing swine
anti-rabbit-FITC labeled F(ab)2, fragment alone and an irrelevant di-
rectly PE labelled monoclonal were used as controls.

Peritoneal fluid macrophage migration in response to VEGF.
Chemotaxis assays were performed with a chemotaxis chamber using
PVP free polycarbonate membranes (5 µm; Nucleoprobe, Pleasan-
ton, CA). Peritoneal fluid macrophages, from both patient groups
were suspended in M199 media containing 0.25% BSA. The lower
chamber contained the indicated amount of VEGF. The chamber was

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incubated at 37°C for 5 min before the cells were added to the top chamber (50 μl). Assays were performed in triplicate over a 90-min incubation period at 37°C, after which non-migrating cells were removed, membranes fixed and cells which have migrated through the membrane were visualized with Diff-quik staining kit (Hannheim, Germany). The number of migrated cells were counted and the results were expressed as the chemotactic index. This calculated as the number of cells which have migrated through the membrane after VEGF treatment divided by the number of migrated untreated cells. N-formylmethionylleucylalanine (FMLP) was used as a positive control (5 × 10⁻⁸ M), whilst M199 media containing 0.25% BSA was the negative.

Statistical analysis. Statistical significance was determined on original data from samples using the nonparametric Wilcoxon’s signed rank test for paired data and Mann-Whitney U test for unpaired data. Statistical significance was accepted at P < 0.05.

Figure 1. (A and B) Immunohistochemical staining for VEGF protein in endometriotic tissue showing immunoreactivity in isolated cells in the stroma. A is the negative control after staining with the peptide preabsorbed anti-VEGF antibody. B is the anti-VEGF polyclonal antibody. Individual cells, within the stroma, are intensely stained for VEGF. Diffuse staining in the area of the VEGF positive cells is also evident. All eight sections (four proliferative, four secretory) are counterstained with haemalum. (×250). (C and D) VEGF-positive macrophages in the stroma of endometriotic tissue after double immunofluorescence staining for the monocyte/macrophage marker LeuM3 (CD14)(C) and VEGF (D) (×400). (E and F) Isolated peritoneal fluid macrophages from patients with endometriosis were immunofluorescently stained for the macrophage activated marker HLA-DR (E) and VEGF (F). HLA-DR-positive macrophages were immunopositive for VEGF (×400).
Results

Localization of VEGF in ectopic endometrium. In intrauterine endometrium VEGF mRNA (5) and protein (Sharkey et al., personal communication) is expressed in both glandular epithelium and stroma in the proliferative phase of the cycle and in glandular epithelium in the secretory and menstrual phases. Surprisingly this was not the case with ectopic endometrium. VEGF immunoreactivity was localized mainly on isolated cells within the ectopic stroma and only light staining was present on the glandular epithelium (Fig. 1 B). In situ hybridization to localize VEGF mRNA in ectopic endometrium showed a similar pattern (data not shown). This pattern of staining was seen in six out of the eight endometriotic samples stained. In the remaining two samples, one from each phase of the cycle, no VEGF positive cells were observed. Double immunofluorescence staining with the macrophage marker CD14 (Fig. 1 C) and VEGF (Fig. 1 D) showed that individual VEGF-positive cells, within the stroma, were macrophages.

Localization of VEGF on activated peritoneal fluid macrophages. Isolated peritoneal fluid macrophages from women with and without endometriosis were immunostained for the macrophage activation marker HLA-DR (MHC class II) (Fig. 1 E) and VEGF (Fig. 1 F). In both groups the majority (＞90%) of VEGF expressing cells were HLA-DR positive. Increased activation of peritoneal fluid macrophages in endometriosis was confirmed after the assessment of the acid phosphatase activity of the peritoneal fluid, this being a marker for macrophage activation. Acid phosphatase activity in peritoneal fluid from women with endometriosis (n=15) was significantly higher (P ＜ 0.01) (15.7±2.2 U/ml) than in women with no endometriosis (n=10) (8.55±2.3 U/ml).

VEGF-dependent increase in endothelial cell proliferation in endometriosis. The effect of media conditioned by peritoneal macrophages on endothelial cell proliferation was determined by assessment of [3H]thymidine incorporation. Neutralizing experiments using a specific anti-VEGF antibody were carried out in parallel. Peritoneal fluid macrophage conditioned media from patients with endometriosis resulted in a significantly greater (P ＜ 0.05) incorporation of [3H]thymidine than that of media conditioned by macrophages from normal women (Fig. 2). This additional incorporation was completely blocked by the inclusion of anti-VEGF neutralizing mAb to the culture medium. This reduced the incorporation of [3H]thymidine to levels comparable to those seen in medium conditioned by macrophages removed from women without endometriosis (Fig. 2). Thus the additional endothelial cell proliferation induced by the peritoneal fluid macrophages in endometriosis was attributable to VEGF. Studies using antibody alone in normal media had no effect on the incorporation of [3H]thymidine. No phase of cycle differences were seen, for either patient group.

