

# The relationship between microvessel density, proliferative activity and expression of vascular endothelial growth factor-A and its receptors in eutopic endometrium and endometriotic lesions

V Bourlev, N Volkov, S Pavlovitch, N Lets, A Larsson<sup>1</sup> and M Olovsson<sup>2</sup>

Research Centre of Obstetrics, Gynaecology and Perinatology, Russian Academy of the Medical Sciences, Moscow, Russia, <sup>1</sup>Department of Medical Sciences, Section for Clinical Chemistry and <sup>2</sup>Department of Women's and Children's Health, The Centre for Reproductive Biology in Uppsala, Uppsala University, S-751 85 Uppsala, Sweden

Correspondence should be addressed to M Olovsson; Email: matts.olvsson@kbh.uu.se

## Abstract

Studies were performed to elucidate the possible relationship between microvessel density, proliferative activity and angiogenesis in eutopic endometrium from women with and without endometriosis and peritoneal endometriotic lesions. The question whether changes in these parameters in endometriotic lesions were reflected by the level of vascular endothelial growth factor-A (VEGF-A) in serum and peritoneal fluid was also studied. Biopsy specimens of both eutopic endometrium and peritoneal endometriotic lesions from women with endometriosis ( $n=25$ ) as well as eutopic endometrium from women without endometriosis ( $n=14$ ) were analysed immunohistochemically regarding microvessel density, proliferative activity, and expression of VEGF-A and its receptors vascular endothelial growth factor receptors 1 and 2 (VEGFR-1 and VEGFR-2) in stroma, glands and blood vessels. The VEGF-A concentration was measured in peritoneal fluid and serum. Secretory phase eutopic endometrium from women with endometriosis had significantly higher microvessel density, expression of VEGF-A in glandular epithelium and VEGFR-2 in endometrial blood vessels than those from women without endometriosis. Endometriotic lesions with high proliferative activity had a higher microvessel density and showed higher vascular expression of VEGFR-2 as well as being accompanied by higher levels of VEGF-A in peritoneal fluid and serum, compared with lesions with low proliferative activity. In conclusion, there seems to be a dysregulation of angiogenic activity in the eutopic endometrium of women with endometriosis and endometriotic lesions with high proliferative activity were accompanied by higher local angiogenic activity and higher levels of VEGF in serum and peritoneal fluid.

*Reproduction* (2006) **132** 501–509

## Introduction

Endometriosis is a common gynaecological disease, occurring in about 10% of all women and up to 60–80% of women with infertility problems or pelvic pain (Goldman & Cramer 1990, Koninckx *et al.* 1991). Despite a number of theories concerning the origin of endometriosis (Vinatier *et al.* 2001), the precise pathogenetic mechanisms remain enigmatic. Proposed mechanisms include genetic predisposition (Stefansson *et al.* 2002, Simpson 2003), involvement of the immune system (Berkanoglu & Arici 2003), changes in the peritoneal fluid, peritoneum and endometrium, and retrograde menstruation (Valle 2002). The ability of endometrial fragments to implant is of particular interest in the pathogenesis of peritoneal endometriosis. A number of studies, both *in vivo* and *in vitro*, indicate that endometrial fragments in the menstrual fluid might survive and adhere

to an intact peritoneal surface (Witz *et al.* 2001). Little is known about the regulation of growth of the ectopic endometrium and the recruitment of blood vessels to the endometriotic lesions. It is known, however, that down-regulation of ovarian activity by use of contraceptive pills, gestagens or GnRH analogues usually reduces the activity of endometriotic lesions and relieves the pain (Rice 2002). The effects of these treatments are thought to be due to a reduction in the levels of oestradiol in the pelvic region (DeMayo *et al.* 2002).

It has been shown that eutopic endometrium of women with endometriosis differ from that of healthy women without the disease (Garrido *et al.* 2002). This includes aromatase activity in the eutopic endometrium from women with moderate to severe endometriosis, but not in those of disease-free women (Noble *et al.* 1996). There is also a higher VEGF-A content in the secretory phase

eutopic glandular epithelium (Donnez *et al.* 1998) and an elevated endothelial cell proliferation in women with endometriosis (Wingfield *et al.* 1995), compared with those without. Accumulated data thus suggest that eutopic endometrium of women with endometriosis behaves differently from the endometrium of women without the disease.

An adequate degree of vascularization is a major prerequisite for normal endometrial function (Nisolle *et al.* 1993). It has also been shown that neovascularization is necessary for the survival of tumour implants larger than 2–3 mm<sup>3</sup> (Folkman 1995) and endometriotic lesions recruit blood vessels by inducing angiogenesis (Taylor *et al.* 2002). Several angiogenic growth factors, such as vascular endothelial growth factor-A (VEGF-A), are present in human ectopic and eutopic endometrium (McLaren 2000, Möller *et al.* 2001). VEGF-A is considered important both for angiogenesis in general and endometrial angiogenesis (Smith 1998, Papetti & Herman 2002). VEGF-A exists in several isoforms, all of which are potent stimulators of angiogenesis (Robinson & Stringer 2001). Alternative splicing of a single gene generates six isoforms of VEGF-A composed of 121, 145, 165, 183, 189 and 206 amino acid residues, respectively. The larger isoforms are cell-associated or bound to extracellular matrix components, while VEGF<sub>121</sub> and VEGF<sub>165</sub> are freely diffusible in the extracellular environment and might therefore be identifiable in biological fluids, such as peritoneal fluid or blood. VEGF-A exerts its biological effects by binding to either of its two tyrosine kinase receptors, vascular endothelial growth factor receptors 1 or 2 (VEGFR-1 and VEGFR-2). Among the most important regulators of VEGF gene expression are hypoxia, sex steroids and different cytokines (Shifren *et al.* 1996, Sharkey *et al.* 2000). VEGF-A has been detected in serum and peritoneal fluid from patients with endometriosis (McLaren *et al.* 1996a, Gagne *et al.* 2003).

