

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

J. McLaren,* A. Prentice,* D.S. Charnock-Jones,* S.A. Millican,† K.H. Müller,‡ A.M. Sharkey,* and S.K. Smith*

*Reproductive Molecular Research Group, Department of Obstetrics & Gynaecology, University of Cambridge, The Rosie Maternity Hospital, Cambridge CB2 2NS; †Department of Pathology, University of Cambridge, Cambridge CB2 1QJ, United Kingdom

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 chorioallantoic 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-1 β (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-

Address correspondence to Dr. John McLaren, Department of Obstetrics & Gynaecology, University of Leicester, School of Medicine, Robert Kilpatrick Clinical Sciences Building, Leicester Royal Infirmary, P.O. Box 65, Leicester LE2 7LX, United Kingdom. Phone: 116-232-3161; FAX: 116-252-3154.

Received for publication 5 January 1996 and accepted in revised form 11 April 1996.

J. Clin. Invest.

© The American Society for Clinical Investigation, Inc.

0021-9738/96/07/0482/08 \$2.00

Volume 98, Number 2, July 1996, 482–489

1. Abbreviations used in this paper: flt, fms-like tyrosine kinase; HUVEC, human umbilical vein endothelial cell; KDR, kinase domain receptor; RT, reverse transcription; VEGF, vascular endothelial growth factor.

lature and the subsequent maintenance of endometriotic explants.

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

Methods

Patient details. Women between 24 and 44-yr-old who were undergoing either diagnostic laparoscopy for dysmenorrhoea or elective laparoscopy for infertility were recruited for this study. Patients were diagnosed 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 non-endometriotic patients ($n = 34$). The extent of endometriosis was determined 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 aspirated from the posterior cul-de-sac. Peritoneal fluid macrophages were isolated by the method of Halme et al. (32, 33), and this method has been extensively characterized. It results in an enriched population 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 macrophages for immunohistochemistry were spotted onto untreated slides and the purity of the preparation was determined using immunohistochemical 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 peroxidase 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, Cambridge, UK). Binding was visualized after incubation with a biotinylated goat anti-rabbit antibody (1:200) for 1 h, and subsequent complexing with avidin-biotin peroxidase (Vector, Peterborough, UK). The complex was detected with diaminobenzidine and hydrogen peroxide in 0.1 M Tris-HCl, pH 7.5. Positive controls included the detection of immunostaining in the placental chorionic plate (35).

Fluorescence double-staining for VEGF-positive macrophages was carried out on both peritoneal fluid macrophage slides and ectopic tissue sections. VEGF was identified using the rabbit polyclonal VEGF antibody (1/50 dilution), while activated peritoneal fluid macrophages were visualized by the macrophage priming and activation marker HLA-DR (1/50 dilution) (MHC class II) (Dako). Ectopic tissue macrophages were identified using the macrophage marker Leu M3 (CD14) (1/50 dilution) (Dako). Slides were treated as above, before incubation for 1 h with the appropriate antibody. An isotype-matched irrelevant mouse monoclonal was used as a control for these mAbs and peptide preabsorbed anti-VEGF polyclonal for the VEGF. The slides were then incubated for 1 h, in the dark, with a 1/200 dilution of FITC-conjugated goat anti-mouse Ig or tetramethylrhodamine isothiocyanate (TRITC)-conjugated goat anti-rabbit Ig. Slides were washed and mounted in aqueous mounting medium and viewed immediately.

