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# Expression of the angiogenic growth factors VEGF, FGF-2, EGF and their receptors in normal human endometrium during the menstrual cycle

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**Angiogenesis is an important but poorly understood process of the cycling endometrium. Endometrial angiogenesis is believed to be regulated by angiogenic growth factors under the influence of ovarian steroids. Vascular endothelial growth factor (VEGF) and its receptors VEGFR-1 and VEGFR-2, fibroblast growth factor 2 (FGF-2) and its receptors FGFR-1 and FGFR-2, as well as epidermal growth factor (EGF) and its receptor EGFR are believed to be important in the control of angiogenesis in the human endometrium. Their expression was examined by immunohistochemistry in endometrial biopsies obtained from 16 healthy women with proven fertility. Western blot analysis showed that the primary antibodies used were specific for their epitopes. We found that VEGF, FGF-2, EGF and their receptors were all expressed, especially in and/or around blood vessels, thus supporting the hypothesis that these peptides contribute to the regulation of angiogenesis and blood vessel function in the human endometrium. The receptors VEGFR-1, VEGFR-2, FGFR-2 and EGFR were co-expressed and exhibited their strongest expression during the beginning of the secretory phase, coinciding with the developing endometrial oedema and formation of a complex subepithelial capillary plexus. No correlation was seen between receptor expression and stromal blood vessel density.**

*Key words:* angiogenesis/EGF/endometrium/FGF-2/VEGF

## Introduction

Ovarian steroids are the prime modulators of the cyclic endometrial changes and they do this by interacting with local growth factors to regulate growth and differentiation of the endometrium (Giudice, 1994). Following the onset of menstrual bleeding, the upper functional layer of the endometrium is shed, and at about this time endometrial regeneration commences. The growth of the endometrium includes the growth of glands, stroma and vessels. Bleeding control and fertility depend on correct regeneration (Cameron *et al*., 1998).

Angiogenesis is defined as the formation of new capillary blood vessels from existent micro-vessels by sprouting, i.e. cellular outgrowth (Folkman, 1985). Other mechanisms by which angiogenesis occurs include intussusception of endothelial cells and vessel elongation (Rogers *et al*., 1998b). Angiogenesis and blood vessel function depend on the interaction between endothelial cells, their basal membrane and stromal tissues surrounding capillary vessels (Battegay, 1995; Risau, 1997). During the early proliferative phase, there is a gradual increase in length, branching and coiling of the spiral arteries, and during the late proliferative and early secretory phases, there is development of a complex subepithelial capillary plexus (Johannisson, 1990). Some previously obtained results (Rogers *et al*., 1993) have shown that endometrial vascular density does not differ during the normal menstrual cycle while others (Morgan *et al*., 1996) have found a significantly higher micro-vascular density in mid-secretory phase stroma compared with the proliferative phase. These studies do not state precisely when angiogenesis occurs but indirectly indicate that the angiogenic activity must be high in order for vascular density to keep pace with endometrial thickening during the proliferative and early secretory phases. However, it has not been possible to demonstrate any peaks of endothelial cell mitotic activity during the menstrual cycle (Goodger and Rogers, 1994).

Several growth factors are present in human endometrium (Abulafia and Sherer, 1999). Among these are vascular endothelial growth factor (VEGF), fibroblast growth factor 2 (FGF-2) and epidermal growth factor (EGF), which appear to be important for both angiogenesis and blood vessel function in

general (Smith, 1995; Pepper *et al*., 1996; Torry and Torry, 1997) and are thus probably also important in the human endometrium (Rogers *et al*., 1992; Smith, 1995, 1997; Risau, 1997; Abulafia and Sherer, 1999). VEGF is a growth factor that stimulates angiogenesis via its receptors VEGFR-1 (flt-1) and VEGFR-2 (KDR), both of which have tyrosine kinase activity (Klagsbrun and D'Amore, 1996). Four different splice variants of VEGF and their two receptors are known to be present in human endometrium (Charnock-Jones *et al*., 1993; Krussel *et al*., 1999; Naresh *et al*., 1999). FGF-2 stimulates angiogenesis by binding to either of its two receptors, FGFR-1 and FGFR-2, both of which have tyrosine kinase activity (Friesel *et al*., 1989). Previous data show that both FGF-2 and its two receptors are expressed in human endometrium (Ferriani *et al*., 1993; Anania *et al*., 1997; Sangha *et al*., 1997). EGF is ascribed with a variety of biological effects including a mitogenic effect, stimulating proliferation of many cell types including human microvascular endothelial cells (Nezu *et al*., 1992; Ushiro *et al*., 1996) and lymphatic endothelial cells (Liu and He, 1997). EGF as well as its receptor (EGFR) have been found in human endometrium (Imai *et al*., 1995; Niikura *et al*., 1996; McBean *et al*., 1997).

