

# Expression of vascular endothelial growth factor (VEGF) and its receptors in human endometrium throughout the menstrual cycle and in early pregnancy

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Immunohistochemistry for vascular endothelial growth factor (VEGF) and its receptors, fms-like tyrosine kinase (flt-1) and kinase insert domain-containing region (KDR), was performed on human endometrium obtained from patients with normal menstrual cycles, patients given oestrogen and progesterone, and women in early pregnancy. Intense immunostaining of VEGF was observed in both glandular epithelial and stromal cells during the mid-secretory phase; the immunostaining intensity was increased by administration of oestrogen plus progesterone and strong immunostaining was observed in decidual cells of early pregnancy. In addition to the immunostaining in vascular endothelial cells, strong KDR immunostaining was observed in glandular epithelial cells and in decidualized stromal cells induced by administration of oestrogen

plus progesterone, whereas flt-1 immunostaining was negligible. Strong immunostaining for flt-1 and KDR was found in both vascular endothelial cells and decidual cells in early pregnancy. Endometrial stromal cells isolated from proliferative phase endometrium were incubated with oestrogen ( $10^{-8}$  mol l<sup>-1</sup>) and medroxyprogesterone acetate (MPA;  $10^{-6}$  mol l<sup>-1</sup>) for 18 days to study the regulation of VEGF, flt-1 and KDR in endometrial stromal cells by oestrogen and progesterone. Expression of VEGF and KDR mRNAs was increased significantly by oestrogen and MPA, accompanied by decidualization, whereas flt-1 mRNA expression was not affected. In conclusion, VEGF and its receptors may play important roles in implantation and maintenance of pregnancy.

## Introduction

Angiogenesis is important for development and differentiation of human endometrium, which are necessary for implantation, as well as for maintenance of pregnancy (Klauber *et al.*, 1997; Smith, 1998; Hickey and Fraser, 2000). Vascular endothelial growth factor (VEGF) is a protein that has the potential to play a dynamic role in the regulation of vascular endothelial growth, angiogenesis and vascular permeability (Ferrara and Davis-Smyth, 1997; Kashida *et al.*, 2001). Recent studies have shown that VEGF is expressed in the endometrium and probably participates in the increased angiogenesis and vascular permeability necessary for successful implantation (Chakraborty *et al.*, 1995; Das *et al.*, 1997). However, studies on the change in endometrial VEGF expression during the menstrual cycle have not given consistent results and, in particular, expression of VEGF in endometrial stromal cells is controversial. It is reported that diffuse and intense immunostaining of VEGF in the stroma is found during the secretory phase but not during the proliferative phase (Greb *et al.*, 1995; Shifren *et al.*, 1996). A recent *in vivo* study in monkeys showed that VEGF immunostaining in endometrial stromal cells was increased by progesterone treatment (Greb *et al.*, 1997). In

contrast, Charnock-Jones *et al.* (1993) and Li *et al.* (1994) reported that VEGF mRNA signals in *in situ* hybridization or VEGF immunostaining in the stroma were higher in the proliferative phase than in the secretory phase. In addition, there are some reports that VEGF expression showed no significant change in the stroma during the menstrual cycle (Torry *et al.*, 1996; Lau *et al.*, 1998; Macpherson *et al.*, 1999). Thus, regulation of stromal VEGF expression by ovarian steroids is still unclear.

VEGF activities are mediated by two known receptors: the fms-like tyrosine kinase (flt-1: VEGFR-1) and the kinase insert domain-containing region (KDR: VEGFR-2), which are generally found on vascular endothelial cells. Recent studies using immunohistochemistry have shown that flt-1 and KDR are localized mainly to vascular endothelial cells in human endometrium (Meduri *et al.*, 2000); however, expression was also observed in the glandular epithelium and stroma (Nayak *et al.*, 2000; Möller *et al.*, 2001). In addition, mRNA expression for the two receptors was found in both epithelial and stromal cells isolated from human endometrium (Krüssel *et al.*, 1999). However, little is known about the expression and regulation of VEGF receptors in human endometrium. Therefore, the aim of the present study was to investigate the localization and hormonal regulation of VEGF, flt-1 and KDR in human endometrium. For this purpose, endometrium obtained

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from patients with normal menstrual cycles, from patients who had been treated with oestrogen and progesterone, and from women in early pregnancy, were used. The regulation of VEGF, flt-1 and KDR by ovarian steroids was examined further using isolated endometrial stromal cells *in vitro*.

## Materials and Methods

This project was reviewed and approved by the committee on investigations involving human subjects of Yamaguchi University School of Medicine. Informed consent from each patient was obtained before collection of any tissue samples for this study.