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 L-glutamine, containing 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 previously 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 using a specific anti-human VEGF neutralizing antibody (R&D Systems, Abingdon, UK) were carried out in parallel at a concentration of 800 ng/ml. After 24 h, [³H]thymidine (1 μCi/ml) (Amersham International, Little Chalfont, UK) was added and incorporation 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 peritoneal fluid macrophages was extracted and analyzed for mRNA encoding the flt and KDR receptors by RT-PCR as previously described (37). The presence of mRNA encoding the estrogen and progesterone receptors in peritoneal fluid macrophages was determined using forward and reverse primers synthesized to anneal with cDNA for estrogen (38) and progesterone (39) receptors. Amplification was in two stages: Estrogen receptor primers 1 (GGAGACATGAGAGCTGCCAA) and 2 (CCAGCAGCATGTCTGAAGATC) for 95°C, 90 s (95°C, 30 s; 59°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 (CTTTGGCCAAGCCCGCTC) and 4 (TCATCATGCGGAACCGAGAT) and reamplified using the above conditions but at a lower annealing temperature (58°C). Progesterone receptor primers 1 (GGTCTTCCTTAAGAGGACA) and 2 (GACTTCGTAGCCCTTCCA) for 95°C, 90s [95°C, 30 s; 53°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 (GGAAGGGCAGCACA ACTA) and 4 (AAGGAATGTATTAAGAAGTAA) and reamplified using the above conditions 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 macrophages. Isolated peritoneal fluid macrophages from women without 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 (10⁻⁶ 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 before 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 for flt and KDR. Isolated peritoneal fluid macrophages from both patient groups were incubated with a 1:50 dilution of flt or KDR rabbit polyclonal (Santa Cruz Biotechnology, Hatfield, 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 fluorescence activated cell sorting (FACS®) analysis. Controls comprised of the same polyclonal antibodies which had previously being preabsorbed a relevant control peptide. In addition, tubes containing swine anti-rabbit-FITC labeled F(ab)₂ fragment alone and an irrelevant directly 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, Pleasanton, 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