The main objective of this study was to examine the co-expression and co-variation of VEGF, FGF-2 and EGF and their respective receptors, and the blood vessel density, in endometrial biopsies obtained from different menstrual cycle phases using immunohistochemistry.

## Materials and methods

### *Patient recruitment*

Sixteen patients aged 32–45 (average age 37) years were included, seven were in the proliferative phase and nine were in the secretory phase. They had no gynaecological disease or any other known disease, used no medication and had regular (27–30 day cycles), menstruation lasting 3–6 days and no excessive pain during menstruation. All patients had proven fertility with no previous fertility problems and they had delivered between two and five live infants with the last delivery 1–3 years prior to biopsy. They had not received any hormonal medication or used any intrauterine device for at least 6 months. None of these women have been smokers. Biopsies were collected at surgical sterilization after cervical dilatation. The protocol of the study was approved by the Ethical Committee of the University of Uppsala and the patients were informed and gave their consent before surgery.

Prior to pre-medication, a venous blood sample was taken for analyses of serum concentrations of oestradiol and progesterone. Hormone analysis were performed using a method based on enzyme-amplified chemiluminescence (Immulite®; Diagnostic Products Corporation, CA, USA) according to supplier's instructions. Biopsies were collected by one blind sharp curettage of the anterior wall and one of the posterior wall of the corpus part of the uterine cavity. Each sample rendered a 5–15 mm strip of endometrium that was immediately cut into smaller pieces, separately mounted in cryo glue for immunohistochemistry and put into cryo tubes for Western blot analysis. The biopsy samples were snap-frozen in –70°C isopentane (2-methyl butane) and stored in liquid nitrogen until used. Menstrual cycle phase was determined by combining menstrual history data, histological criteria (Noyes *et al*., 1950) and serum concentrations of oestradiol and progesterone. The samples were dated as the number of days from estimated time of ovulation (LH ± 0).

### *Immunohistochemistry*

Serial 5 µm cryo-sections were prepared from biopsies from both the anterior and posterior walls of the uterus. When used for staining of VEGF, VEGFR, FGF-2 and FGFR the sections were fixed with Zamboni's fixative (phosphate buffer with paraformaldehyde 2% w/v and picric acid 57 mmol/l, pH 7.3) for 2 min and endogenous peroxidase enzyme activity was quenched with 0.6% hydrogen peroxide (v/v) in phosphate-buffered saline (PBS) (Merck, Darmstadt, Germany). Prior to staining for EGF and EGFR the sections were fixed for 10 min in acetone at –20°C and endogenous peroxidase enzyme activity was quenched with 0.3% hydrogen peroxide (v/v) in PBS. All biopsies included in the study were run simultaneously and in three separate stainings for each primary antibody. Incubations were done in a humified chamber and between each step the sections were rinsed three times for 5 min with PBS. Non-specific binding was blocked with bovine serum albumin fraction V (BSA; Sigma, St Louis, MO, USA) for 1 h at room temperature. Incubations with primary antibodies were done overnight at 4°C. Rabbit anti-VEGF at 2 µg/ml (polyclonal, Ab-2, PC37; Oncogene Science Inc., Cambridge, MA, USA), rabbit anti-VEGFR-1/Flt-1 at 0.5 µg/ml (polyclonal, C-17, sc-316; Santa Cruz Biotechnology Inc., CA, USA), mouse anti-VEGFR-2/KDR at 0.5 µg/ml ($IgG_1$, A-3, sc-6251; Santa Cruz Biotechnology), mouse anti-FGF-2 at 0.1 µg/ml ($IgG_1$, Ab-3, GF22; Santa Cruz Biotechnology), rabbit anti-Flg/FGFR-1 at 0.1 µg/ml (polyclonal, C-15, sc-121; Santa Cruz Biotechnology), rabbit anti-Bek/FGFR-2 at 0.1 µg/ml (polyclonal, C-17, sc-122; Santa Cruz Biotechnology), rabbit anti-EGF at 5 µg/ml (polyclonal, Ab-3; Oncogene Science) and rabbit anti-EGFR at 1 µg/ml (polyclonal, Ab-1, Oncogene Science) were used as primary antibodies. After rinsing, the sections were incubated with a biotinylated secondary antibody (Multi-link, E 0453; Dako A/S, Glostrup, Denmark) for 30 min on a rocking table at room temperature. The sections were stained by the highly sensitive biotin-streptavidin horseradish peroxidase method using the Elite ABC kit (Vector Laboratories, Burlingame, CA, USA) according to the kit instructions. After rinsing, the sections were exposed to chromogen solution (6 ml dimethylsulphoxide, 10 mg 3-amino-9-ethylcarbazol, 50 ml PBS and 10 µl 30% $H_2O_2$) for 5 min at room temperature. Counter-staining was done with Mayer's haematoxylin solution. The sections were rinsed first with tap water and then with deionized water prior to mounting with glycerol-gelatin at 37°C. Negative control staining was performed by omitting the primary antibody and by using 1 µg/ml rabbit polyclonal Ig mix (cat. no. X0903; Dako A/S) instead of anti-VEGF, -VEGFR-1, anti-FGFR-1, -FGFR-2, -EGF and -EGFR antibodies, or 0.5 µg/ml mouse $IgG_1$ (cat. no. X0931; Dako A/S) instead of anti-VEGFR-2 and anti-FGF-2 antibodies. Non-specific staining was not detected in biopsies from different parts of the menstrual cycle. Examination was conducted using a Nikon microscope. Immunostaining of biopsies from the anterior and posterior aspects of the uterine cavity was graded by two independent observers as (0) when comparable to negative control, (1) when weak (clearly visible but no more), (2) when in-between weak and strong and (3) when strong. Series of all samples and controls were run at least twice.