Control of VEGF secretion by peritoneal fluid macrophages. To investigate the control of VEGF secretion by peritoneal fluid macrophages we measured VEGF secretion by activated (LPS 10 ng/ml) and nonactivated peritoneal fluid macro-

![Figure 2. Effect of peritoneal fluid macrophage conditioned media on HUVEC proliferation. [3H]thymidine incorporation was determined in HUVECs exposed to media conditioned by macrophages. Neutralization studies were carried out using an anti-human VEGF neutralizing antibody. Results are expressed as percentage of control (mean±SD). Nonmacrophage-conditioned media equals 100%. Statistics were carried out on the original data. * Significantly greater compared to nonmacrophage-conditioned media for each group. (P ＜ 0.05). ** Significantly greater compared to media conditioned by normal (nonendometriotic) macrophages. ' Significantly less than media conditioned by macrophages from women with endometriosis (P ＜ 0.05). Eight patients were sampled from each patient group, with four from the proliferative and four from the secretory phase of the cycle.](image)

![Figure 3. VEGF production from activated and/or steroidally treated peritoneal fluid macrophages. Isolated peritoneal fluid macrophages from six women without endometriosis (three proliferative and three secretory phase samples), untreated or activated by LPS (10 ng/ml), were incubated with either estradiol-17β (10⁻⁸ M) or progesterone (10⁻⁸ M) for 24 h, and the resultant conditioned media was assayed for VEGF by ELISA. * Significantly greater compared to untreated non-activated macrophage media, within each treatment group. (P ＜ 0.05). ** Significantly greater compared to untreated LPS activated macrophage media.](image)
vated in peritoneal fluid (40), and data exist to show that these fluid macrophages.

Ovarian steroid concentrations are elevated in peritoneal fluid (40), and data exist to show that these cells to steroids.

VEGF receptors on peritoneal fluid macrophages. Peritoneal fluid macrophages, from both patient groups, contained mRNA encoding the VEGF receptors flt and KDR and subjected to FACS (Fig. 4 A). No phase of cycle dependent variation in the expression of these receptor mRNAs was evident for either group. Direct stimulation of primary cultures of peritoneal fluid macrophages with estradiol-17β and progesterone (Fig. 3), resulted in an approximate twofold increase in the secretion of VEGF by peritoneal fluid macrophages. Previous activation of the cells with LPS (10 ng/ml) resulted in a further elevation in the steroid-stimulated levels of macrophage secreted VEGF (Fig. 3). There was no significant difference in response between estradiol-17β, nor progesterone or estradiol-17β/ progesterone in combination (data not shown). No phase of cycle differences were seen. Thus peritoneal fluid macrophages are directly stimulated to produce VEGF by estradiol-17β and progesterone and activation enhanced the response of these cells to steroids.

We therefore investigated the regulation of VEGF secretion from peritoneal fluid macrophages by ovarian steroids. RT-PCR for the estrogen and progesterone receptors revealed that peritoneal fluid macrophages isolated from both patient groups contained the mRNAs encoding for these receptors (Fig. 4 A). No phase of cycle dependent variation in the expression of these receptor mRNAs was evident for either group. Direct stimulation of primary cultures of peritoneal fluid macrophages with estradiol-17β and progesterone (Fig. 3), resulted in an approximate twofold increase in the secretion of VEGF by peritoneal fluid macrophages. Previous activation of the cells with LPS (10 ng/ml) resulted in a further elevation in the steroid-stimulated levels of macrophage secreted VEGF (Fig. 3). There was no significant difference in response between estradiol-17β, nor progesterone or estradiol-17β/ progesterone in combination (data not shown). No phase of cycle differences were seen. Thus peritoneal fluid macrophages are directly stimulated to produce VEGF by estradiol-17β and progesterone and activation enhanced the response of these cells to steroids.

### Figure 4

Expression of mRNA encoding for oestrogen, progesterone, flt and KDR, receptors in isolated peritoneal fluid macrophages as assayed by RT-PCR. Ethidium-stained agarose gels showing representative products amplified from cDNA derived from peritoneal fluid macrophages. (A) Estrogen and progesterone receptors. Lane 1, 100-bp mol wt marker. Lane 2, positive control using placental cDNA. Lane 3, negative control (no cDNA). Lane 4, macrophage cDNA from a normal patient (proliferative phase). Lane 5, macrophage cDNA from a normal patient (secretory phase). Lane 6, macrophage cDNA from an endometriotic patient (proliferative phase). Lane 7, macrophage cDNA from an endometriotic patient (secretory phase). (B) VEGF receptors KDR and flt. Lane 1, 100-bp mol wt marker. Lane 2, negative control (no cDNA). Lane 3, positive control using placental cDNA. Lane 4, macrophage cDNA from a normal patient (proliferative phase). Lane 5, macrophage cDNA from an endometriotic patient (proliferative phase). Lane 6, macrophage cDNA from an endometriotic patient (proliferative phase). Lane 7, macrophage cDNA from an endometriotic patient (secretory phase). Calculated molecular weights of product bands are indicated.