### Materials

Phenol red-free Dulbecco's modified Eagle's medium (DMEM) and glutamine were purchased from ICN Bio-medicals Inc. (Aurora, OH). Streptomycin, penicillin,  $1 \times$  trypsin-EDTA, deoxynucleotide triphosphates and Moloney murine leukaemia virus reverse-transcriptase were from Life Technologies Inc. (Grand Island, NY). Collagenase, medroxyprogesterone acetate (MPA) and oestradiol were from Sigma Chemical Co. (St Louis, MO). Fetal calf serum (FCS) was from PAA Laboratories GmbH (Linz). Tissue flasks and nylon mesh were from Becton Dickinson Co. (Franklin Lakes, NJ). Random Hexamer and *Taq* DNA polymerase were from Perkin-Elmer Co. (Foster City, CA). [ $\alpha$ - $^{32}$ P]deoxycytidine triphosphate (dCTP) was from Amersham (Arlington Heights, IL). Isogen was from Wako Pure Chemical Industries Ltd (Osaka).

### Tissue samples

Endometrial tissues were obtained from patients aged 39–49 years who were undergoing hysterectomy for myoma uteri. As patients with submucous myoma were excluded from the study, endometrial samples were not close to leiomyoma. All tissue samples used in this study were shown to be histologically normal. Endometrial samples from patients with normal menstrual cycles were classified into five different groups according to the criteria of Noyes *et al.* (1950): early proliferative phase (days 4–7), mid- to late proliferative phase (days 8–14), early secretory phase (days 15–18), mid-secretory phase (days 19–23) and late secretory phase (days 24–28). Three women received oral administration of ethinyloestradiol (0.05 mg) plus norgestrel (0.5 mg) (Planovar; Weis-Ezai Co. Ltd, Tokyo) daily for 7–10 days from the mid-luteal phase onwards to avoid the onset of menstruation at surgery. Gestational endometria were obtained from three women undergoing artificial (surgical) abortion at weeks 6–8 of gestation.

### Immunohistochemistry

Immunohistochemical study was performed on three different tissue samples from each of five stages of the menstrual cycle, three patients given oestrogen and

progesterone, and three women in the early stages of pregnancy. Endometrial tissues were fixed in Carnoy solution (ethanol:chloroform:glacial acetic acid, 6:3:1), embedded in paraffin wax and cut into sections (4  $\mu$ m thickness). The tissue sections were deparaffinized in xylene and dehydrated in a graded series of ethanol. Immunohistochemistry for VEGF and flt-1 was performed with a peroxidase-anti-peroxidase method (DAKO PAP kit; DAKO Japan Co. Ltd, Tokyo) using rabbit anti-human VEGF polyclonal antibodies (RB-222-p; Neomarkers, Inc., Union City, CA) or rabbit anti-human flt-1 polyclonal antibodies (C-17, sc-316; Santa Cruz Biotechnology Inc., Santa Cruz, CA), as reported by Sugino *et al.* (2000a). After inhibition of endogenous peroxidase activity with 0.3% (v/v)  $H_2O_2$  for 50 min, the sections were incubated with 10% (v/v) normal swine serum for 10 min at room temperature to avoid non-specific binding. The sections were incubated with the primary antibodies at a dilution of 1:50 in PBS-BSA (1% (w/v)) overnight at 4°C. After three washes with PBS for 5 min each, the sections were incubated with swine anti-rabbit immunoglobulin for 30 min at room temperature, washed three times with PBS for 5 min each, and reacted with rabbit-PAP for 40 min at room temperature. Immunohistochemistry for KDR was performed with a streptavidin-biotin-peroxidase complex method (SAB-PO kit; Nichirei Co. Ltd, Tokyo) using mouse anti-human KDR monoclonal antibodies (A-3, sc-6251; Santa Cruz Biotechnology Inc.) as reported by Sugino *et al.* (2000a). After inhibition of endogenous peroxidase activity with 0.3% (v/v)  $H_2O_2$  for 50 min, the sections were incubated with 10% (v/v) normal rabbit serum for 10 min at room temperature to avoid non-specific binding. The sections were incubated with the primary antibody at a dilution of 1:50 in PBS-BSA (1% (w/v)) overnight at 4°C. After three washes with PBS for 5 min each, the sections were incubated with biotinylated rabbit anti-mouse immunoglobulin for 10 min at room temperature, washed three times with PBS for 5 min each, and reacted with peroxidase-conjugated streptavidin for 5 min at room temperature. Peroxidase activity was visualized by incubating the sections with 3,3'-diaminobenzidine-4-HCl (Nacalai Tesque Co. Ltd, Tokyo) in 0.05 mol Tris-HCl buffer  $I^{-1}$  (pH 7.6) containing 0.01% (v/v)  $H_2O_2$  for 2–3 min. For controls, sections were incubated with normal rabbit serum for VEGF and flt-1, or normal mouse serum for KDR, or the anti-flt-1 antibody was pre-absorbed with an excess of flt-1 blocking peptide (sc-316p; Santa Cruz Biotechnology Inc.) overnight at 4°C. In addition, the specificities of the antibodies for VEGF, flt-1 and KDR have been established by western blotting and immunoprecipitation performed by the manufacturers. Counterstaining was performed with Meyer's haematoxylin.