Proliferating cells can be identified by studying the nuclear or perinuclear expression of Ki-67, a protein that is expressed during all phases of the cell cycle except the G<sub>0</sub> phase. A number of studies have shown good correlation between expression of Ki-67 and the number of mitotic cells (Ueda *et al.* 1989). It has been reported that the mitotic activity changes in both eutopic and ectopic endometrium during the menstrual cycle (Nisolle *et al.* 1993, Jones *et al.* 1995). Recently, it has also been shown that there are differences in the mitotic activity between red, black and white endometriotic lesions and the proliferative activity seems to correlate with the content of VEGF in the peritoneal fluid (Khan *et al.* 2004).

We hypothesise that a high microvessel density should be reflected by a high endothelial cell mitotic activity and also a high expression of the angiogenic growth factor VEGF-A and its two receptors. The main objective of the present study therefore was to examine the relationship between microvessel density, mitotic activity in blood vessels and expression of VEGF-A and

its receptors VEGFR-1 and VEGFR-2 in eutopic endometrium from women with and without endometriosis and in peritoneal endometriotic lesions. We also wanted to determine whether differences between lesions regarding local proliferative activity could be detected by analysing VEGF in serum and peritoneal fluid.

## Materials and Methods

### Patient recruitment

Twenty-five patients of 22–44 years (mean 32.7) with peritoneal endometriosis, and 14 patients of 20–37 years (mean 29.0) without endometriosis, were recruited. The diagnosis of peritoneal endometriosis was confirmed by laparoscopy and histological examination of peritoneal biopsy specimens collected from areas with suspected lesions. The extent of the endometriosis was scored according to the revised American Society for Reproductive Medicine Classification (American Society for Reproductive Medicine 1996) and was equal to stage II–III in all cases of the endometriosis group.

Patients without endometriosis were recruited among women undergoing laparoscopic sterilization. The women were assessed as being in the proliferative (13 with and 6 without endometriosis) or secretory (12 with and 8 without endometriosis) phase of the menstrual cycle on the basis of the menstrual cycle pattern, histological examination of the eutopic endometrium (Noyes *et al.* 1975) and the serum concentrations of oestradiol and progesterone. Hormone analysis was performed with a method based on enzyme-amplified chemiluminescence (Immulite, Diagnostic Products Corporation, CA, USA) in accordance with the supplier's instructions. The mean serum concentrations of progesterone and oestradiol were 5.8 ± 0.5 nM (3.6–7.0 nM) and 307.3 pM (87–581 pM) respectively in the proliferative phase and 23.0 nM (9.5–41.8 nM) and 337.9 pM (91.0–709.0 pM) respectively in the secretory phase. Patients with irregular menstrual cycles or gynaecological disorders other than endometriosis and those who had received hormonal treatment within the last 3 months were excluded. The Ethics Committee at the Research Centre for Obstetrics, Gynaecology and Perinatology in Moscow approved the study protocol and informed consent was obtained from all patients. The protocol of the study was also approved by the Ethics Committee of Uppsala University.

### Collection of tissue specimens, serum and peritoneal fluid

Samples of eutopic endometrium were obtained prior to laparoscopy by sharp curettage of the corpus of the uterine cavity of both healthy controls and women with endometriosis. Samples of ectopic endometrium were obtained during laparoscopy by carefully removing at

least three suspected peritoneal endometriotic lesions from each patient with laparoscopically verified pelvic endometriosis. The majority of the collected endometriotic specimens appeared mainly as black during laparoscopy. Of the collected lesions were 44% black, 52% black and partly red or white and 4% red. Each biopsy specimen was divided into two parts: one was prepared for histopathological investigation and the other was snap-frozen in liquid nitrogen and stored at  $-70^{\circ}\text{C}$  until analysed. Histopathologically, all endometrial biopsy samples appeared normal and all samples of ectopic endometrium contained endometrial glandular epithelium surrounded by stromal tissue.

Venous blood samples were collected in the morning prior to surgery. Peritoneal fluid samples were collected by aspiration during surgery if there was no contamination with blood. Both the serum and the peritoneal fluid samples were cooled with ice water and centrifuged at 1500 *g* for 10 min in a refrigerated centrifuge within 30–60 min after collection. Supernatants of peritoneal fluid and serum samples were stored at  $-70^{\circ}\text{C}$  until analysed.

### **Immunohistochemical staining**

Immunostaining was performed by applying the avidin–biotin peroxidase complex (ABC) method. Serial cryosections of 5  $\mu\text{m}$  were prepared, air-dried, and fixed in Zamboni's fixative (pH 7.3). Endogenous peroxidase activity was blocked with 0.6%  $\text{H}_2\text{O}_2$  in distilled water. Unspecific binding was blocked by incubating the sections with 2% BSA in PBS (pH 7.4) for 30 min. The sections were incubated with primary antibody for 1 h at  $37^{\circ}\text{C}$  and rinsed three times for 5 min each in PBS prior to incubation with secondary antibody for 60 min at room temperature. Sections were then rinsed for three times, 5 min each in PBS and incubated with avidin–biotin complex (ABC Elite Kit, Vector Laboratories, CA, USA) for 30 min at room temperature. Specific staining was visualized by applying diaminobenzidine substrate kit (Vector Laboratories) for 3–5 min. After washing, the sections were counterstained with Mayer's haematoxylin, dehydrated and mounted in mounting medium (Pertex, Histolab products AB, Gothenburg, Sweden). Series of all samples and controls were run at least twice.

Image analysis was used to count the number of blood vessels and proliferating cells (Ki-67) and as an aid in grading the intensities of staining for VEGF-A and its receptors. Basically, five to ten non-overlapping, randomly chosen pictures of the endometrial sections were taken at  $400\times$  magnification with a JVC 3-CCD video camera attached to a light field microscope (Nikon Labophot, Nikon, Tokyo, Japan). Images were digitised in a PC and subsequently assessed using the image analysis software program Image pro-plus (Media Cybernetics, Massachusetts, CA, USA).