### Figure 5

Peritoneal fluid macrophages immunostained for the VEGF receptors flt and KDR and subjected to FACS. (A) Histogram of the percentage of flt positive peritoneal fluid macrophages isolated from a patient with endometriosis who was in the luteal phase of the menstrual cycle, stained for flt and KDR, respectively. These graphs show a high percentage of VEGF receptor positive peritoneal fluid macrophages. (Graph B) Histogram of the percentage of flt-positive peritoneal fluid macrophages from women with and without endometriosis. No significant difference in the percentage of flt positive macrophages was seen between the two patient groups, nor was there any cycle specific variation within each patient group. (Graph D) Histogram showing the percentage of KDR positive peritoneal fluid macrophages found throughout the cycle for both patient groups. "Significantly greater (P < 0.01) compared to normal proliferative phase; "* significantly greater (P < 0.05) compared to endometriotic proliferative phase; "significantly greater (P < 0.05) compared to normal secretory phase. Twelve patients were sampled from each patient group for both VEGF receptors, with six from the proliferative and six from the secretory phase of the cycle.
However, cyclical differences in the expression of KDR were evident in both groups of patients (Fig. 5 D). The percentage of KDR positive peritoneal fluid macrophages was significantly higher in the secretory phase of the cycle for both normal (32±20%) (P < 0.01) and endometriotic patients (80±15%) (P < 0.05) compared to the numbers in the proliferative phase of the cycle (6±3% and 23±12%, respectively) (Fig. 5 D). In endometriosis the number of KDR-positive macrophages detected during the secretory phase of the cycle was significantly higher than that seen in the normal group (P < 0.05) (Fig. 5 D).

**VEGF-mediated migration of peritoneal fluid macrophages.** The presence of VEGF receptors might be expected to enable these cells to respond to exogenous VEGF. The chemotactic response to VEGF of peritoneal fluid macrophages, from both patient groups, was therefore determined. Only peritoneal fluid macrophages taken in the luteal phase of the cycle from women with endometriosis showed a significant (P < 0.05) dose-dependent chemotactic response to exogenous VEGF (Fig. 6). This coincides with the increased number of KDR-positive macrophages (Fig. 5 D). No significant chemotaxis was observed with macrophages taken from women without endometriosis, independent of the time of cycle.

**Discussion**

In this study we have demonstrated that peritoneal fluid macrophages are the principal source of the angiogenic growth factor VEGF and that the enhanced endothelial cell proliferation induced by conditioned medium from macrophages, isolated from the peritoneal cavity of women with endometriosis, was abolished by anti-VEGF antibody. Peritoneal fluid macrophages were directly stimulated to secrete VEGF by ovarian steroids, a response which is enhanced if the cells have been previously activated with LPS. In addition, peritoneal fluid macrophages expressed both VEGF receptors flt and KDR with the expression of the latter being cycle specific suggesting that steroids may influence the autocrine regulation of macrophage function.

In normal endometrium VEGF expression is present in both glandular epithelium and stroma, depending on the phase of the cycle (5, 44). However in ectopic tissue there was limited expression of VEGF. Intense immunopositive staining for VEGF was only seen in individual tissue macrophages distributed throughout the stroma (Fig. 1, C and D). This suggests that ectopic endometrium is not the primary source of VEGF in the peritoneal cavity of women with endometriosis.

Several growth factors which may have angiogenic activity such as IL-8 (45) and TNF-α (27) have been found to be elevated in the peritoneal fluid of women with endometriosis. However, these studies did not identify the origin of these factors, nor fully demonstrated their angiogenic potential. Previous work in our laboratory has shown elevated levels of VEGF in the peritoneal fluid of women with endometriosis (25). VEGF is also a potent inducer of vascular permeability (14), and since VEGF is elevated in the peritoneal fluid of women with endometriosis, it might be expected that there would be a concomitant increase in vascular permeability activity. However, there is no direct evidence to support this, but increases in the volume of peritoneal fluid seen in women with endometriosis may reflect this. In this study we have shown VEGF is synthesized and secreted by activated peritoneal fluid macrophages, by double immunohistochemical staining, (Fig. 1 E and F) and ELISA. The ability of macrophage-derived VEGF to induce one of the critical events in angiogenesis, endothelial cell proliferation, was confirmed using incorporation of [H]thymidine in HUVECs (Fig. 2). A significantly greater increase was achieved by media conditioned by macrophages obtained from women with endometriosis. This effect was completely inhibited by incubation with a specific anti-VEGF monoclonal antibody. This suggests that most of the enhanced angiogenic activity found in the peritoneal fluid of patients with endometriosis is due to VEGF. Since the addition of the neutralizing antibody had no effect on the basal levels of [H]thymidine incorporation, it also suggests that normally the levels of bioactive VEGF in peritoneal fluid are low.