### Endometrial stromal cell isolation and cell culture

Human endometrium was obtained at hysterectomy from patients with normal menstrual cycles. As the cases with submucous myoma were excluded from the study,

endometrial samples were not close to leiomyoma. All tissue samples used in this study were histologically normal. Proliferative phase endometrium, which was diagnosed histologically according to the criteria of Noyes *et al.* (1950), was used for cell culture. Tissue samples were washed with phenol red-free DMEM containing 4 mmol glutamine l<sup>-1</sup>, 50 mg streptomycin l<sup>-1</sup> and 5 × 10<sup>4</sup> U penicillin l<sup>-1</sup>, and were minced into small pieces of < 1 mm<sup>3</sup>. Endometrial stromal cells were isolated as reported by Sugino *et al.* (2000b). In brief, after the enzymatic digestion of minced tissues with 0.2% (w/v) collagenase in a shaking waterbath for 2 h at 37°C, stromal cells were separated by filtration through a 70 µm nylon mesh. The filtrates were washed three times and the number of viable cells was counted by trypan blue exclusion. The homogeneity of the stromal cell preparation was verified by immunocytochemistry for the stromal-reacting antibody (vimentin) and > 98% of the cells were vimentin positive (data not shown). Cells were seeded at 10<sup>5</sup> cells cm<sup>-2</sup> in 75 cm<sup>2</sup> tissue culture flasks and incubated in phenol red-free DMEM containing glutamine, antibiotics and 10% (v/v) dextran-coated charcoal-stripped FCS at 37°C, 95% air and 5% CO<sub>2</sub>. At confluence, cells were trypsinized by 1 × trypsin-EDTA and subcultured in 25 cm<sup>2</sup> tissue culture flasks. At approximately 80% confluence after first passage, the cell culture medium was changed to phenol red-free DMEM supplemented with glutamine, antibiotics and 2% (v/v) stripped FCS, with or without oestradiol (10<sup>-8</sup> mol l<sup>-1</sup>) plus MPA (10<sup>-6</sup> mol l<sup>-1</sup>). The endometrial stromal cells were incubated for 18 days at 37°C in 95% air and 5% CO<sub>2</sub> to induce decidualization, as reported by Sugino *et al.* (2000b). The medium was changed every 2 days. A single incubation was performed in triplicate on cells from a single hysterectomy sample. The samples from three individuals were used and, hence, three different incubations were performed.

#### Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from endometrial stromal cells with Isogen by the method provided by the manufacturer. For mRNA analysis, RT-PCR was performed as reported by Sugino *et al.* (1998) with the oligonucleotide primers for VEGF (5'-CACATAGGAGAGATGAGCTTC-3' and 5'-CCGCCTCGGCTTGTACAT-3'), flt-1 (5'-CTAGGATCGTGACTTATTTTTCTCAACAAGG-3' and 5'-CTCGAATTGATCTCCATAGTGATGGGCTC-3') and KDR (5'-CGTGGATCCACCAAGGGGACGATCCGTC-3' and 5'-CTCGAATTCTGTAACAGATGAGATGCTCCAAGG-3') designed by Athanassiades *et al.* (1998). Direct sequence analyses of the PCR products were performed for sequence verification. Two oligonucleotide primers (5'-CTGAAGGTCAAAGGGAATGTG-3' and 5'-GGACAGAGTCTTGATGATCTC-3') were used to amplify ribosomal protein L19 as an internal control (Sugino *et al.*, 1998). In brief, 3 µg total RNA was reverse-transcribed at 42°C in a reaction mixture (single-strength PCR buffer, 2.5 mmol deoxynucleotide