### **Microvessel density**

With a few modifications, the measurements were made according to a technique described earlier (Goddard *et al.* 2002). The primary antibody used was a monoclonal anti-human CD31 (DAKO A/S, Glostrup, Denmark) at 0.3  $\mu\text{g}/\text{ml}$ . Relevant class-matched IgG was used as negative control. As secondary antibody, we used a biotinylated horse anti-mouse antibody at 1.0  $\mu\text{g}/\text{ml}$  (Vector laboratories, Burlingame, CA, USA). Positive controls were performed by staining human placenta and human lymph node that have a known expression of the corresponding epitope. Five randomly chosen fields measuring 0.109  $\text{mm}^2$  were counted, the mean values calculated, and the results expressed as the number of microvessels per millimetre square.

### **Proliferative activity**

Monoclonal mouse anti-Ki-67 (MIB-1, Immunotech, Marseille, France; diluted 1:300) was used as primary antibody and biotinylated horse anti-mouse antibody (Vector Laboratories) as secondary. Immunohistochemical staining of cryosections of a human ovarian carcinoma served as positive control. The number of Ki-67-positive cells was evaluated separately in stroma, glands and blood vessels. A proliferative index (PI) was calculated as the percentage of cells that were Ki-67 positive.

### **Angiogenic growth factor VEGF-A and its receptors VEGFR-1 and VEGFR-2**

The primary antibodies used were polyclonal mouse anti-VEGF (AB-2, PC-37, Oncogene Science, Inc., Cambridge, MA, USA; 1:500), rabbit anti-VEGFR-1 (c-17, sc-316, Santa Cruz Biotechnology, CA, USA; 1:500) and a monoclonal mouse anti-VEGFR-2 (IgG1, A-3, sc-6251, Santa Cruz Biotechnology; 1:500). As secondary antibody, either horse anti-mouse IgG (1:200) or goat anti-rabbit IgG (1:200, Vector Laboratories) was used. Positive controls were performed for all antibodies by staining tissues with known expression of the corresponding epitopes (human placenta and human lymph node). Omission of the primary antibody served as negative control. Non-specific staining was not detected.

The intensity of the staining was evaluated by two independent observers (V B and S P) and graded as 0, when comparable to the negative control; 1, when weak (clearly visible but no more); 2, when between weak and strong and 3, when strong.

### **Quantitative determination of VEGF-A in serum and peritoneal fluid**

For analysis of samples, commercially available ELISA kits for VEGF-A (DVE00, R&D Systems, Minneapolis, MN, USA) were used according to the supplier's

instructions. The lower detection limit for VEGF-A was set at 5.0 pg/ml.

### Statistical analysis

Values are reported as mean  $\pm$  s.e.m. Unpaired two-tailed *t*-tests or Wilcoxon rank-sum tests were used for comparing results. For analysis, SPSS for Windows (Release 10.0.7) was used. The level of statistical significance was set at  $P < 0.05$ .

## Results

### Microvessel density in eutopic and ectopic endometrium

The data are presented in Table 1. The eutopic endometrium from women without endometriosis had an average microvessel density of 229/mm<sup>2</sup>, with no significant difference between proliferative and secretory phases of the menstrual cycle. In women with endometriosis, the eutopic endometrium had a higher microvessel density in the secretory than the proliferative menstrual phase. Secretory phase eutopic endometrium from women with endometriosis had a higher microvessel density than that from women without endometriosis. Ectopic endometrium (endometriotic lesions) showed the opposite pattern, with a higher microvessel density in the proliferative than in the secretory phase. In the proliferative phase, ectopic endometrium with high proliferative activity had a higher microvessel density than that with low proliferative activity.

### Proliferative activity in eutopic and ectopic endometrium

In eutopic endometrium (Table 2) from women without endometriosis, the proliferative index (PI) was higher in glandular epithelium during the proliferative phase compared with the secretory phase. No such difference was seen for stromal and vascular cells.

In eutopic endometrium from women with endometriosis, the proliferative index (PI) again was higher in glandular epithelium during the proliferative phase compared with the secretory phase. Vascular cells, however, had a significantly higher PI during the secretory phase compared with the proliferative phase.

In ectopic endometrium (Table 3), the PI varied from almost 0 to a fairly high value in both stromal and glandular epithelial cells. The three or more samples from each patient showed the same degree of proliferative activity and the mean PI value was calculated for each patient. The patients were subsequently divided into two groups based on PI in the glandular epithelial cells. Lesions with PI  $< 1$  were denoted as having low proliferative activity ( $n = 8$ ), and those with PI above 1, as having high proliferative activity ( $n = 17$ ). Lesions classified as having low proliferative activity had a significantly lower PI in both the stroma ( $P < 0.001$ ) and the glandular epithelial cells ( $P < 0.0001$ ) than those with higher proliferative activity. There was, however, no significant difference in PI for blood vessels between women with lesions with low and high proliferative activities.

### Expression of VEGF-A and its receptors in eutopic and ectopic endometrium

In eutopic endometrium from women without endometriosis, the expression of VEGF-A was higher in glandular epithelium during the proliferative than the secretory menstrual phase (Table 2). This difference was not seen in eutopic endometrium from women with endometriosis. There were no menstrual cycle phase-related differences for VEGFR-1 and VEGFR-2 in eutopic endometrium. Concerning the expression of VEGF-A, it was higher in endometrial glandular epithelial cells from women with endometriosis than those without, during the secretory phase. The expression of VEGFR-1 and VEGFR-2 was lower in stromal cells from women with endometriosis in both proliferative and secretory menstrual phases, while the expression of VEGFR-2 was

**Table 1** Microvessel density (MVD) expressed as the number of intersecting microvessels per millimetre squared.

Types of endometrium	All patients 1	Proliferative phase 2	Secretory phase 3	$P < 0.05$
A	228.9 $\pm$ 72.7 <i>n</i> = 14	192.1 $\pm$ 37.9 <i>n</i> = 6	243.9 $\pm$ 80.8 <i>n</i> = 8	
B	315.3 $\pm$ 196.5 <i>n</i> = 25	152.8 $\pm$ 25.2 <i>n</i> = 13	452.8 $\pm$ 170.1 <i>n</i> = 12	2–3
C	225.1 $\pm$ 154.2 <i>n</i> = 25	295.6 $\pm$ 172.7 <i>n</i> = 13	132.2 $\pm$ 40.9 <i>n</i> = 12	2–3
D	261.1 $\pm$ 87.3 <i>n</i> = 17	321.1 $\pm$ 190.1 <i>n</i> = 8	134.1 $\pm$ 48.3 <i>n</i> = 9	2–3
E	161.6 $\pm$ 57.6 <i>n</i> = 8	225.9 $\pm$ 98.8 <i>n</i> = 5	127.1 $\pm$ 48.4 <i>n</i> = 3	
$P < 0.05$ of importance	D–E	C–E; D–E	A–B	

(A) Eutopic endometrium from women without endometriosis. (B–E) Biopsy specimens from women with endometriosis: (B) eutopic endometrium, (C) ectopic endometrium, (D) ectopic endometrium with high proliferative activity, (E) ectopic endometrium with low proliferative activity. *n*, Number of patients. Data are expressed as mean values  $\pm$  s.d.