### *Blood vessel density*

This was estimated by counting blood vessels stained with a monoclonal mouse anti-CD31 (PECAM-1) (Sigma). Staining procedure was the same as described above with fixation in Zamboni's fixative. Five random fields each 0.112 $mm^2$ of the functional layer were counted and the median value was calculated. Double staining

(EnVision; Dako A/S) with monoclonal mouse anti-human CD68 (Dako A/S) was done to avoid counting monocytes stained with anti-CD31. Negative control staining was performed by using mouse $IgG_1$ (cat. no. X0931; Dako A/S) instead of anti-CD31 and anti-CD68 antibodies.

### *Western blot analyses*

These were done to test the specificity of primary antibodies used for immunohistochemistry. Fresh frozen tissues were homogenized in lysis buffer (20 mmol/l Tris–HCl, pH 7.4, 0.1 mol/l NaCl, 5 mmol/l $MgCl_2$, 1% Nonidet P-40, 0.5% sodium deoxycholate and 2 kallikrein inhibitor units/ml aprotinin). Tissue samples were centrifuged at 13 000 *g* for 20 min and the supernatants were prepared for electrophoresis. The protein concentration was determined by using the Bio-Rad Protein Assay (BioRad Laboratories, Hercules, CA, USA). Each lane of a sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS–PAGE) gel was loaded with 100 µg of proteins and run under standard conditions. Proteins were electroblotted onto nitrocellulose membranes (Hybond-C extra; Amersham Life Science, Bucks, UK). Non-specific binding was blocked by incubating the membranes with 5% BSA for 2 h. The blots were incubated with the same primary antibodies as were used for immunohistochemistry (see previous section). The membranes were washed in PBS with 0.05% Tween 20 (Merck, Hohenbrunn, Germany) and allowed to react with a secondary antibody that corresponded to the primary antibody, goat anti-rabbit Ig or anti-mouse Ig (Dako A/S) for 60 min at room temperature. Immunoreactive proteins were visualized with NBT/BCIP (4-nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate), a modified alkaline phosphatase substrate. Negative control staining was performed by omitting the primary antibody and by using 1 µg/ml rabbit polyclonal Ig-mix (cat. no. X0903; Dako A/S) instead of anti-VEGF, -VEGFR-1, -FGFR-1, -FGFR-2, -EGF and -EGFR antibodies, or 0.5 µg/ml mouse $IgG_1$ (cat. no. X0931; Dako A/S) instead of anti-VEGFR-2 and -FGF-2 antibodies. Non-specific staining was not detected. Kaleidoscope pre-stained standards (BioRad Laboratories) were used as molecular weight markers.

## Results

Mean serum concentrations of progesterone and oestradiol were 2.99 (0.4–5.9) nmol/l and 328 (85–748) pmol/l respectively in the proliferative phase and 33.9 (12.2–52.8) nmol/l and 490 (262–725) pmol/l respectively in the secretory phase.

### *Immunohistochemistry*

Within each patient we found similar results in samples from anterior and posterior aspects of the uterine cavity.

VEGF was detected in endometrial gland epithelium, luminal epithelium, stroma and blood vessels with no significant changes during the menstrual cycle (Figure 2A). Blood vessels exhibited the most pronounced expression (Figure 1A). VEGFR-1 showed a weak to moderate expression in the stroma and in the apical aspect of some but rather few endometrial gland cells in some samples. Most of the blood vessels showed a weak to strong expression in endothelial cells with the strongest and most constant expression from LH+3 until LH+11 (Figures 1B and 2C). VEGFR-2 showed a moderate to strong expression in luminal and glandular epithelium while the expression in stroma varied from none to weak. Glandular epithelial cells were stained mainly in their apical region. In blood vessels (Figure 1C), the expression varied from none to moderate with an apparent maximum of expression during days LH+3, LH+4 and LH+5 (Figure 2E).