triphosphates l<sup>-1</sup>, 5 µmol random hexamer primer l<sup>-1</sup>, 1.5 mmol MgCl<sub>2</sub> l<sup>-1</sup> and 200 U Moloney murine leukaemia virus reverse transcriptase). The reverse-transcribed product was divided into equal aliquots in two tubes for VEGF, flt-1 or KDR primers, and L19 primers, and PCR was performed. For PCR amplification, a mixture containing the oligonucleotide primers (50 pmol), [ $\alpha$ -<sup>32</sup>P]dCTP (2 µCi at 3000 Ci mmol<sup>-1</sup>) and *Taq* DNA polymerase (2.5 U) was added to each reaction. Amplification was carried out for 25 cycles consisting of 94°C (1 min), 57°C (1 min) and 72°C (1 min) for VEGF, 28 cycles consisting of 94°C (1 min), 60°C (1 min) and 72°C (1 min) for flt-1, and 25 cycles consisting of 94°C (1 min), 60°C (1 min) and 72°C (1 min) for KDR, followed by 10 min of final extension at 72°C in a programmed temperature control system PC-800 (ASTECC, Fukuoka). The predicted sizes of the PCR-amplified products were 98 bp for VEGF121, 228 bp for VEGF165, 230 bp for flt-1, 209 bp for KDR and 194 bp for L19. A linear curve was plotted using number of cycles of amplification versus densitometric values of the PCR products and measured with a BAS2000 (Fuji Photo Film Co., Tokyo). The optimal number of cycles for amplification that fitted within the linear range was chosen for each set of primers of VEGF, flt-1, KDR and L19 (data not shown). Reaction products were separated by electrophoresis on an 8% (w/v) polyacrylamide non-denaturing gel. After autoradiography, band intensities were analysed using a bioimaging analyser BAS2000. For quantification, the densities of VEGF, flt-1 and KDR were normalized to that of the internal control L19.

#### Statistical analysis

Data were examined by ANOVA and Student's *t* test. Differences were considered significant at *P* < 0.05.

## Results

#### VEGF immunohistochemistry

During the menstrual cycle, VEGF immunostaining was observed in both glandular epithelial cells and stromal cells, with the highest intensity during the mid-secretory phase (Fig. 1a,b). At the premenstruation stage, the glandular immunostaining intensity decreased (data not shown). Endometrium from patients who had been treated with oestrogen and progesterone was used to study the regulation of VEGF expression by oestrogen and progesterone. The immunostaining intensity increased in the patients who received oestrogen and progesterone, especially in the stromal cells associated with decidualization (Fig. 1c). Decidual cells in early pregnancy also showed strong immunostaining (Fig. 1d).

#### flt-1 immunohistochemistry

flt-1 immunostaining of vascular endothelial cells was negligible during the entire menstrual cycle except for



