

## Ovarian steroid and cytokine modulation of human endometrial angiogenesis

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**A key mechanism underlying the cyclical growth of the endometrium is its ability to regenerate a vascular capillary network. In normal cycling human endometrium, angiogenesis is influenced by both endocrine and paracrine factors. Hormonal manipulation of the endometrium, such as that occurring during the use of steroidal contraception, appears to result in capillary proliferation and fragility. As a consequence of these vascular changes, contraceptive users may be predisposed to unpredictable uterine bleeding, which is responsible for the high frequency of contraceptive discontinuation. In this paper we address mechanisms responsible for vascular endothelial cell proliferation in normal and contraceptive steroid-exposed endometria. We propose that regulation of endometrial angiogenesis is mediated indirectly, via steroid and cytokine actions on vascular endothelial growth factor (VEGF), and we present data indicating that VEGF expression in normal endometrial stromal cells is increased by oestrogens and progestins. Three proinflammatory cytokines with angiogenic effects in other systems (i.e. interleukin-1 $\beta$ , tumour necrosis factor- $\alpha$  and interferon- $\gamma$ ) do not appear to up-regulate VEGF expression in normal endometrial stromal cells. Well-characterized in-vitro models in conjunction with immunohistochemistry provide useful experimental systems to study endometrial neovascularization under physiological**

**conditions and in those potentially perturbed via the use of contraceptive steroids.**

*Key words:* bleeding/Norplant/progestins/uterus/VEGF

### Introduction

Contraceptive effectiveness and safety are high priority public health agenda items in an era when world population growth exceeds the rate of economic productivity. Among the most important practical issues are the acceptability, compliance and continuance of specific contraceptive methods. Uterine bleeding pattern constitutes an important factor determining the acceptability of hormonal methods of birth control. In an international survey (Snowden and Christian, 1983) it was found that most women desire a contraceptive method that yields a regular pattern of bleeding. Of women who discontinue depot medroxyprogesterone acetate (DMPA) or Norplant<sup>®</sup> contraception during the first year of therapy, >60% cite abnormal bleeding patterns as their primary reason for abandoning parenteral progestin methods (Said, 1986; Olsson and Odland, 1988).

DMPA administration is commonly associated with unpredictable bleeding. During the first 9 months of treatment, only 10% of subjects reported 'no disturbance' in their bleeding pattern (Belsey, 1988). The bleeding pattern in the majority of women using Norplant is reported as frequent,

irregular and/or prolonged, particularly during the first year of use (Faundes *et al.*, 1978). In one study of women using Norplant, 16% reported regular cycles, 70% had irregular bleeding, and amenorrhoea occurred in 14% (Shoupe *et al.*, 1991). A variety of factors is involved in the aberrant bleeding seen with parenteral progestin contraceptives. Generally these relate to feedback effects on the hypothalamic–pituitary–ovarian axis and direct effects of these hormones on the endometrium *per se*.

#### ***Cell biology of endometrial vascularization: menstrual cycle effects***

The classical description of histological changes in the cycling human endometrium *in situ* were reported by Noyes *et al.*, in 1950. A decade earlier, Markee described similar morphological changes in real-time, by direct observation of full-thickness biopsies of primate endometrium transplanted into the anterior chamber of the rhesus eye (Markee, 1940). The uterine endometrium grows from a thickness of 1–2 mm during the early proliferative phase to a maximum thickness of >5 mm at the time of ovulation. The number of endometrial glands in the functional layer remains relatively constant throughout the cycle at ~20/mm<sup>2</sup>; however, an increase in epithelial and stromal cell DNA synthesis and mitosis occurs under the influence of follicular phase oestradiol secretion. Studies now indicate that much of the mitotic activity is mediated via the expression of epidermal growth factor (EGF) (Nelson *et al.*, 1991) and its receptor (Lingham *et al.*, 1988).

The functional layer of endometrium is supplied by an end-arteriole referred to as the spiral artery. Each artery is responsible for the perfusion of ~4–7 mm<sup>2</sup> of endometrium. These vessels, unlike the radial and basal arteries that feed them, are highly sensitive to ovarian steroids (Markee, 1940). The endometrial vascular architecture changes throughout the menstrual cycle, paralleling changes in the epithelium and stroma. From day 0 to day 25, there is a gradual increase in branching and coiling of spiral arteries, corresponding to an increase in the length and coiling of endometrial glands. Most studies indicate that endometrial microvessel proliferation also is modulated during the ovulatory cycle.