FGF-2 was detected in luminal and glandular epithelium, blood vessels and stroma. It was found only in the basal part of luminal and glandular epithelial cells and/or in their basement membrane in most (but not all) samples, and its expression was more intense during the proliferative phase. In blood vessels, the expression was concentrated in endothelial cells and/or their basement membrane (Figure 1D), with the most intense expression during the menstrual and proliferative phases (Figure 2B). In stroma, the expression was rather weak during the menstrual cycle except at menstruation and in the early proliferative phase. FGFR-1 was expressed in glandular and luminal epithelium and arteries but not in veins or stroma. In glandular epithelium, the expression was strong during the late proliferative and secretory phases. In arteries, there was a weak expression in samples from LH+8 and LH+11 (Figures 1E and 2D). FGFR-2 was expressed in endometrial glands in about 50% of the samples with no change during the menstrual cycle. Blood vessels showed a weak or moderate expression in endothelial cells only in the early and late secretory phases (Figures 1F and 2F). Both FGFR-1 and FGFR-2 were expressed in arteries but not in veins in the late secretory phase.

EGF was expressed mainly in luminal and glandular epithelium and in stroma. In glandular epithelium, the expression was intense in mid-proliferative phase while the expression was weak or absent in the secretory and menstrual phases (Figure 2G). In stroma, the expression showed a rather variable pattern. In arteries, there was a weak expression in biopsies from certain patients (Figure 1G) but expression was absent in the remainder, and there was no expression in veins. EGFR was expressed in luminal and glandular epithelium, blood vessels and stroma. Glandular and luminal epithelium showed strong expression throughout the menstrual cycle. In stroma, there was no or weak staining in all samples between LH–9 and LH+7 whereas the expression was moderate in the late secretory phase and strong in the samples collected at LH–12 and LH–10. In arteries, there was no or weak expression except for an intense expression at LH–12 and a moderate expression in samples from LH+4, LH+5 and LH+6 (Figures 1H and 2H).

In blood vessels, there was co-expression and co-variation of VEGFR-1, VEGFR-2, FGFR-2 and EGFR during the early secretory phase whereas FGFR-1 was only expressed prior to menstruation (Figure 2C, E, F and H). The corresponding growth factors were found either in the blood vessel wall or in adjacent stroma.

### *Blood vessel density*

There was a rather inter-individual pattern with no clear tendency during the menstrual cycle (Figure 3). Two individual values were higher than the others, one from a menstruating women (345 vessels/$mm^2$) and one in late secretory phase (304 vessels/$mm^2$) while the others varied between 99 and 251 vessels/$mm^2$. Biopsies from anterior and posterior walls of the same uterus showed similar results. The average values are presented.