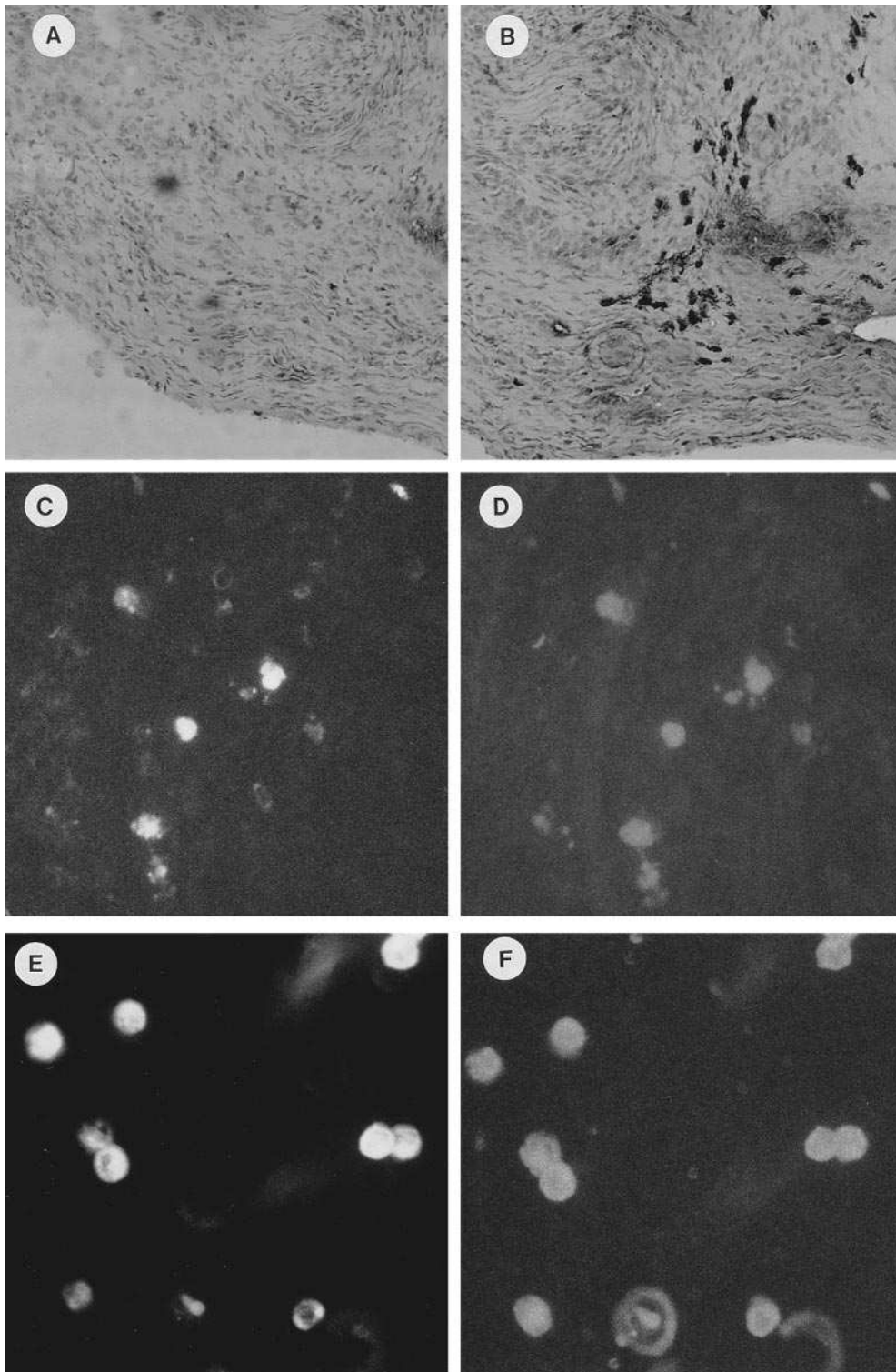


Figure 1. (A and B) Immunohistochemical staining for VEGF protein in endometriotic tissue showing immunoreactivity in isolated cells in the stroma. A is the negative control after staining with the peptide preabsorbed anti-VEGF antibody. B is the anti-VEGF polyclonal antibody. Individual cells, within 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. ($\times 250$). (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) ($\times 400$). (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 ($\times 400$).

incubated at 37°C for 5 min before the cells were added to the top chamber ($50\ \mu\text{l}$). 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-quick 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 ($5 \times 10^{-8}\ \text{M}$), 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 $P < 0.05$.

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 [3 H]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 [3 H]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 [3 H]thymidine to levels comparable to those seen with 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 [3 H]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-

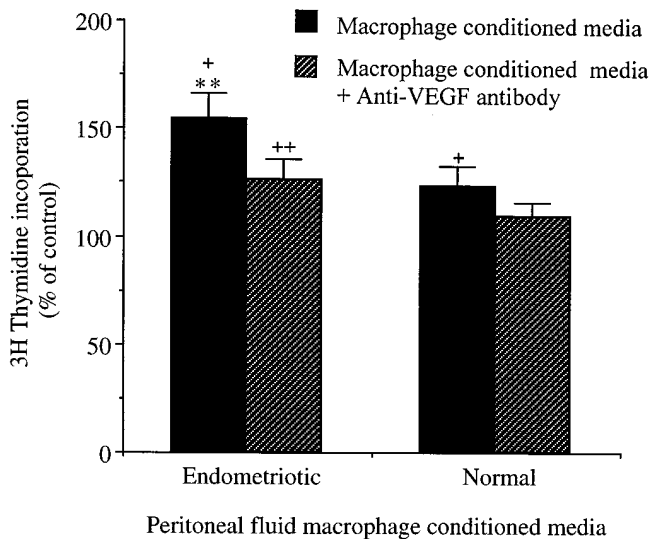


Figure 2. Effect of peritoneal fluid macrophage conditioned media on HUVEC proliferation. [3 H]thymidine incorporation was determined in HUVECs exposed to media conditioned by macrophages. Neutralization studies were carried out using an anti-human VEGF neutralizing antibody. Results are expressed as percentage of control (mean \pm 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.