During the late proliferative phase and throughout the secretory phase a complex subepithelial capillary plexus develops, achieving a maximal density between cycle days 18–22 (Fanger and Barker, 1961). Endometrial endothelial cell proliferation, assessed by [<sup>3</sup>H]thymidine incorporation in tissue explants (Ferenczy *et al.*, 1979) was maximal between days 19 and 22 of the cycle. Human secretory endometrial explants also secrete more angiogenic activity than tissue samples from other phases of the ovulatory cycle (Rogers *et al.*, 1992). Thus, under endocrine conditions where both oestrogen and progesterone levels are elevated, human endometrial angiogenesis appears to be most active. Similar observations have been made in the subhuman primate (Kaiserman and Padykula, 1989). On days 25–28, perivascular and stromal predecidualization is the harbinger for the onset of menses. On cycle days 1–4 (menses), diffuse necrosis, inflammation and vascular thrombosis occur (Noyes *et al.*, 1950) resulting in the predictable, discrete menstrual period observed clinically in ovulatory women.

#### ***Effects of contraceptive steroids on the endometrial vasculature***

Several studies suggest that the endometrial microvessels in the subepithelial capillary plexus also are sensitive to the effects of exogenous steroid hormones. In contrast to normal menstruation, where spiral artery constriction is the critical step leading to the initiation of bleeding, it is probable that progestin-associated bleeding disturbances begin in dilated capillary sinusoids (Martinez-Manautou *et al.*, 1975; Hourihan *et al.*, 1991), which can be observed hysteroscopically in chronic progestin users (Fraser and Peek, 1992). Progestin effects on endometrial capillaries include endothelial cytoplasmic contraction, increased electron density and increased numbers of plasmalemmal vesicles. These findings were temporally correlated with abnormal bleeding patterns (Johannisson *et al.*, 1982). Bleeding problems on Norplant tend to be worst during the first 12 months after insertion of the devices, when suppression of ovarian function is most complete, and levels of oestadiol are lowest. In patients with irregular bleeding on Norplant, the addition of exogenous oestadiol has

been shown to be effective in a blinded, placebo-controlled trial (Diaz *et al.*, 1990).

It was reported that endometrial veins were increased in women taking oral progestins, whereas other endometrial vessels tended to be decreased in number (Hourihan *et al.*, 1986). Unfortunately, the ability to distinguish venules from lymphatic channels is difficult by light microscopy. A more sensitive immunohistochemical staining technique for the CD34 glycoprotein expressed on the plasma membranes of human haematopoietic and vascular endothelial cells has been used (Rogers *et al.*, 1993). These workers reported that microvascular density (expressed as vessels/mm<sup>2</sup>) was increased in Norplant users compared to normal controls biopsied throughout the cycle. Whether the apparent increase in vessel density in women exposed to chronic levonorgestrel is an effect on absolute vessel number *per se* or reflects atrophic changes in endometrial volume will require further investigation.

Some evidence exists to support a role of certain progestins as anti-angiogenic factors *in vitro*. Using the chick chorioallantoic membrane assay for neo-vascularization, it was demonstrated that steroids containing a pregnane nucleus inhibited angiogenesis (Crum *et al.*, 1985). This effect was independent of mineralocorticoid or glucocorticoid activity. Vascular effects of progestins have also been evaluated using endothelial cell cultures. Medroxy-progesterone acetate (MPA) had no effect on bovine endothelial cell growth *in vitro*, but this progestin dramatically reduced endothelial cell plasminogen activator activity with an EC<sub>50</sub> = 10 nmol/l (Ashino-Fuse *et al.*, 1989). The concentration dependence and EC<sub>50</sub> of the MPA effect are similar to those (Ryan *et al.*, 1994) observed for the induction of prolactin secretion by human endometrial stromal cells *in vitro* (Tabanelli *et al.*, 1992), suggesting that these actions may be transduced via progesterone receptors. In primate studies, an anti-progestin (ZK 137,316) had suppressive effects on endometrial development, including inhibition of spiral arteriole formation (Slayden *et al.*, 1998).