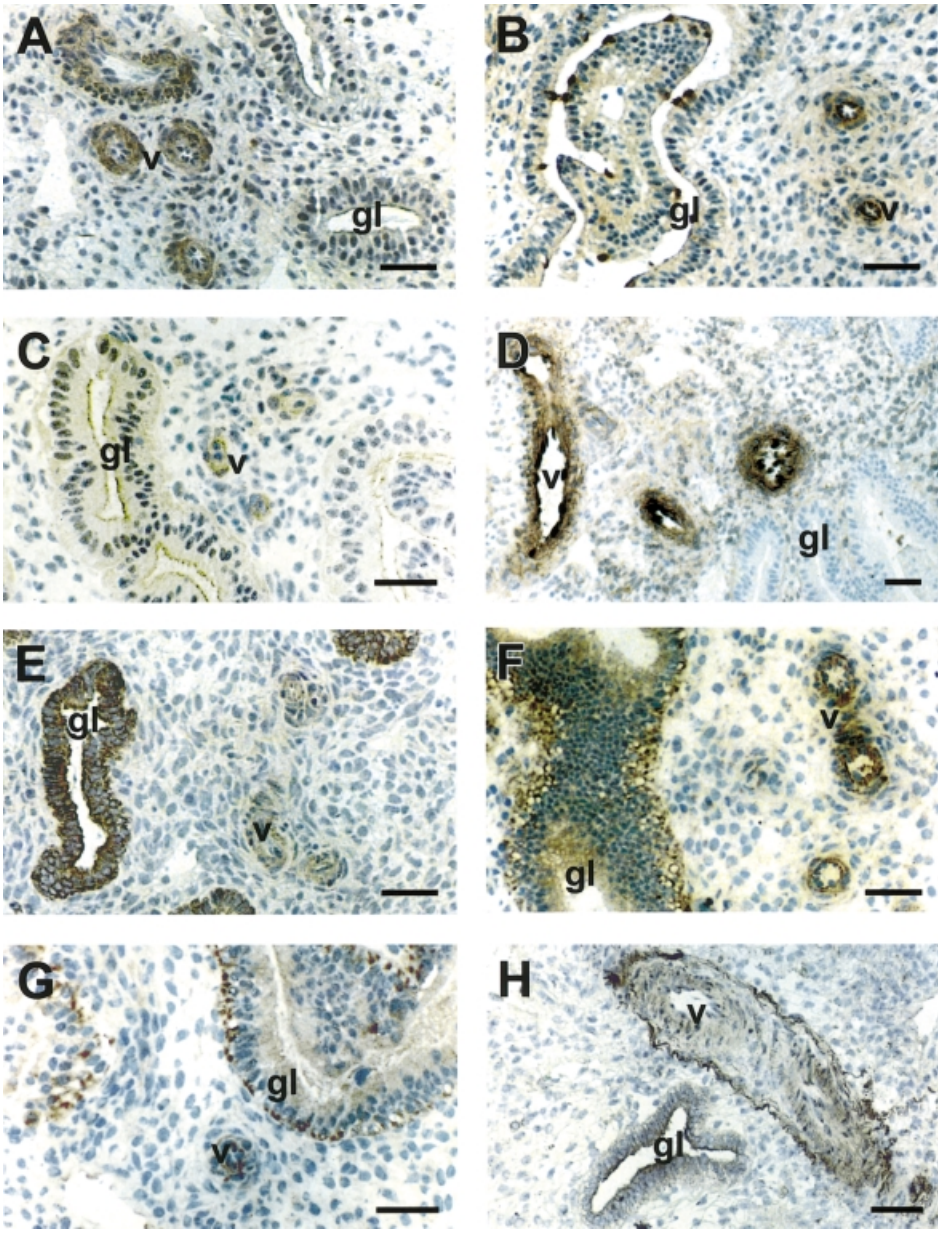


**Figure 1.** Immunohistochemical staining of human endometrium showing the expression in blood vessels (v), glandular epithelial cells (gl), and stromal cells. Vascular endothelial growth factor (VEGF) (LH+2) (**A**), VEGF receptor (VEGFR)-1 (LH+2) (**B**) and VEGFR-2 (LH+5) (**C**) were all found in both blood vessels (v) and glandular epithelial cells (gl). VEGF was expressed in all components of the blood vessel whereas VEGFR-1 and VEGFR-2 were expressed mainly in the endothelial cells. Fibroblast growth factor (FGF)-2 (LH+2) (**D**), FGF receptor (FGFR)-1 (LH+13) (**E**) and FGFR-2 (LH+13) (**F**) were all expressed in blood vessels (v) and sometimes in glandular epithelium. FGF-2 and FGFR-2 seem to be expressed mainly in the endothelial cells or their basement membrane. Epidermal growth factor (EGF) (LH+6) (**G**) and EGFR (LH+2) (**H**) were expressed in some of the blood vessels (v). EGF was also expressed in the basal part of glandular epithelium while EGFR was expressed in the apical part of the glandular epithelial cells (gl). Scale bar = 25 μm.

### *Western blot analysis*

This revealed that the antibodies recognized bands containing molecules with molecular weights corresponding to the desired epitopes (Figure 4). The anti-VEGF antibody stained bands corresponding to $VEGF_{121}$, $VEGF_{165}$ and $VEGF_{189}$, anti-VEGFR-1 to a soluble form at 150 kDa and a membrane bound form at 180 kDa, and anti-VEGFR-2 to a band corresponding to the full length receptor at 220 kDa. Anti-FGF-2 stained bands at both 16 and 18 kDa, where the 16 kDa band represent a degradation product of FGF-2. Antibodies against FGFR-1 and FGFR-2 recognized bands at 150 and 125 kDa respectively and antibodies against EGF and EGFR recognized their epitopes at about 6 and 170 kDa respectively.

## Discussion

Previous studies on the expression of different growth factors and growth factor receptors in human endometrium have usually been focused on one ligand or receptor. A rather high degree of regional variability has been demonstrated within the endometrium of a given individual (Rogers, 1998a) and between individuals in the same menstrual cycle phase