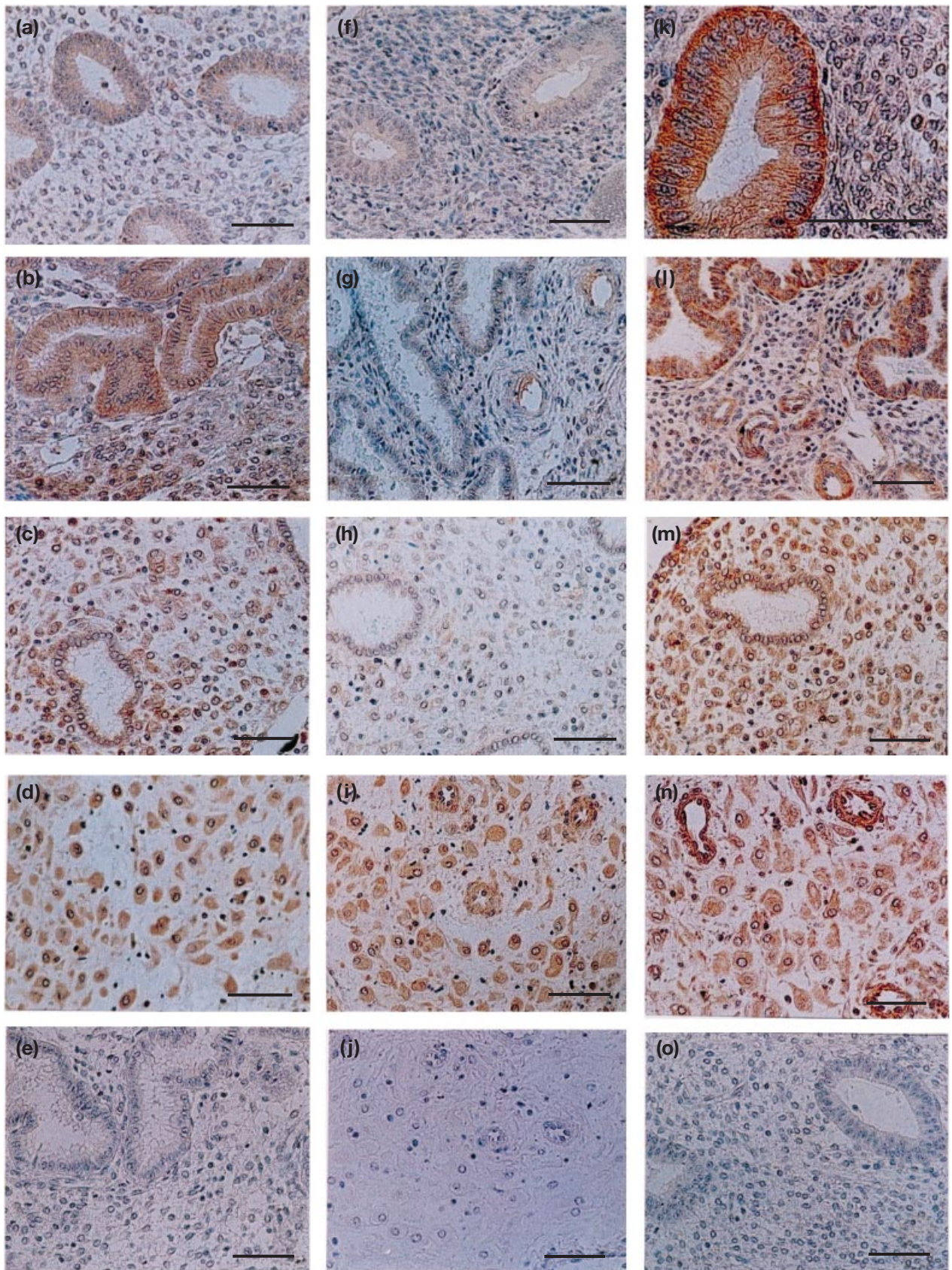


Fig. 1. For legend see facing page.



**Table 1.** Summary of the immunohistochemical experiments identifying VEGF, flt-1 and KDR in human endometrium

	Proliferative phase		Secretory phase			Treatment with oestrogen and progesterone	Early pregnancy
	Early	Mid- to late	Early	Mid-	Late		
<b>Epithelium</b>							
VEGF	+	+	+	++	-	++	++
flt-1	-	-	-	-	-	-	-
KDR	+++	+++	++	+++	+++	+++	+++
<b>Stroma</b>							
VEGF	+	+	+	++	+	+++	+++
flt-1	-	-	-	-	-	-	+++
KDR	-	-	+	+	+	+++	+++
<b>Blood vessel</b>							
VEGF	-	-	-	-	-	-	+
flt-1	-	-	-	-	-	-	+++
KDR	+	+	+	++	++	++	+++

VEGF: vascular endothelial growth factor; flt-1: fms-like tyrosine kinase; KDR: kinase insert domain-containing region.

Relative intensities of signals: from + (weakly positive) to +++ (strongly positive). -: no signal.

strong immunostaining in early pregnancy (Fig. 1i). Immunostaining for flt-1 in glandular epithelial cells and stromal cells was negligible during the menstrual cycle (Fig. 1-f,g) and in the endometrium of patients given oestrogen and progesterone (Fig. 1h). However, decidual cells in early pregnancy showed strong immunostaining (Fig. 1i). No flt-1 immunoreactivity was observed in the decidua after pre-absorption of the anti-flt-1 antibody with an excess of flt-1 blocking peptide (Fig. 1j).

#### KDR immunohistochemistry

Immunostaining for KDR was observed in endothelial cells and smooth muscle cells of blood vessels during the menstrual cycle and in early pregnancy (Fig. 1l,n). Strong immunostaining was also detected in glandular epithelial cells in all stages of endometrium studied (Fig. 1k,l). In contrast, negligible immunostaining was observed in the stroma during the proliferative phase (Fig. 1k), but weak and diffuse immunostaining was found in the stroma during the secretory phase (Fig. 1l). The immunostaining intensity increased in decidualized stromal cells of the endometrium treated with oestrogen and progesterone (Fig. 1m). Decidual cells in early pregnancy showed strong immunostaining (Fig. 1n).

These observations for VEGF, flt-1 and KDR are summa-

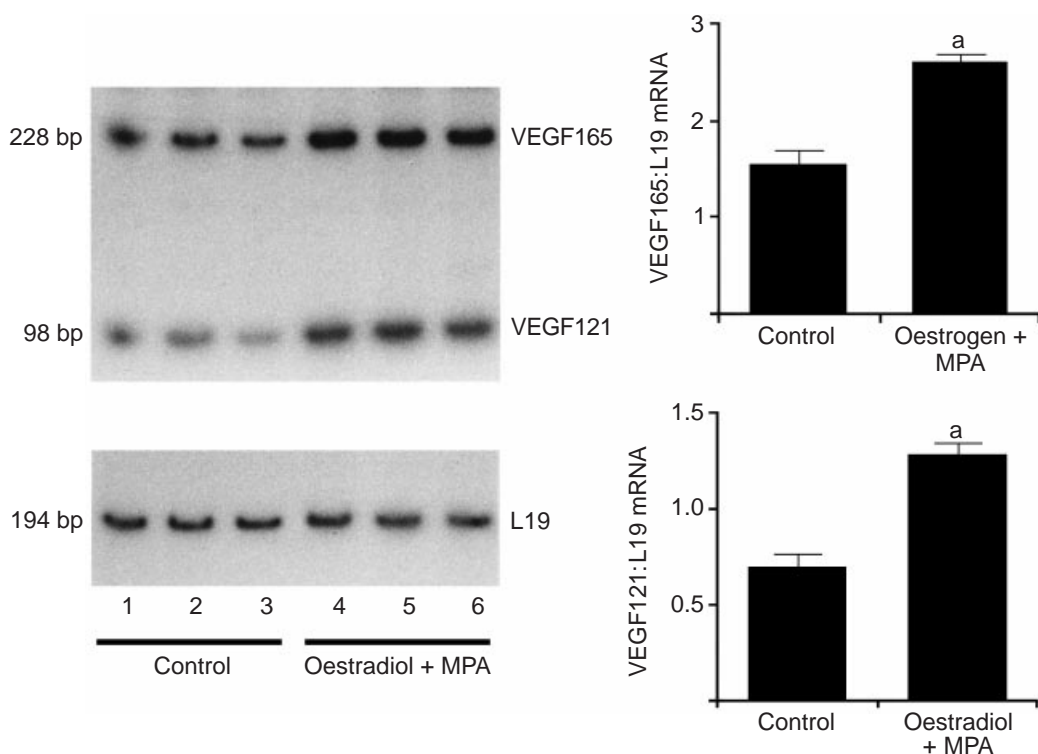
rized (Table 1). There was little variation in the tissue samples used in this study.