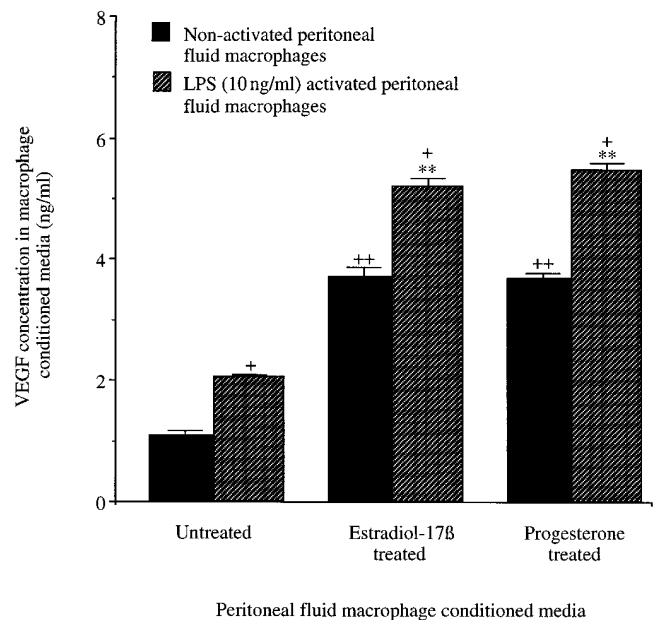


Figure 3. VEGF production from activated and/or steroidally treated peritoneal fluid macrophages. Isolated peritoneal fluid macrophages from six women without endometriosis (three proliferative and three secretory phase samples), untreated or activated by LPS (10 ng/ml), were incubated with either estradiol-17 β (10^{-8} M) or progesterone (10^{-6} 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 nonactivated macrophage media ($P < 0.05$). ^{**}Significantly greater than untreated LPS activated macrophage media.

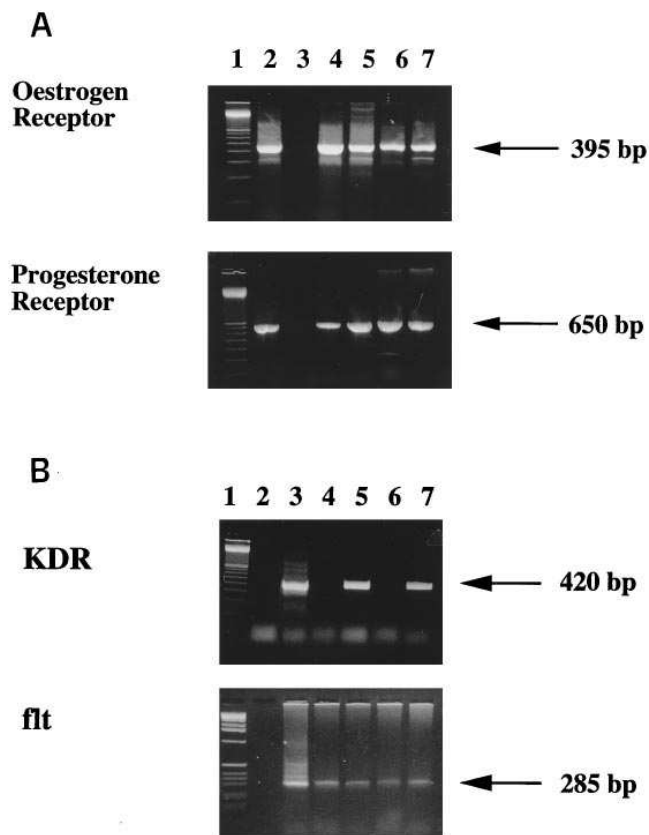


Figure 4. Expression of mRNA encoding for oestrogen, progesterone, flt and KDR, receptors in isolated peritoneal fluid macrophages as assayed by RT-PCR. Ethidium-stained agarose gels showing representative products amplified from cDNA derived from peritoneal fluid macrophages. (A) Estrogen and progesterone 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 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). Calculated molecular weights of product bands are indicated.

phages, isolated from women without endometriosis. Macrophages from women with endometriosis were not used in order to eliminate any problems due to differences in the basal activation status of these cells. VEGF was secreted from both activated and nonactivated peritoneal fluid macrophages (Fig. 3). However, activation significantly ($P < 0.05$) elevated the amount of VEGF secreted by these cells (Fig. 3). No phase of cycle differences were seen.

Steroid stimulation of VEGF production by peritoneal fluid macrophages. Ovarian steroid concentrations are elevated in peritoneal fluid (40), and data exist to show that these steroids can regulate the secretion of a number of factors from both animal and human peritoneal fluid macrophages (41–43).