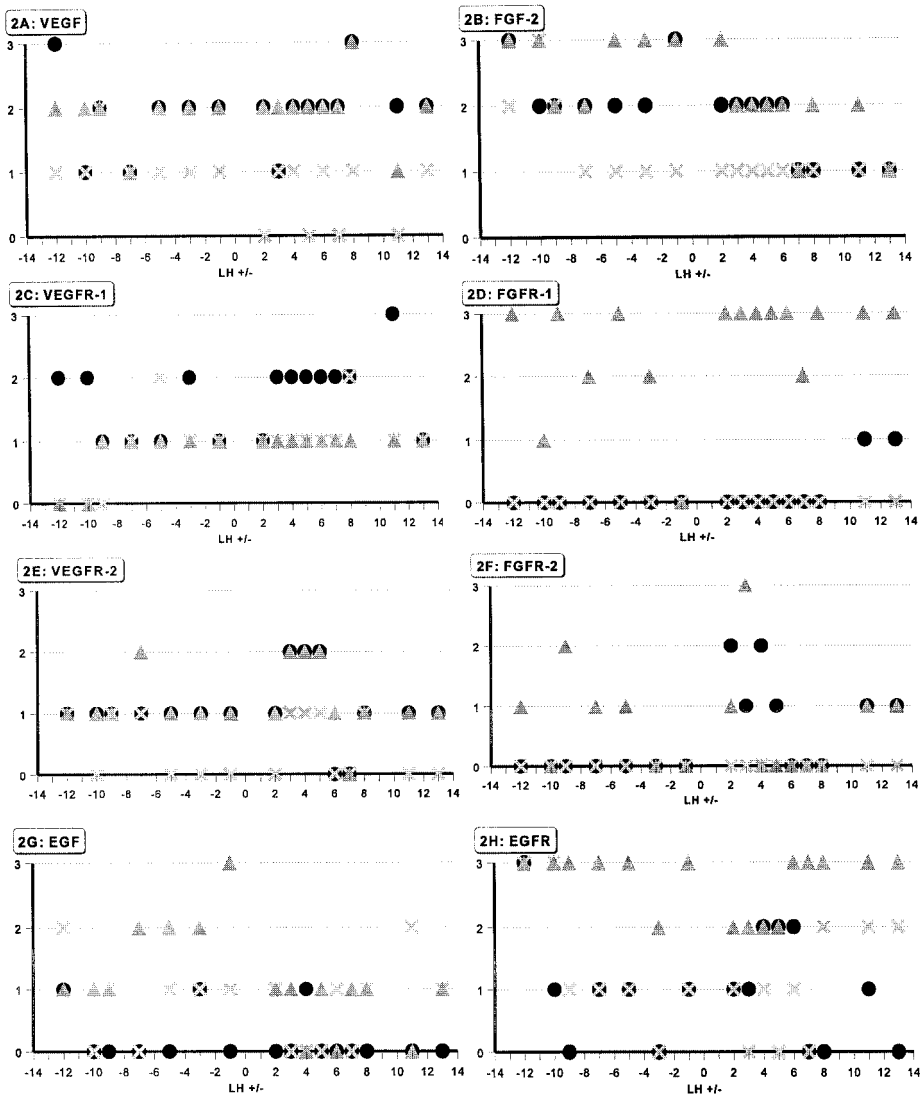


**Figure 2.** Immunostaining of endometrial blood vessels (●), stroma (×) and epithelium (▲) in endometrial biopsies that were dated as time of ovulation (LH) ± days (*x*-axis). Staining was graded from 0 to 3 (*y*-axis) and each dot represents one patient. VEGF (**A**), FGF-2 (**B**), VEGFR-1 (**C**), FGFR-1 (**D**), VEGFR-2 (**E**), FGFR-2 (**F**), EGF (**G**) and EGFR (**H**). See Figure 1 for abbreviations.

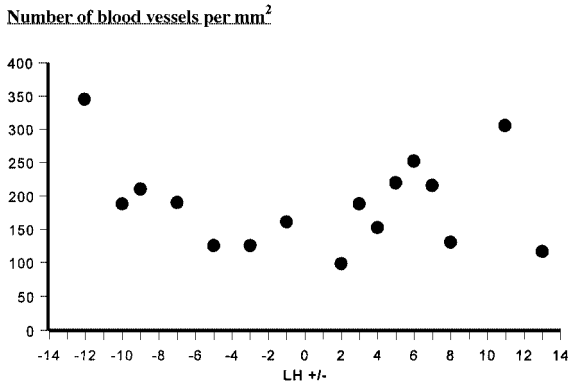


**Figure 3.** Number of endometrial blood vessels per $mm^2$ in the stroma of endometrial biopsies that were dated as time of ovulation (LH) ± days (*x*-axis). Each dot represents one patient.

(Goodger and Rogers, 1994) making it hazardous to interpret data by combining results from different studies. To reduce these problems, we studied the co-expression and co-variation of VEGF, FGF-2 and EGF and their receptors in a set of biopsies from women with normal endometrium.