#### RT-PCR

From the results of the immunohistochemistry, it was decided to examine the effect of decidualization on expression of VEGF and its receptors in endometrial stromal cells. Endometrial stromal cells isolated from proliferative phase endometrium were cultured in the presence or absence of oestradiol ( $10^{-8}$  mol l<sup>-1</sup>) and MPA ( $10^{-6}$  mol l<sup>-1</sup>) for 18 days, reported by Sugino *et al.* (2000b) to induce decidualization by oestrogen and progesterone. Autoradiographs of RT-PCR for mRNA expression of VEGF, KDR and flt-1 are shown (Figs 2, 3 and 4, respectively). *In vitro* decidualization by oestradiol and MPA was confirmed by expression of insulin-like growth factor-binding protein-1 (IGFBP-1) mRNA, a specific marker of decidualization, as reported by Sugino *et al.* (2000b) (data not shown).

Four VEGF splice variants of 121, 165, 189, and 206 amino acids have been identified in human tissues (Ferrara and Davis-Smyth, 1997). RT-PCR analysis showed that the bands corresponding to VEGF121 and VEGF165 were in the isolated endometrial stromal cells (Fig. 2). The mRNA expression of both isoforms of VEGF was increased significantly by incubation with oestradiol and MPA (Fig. 2).

**Fig. 1.** Immunohistochemical staining for (a–e) vascular endothelial growth factor (VEGF), (f–j) fms-like tyrosine kinase (flt-1) and (k–o) kinase insert domain-containing region (KDR) in human endometrium from the proliferative phase ((a) late proliferative phase; (f,k) mid-proliferative phase), the mid-secretory phase (b,g,l), the patients given oestrogen plus progesterone (c,h,m), and early pregnancy (d,l,n). (e) Mid-secretory phase; (j) early pregnancy; and (o) late proliferative phase are negative controls for VEGF, flt-1 and KDR, respectively. No VEGF and KDR immunostaining was seen in the control section (e,o) and no flt-1 immunostaining was observed after pre-absorption of the anti-flt-1 antibody with an excess of flt-1 blocking peptide (j). The negative controls were similar regardless of whether normal rabbit serum or normal mouse serum was used. Scale bars represent 50 µm.



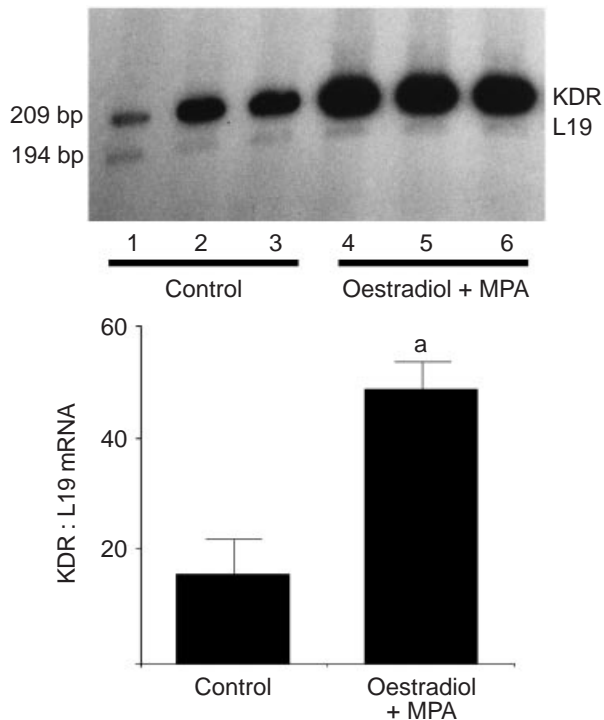
**Fig. 2.** Effects of oestrogen and progesterone on expression of vascular endothelial growth factor (VEGF) mRNA in human endometrial stromal cells *in vitro*. Endometrial stromal cells isolated from proliferative phase endometrium were incubated in the presence (lanes 4, 5 and 6) or absence (lanes 1, 2 and 3) of oestradiol ( $10^{-8}$  mol  $l^{-1}$ ) and medroxyprogesterone acetate (MPA:  $10^{-6}$  mol  $l^{-1}$ ) for 18 days. After incubation, total RNA was isolated and mRNA content was determined by RT-PCR. A single incubation was performed in triplicate on cells from a single hysterectomy sample. The tissue samples from three individuals were used and, hence, three different incubations were performed. Each lane is from a culture derived from a single individual. The intensity of the signals of VEGF165 or VEGF121 was normalized to that of the internal control L19. The quantification data (the ratio of VEGF165 or VEGF121 to L19) represent the mean  $\pm$  SEM of three different samples. <sup>a</sup> $P < 0.01$  versus control.