**Table 2** Immunohistochemical staining of eutopic endometrium from women without (N) and with (E) pelvic endometriosis.

		Proliferative menstrual phase (N) n=6 (E) n=13			Secretory menstrual phase (N) n=8 (E) n=12			P values of importance
		Stromal cells 1	Glandular epithelium 2	Blood vessels 3	Stromal cells 4	Glandular epithelium 5	Blood vessels 6	
Ki-67	N	10.9±4.8	25.0±3.8	1.3±0.2	10.5±4.8	12.9±5.2	3.5±0.5	2-5 <0.05
	E	8.4±1.4	27.9±3.1	1.8±0.4	9.6±1.8	15.1±3.5	3.9±0.6	2-5 <0.05 3-6 <0.05
P value		NS	NS	NS	NS	NS	NS	
VEGF-A	N	1.2±0.3	2.3±0.3	0.2±0.2	0.5±0.3	0.5±0.3	0.2±0.2	2-5 <0.01
	E	0.8±0.1	2±0.2	0.3±0.2	0.5±0.1	1.5±0.2	0.4±0.2	
P value		NS	NS	NS	NS	<0.01	NS	
VEGFR-1	N	1.2±0.2	2.0±0.3	1.2±0.7	1.2±0.2	2.0±0.2	1.2±0.6	
	E	0.4±0.2	1.8±0.2	1.1±0.5	0.5±0.2	1.8±0.2	1.3±0.4	
P value		<0.01	NS	NS	<0.01	NS	NS	
VEGFR-2	N	1.2±0.2	2.4±0.6	1.6±0.5	1.2±0.2	2.5±0.2	1.3±0.2	
	E	0.7±0.2	1.8±0.2	1.8±0.4	0.6±0.2	1.8±0.2	1.9±0.2	
P value		<0.01	NS	NS	<0.01	<0.05	<0.05	

Proliferative index is expressed as percentage of cells expressing Ki-67. Staining intensities for VEGF-A and its receptors VEGFR-1 and VEGFR-2 in endometrial stroma, glands and blood vessels were graded from 0 to 3. NS, not statistically significant. Data are expressed as mean values ±s.d.

higher in blood vessels of patients with endometriosis in the secretory phase.

Table 3 and Fig. 1 present results of samples of ectopic endometrium with either high or low proliferative activity in glandular epithelial cells. There were no significant differences in the proliferative activity in blood vessels between these two groups. Samples with high proliferative activity in glandular epithelial cells showed higher expression of VEGF-A in both stromal ( $P<0.01$ ) and epithelial ( $P<0.001$ ) cells than samples with low proliferative activity. The expression of VEGFR-1 was similar in samples with high and low proliferative activities. Samples with high proliferative activity in glandular epithelial cells displayed higher expression of VEGFR-2 in stromal ( $P<0.05$ ), glandular epithelial ( $P<0.01$ ) and vascular ( $P<0.05$ ) cells than those with low proliferative activity.

**Serum and peritoneal fluid concentrations of VEGF-A**

Women with endometriosis had a higher content of VEGF-A in peritoneal fluid ( $282 \pm 65$  pg/ml) compared with

women without endometriosis ( $125 \pm 17$  pg/ml;  $P<0.05$ ) in the secretory phase. There was no difference in the serum content of VEGF between women with and without endometriosis. There were no significant differences in VEGF content in serum and peritoneal fluid between proliferative and secretory menstrual phases.

Women with endometriotic lesions showing high proliferative activity had a higher level of VEGF-A in both peritoneal fluid and serum than women with endometriotic lesions showing low proliferative activity ( $287 \pm 46$  vs  $140 \pm 17$ ,  $P<0.01$  and  $136 \pm 24$  vs  $61 \pm 17$ ,  $P<0.02$  respectively).