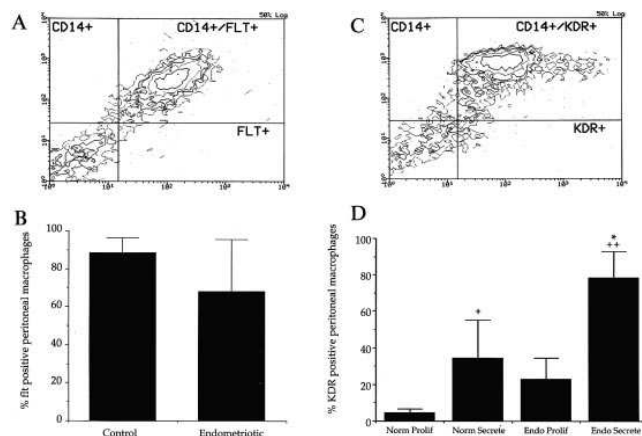


Figure 5. Peritoneal fluid macrophages immunostained for the VEGF receptors flt and KDR and subjected to FACS. Graphs A and C. Representative contour plots of peritoneal fluid macrophages isolated from a patient with endometriosis who was in the luteal phase of the menstrual cycle, stained for flt and 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.

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-17 β 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-17 β , nor progesterone or estradiol-17 β /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-17 β and progesterone and activation enhanced the response of 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 as assessed by RT-PCR (Fig. 4 B) and demonstrated immunoreactivity for the receptors as shown by FACS (Fig. 5). No difference in the percentage of flt-positive macrophages was seen between the normal ($87 \pm 9\%$) and endometriotic group ($68 \pm 30\%$) (Fig. 5 B).

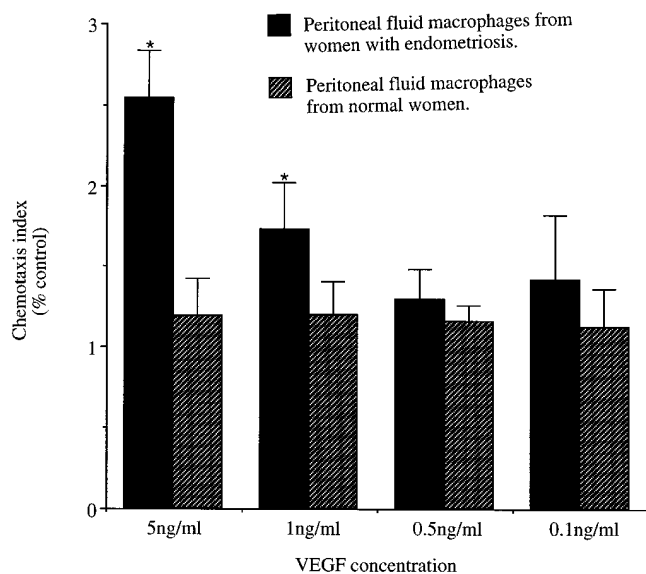


Figure 6. Effect of VEGF on peritoneal fluid macrophage migration. Peritoneal fluid macrophages were added to the upper compartment of a chemotaxis chamber and migration was initiated by placing VEGF, at the indicated concentration, in the lower well. Only peritoneal fluid macrophages isolated from the luteal phase of women with endometriosis demonstrated significant migration in response to VEGF. Results are expressed as mean \pm SD. *Significantly greater compared to normal luteal phase macrophages ($P < 0.01$). Eight patients were sampled from each patient group, with four from the proliferative and four 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 ($32 \pm 20\%$) ($P < 0.01$) and endometriotic patients ($80 \pm 15\%$) ($P < 0.05$) compared to the numbers in the proliferative phase of the cycle ($6 \pm 3\%$ and $23 \pm 12\%$, 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 [3 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 [3 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-17 β (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-17 β , 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 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. 5D) 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).