Expression of KDR and flt-1 mRNA was also detected in the isolated endometrial stromal cells, and the treatment with oestradiol and MPA increased expression of KDR mRNA, but not flt-1 mRNA, significantly (Figs 3 and 4).

### Discussion

The results of the present study indicate that during the normal menstrual cycle VEGF immunostaining of both glandular epithelial cells and stromal cells increased during the mid-secretory phase compared with the proliferative phase. A number of reports have shown that epithelial VEGF expression is highest during the mid-secretory phase and is probably under the control of ovarian steroids (Charnock-Jones *et al.*, 1993, 2000; Li *et al.*, 1994; Greb *et al.*, 1995; Shifren *et al.*, 1996; Torry *et al.*, 1996; Macpherson *et al.*, 1999), whereas other workers have reported no significant change during the menstrual cycle (Möller *et al.*, 2001) or high expression at the onset of menstruation (Graubert *et al.*, 2001). However, results on

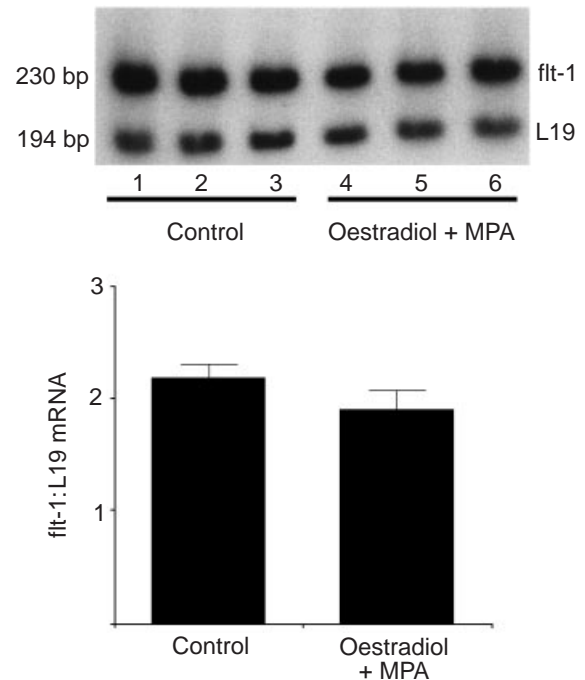
stromal VEGF expression have been more controversial. Therefore, in the present study, endometrium from patients who had been treated with oestrogen and progesterone was used and, in addition, isolated endometrial stromal cells were cultured with oestrogen and progesterone. Our findings indicate that oestrogen and progesterone increased not only VEGF immunostaining in stromal cells *in vivo* but also expression of VEGF mRNA in isolated endometrial stromal cells *in vitro* accompanied by decidualization. These findings are consistent with previous reports showing that VEGF expression is upregulated by oestrogen and progesterone in endometrial stromal cells (Shifren *et al.*, 1996; Huang *et al.*, 1998; Lebovic *et al.*, 2000). In the decidua of early pregnancy, strong VEGF expression in decidual cells and strong flt-1 and KDR expression in vascular endothelial cells were found. The presence of VEGF and its receptors has been reported in peri-implantation uteri in mice (Chakraborty *et al.*, 1995). Collectively, it is likely that VEGF contributes to successful implantation and maintenance of pregnancy through the



**Fig. 3.** Effects of oestrogen and medroxyprogesterone acetate (MPA) on expression of kinase insert domain-containing region (KDR) mRNA in human endometrial stromal cells *in vitro*. Expression of KDR mRNA was examined by RT-PCR using the same total RNA as in Fig. 2. The quantification data (the ratio of KDR:L19) represent the mean  $\pm$  SEM. <sup>a</sup> $P < 0.01$  versus control.

increase in vascular permeability or the formation of the dense vascular network in the decidua.