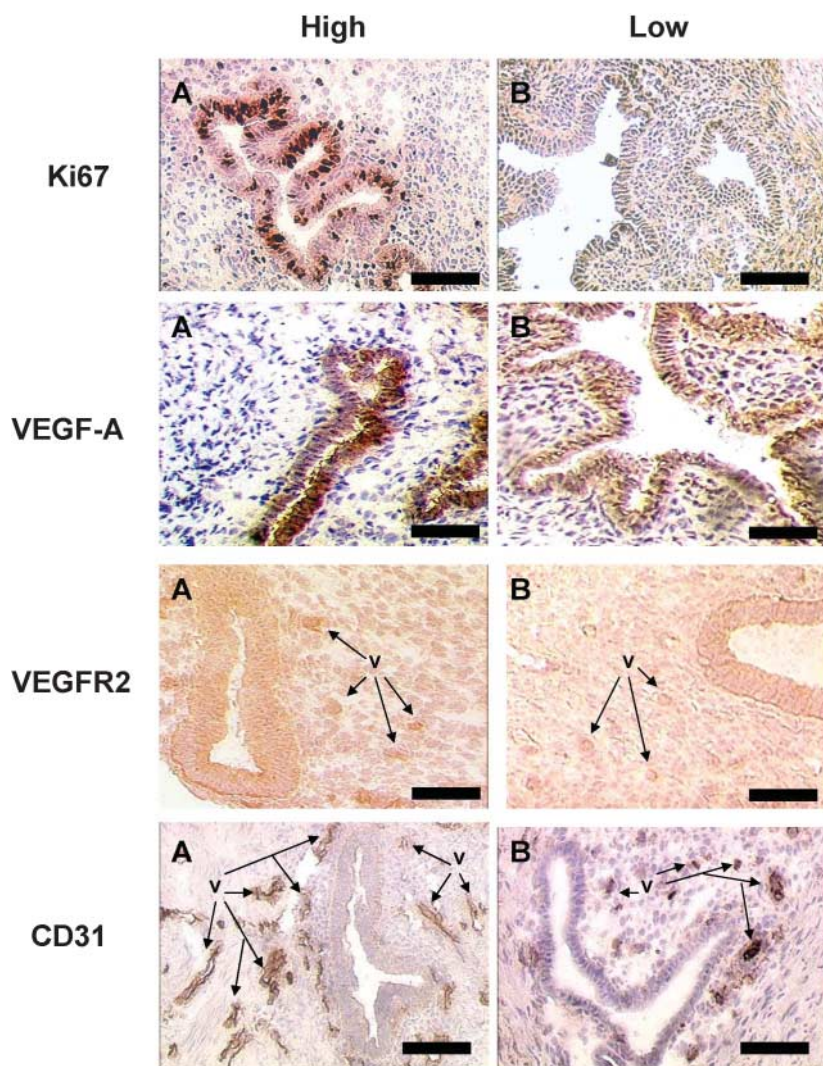
**Discussion**

This study has comprised an integrated analysis of the proliferative activity, microvessel density and expression of the angiogenic growth factor VEGF and its receptors VEGFR-1 and VEGFR-2 in biopsy samples of eutopic and ectopic endometrium. Further, the concentration of VEGF-A was measured in both peritoneal fluid and serum from the same patients. We found a relation

**Table 3** Immunohistochemical staining of ectopic endometrium.

	High proliferative activity (n=17)			Low proliferative activity (n=8)			P values of importance
	Stromal cells 1	Glandular epithelium 2	Blood vessels 3	Stromal cells 4	Glandular epithelium 5	Blood vessels 6	
Ki-67	3.8±3.3	8.9±6.4	1.8±1.0	0.9±0.9	0.5±0.3	1.3±0.6	1-4 <0.0001 2-5 <0.001
VEGF-A	0.8±0.1	1.7±0.2	0.1±0.1	0.5±0.1	0.7±0.1	0.4±0.4	1-4 <0.01 2-5 <0.001
VEGFR-1	0.7±0.1	1.4±0.2	1.75±1.0	0.7±0.1	1.2±0.2	0.75±0.4	
VEGFR-2	0.8±0.1	1.9±0.1	2.14±0.6	0.5±0.1	0.8±0.2	1.25±0.3	1-4 <0.05 2-5 <0.01 3-6 <0.05

The samples were sub-grouped according to their proliferative activity in glandular epithelial cells. Proliferative index is expressed as percentage of cells expressing Ki-67. Staining intensities for VEGF-A and its receptors VEGFR-1 and VEGFR-2 in endometrial stroma, glands and blood vessels were graded from 0 to 3. Data are expressed as mean values ±s.d.



**Figure 1** Immunohistochemical staining of ectopic endometrium. Proliferating cells were identified by staining for Ki-67. The angiogenic growth factor VEGF-A and its receptor VEGFR-2 were found in stroma, glands and blood vessels (v). Blood vessels were recognised by staining for CD31. Pictures on the left (A) represent samples with a high mitotic activity in the glandular epithelial cells and those on the right (B) represent samples with a low proliferative activity. Scale bar is 25  $\mu$ m.

between a higher microvessel density in the secretory phase eutopic endometrium from women with endometriosis with a higher expression of VEGF-A in secretory phase glandular epithelium and VEGFR-2 in endometrial blood vessels. We were also able to show a correlation between the proliferative and the angiogenic activity in endometriotic lesions and this was also reflected by the VEGF-A content in serum and peritoneal fluid.

In our study, we used lesions where the majority appeared black or mainly black during laparoscopy. These black lesions are considered to be more established and mature compared with the red ones (Nisolle *et al.* 1993, Brosens 1994). However, it has been shown that black lesions have lower proliferative activity than the red ones (Nisolle *et al.* 1993, Khan *et al.* 2004). In spite of the histological homogeneity in our material, we found large differences in cell mitotic activity in both stroma and glandular epithelium, ranging from no

detectable mitoses to numerous ones. The stromal mitotic activity was higher in samples with high mitotic activity in the glandular epithelium, but the main difference in mitotic activity was attributable to the glandular epithelial cells. On the basis of this finding, we sub-grouped the samples into those with high and low mitotic activities in the glandular epithelial cells. PI was chosen as a cut when the samples were sub-grouped because it was required to have a group of samples with very low PI to compare with those with a significantly higher PI.

Angiogenesis is believed to be important for the establishment and the survival of endometriotic lesions as well as for the eutopic endometrium. A number of studies on women with endometriosis have shown differences from healthy controls both in the peritoneal fluid composition, and the eutopic and ectopic endometrium that relate to the regulation of angiogenesis (McLaren *et al.* 1996b, Healy *et al.* 1998, McLaren

2000, Sharkey *et al.* 2000, Garrido *et al.* 2002). VEGF-A seems to be one of the most important angiogenic growth factors in the regulation of angiogenesis in general and probably also in both eutopic and ectopic endometrium (Li *et al.* 1994, McLaren *et al.* 1996b, Shifren *et al.* 1996, Torry *et al.* 1996, Donnez *et al.* 1998, Möller *et al.* 2001). We can show that VEGF-A and its receptors VEGFR-1 and VEGFR-2 are present in both eutopic and ectopic endometrium, and their relation to microvessel density and proliferative activity will be discussed later.