#### ***Oestrogen and progesterone receptors in vascular cells***

While changes in endometrial capillary proliferation are modulated during the ovarian cycle and

with contraceptive steroids, endothelial cells *per se* may not be direct targets of oestadiol or progesterone. Receptors for these steroids are present in human uterine artery smooth muscle, but have not been identified convincingly in endothelium (Perrot-Appianat *et al.*, 1988; Taylor *et al.*, 1999). Using the highly sensitive reverse transcription-polymerase chain reaction approach, we (Baker *et al.*, 1997) and others (Jensen *et al.*, 1998) were unable to detect either oestrogen receptor- $\alpha$  or - $\beta$  mRNA in human umbilical vein endothelium. However, functional evidence of oestrogen receptor-mediated gene activation and gel mobility shift assays have been reported by some investigators using these cells (Kim-Schulze *et al.*, 1996). Endothelial cell nuclear progesterone receptors have been demonstrated in murine vessels and were found to respond to progesterone by inhibiting proliferation (Vazquez *et al.*, 1999).

In general, it is believed that oestrogen and progestin effects on angiogenesis are mediated indirectly, predominantly via the paracrine actions of prostaglandins (Schatz *et al.*, 1987; White *et al.*, 1991) or polypeptide growth factors (Presta, 1988; Nelson *et al.*, 1991) generated from nearby endometrial epithelial or stromal cells.

#### ***VEGF as a prototypical angiogenic factor***

Given these observations we postulated that neo-vascularization in normal endometrium was indirectly modulated by ovarian steroids or cytokines, via the production of locally active angiogenic factors (Taylor *et al.*, 1997). Various pleiotropic growth factors [e.g. basic fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), transforming growth factor (TGF)- $\beta$ ] have been shown to have angiogenic activities (Folkman and Klagsburn, 1987). By contrast, the 43 kDa heparin-binding glycoprotein VEGF is specifically mitogenic for endothelial cells (Ferrara *et al.*, 1992) and at present VEGF receptors have only been found on endothelial, myeloid and trophoblast cells. Five molecular species of VEGF have been identified to date (VEGF121, VEGF145, VEGF165, VEGF189, VEGF201), named for the length of their amino acid sequences. The different proteins are generated by alternative splicing of a single primary mRNA transcript. The dominant VEGF mRNA transcripts

expressed by human endometrium and primary human endometrial stromal cells encode the VEGF165 (Torry *et al.*, 1996) and VEGF121 (Huang *et al.*, 1998) proteins.

Our laboratory has investigated the role of two classes of steroid hormones (oestrogens and progestins) and three proinflammatory cytokines [interleukin (IL)-1 $\beta$ , tumour necrosis factor (TNF)- $\alpha$  and interferon (IFN)- $\gamma$ ] as potential mediators of endometrial neovascularization. We postulated that abnormal VEGF expression, mediated by these effectors, is partly responsible for the disordered endometrial capillary proliferation, permeability and fragility observed in users of parenteral progestin contraceptive methods (e.g. Norplant and DMPA).

## Materials and methods

### *Subjects and specimens*

Normally menstruating women undergoing laparoscopy for various indications were recruited. Women who had taken oral contraceptives, hormonal supplements or gonadotrophin-releasing hormone (GnRH) analogues over the previous 3 months were excluded. Twelve control subjects were identified at laparoscopy for tubal sterilization or for assessment of pelvic pain in whom no visible evidence of pelvic pathology was found.

Control endometrial biopsies were obtained under anaesthesia during the operative procedure. Four women each using DMPA or Norplant II contraceptives provided endometrial biopsies for immunohistochemical studies (see below). All subjects provided written informed consent under a protocol approved by the Committee on Human Research at the University of California, San Francisco.

### *Immunohistochemistry*

Endometrial tissue specimens were fixed for 24 h in Histochoice MB<sup>®</sup> (Amresco, Solon, OH, USA), paraffin-embedded and cut in serial sections of 8  $\mu$ m. Sections were stained with a rabbit polyclonal anti-human VEGF antiserum (Santa Cruz Antibodies, Santa Cruz, CA, USA) at a concentration of 10  $\mu$ g/ml using a Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA,

USA). Negative controls for the immunostaining were performed using normal rabbit serum at the identical protein concentration (10  $\mu$ g/ml) as described previously (Shifren *et al.*, 1996). Immunostaining of endometrial capillaries was performed using mouse monoclonal antibodies against human CD34 (2  $\mu$ g/ml; Zymed, South San Francisco, CA, USA). The chromagen signal was enhanced with NiSO<sub>4</sub> using the Liquid Diaminobenzidine–Black Substrate kit (Zymed). Negative controls for the immunostaining were performed using an irrelevant, isotype-specific mouse monoclonal antibody (anti-synaptophysin) at the identical protein concentration (2  $\mu$ g/ml) as described previously (Hornung *et al.*, 1997).