References

1. Strathy, J.H., C.A. Molgaard, and C.B. Coulman. 1982. Endometriosis and infertility: a laparoscopic study of endometriosis among fertile and infertile women. *Fertil. Steril.* 38:667–672.
2. Thomas, E.J., and A. Prentice. 1992. The aetiology and pathogenesis of endometriosis. *Reprod. Med. Rev.* 1:21–36.
3. Nisolle, M., F. Casanas-Roux, V. Anaf, J.-M. Mine, and J. Donnez. 1993. Morphometric study of the stromal vascularisation in peritoneal endometriosis. *Fertil. Steril.* 59:681–684.
4. Folkman, J., and M. Klagsbrun. 1987. Angiogenic factors. *Science (Wash. DC)*. 235:442–446.
5. Charnock-Jones, D.S., A.M. Sharkey, J. Rajput-Williams, D. Burch, J.P. Schofield, S.A. Fountain, C.A. Boocock, and S.K. Smith. 1993. Identification and localisation of alternately spliced mRNAs for vascular endothelial growth factor in human uterus and estrogen regulation in endometrial carcinoma cell line. *Biol. Reprod.* 48:1120–1128.
6. Ravindranath, N., L. Little-Ihrig, H.S. Phillips, N. Ferrara, and J. Zeleznik. 1991. Vascular endothelial growth factor messenger ribonucleic acid expression in the primate ovary. *Endocrinology*. 131:254–260.
7. Breier, G., U. Albrecht, S. Sterrer, and W. Risau. 1992. Expression of vascular endothelial growth factor during embryonic angiogenesis and endothelial cell differentiation. *Development (Camb.)*. 114:521–532.
8. Potgens, A.J.G., N.H. Lubsen, M.C. Van Altena, J.G.G. Schoenmakers, D.J. Ruiter, and R.M.W. De Waal. 1995. Vascular permeability factor expression influences tumour angiogenesis in human melanoma lines xenografted to nude mice. *Am. J. Pathol.* 146:147–209.
9. Senger, D.R., L. Van De Water, L.F. Brown, J.A. Nagy, K.-T. Yeo, T.-K. Yeo, B. Berse, R.W. Jackman, A.M. Dvorak, and H.F. Dvorak. 1993. Vascular permeability factor (VPF, VEGF) in tumour biology. *Cancer Metastasis Rev.* 12:303–324.
10. Kim, K.J., B. Li, J. Winer, M. Armanini, N. Gillet, H.S. Phillips, and N. Ferrara. 1993. Inhibition of vascular endothelial growth factor induced angiogenesis suppresses tumour growth in vivo. *Nature (Lond.)*. 362:841–844.
11. Koch, A.E., L.A. Harlow, G.K. Haines, E.P. Amento, E.N. Unemori, W.L. Wong, R.M. Pope, and N. Ferrara. 1995. Vascular endothelial growth factor. A cytokine modulating endothelial function in rheumatoid arthritis. *J. Immunol.* 152:4149–4156.
12. Ross, R. 1993. The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature (Lond.)*. 362:801–809.
13. Houck, K.A., N. Ferrara, J. Winer, G. Cachianes, B. Li, and D.W. Leung. 1991. The vascular endothelial growth factor family: identification of a fourth molecular species and characterisation of alternative splicing of RNA. *Mol. Endocrinol.* 5:1806–1814.
14. Connolly, D.T., D.M. Heuvelman, R. Nelson, J.V. Olander, B.L. Eppley, J.J. Bellino, N.R. Siegel, R.M. Leimgruber, and J. Feder. 1994. Tumor vascular permeability factor stimulates endothelial cell growth and angiogenesis. *J. Clin. Invest.* 84:213–217.
15. Ferrara, N., K. Houck, L. Jakeman, and D.W. Leung. 1992. Molecular and biological properties of the vascular endothelial growth factor family of polypeptides. *Endocr. Rev.* 13:18–32.
16. Leung, D.W., G. Cachianes, W.-J. Kuang, D.V. Goeddel, and N. Ferrara. 1989. Vascular endothelial growth factor is a secreted angiogenic mitogen. *Science (Wash. DC)*. 246:1306–1309.
17. Phillips, G.D., A.-M. Stone, B.D. Jones, J.C. Schultz, R.A. Whitehead, and D.R. Knighton. 1994. Vascular endothelial growth factor (rhVEGF165) stimulates direct angiogenesis in the rabbit cornea *in vivo* (Attiki). 8:961–965.
18. Wilting, J., B. Christ, M. Bokeloh, and H.A. Weich. 1993. In vivo effects of vascular endothelial growth factor on chicken chorioallantoic membrane. *Cell Tissue Res.* 274:163–172.
19. de Vries, C., J.A. Escobedo, H. Ueno, K. Houck, N. Ferrara, and L.T. Williams. 1992. The fms-like tyrosine kinase, a receptor for vascular endothelial growth factor. *Science (Wash. DC)*. 255:989–991.
20. Terman, B.I., M. Dougher-Verzamen, M.E. Carrion, D. Dimitrov, D.C. Armellino, D. Gospodarowicz, and P. Bohlen. 1993. Identification of the KDR tyrosine kinase as a receptor for vascular endothelial cell growth factor. *Biochem. Biophys. Res. Commun.* 187:1579–1586.
21. Jakeman, L.B., J. Winer, G.L. Bennett, A. Altar, and N. Ferrara. 1992. Binding sites for vascular endothelial growth factor are localized on endothelial cell in adult rat tissues. *J. Clin. Invest.* 89:244–253.