The present study also showed intense KDR immunostaining in glandular epithelial cells during the menstrual cycle; this finding is consistent with reports by Möller *et al.* (2001) and Meduri *et al.* (2000). Significant KDR immunostaining was observed in the stroma during the secretory phase, especially in the decidualized stromal cells after treatment with oestrogen and progesterone. The immunohistochemical findings in the stroma are supported by the RT-PCR analysis, which showed that KDR mRNA content in the isolated endometrial stromal cells was increased by oestrogen and progesterone treatment. In contrast, Nayak *et al.* (2000) demonstrated immunohistochemical KDR expression in the stroma during the premenstrual phase and that progesterone suppresses stromal KDR expression in macaque endometrium. In addition, Graubert *et al.* (2001) demonstrated high expression of KDR and *flt-1* mRNAs during the menstrual phase, and Krüssel *et al.* (1999) reported that KDR and *flt-1* mRNAs were present at almost constant values throughout the menstrual cycle. In contrast to KDR, immunostaining for *flt-1* in glandular epithelial cells and stromal cells was negligible during the menstrual cycle in the present study, whereas *flt-1* immunostaining in epithelial and stromal cells has been observed in



**Fig. 4.** Effects of oestrogen and medroxyprogesterone acetate (MPA) on expression of *fms*-like tyrosine kinase (*flt-1*) mRNA in human endometrial stromal cells *in vitro*. Expression of *flt-1* mRNA was examined by RT-PCR using the same total RNA as in Fig. 2. The quantification data (the ratio of *flt-1*:L19) represent the mean  $\pm$  SEM.

other studies (Meduri *et al.*, 2000; Möller *et al.*, 2001). Thus, the results on *flt-1* and KDR expression in the human endometrium are still controversial. It is hard to explain the difference, but it may be due to differences in experimental technique, sample preparation and the antibodies used.

KDR expression in endometrial stromal cells was increased by oestrogen and progesterone *in vivo* and *in vitro*, and strong KDR expression was observed in decidual cells. As the gene promoter of human KDR has the binding sites for glucocorticoid receptor/progesterone receptor (GR/PR) but not for oestrogen receptor (Patterson *et al.*, 1995), KDR appears to be under the control of progesterone. In contrast, stromal *flt-1* expression was not affected by oestrogen and progesterone, whereas *flt-1* was expressed strongly in decidual cells. Decidual *flt-1* expression may be influenced by a local factor derived from the conceptus, as there is a report suggesting the involvement of a conceptus factor to complete the decidualization process of endometrial stromal cells in primates (Tarantino *et al.*, 1992). In addition, there is a possibility that VEGF receptor expression may be modified by factors other than ovarian steroids, for example, cytokines or hypoxia (Patterson *et al.*, 1996; Sharkey *et al.*, 2000). These different expression patterns and regulation of *flt-1* and KDR indicate that they may play different roles in the regulation of endometrial function, as suggested by Smith (1998) and Waltenberger *et al.* (1994).

Further studies are needed to determine their specific roles. In the present study, KDR was also localized in vascular smooth muscle cells. As there is a report that uterine smooth muscle cells express KDR and proliferate in response to VEGF (Brown *et al.*, 1997), the VEGF system may be involved in the proliferation of vascular smooth muscle cells.

The present study raises the possibility that VEGF may regulate the function of endometrial stromal cells and glandular epithelial cells in an autocrine–paracrine manner, as VEGF receptors were expressed in those cells. It has been reported that VEGF receptors are expressed in a variety of non-endothelial cells (Ergun *et al.*, 1997; Krüssel *et al.*, 1999; Meduri *et al.*, 2000; Sugino *et al.*, 2000a; Möller *et al.*, 2001) and that they are functional (Barleon *et al.*, 1996; Clauss *et al.*, 1996; Ahmed *et al.*, 1997; Brown *et al.*, 1997). It is of special interest to note that VEGF can act as a survival factor and inhibit apoptosis in endothelial cells (Spyridopoulos *et al.*, 1997; Gerber *et al.*, 1998). Recent evidence indicates that apoptosis may play an important role in the determination of the lifespan of decidual cells and glandular epithelial cells (Akcali *et al.*, 1996; Kokawa *et al.*, 1996, 1998; Dai *et al.*, 2000). In addition, the redox system that may control apoptosis is present in human endometrium (Sugino *et al.*, 1996, 2000b,c, 2001). The high expression of VEGF and its receptors in decidualized cells and glandular epithelial cells, and the upregulation of VEGF and KDR by oestrogen and progesterone indicate that VEGF may be involved in the prolongation of the lifespan of endometrial cells for successful pregnancy.

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