### *Cell cultures*

The techniques for isolation and culture of human endometrial stromal cells *in vitro* have been discussed in detail elsewhere (Ryan *et al.*, 1994). Briefly, follicular phase endometrial biopsies from control women were used to prepare cultures of endometrial stroma. The specimens were minced, digested with collagenase, and then serially filtered through narrow gauge sieves with apertures of 38–105  $\mu$ m to trap the glandular epithelium. Stromal cells were plated and allowed to adhere to plastic cell-culture dishes for 30 min, at which time contaminating epithelial and blood cells and tissue debris were rinsed free. Cultures were allowed to proliferate in minimum essential medium (MEM)- $\alpha$  supplemented with 10% fetal bovine serum, nucleosides, and non-essential amino acids and subcultured to eliminate contamination by immunocytes. All experiments were performed with cells at passage two, within 14 days of initial isolation.

### *Steroid and cytokine treatment*

Prior studies documented the presence of oestrogen receptor (ER)- $\alpha$  and - $\beta$  mRNA and ER and progesterone receptor (PR) proteins in endometrial stromal cells cultured *in vitro* (Brandenberger *et al.*, 1999). The effects of oestradiol, MPA or a combination of the two steroids on VEGF mRNA and protein synthesis were analysed by Northern hybridization and enzyme-linked immunosorbent assay (ELISA), respectively, as described previously (Shifren *et al.*, 1996). Briefly, confluent

cultures of endometrial stromal cells in 24-well dishes were incubated overnight in low-serum medium (phenol-red-free MEM $\alpha$  supplemented with 2.5% FBS, nucleosides, antibiotics and non-essential amino acids) and then exposed to 10 nmol/l 17 $\beta$ -oestradiol, 10 nmol/l oestradiol + 100 nmol/l MPA, or vehicle (ethanol) control for an additional 24 h. Additional experiments were performed using 10 ng/ml IL-1 $\beta$  (R&D Systems, Minneapolis, MO, USA) or 100 ng/ml TNF $\alpha$  and IFN $\gamma$  (Sigma Chemical Co., St Louis, MO). All the cytokines were recombinant human proteins, to which the endometrial stromal cells were exposed for up to 24 h. These doses previously were shown to optimally induce IL-6 (Tseng *et al.*, 1996) and RANTES (Regulated on Activation, Normal T cell Expressed and Secreted) (Hornung *et al.*, 1998) expression in endometrial stromal cell cultures.

#### **Quantification of VEGF mRNA and protein**

At the end of each experiment, conditioned media were aspirated, centrifuged to pellet floating cells, and the supernatants assayed for secreted VEGF<sub>165</sub> using a sensitive sandwich ELISA developed at Genentech, Inc. (South San Francisco, CA, USA) (Shifren *et al.*, 1996). The assay was linear for VEGF in conditioned medium and was sensitive to 0.03 ng/ml. The cells were lysed in TriZOL reagent (Gibco-BRL, Gaithersburg, MD, USA), total RNA was isolated and subjected to formaldehyde gel electrophoresis. Northern blotting with random-primed [ $\alpha$ -<sup>32</sup>P]dCTP-labelled VEGF and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probes were performed as described (Shifren *et al.*, 1996).

#### **Data analysis and statistics**

The data are expressed as the mean  $\pm$  SE of *n* independent determinations. Analysis of variance (ANOVA) and Student's *t*-tests were used. Statistical significance was accepted at *P* < 0.05 for two-tailed analyses.

#### **Results**

Immunohistochemical staining of mid-secretory phase endometrial biopsies from normal controls (Figure 1A) revealed prominent epithelial and