22. Pepper, M.S., N. Ferrara, L. Orcil, and R. Montesano. 1991. Vascular endothelial growth factor (VEGF) induces plasminogen activators and plasminogen activator inhibitor-1 in microvascular endothelial cells. *Biochem. Biophys. Res. Commun.* 181:902–906.
23. Unemori, E.N., N. Ferrara, E.A. Bauer, and E.P. Amento. 1992. Vascular endothelial growth factor induces interstitial collagenase expression in human endothelial cells. *J. Cell. Physiol.* 153:557–562.
24. Oosterlynck, D.J., C. Meuleman, H. Sobis, M. Vandeputte, and P.R. Koninckx. 1993. Angiogenic activity of peritoneal fluid from women with endometriosis. *Fertil. Steril.* 59:778–782.
25. McLaren, J., A. Prentice, D.S. Charnock-Jones, and S.K. Smith. 1995. Vascular endothelial growth factor concentrations are elevated in peritoneal fluid of women with endometriosis. *Hum. Reprod. (Oxf.)* 11:220–223.
26. Eischen, A., B. Duclos, M. Schmitt-Gognel, N. Rouyer, J.P. Bergerat, M. Hummel, R. Oskam, and Fr. Oberling. 1994. Human resident peritoneal macrophages: phenotype and histology. *Br. J. Haematol.* 88:712–722.
27. Halme, J. 1989. Release of TNF- α by human peritoneal macrophages in vivo and in vitro. *Am. J. Obstet. Gynecol.* 161:1718–1725.
28. Polan, M.L., J. Loukides, P. Nelson, S. Carding, M. Diamond, A. Walsh, and K. Bottomly. 1989. Progesterone and estrogen modulate interleukin-1b mRNA levels in cultured human peripheral monocytes. *J. Clin. Endocrinol. & Metab.* 69:1200–1206.
29. Simoni, J., G. Simoni, C.D. Lox, D.E. McGunagle, and M. Feola. 1994. Cytokines and PAF release from human monocytes and macrophages. Effects of hemoglobin and contaminants. *Artif. Cells Blood Substit. Immobil. Biotechnol.* 22:523–534.
30. Rier, S.E., A.K. Parsons, and J.L. Becker. 1994. Altered interleukin-6 production by peritoneal leukocyte from patients with endometriosis. *Fertil. Steril.* 61:294–299.
31. Keenan, J.A., T.T. Chen, N.L. Chadnell, D.S. Torry, and M.R. Caudle. 1994. Interferon- γ and interleukin-6 in peritoneal fluid and macrophages conditioned media of women with endometriosis. *Am. J. Reprod. Immunol.* 32:180–183.
32. Halme, J., S. Becker, M.G. Hammond, M.H.G. Raj, and S. Raj. 1983. Increased activation of pelvic macrophages in infertile women with mild endometriosis. *Am. J. Obstet. Gynecol.* 145:333–337.
33. Halme, J., S. Becker, and S. Haskill. 1987. Altered maturation and function of peritoneal macrophages: possible role in pathogenesis of endometriosis. *Am. J. Obstet. Gynecol.* 156:783–789.
34. Berse, B., L.F. Brown, L. Van De Water, H.F. Dvorak, and D.R. Senger. 1992. Vascular permeability factor (VEGF) gene is expressed differentially in normal tissue, macrophages and tumours. *Mol. Biol. Cell.* 3:211–220.
35. Sharkey, A.M., J.D. Charnock, C.A. Boocock, K.D. Brown, and S.K. Smith. 1993. Expression of mRNA for vascular endothelial growth factor in human placenta. *J. Reprod. Fertil.* 99:609–615.
36. Jaffe, E.A., R.L. Nachman, C.G. Becker, and C.R. Minick. 1973. Culture of human endothelial cells derived from umbilical veins. *J. Clin. Invest.* 52:2745–2756.
37. Boocock, C.A., D.S. Charnock-Jones, A.M. Sharkey, J. McLaren, P.J. Barker, K.A. Wright, P.R. Twentyman, and S.K. Smith. 1995. Expression of vascular endothelial growth factor and its receptors flt and KDR in ovarian carcinoma. *J. Natl. Cancer Inst.* 87:506–516.