Regarding the microvessel density in eutopic endometrium from women without endometriosis, our data were consistent with the previous reports (Rogers *et al.* 1993, Goodger & Rogers 1994, Charnock-Jones *et al.* 2000, Möller 2004) that the number of intersecting microvessels per millimetre squared seems to be fairly constant during the menstrual cycle (Table 1). The significantly higher microvessel density in the secretory phase eutopic endometrium from women with endometriosis, compared with women without endometriosis, was reflected by a significantly higher expression of VEGF-A in glandular epithelium and of VEGFR-2 in blood vessels as well as a higher level of VEGF-A in peritoneal fluid. Thus, there seems to be a more pro-angiogenic situation in eutopic endometrium from women with endometriosis compared with women without the disease. We were also able to demonstrate a significantly higher proliferative activity in blood vessels during the secretory, compared with the proliferative menstrual phase in women with endometriosis. It has previously been shown that the VEGF-A content was significantly higher in the eutopic glandular epithelium of endometriotic patients during the secretory phase (Donnez *et al.* 1998) and that the peritoneal fluid level of VEGF-A is elevated in women with endometriosis, with the highest levels during the secretory phase (McLaren *et al.* 1996a). The higher microvessel density in the secretory phase eutopic endometrium from women with endometriosis might be due to a higher production of VEGF-A within the eutopic endometrium (McLaren *et al.* 1996b) or an extensive endometrial exposure of VEGF-A that is produced and contained in the peritoneal fluid. There are two possible ways by which peritoneal fluid VEGF-A can reach the eutopic endometrium and stimulate endometrial angiogenesis: (1) by transport of peritoneal fluid via the uterine tubes to the uterine cavity and (2) through the local pelvic network of lymph and blood vessels. There is also a possibility that substances other than VEGF-A in the peritoneal fluid might stimulate endometrial angiogenesis. In any case, there seems to be increased angiogenic activity in the secretory phase eutopic endometrium in women with endometriosis. A widely accepted theory concerning the pathogenesis of endometriosis includes retrograde menstruation and implantation of endometrial fragments on peritoneal

surfaces (Sampson 1927). Such endometrial implants will soon depend on the formation of new blood vessels through angiogenesis. It has been proposed that high microvessel density and angiogenic activity in endometrial fragments seeded into the peritoneal cavity might be of advantage during the establishment and growth of the resulting peritoneal lesions, as the large amount of microvessels and endothelial cells would be able to take part in neovascularization (Healy *et al.* 1998). The high vascular density and increased pro-angiogenic properties found in eutopic secretory phase endometrium from women with endometriosis might thus at least be a supportive factor in the pathogenesis of endometriosis.

Ectopic endometrium showed a higher microvessel density in the proliferative phase, in contrast to the finding in eutopic endometrium. Since we found no differences in the expression of VEGF-A or its receptors (data not shown), or in the content of VEGF-A in peritoneal fluid or serum, between the proliferative and secretory menstrual phases, other reasons for this may need to be sought. Possible explanatory mechanisms include the ectopic site of growth, the presence of endogenous aromatase activity in the endometriotic lesions (Bulun *et al.* 2001) and the continuous exposure to peritoneal fluid with its content of pro-inflammatory and pro-angiogenic substances, factors that might override or bias the control of proliferation and function that is normally exerted by the ovarian sex steroid hormones. In a previous study, no difference was found in the microvessel density of endometriotic lesions between the different phases of the menstrual cycle (Matsuzaki *et al.* 1998).

It has previously been shown that red lesions with high mitotic activity also had higher microvessel density than white lesions with lower proliferative activity (Nisolle *et al.* 1993). We found similar differences between ectopic endometrium with high and low proliferative activities in epithelial and stromal cells, where the microvessel density was higher in samples with high proliferative activity in the proliferative, but not the secretory phase of the menstrual cycle (Table 1). We have now been able to show that endometriotic lesions with high proliferative activity are also accompanied by high angiogenic activity, as reflected by higher expression of VEGF-A in stroma and glandular epithelium and of VEGFR-2 in blood vessels (Table 3), as well as higher levels of VEGF-A in both serum and peritoneal fluid. Moreover, the mitotic activity in blood vessels seemed to be higher in lesions with high proliferative activity, but the difference was not statistically significant. This may not be surprising, however, since it has been shown that angiogenic activity in eutopic endometrium assessed in terms of the number of proliferating endothelial cells is fairly constant during the menstrual cycle (Rogers *et al.* 1993), although there are reports on peaks of blood vessel formation during the

menstrual cycle (Gargett *et al.* 1999, Gargett & Rogers 2001). Several studies also indicate that endometrial angiogenesis does not occur according to the classical mechanism with sprouting of new capillary blood vessels from maternal blood vessels. It has been suggested that sprouting is of minor importance, since markers for and formations of sprouts have not been observed in eutopic human endometrium (Rogers & Gargett 1998). It is believed, rather, that angiogenesis in eutopic endometrium occurs mainly through vessel elongation and to some extent also via intussusceptive microvascular growth and incorporation of circulating endothelial progenitor cells into the growing blood vessels (Gambino *et al.* 2002). However, it is not known whether the same mechanisms are responsible for angiogenesis in ectopic endometrium. It has previously been shown that higher the concentration of VEGF in the peritoneal fluid is, the more advanced is the endometriosis (Mahnke *et al.* 2000) and that the amount of vascularization in and around the endometriotic lesion correlates with the mitotic activity within the lesion (Nisolle *et al.* 1993).

We thus found a correlation between high mitotic activity in the endometriotic lesions and high levels of VEGF in serum and peritoneal fluid. It has previously been shown that there is a correlation between the mitotic activity in endometriotic lesions and the serum level of CA-125 in women with stage III–IV peritoneal endometriosis (Toki *et al.* 2000). Similarly, a correlation has been observed between the mitotic activity in endometriotic lesions and the peritoneal fluid levels of hepatocyte growth factor, VEGF, interleukin-6 and monocyte chemoattractant protein-1 (Khan *et al.* 2004). The clinical implications of high mitotic activity in endometriotic lesions remain unclear, but this finding might be relevant for the intensity of pain or the question of whether the lesions are growing on the peritoneal surface or are infiltrative. Diagnosis of endometriosis by laboratory testing of VEGF is not possible according to our results, as also has been concluded by others (Gagne *et al.* 2003, Bedaiwy & Falcone 2004, Potlog-Nahari *et al.* 2004). Like others (Gagne *et al.* 2003), we, in serum, found no differences in VEGF levels between women with and without endometriosis. An analysis of the serum level of VEGF might, however, be a useful tool to determine whether endometriotic lesions have high or low proliferative activity, a test that might be meaningful when attempting to monitor a response to treatment.