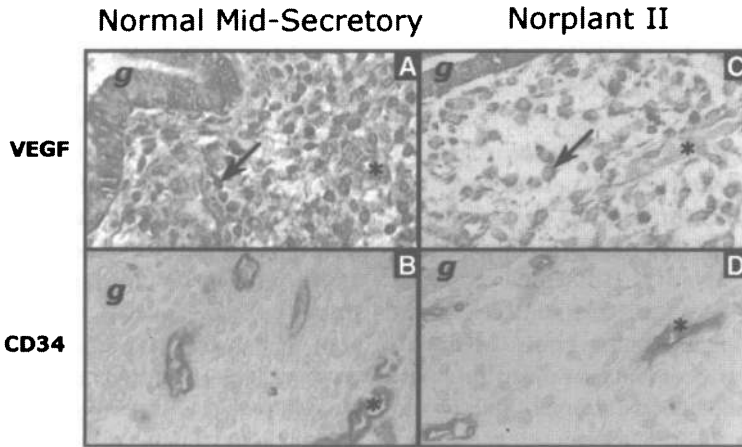
stromal expression of VEGF as we and others have reported previously (Shifren *et al.*, 1996; Donnez *et al.*, 1998). Capillaries within the stromal compartment were immunopositive for CD34 (Figure 1B). Biopsies obtained from Norplant (Figure 1C and D) and DMPA users (data not shown) demonstrated a mixed histological pattern of both proliferative and secretory characteristics (Darney *et al.*, 1996; I.P.Ryan *et al.*, unpublished observations), including stromal oedema, associated with prominent glandular and stromal immunoreactivity for VEGF (Figure 1C). Microvascular morphology in the biopsies was highlighted by anti-CD34 antibodies (Figure 1D). The negative controls showed no immunopositive reaction (data not shown).

Endometrial stromal cell cultures derived from proliferative phase biopsies of normal subjects were developed as a model for the investigation of hormone and cytokine effects on these cells. The combination of oestradiol and progestin (oestradiol+MPA), designed to mimic the endocrine milieu of the normal secretory phase and that of endometrium exposed to chronic progestin administration, caused a marked increase in VEGF mRNA accumulation (Figure 2A). The diffuse bands in the Northern blots are typical for VEGF mRNA and reflect alternative splicing of the primary transcript. A significant stimulation of VEGF protein secretion over control levels also was observed (*P* < 0.05, Table I).

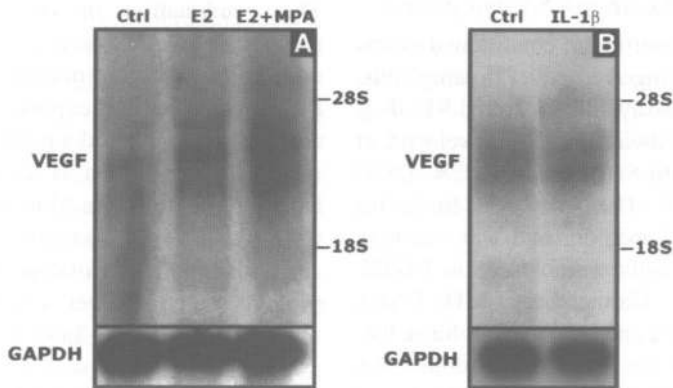
By contrast, exposure of normal endometrial stromal cells to IL-1 $\beta$  had no effect on steady-state VEGF transcript concentrations (Figure 2B) and failed to increase VEGF secretion by these cells (Table II). Likewise, treatment of the stromal cells with TNF $\alpha$  and IFN $\gamma$  for 24 h failed to induce VEGF protein secretion (Table II).

#### **Discussion**

The findings in the current study confirm previous results from ourselves (Shifren *et al.*, 1996) and others (Huang *et al.*, 1998; Smith, 1998) that VEGF gene expression in isolated normal human endometrial stromal cells is up-regulated by ovarian steroid hormones. In particular, the combination of oestrogen and progestin exerts a potent effect on VEGF mRNA accumulation [4.7-fold



**Figure 1.** Immunohistochemistry of vascular endothelial growth factor (VEGF) expression in human endometrial tissues. (A) Mid-secretory phase endometrium from control subject. Black peroxidase reaction denotes VEGF protein present throughout the endometrium functionalis stroma (arrow) and areas of glandular epithelial cytoplasmic immunolocalization (g). (B) CD34 staining on an adjacent section identifies endometrial capillaries (\*). (C) Representative endometrial biopsy from woman using Norplant II for 8 months. Biopsy demonstrated a mixed histological pattern, with prominent glandular (g) and stromal (arrow) cytoplasmic VEGF immunostaining and stromal oedema. (D) Corresponding section showing CD34 positive vascular endothelium in endometrial capillaries (\*).