The female reproductive tract undergoes a number of cyclical changes including the development and the decline of ovarian and uterine structures under the influence of steroid hormones (46). These steroids are essential for the maintenance of ectopic endometrium (47). To date it was assumed that this was a consequence of the direct actions of steroids on the endometriotic tissue. Indirect actions of the steroids on cells within the peritoneal fluid have generally been overlooked. We therefore investigated the regulation of VEGF secretion from human peritoneal fluid macrophages by ovarian steroids. These cells contain the mRNAs encoding both ER and PR (Fig. 4 A) and show strong nuclear staining with anti-receptor monoclonal antibodies (data not shown). Most significantly, the secretion of VEGF from isolated peritoneal fluid macrophages was greatly enhanced by treatment, in culture, with estradiol-17β (10⁻⁸ M) and progesterone (10⁻⁶ M) (Fig. 3).
While these doses are not in the range normally found in the peripheral blood of endometriotic patients (40) they are present in the peritoneal fluid after ovulation.

The findings from this study provide, for the first time, strong evidence that ovarian steroids directly regulate the secretion of a potent angiogenic growth factor from human peritoneal fluid macrophages. This steroidal stimulation of VEGF secretion was greater from activated macrophages, which are more frequently found in endometriotic patients (32, 33). These findings may provide an alternative explanation for the regression of ectopic endometrium after treatment to reduce or antagonize the action of estradiol-17β, by causing reductions in macrophage derived VEGF levels leading to reduced angiogenesis or maintenance of existing blood vessels. Recent evidence suggests that VEGF is not only a potent angiogenic growth factor but may also act as an endothelial cell survival factor (48).

The VEGF receptors flt and KDR are tyrosine kinase receptors expressed predominately on endothelial cells (21, 49). The binding of VEGF to these receptors results in a number of responses including increased mitogenesis, changes in cell morphology, enhanced migration and the release of various proteolytic enzymes (14–16). VEGF receptors are also found on non-endothelial cells including peripheral blood monocytes (50) (the cellular precursors of macrophages), malignant melanoma cell lines (51), ovarian carcinoma tumour cells (37), and trophoblasts (52). These cells are all highly invasive. Here we show for the first time the presence of both types of VEGF receptors on peritoneal fluid macrophages by RT-PCR (Fig. 4B) and fluorescence activated cell sorting (Fig. 5). Coexpression of VEGF and its receptors on these cells raises the possibility of an autocrine stimulation and of therapeutic strategies targeting this receptor-ligand interaction. Steroidal regulation of KDR expression, suggested by the cycle dependent expression of KDR, (Fig. 5 D) indicates further steroidal regulation of macrophage function. What effect the presence of increased numbers of KDR-positive macrophages might have on the course of endometriosis is unclear. However, the finding that VEGF only stimulated migration of peritoneal fluid macrophages from women with endometriosis in the luteal phase, when they express KDR (Fig. 6) may explain, in part, the enhanced number of macrophages found in the endometriotic explants and peritoneal fluid of women with endometriosis.

Previously, therapeutic strategies for endometriosis have modulated the steroidal support assumed to be needed for the maintenance of ectopic endometrium. However, this study suggests that VEGF plays a critical role in the aetiology of endometriosis and that it is derived not from ectopic endometrium but from activated peritoneal fluid macrophages. That its expression in these cells is regulated by ovarian steroids which in addition modulate the autocrine response to VEGF provides evidence for an alternative explanation of the disease. Since endometriosis is characterized by pronounced vascularization both within and surrounding the ectopic tissue, elevated levels of the potent angiogenic factor VEGF in the peritoneal fluid and the presence of VEGF-positive macrophages within the ectopic tissue will be of critical clinical importance in this disease. VEGF-induced angiogenesis may therefore be a critical aspect of the pathophysiology of this disease, consequently a new clinical approach to this disease may be required with anti-angiogenic therapies being the way forward for the treatment of endometriosis.

Acknowledgments

We would like to thank the consultants, junior doctors, and theatre staff for their invaluable contributions to this work. In addition we would like to thank Mrs. Sue Dunn for the collection of samples and organization of the tissue bank.

This work was generously supported by a grant from the Wellcome Trust (037011/Z/92).

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