Taken together, our results show that there seems to be a dysregulation of angiogenic activity in the eutopic endometrium of women with endometriosis and a correlation between the proliferative and angiogenic activities in endometriotic lesions, which was also reflected by the VEGF-A content in serum and peritoneal fluid. An abnormal control of endometrial angiogenesis

in women with endometriosis might have an impact on bleeding control and fertility.

## Acknowledgements

The authors thank Dr Denis Stygar, The Research Centre of Obstetrics, Gynaecology and Perinatology, Moscow, for his assistance in collecting the samples and Björn Möller, Margareta Nordling and Ulla Geifalk, The Department of Women's and Children's Health, Uppsala University, for their technical assistance. This investigation was supported by grants from the Russian Academy of Medical Sciences, the Royal Swedish Academy of Sciences, the Uppsala Family Planning Foundation, the Swedish Society of Medicine and the Swedish Research Council (Project no. 8683). The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

## References

- American Society for Reproductive Medicine** 1996 Revised American society for reproductive medicine classification of endometriosis. *Fertility and Sterility* **67** 817–821.
- Bedaiwy MA & Falcone T** 2004 Laboratory testing for endometriosis. *Clinica Chimica Acta* **340** 41–56.
- Berkkanoglu M & Arici A** 2003 Immunology and endometriosis. *American Journal of Reproductive Immunology* **50** 48–59.
- Brosens IA** 1994 Is mild endometriosis a disease? Is mild endometriosis a progressive disease? *Human Reproduction* **9** 2209–2211.
- Bulun SE, Yang S, Fang Z, Gurates B, Tamura M, Zhou J & Sebastian S** 2001 Role of aromatase in endometrial disease. *Journal of Steroid Biochemistry and Molecular Biology* **79** 19–25.
- Charnock-Jones DS, Macpherson AM, Archer DF, Leslie S, Makkink WK, Sharkey AM & Smith SK** 2000 The effect of progestins on vascular endothelial growth factor, oestrogen receptor and progesterone receptor immunoreactivity and endothelial cell density in human endometrium. *Human Reproduction* **15** 85–95.
- DeMayo FJ, Zhao B, Takamoto N & Tsai SY** 2002 Mechanisms of action of estrogen and progesterone. *Annals of the New York Academy of Sciences* **955** 48–59.
- Donnez J, Smoes P, Gillerot S, Casanas-Roux F & Nisolle M** 1998 Vascular endothelial growth factor (VEGF) in endometriosis. *Human Reproduction* **13** 1686–1690.
- Folkman J** 1995 Clinical applications of research in angiogenesis. *New England Journal of Medicine* **333** 1757–1764.
- Gagne D, Page M, Robitaille G, Hugo P & Gosselin D** 2003 Levels of vascular endothelial growth factor (VEGF) in serum of patients with endometriosis. *Human Reproduction* **18** 1674–1680.
- Gambino LS, Wreford NG, Bertram JF, Dockery P, Lederman F & Rogers PA** 2002 Angiogenesis occurs by vessel elongation in proliferative phase human endometrium. *Human Reproduction* **17** 1199–1206.
- Gargett CE & Rogers PA** 2001 Human endometrial angiogenesis. *Reproduction* **121** 181–186.
- Gargett CE, Lederman FL, Lau TM, Taylor NH & Rogers PA** 1999 Lack of correlation between vascular endothelial growth factor production and endothelial cell proliferation in the human endometrium. *Human Reproduction* **14** 2080–2088.
- Garrido N, Navarro J, Garcia-Velasco J, Remohi J, Pellicer A & Simon C** 2002 The endometrium versus embryonic quality in endometriosis-related infertility. *Human Reproduction Update* **8** 95–103.
- Goddard JC, Sutton CD, Furness PN, Kockelbergh RC & O'Byrne KJ** 2002 A computer image analysis system for microvessel measurement in solid tumours. *Angiogenesis* **5** 15–20.