**Figure 2.** Northern blotting analysis of vascular endothelial growth factor (VEGF) mRNA transcripts in human endometrial stromal cells. (A) Cells exposed to vehicle (control, Ctrl), 10 nmol/l oestradiol or 10 nmol/l oestradiol + 100 nmol/l medroxyprogesterone acetate (oestradiol + MPA). Note up-regulation of multiple VEGF mRNA bands [3.1-fold (oestradiol) and 4.7-fold (oestradiol + MPA) induction over the control], corresponding to multiple transcripts induced by the steroid hormones [adapted from Shifren *et al.* (1996)]. (B) Endometrial stromal cells exposed to vehicle (control, Ctrl) or 10 ng/ml interleukin-1 $\beta$  (IL-1 $\beta$ ) both exhibited basal expression of VEGF mRNA transcripts with no evidence of cytokine-induced up-regulation. Migration of 28S and 18S ribosomal RNA bands are indicated. As a control for equal RNA loading, the constitutive glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene product also was probed.

increase (Shifren *et al.*, 1996)] and protein production [2.3-fold increase (Table I)] relative to untreated cells ( $P < 0.05$ ). This endocrine milieu is similar to those observed in the luteal phase of the menstrual cycle, in the decidua of early human pregnancy, and in the histologically mixed endometria of users of parenteral progestin contraceptives. Clinical studies indicate that VEGF mRNA in

endometrium *in vivo* is increased in all three of these conditions (Shifren *et al.*, 1996; Sharkey *et al.*, 1998; Macpherson *et al.*, 1999). In addition, circulating VEGF concentrations and uterine artery blood flow were noted to be greatest during the luteal phase of the menstrual cycle (Agrawal *et al.*, 1999). Although microvascular growth occurs within the VEGF-positive endometrial stroma

**Table I.** Steroid hormone effects on vascular endothelial growth factor secretion by endometrial stromal cells

Treatment	Experiments (n)	VEGF secretion (pg/ml)
Control (0.1% ethanol)	6	44 ± 9
17β-Oestradiol (10 nmol/l)	6	66 ± 12
17β-Oestradiol (10 nmol/l) + MPA (100 nmol/l)	5	103 ± 22 <sup>a</sup>

<sup>a</sup>Differs significantly from control by analysis of variance with Scheffé's post-hoc test ( $P < 0.05$ ).  
MPA = medroxyprogesterone acetate.

**Table II.** Interleukin 1β (IL-1β), tumour necrosis alpha factor-α (TNF-α) and interferon-γ (IFN-γ) effects on vascular endothelial growth factor secretion by endometrial stromal cells

Treatment	Experiments (n)	VEGF secretion (pg/ml)
Control (PBS)	3	64 ± 34
IL-1β (10 ng/ml)	3	45 ± 7 <sup>a</sup>
TNFα and IFNγ (100 ng/ml)	3	29 ± 15 <sup>b</sup>

<sup>a</sup>Not significantly different from control by Student's *t*-test ( $P = 0.76$ ).

<sup>b</sup>Not significantly different from control by Student's *t*-test ( $P = 0.28$ ).

(Figure 1A and B), a clear correlation between these two phenomena was not identified (Gargett *et al.*, 1999).

Using the same in-vitro cell model we were unable to observe effects of IL-1β, TNFα and IFNγ on VEGF expression, despite the fact that all three cytokines have known actions on other bioactive proteins in normal endometrial stromal cells (Tseng *et al.*, 1996; Hornung *et al.*, 1998). While the data indicate that these cytokines do not modulate endometrial stromal VEGF gene expression, our findings do not exclude potential effects of these cytokines on VEGF expression by other cell types within the endometrium. Furthermore, our studies to date have not addressed the potential interactions of steroid hormones and cytokines on VEGF gene regulation. In fact, information from several studies, briefly reviewed below, supports the possible role of these three

proinflammatory cytokines in endometrial neovascularization.

The IL-1 cytokines play a central role in a variety of inflammatory and immune responses. Originally recognized as activators of T and B lymphocytes, other paracrine and endocrine effects of IL-1 also have been characterized in the uterus. Two receptor agonists, IL-1α and -β and an endogenous receptor antagonist (IL-1ra) have been identified in the human endometrium and decidua (Tabibzadeh and Sun, 1992). IL-1β is specifically detected only in endothelial cells of spiral arteries and isolated stromal cells during the mid- to late secretory phase of the menstrual cycle (Simón *et al.*, 1993).