Biopsies were collected from carefully selected women to avoid confounding factors. It is well known that many diseases and pharmaceutical compounds may influence the menstrual cycle and the endometrium. It has been shown that uterine pathology can cause an abnormal expression of different angiogenic growth factors and growth factor receptors (Kooy *et al*., 1996; Anania *et al*., 1997; Sangha *et al*., 1997). Smokers were excluded since cigarette smoking has an anti-oestrogenic effect (Michnovicz *et al*., 1986; Vessey *et al*., 1987; McLaren *et al*., 1990) and because tobacco use causes sub-fertility (Bolumnar *et al*., 1997; Curtis *et al*., 1997) as well as menstrual abnormalities among users of contraceptive pills (Rosenberg *et al*., 1996). All included patients had normal concentrations of oestradiol and progesterone that were in accordance with menstrual data and histological appearance of the tissue sections. These numerous criteria, when selecting patients, made it difficult to obtain samples. Regional variability within the

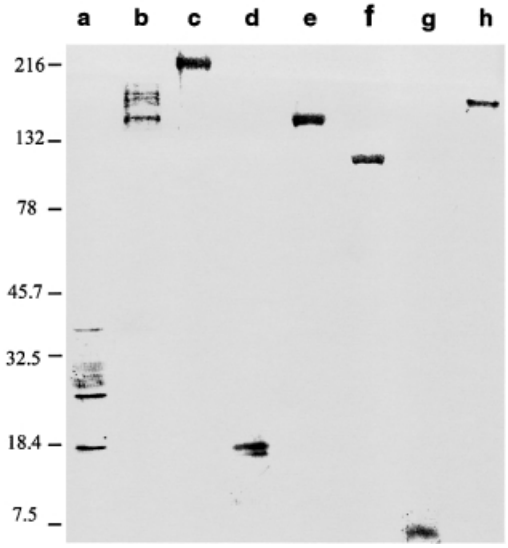


**Figure 4.** Western blot of pooled samples of human endometrium. Stained bands represent in lanes: (**a**) $VEGF_{121}$,$VEGF_{165}$ and $VEGF_{189}$, (**b**) VEGFR-1 (150 and 180 kDa), (**c**) VEGFR-2 (220 kDa), (**d**) FGF-2 (16 and 18 kDa), (**e**) FGFR-1 (150 kDa), (**f**) FGFR-2 (125 kDa), (**g**) EGF (6 kDa) and (**h**) EGFR (170 kDa). See Figure 1 for abbreviations.

endometrium makes it difficult to interpret data obtained from a limited number of patients. To reduce this problem, we examined samples from the anterior and posterior aspects of the corpus part of the uterine cavity from most of the patients. Within each patient we found similar results in the examined samples.

We found expression of VEGF, FGF-2, EGF and their receptors in our material. Their presence in endometrial blood vessels and their surroundings indicates a potential involvement in the regulation of angiogenesis in human endometrium. Western blot analysis showed that the primary antibodies used for immunohistochemistry recognized the desired epitopes.

VEGF expression showed no significant changes during the menstrual cycle either in blood vessels, in stroma or in epithelium. Our findings are thus in agreement with some previously presented data (Li *et al*., 1994; Shifren *et al*., 1996) but not with others (Torry *et al*., 1996; Naresh *et al*., 1999). A menstrual cycle-dependent variation in VEGF activities is expected to be under the control of ovarian steroids (Hyder and Stancel, 2000). Another possibility is that ovarian steroids control VEGF activities by regulating the expression and function of the VEGF receptors. Besides ovarian steroids, it seems as if hypoxia can up-regulate the expression of endometrial VEGF (Sharkey *et al*., 2000). Our results are consistent with (although possibly not for the same reasons) the lack of cyclicity observed in serum concentrations of VEGF during the normal menstrual cycle (Unkila-Kallio *et al*., 2000). The production and expression of VEGF as well as other growth factors is probably a local event that normally is not mirrored in serum concentrations.

The VEGF receptors showed distinct patterns and changes of expression during the menstrual cycle. VEGFR-1 was detected in endometrial glands, blood vessels and in scattered stromal cells. The latter probably represents macrophages (monocytes) which are known to express VEGFR-1 but not VEGFR-2 (Ahmed *et al*., 1995; Clauss *et al*., 1996) and to actively participate in the angiogenic process (Sunderkötter *et al*., 1994). The unexpected finding that VEGFR-1 and VEGFR-2 were expressed in epithelial cells is supported in a study where they found mRNA for the two receptors in both epithelial and stromal cell fractions by using reverse transcription-polymerase chain reaction (Krussel *et al*., 1999). It is known that, in response to VEGF stimulation, VEGFR-2 may be involved in biological functions other than angiogenesis such as a rapid release of nitric oxide from endothelial cells (Kroll and Waltenberger, 1999). VEGFR-2 is also found in human uterine myometrial smooth muscle cells (Brown *et al*., 1996), porcine endometrial epithelial cells (Winther *et al*., 1999) and in several non-uterine tissues such as in pancreatic islet cells (Oberg *et al*., 1994), human glomeruli (Whittle *et al*., 1999), and in several types of tumour cells such as Kaposi's sarcoma (Skobe *et al*., 1999). The precise function in each situation is, however, not known.