- Goodger AM & Rogers PA** 1994 Endometrial endothelial cell proliferation during the menstrual cycle. *Human Reproduction* **9** 399–405.
- Goldman MB & Cramer DW** 1990 The epidemiology of endometriosis. *Progress in Clinical and Biological Research* **323** 15–31.
- Healy DL, Rogers PAW, Hii L & Wingfield M** 1998 Angiogenesis: a new theory for endometriosis. *Human Reproduction Update* **4** 736–740.
- Jones RK, Bulmer JN & Searle RF** 1995 Immunohistochemical characterization of proliferation, oestrogen receptor and progesterone receptor expression in endometriosis: comparison of eutopic and ectopic endometrium with normal cycling endometrium. *Human Reproduction* **10** 3272–3279.
- Khan KN, Masuzaki H, Fujishita A, Kitajima M, Sekine I & Ishimaru T** 2004 Higher activity by opaque endometriotic lesions than in nonopaque lesions. *Acta Obstetrica et Gynecologica Scandinavica* **83** 375–382.
- Koninckx PR, Meuleman C, Demeyere S, Lesaffre E & Cornille FJ** 1991 Suggestive evidence that pelvic endometriosis is a progressive disease, whereas deeply infiltrating endometriosis is associated with pelvic pain. *Fertility and Sterility* **55** 759–765.
- Li XF, Gregory J & Ahmed A** 1994 Immunolocalisation of vascular endothelial growth factor in human endometrium. *Growth Factors* **11** 277–282.
- Mahnke JL, Dawood MY & Huang J-C** 2000 Vascular endothelial growth factor and interleukin-6 in peritoneal fluid of women with endometriosis. *Fertility and Sterility* **73** 166–170.
- Matsuzaki S, Canis M, Darcha C, Dechelotte P, Pouly JL & Bruhat MA** 1998 Angiogenesis in endometriosis. *Gynecologic and Obstetric Investigation* **46** 111–115.
- McLaren J** 2000 Vascular endothelial growth factor and endometriotic angiogenesis. *Human Reproduction Update* **6** 45–55.
- McLaren J, Prentice A, Charnock-Jones DS & Smith SK** 1996a Vascular endothelial growth factor (VEGF) concentrations are elevated in peritoneal fluid of women with endometriosis. *Human Reproduction* **11** 220–223.
- McLaren J, Prentice A, Charnock-Jones DS, Millican SA, Müller KH, Sharkey AM & Smith SK** 1996b Vascular endothelial growth factor is produced by peritoneal fluid macrophages in endometriosis and is regulated by ovarian steroids. *Journal of Clinical Investigation* **98** 482–489.
- Möller B** 2004 Human endometrial angiogenesis. An immunohistochemical study of the endometrial expression of angiogenic growth factors and their corresponding receptors. Acta Universitatis Upsaliensis. *Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine* **1315** 50 (Uppsala. ISBN 91-554-5831-9).
- Möller B, Rasmussen C, Lindblom B & Olovsson M** 2001 Expression of the angiogenic growth factors VEGF, FGF-2, EGF and their receptors in normal human endometrium during the menstrual cycle. *Molecular Human Reproduction* **5** 65–72.
- Nisolle M, Casanas-Roux F, Anaf V, Mine JM & Donnez J** 1993 Morphometric study of the stromal vascularization in peritoneal endometriosis. *Fertility and Sterility* **59** 681–684.
- Noble LS, Simpson ER, Johns A & Bulun SE** 1996 Aromatase expression in endometriosis. *Journal of Clinical Endocrinology and Metabolism* **81** 174–179.
- Noyes RW, Hertig AT & Rock J** 1975 Dating the endometrial biopsy. *American Journal of Obstetrics and Gynecology* **122** 262–263.
- Papetti M & Herman IM** 2002 Mechanisms of normal and tumor-derived angiogenesis. *American Journal of Physiology. Cell Physiology* **282** 947–970.
- Potlog-Nahari C, Stratton P, Winkel C, Widra E, Sinaii N, Connors S & Nieman LK** 2004 Urine vascular endothelial growth factor-A is not a useful marker for endometriosis. *Fertility and Sterility* **81** 1507–1512.
- Rice VM** 2002 Conventional medical therapies for endometriosis. *Annals of the New York Academy of Sciences* **955** 343–352.
- Robinson CJ & Stringer SE** 2001 The splice variants of vascular endothelial growth factor (VEGF) and their receptors. *Journal of Cell Science* **114** 853–865.
- Rogers PAW & Gargett CE** 1998 Endometrial angiogenesis. *Angiogenesis* **2** 287–294.
- Rogers PA, Au CL & Affandi B** 1993 Endometrial microvessel density during the normal menstrual cycle and following exposure to long-term levonorgestrel. *Human Reproduction* **8** 1396–1404.
- Sampson JA** 1927 Peritoneal endometriosis due to the menstrual dissemination of endometrial tissue into the peritoneal cavity. *American Journal of Obstetrics and Gynecology* **14** 422–469.
- Sharkey AM, Day K, McPherson A, Malik S, Licence D, Smith SK & Charnock-Jones DS** 2000 Vascular endothelial growth factor expression in human endometrium is regulated by hypoxia. *The Journal of Clinical Endocrinology and Metabolism* **85** 402–409.
- Shifren JL, Tseng JF, Zaloudek CJ, Ryan IP, Meng YG, Ferrara N, Jaffe RB & Taylor RN** 1996 Ovarian steroid regulation of vascular endothelial growth factor in the human endometrium: implications for angiogenesis during the menstrual cycle and in the pathogenesis of endometriosis. *Journal of Clinical Endocrinology and Metabolism* **81** 3112–3118.
- Simpson JL** 2003 Heritability and candidate genes for endometriosis. *Reproductive Biomedicine Online* **7** 162–169.
- Smith SK** 1998 Angiogenesis, vascular endothelial growth factor and the endometrium. *Human Reproduction Update* **4** 509–519.
- Stefansson H, Geirsson RT, Steinthorsdottir V, Jonsson H, Manolescu A, Kong A, Ingsdottir G, Gulcher J & Stefansson K** 2002 Genetic factors contribute to the risk of developing endometriosis. *Human Reproduction* **17** 555–559.
- Toki T, Kubota J, Lu X & Nakayama K** 2000 Immunohistochemical analysis of CA125, CA19-9, and Ki-67 in stage III or IV endometriosis: positive correlation between serum CA125 level and endometriotic epithelial cell proliferation. *Acta Obstetrica et Gynecologica Scandinavica* **79** 771–776.
- Taylor RN, Lebovic DI & Mueller MD** 2002 Angiogenic factors in endometriosis. *Annals of the New York Academy of Sciences* **955** 89–100.
- Torry DS, Holt VJ, Keenan JA, Harris G, Caudle MR & Torry RJ** 1996 Vascular endothelial growth factor expression in cycling human endometrium. *Fertility and Sterility* **66** 72–79.
- Ueda T, Aozasa K, Tsujimoto M, Ohsawa M, Uchida A, Aoki Y, Ono K & Matsumoto K** 1989 Prognostic significance of ki-67 immunoreactivity in soft tissue sarcomas. *Cancer* **63** 1607–1611.
- Valle RF** 2002 Endometriosis: current concepts and therapy. *International Journal of Gynaecology and Obstetrics* **78** 107–119.
- Vinatier D, Orazi G, Cosson M & Dufor P** 2001 Theories of endometriosis. *European Journal of Obstetrics, Gynecology, and Reproductive Biology* **96** 21–34.
- Wingfield M, Macpherson A, Healy DL & Rogers PA** 1995 Cell proliferation is increased in the endometrium of women with endometriosis. *Fertility and Sterility* **64** 340–346.
- Witz CA, Thomas MR, Montoya-Rodriguez IA, Nadir AS, Centonze VE & Schenken RS** 2001 Short-term culture of peritoneum explants confirms attachment of endometrium to intact peritoneal mesothelium. *Fertility and Sterility* **75** 385–390.

---

Received 15 January 2006

First decision 31 March 2006

Revised manuscript received 8 May 2006

Accepted 9 June 2006