38. Misrahi, M., M. Atger, L. D'Auriol, H. Loosfelt, C. Meriel, F. Fridlansky, A. Guiochon-Mantel, F. Galibert, and E. Milgrom. 1987. Complete amino acid sequence of the human progesterone receptor deduced from cloned cDNA. *Biochem. Biophys. Res. Commun.* 143:740–748.
39. Greene, G.L., P. Gilna, M. Waterfield, A. Baker, Y. Hort, and J. Shine. 1986. Sequence and expression of human estrogen receptor complementary DNA. *Science (Wash. DC)* 231:1150–1154.
40. DeLeon, F.D., R. Vijayakumar, M. Brown, C.H.V. Rao, M.A. Yussman, and G. Schultz. 1986. Peritoneal fluid volume, estrogen, progesterone, prostaglandin, and epidermal growth factor concentrations in patients with and without endometriosis. *Obstet. Gynecol.* 68:189–194.
41. Hu, S.-K., Y.L. Mitcho, and N.C. Rath. 1988. Effect of estradiol on interleukin 1 synthesis by macrophages. *Int. J. Immunopharmacol.* 10:247–252.
42. Frazier-Jessen, M.R., and E.J. Kovacs. 1995. Estrogen modulation of JE/monocyte chemoattractant protein-1 mRNA expression in murine macrophages. *J. Immunol.* 154:1838–1845.
43. Chao, T.-C., P.J. Van Alten, J.A. Greager, and R.J. Walter. 1995. Steroid sex hormones regulate the release of tumor necrosis factor by macrophages. *Cell. Immunol.* 160:43–49.
44. Le, X.-Y., J. Gregory, and A. Ahmed. 1994. Immunohistochemical localisation of vascular endothelial growth factor in human endometrium. *Growth Factors.* 11:277–282.
45. Ryan, I.P., T.F. Tseng, E.D. Schriock, O. Khorram, D.V. Landers, and R.N. Taylor. 1995. Interleukin-8 concentrations are elevated in peritoneal fluid of women with endometriosis. *Fertil. Steril.* 63:929–932.
46. Johnson, M.H., and B.J. Everitt. 1995. Actions of steroid hormones in the adult. In *Essential Reproduction*. M. H. Johnson and B.J. Everitt, editors. Blackwell Science, Oxford. 122–140.
47. Bergqvist, A. 1992. Steroid receptors in endometriosis. In *Modern Approaches to Endometriosis*. E. Thomas and J. Rock, editors. Kluwer Academic Publishers Group, Dordrecht, The Netherlands. 257–274.
48. Alon, T., I. Hemo, A. Itin, J. Pe'er, J. Stone, and E. Keshet. 1995. Vascular endothelial growth factor acts as a survival factor for newly formed retinal vessels and has implications for retinopathy of prematurity. *Nat. Med.* 1:1024–1028.
49. Ferrara, N., and W.J. Henzel. 1989. Pituitary follicular cells secrete a novel heparin-binding growth factor specific for vascular endothelial cells. *Biochem. Biophys. Res. Commun.* 161:851–858.
50. Shen, H., M. Clauss, J. Ryan, A.-M. Schmidt, P. Tijburg, L. Borden, D. Connolly, D. Stern, and J. Kao. 1993. Characterisation of vascular permeability factor/vascular endothelial growth factor receptors on mononuclear phagocytes. *Blood.* 10:2767–2773.
51. Gitay-Goren, H., R. Halaban, and G. Neufeld. 1993. Human melanoma cells but not normal melanocytes express vascular endothelial growth factor receptors. *Biochem. Biophys. Res. Commun.* 190:702–708.
52. Charnock-Jones, D.S., A.M. Sharkey, C.A. Boocock, A. Ahmed, R. Plevin, N. Ferrara, and S.K. Smith. 1994. Vascular endothelial growth factor receptor localisation and activation in human trophoblast and choriocarcinoma cells. *Biol. Reprod.* 51:524–530.