FGF-2 lacks a secretory signal sequence and is therefore thought to be most active at menstruation and in the early proliferative phase, since it is released in significant amounts during this period (Rusnati *et al*., 1993; Sangha *et al*., 1997). However, FGF-2 was expressed throughout the menstrual cycle in blood vessels and their surroundings from where it can be recruited by activated endothelial cells that release proteolytic enzymes into their surroundings. The FGF receptors were not expressed in blood vessels during the major part of the menstrual cycle in our material although we could detect FGFR-2 but not FGFR-1 in early secretory phase, and both FGFR-1 and FGFR-2 were expressed in arteries but not veins in late secretory phase. The significance of expression in arteries but not in veins remains unknown. Previous data show an expression of FGFR-1 in endothelial cells throughout the menstrual cycle (Sangha *et al*., 1997). No previous data have been available on the expression of FGFR-2 protein in blood vessels of normal cycling endometrium, but its mRNA has been shown to be present in both proliferative and secretory endometrium (Siegfried *et al*., 1997). From our data, we conclude that FGFR-1 and FGFR-2 are rather sparsely expressed in and around blood vessels and that they might therefore be less important for the regulation of angiogenesis, except in the secretory phase arteries when they may have a more important role.

EGF was expressed in stroma in most samples. Arteries showed a weak expression in a few samples, but there was no staining in veins. EGFR was also found in endometrial arteries but not in veins. The present EGF and EGFR may thus be able to induce angiogenesis in arteries, either via paracrine or autocrine routes. EGFR may, however, also be used by some of its other five ligands (Pfeiffer *et al*., 1997), thus making it difficult to interpret whether EGF and EGFR actually interact within the endometrium. Our data and previous data (Mukku and Stancel, 1985; Huet-Hudson *et al*., 1990; Ishihara *et al*., 1990; Morishige *et al*., 1993; Adachi *et al*., 1995; Imai *et al*., 1995; McBean *et al*., 1997) suggest that synthesis and expression of EGF and EGFR vary with the stage of the menstrual cycle and that expression of EGF is associated with

the increase in serum oestrogen, while the expression of EGFR is associated with an increase in progesterone concentrations. However, we found the most pronounced menstrual cycle-dependent variations of EGF in epithelium and of EGFR in blood vessels and stroma.

Ovarian steroids primarily modulate cyclic endometrial changes by interacting with local growth factors to regulate growth, differentiation and function of the endometrium. Oestrogen and progesterone might also have a direct impact on endometrial endothelial cells since they express oestrogen and progesterone receptors (Iruela-Arispe *et al*., 1999). However, recent data show a lack of correlation between menstrual cycle stage and endothelial cell proliferation index or endothelial cell expression of integrin $\alpha_v\beta_3$, which suggests that vascular growth, at least, is not under the overall control of oestrogen and progesterone (Rogers *et al*., 1998b). Stromal endometrial vascular density is, according to previous data (Morgan *et al*., 1996), significantly higher in the mid-secretory phase compared to proliferative phase, while others (Rogers *et al*., 1993), found no significant difference across the normal menstrual cycle. These data suggest that there is an ongoing angiogenic activity whenever there is a thickening of the endometrium and that peaks in angiogenic activity should be expected during periods when there is an increase in endometrial thickness, and also when endometrial oedema starts to develop after ovulation. Such peaks of endothelial cell mitotic activity have, however, not been possible to demonstrate (Goodger and Rogers, 1994), probably due to a rather heterogeneous material and a large variation in endothelial cell proliferative activity between individuals within the same menstrual stage. Our results showed two endometria with a rather high vascular density. The first one was during menstruation (LH–12) when the functional layer was shed, thus representing vascular density in the basal layer. If this one is omitted, the median vascular density for the functional layer would be 166 vessels/mm$^2$ in the proliferative phase and 186 vessels/mm$^2$ in the secretory phase, thus indicating a higher vascular density in the secretory phase. There is a cluster of samples representing LH+3 to LH+7 with a higher expression and co-expression of VEGFR-1, VEGFR-2, FGFR-2 and EGFR. A high vascular density was also noted in a sample from LH+11 that showed the highest expression of VEGFR-1, but the significance of this remains unclear. These data are in concordance with previous data showing an intensified expression and co-expression of VEGFR-1 and VEGFR-2 in the secretory phase, coinciding with an increase in microvascular density (Meduri *et al*., 2000).

Our results thus show a co-expression of VEGFR-1, VEGFR-2, FGFR-2 and EGFR in endometrial blood vessels that was stronger during the early secretory phase and thus correlates in time with the development of stromal oedema and an increase in microvascular density. Whether VEGF, FGF-2 or EGF up-regulates angiogenesis via VEGFR-1, VEGFR-2, FGFR-2 and EGFR during this period, however, remains to be shown.

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