TNF are pleiotropic cytokines with a range of beneficial and injurious effects, depending on the local concentration, tissue localization, activity of TNF-binding proteins, and hormonal and cytokine milieu. TNFα is produced by activated leukocytes and other non-haematopoietic cells. TNFα synthesis by endometrial explants is greatest in specimens collected during the secretory phase (Philippeaux and Piguet, 1993) and is localized predominantly in the epithelial cell layer (Tabibzadeh *et al.*, 1995). Although TNFα can increase the expression of VEGF in human glioma cells (Ryuto *et al.*, 1996), the results of the current studies indicate that this cytokine, even when administered in combination with IFNγ, does not induce endometrial stromal cell VEGF protein secretion.

The precise role of IFNγ in human endometrium is relatively unknown; however, it has been shown to be synthesized by secretory phase endometrial epithelial cells (Chiang and Hill, 1997) where it appears to exert an anti-proliferative autocrine effect (Tabibzadeh *et al.*, 1988). Moreover, resident leukocytes in the endometrium express IFNγ (Yeaman *et al.*, 1998). In the mouse, IFNγ-activated monocytes themselves express VEGF (Xiong *et al.*, 1998).

#### VEGF gene promoter

The cloned human VEGF gene promoter consists of 2.4 kb of DNA upstream from the transcription start site and a long 5' untranslated region (Tischer *et al.*, 1991). The initiation of VEGF mRNA

synthesis is modulated by sequence-specific consensus sites that bind transcriptional control factors. Analyses of the primary sequence of the VEGF promoter reveal several common response elements, including: AP-1, AP-2, GATA-6, hypoxia-induced enhancer sequences, and half-palindromic oestrogen (ERE) and progesterone (PRE) response elements (von der Ahe *et al.*, 1985; Klein-Hitpass *et al.*, 1988; Tischer *et al.*, 1991; Garrido *et al.*, 1993; Welter *et al.*, 1995; Davis and Burch, 1996).

Oestrogen-receptor (ER) complexes classically bind as dimers to consensus, 13 bp palindromic ERE (Kumar and Chambon, 1988). While very few natural genes contain perfect palindromic ERE, ER complexes also can activate components of the AP-1 complex (e.g. *c-fos* and *c-jun*), (Paech *et al.*, 1997) or bind imperfect ERE if a GATA-6 sequence is present nearby (Davis and Burch, 1996). While there is no consensus palindromic ERE sequence in the 2.4 kb VEGF promoter, oestradiol treatment increases the VEGF mRNA as early as 1 h following oestrogen administration suggesting a direct regulation of VEGF gene transcription (Shifren *et al.*, 1996). VEGF gene expression also has been shown to be regulated rapidly by oestrogen and progesterone in the rat uterus (Cullinan-Bove and Koos, 1993). The imperfect palindromic ERE in the VEGF promoter has correct 5' and 3' ends of the consensus sequence but is separated by 50 bp. This amounts to 6.7 helical DNA turns from end-to-end of the two ERE. One study has shown that a separation of three helical turns between half-sites did not diminish the oestrogen effect, although 14.4 turns abolished oestrogen inducibility of the gene (Martinez *et al.*, 1987). It is not known how this intermediate separation might affect oestrogen regulation of VEGF transcription.

Apart from our own investigation, specific studies of the effects of progestins on VEGF gene expression are limited. Using the Ishikawa adenocarcinoma cell line, Fujimoto *et al.* reported that progestins inhibited the transient oestradiol-induced secretion of VEGF protein (Fujimoto *et al.*, 1999). By contrast, anti-progestins were shown to have anti-angiogenic effects in primate endometrium (Slayden *et al.*, 1998). Mechanistic studies of progestin-receptor complex activation of VEGF gene transcription are currently underway in our laboratory.

As described above, cytokines such as TNF- $\alpha$  and IL-1 $\beta$  can up-regulate human VEGF in other cell types such as glioma cells (Ryuto *et al.*, 1996). It has been postulated that these cytokine effects are mediated via NF $\kappa$ B motifs located in the VEGF promoter (Royds *et al.*, 1998). Our current studies imply that IL1 $\beta$ , TNF $\alpha$  and IFN $\gamma$  have few direct effects on VEGF expression in normal endometrium themselves, although their ability to modulate ovarian steroid effects on VEGF production was not tested. By contrast, direct or indirect angiogenic effects of cytokines were observed in endometriosis-derived cells (Lebovic *et al.*, 2000). Tissue-specific enhancers of VEGF gene expression have yet to be explored.

#### *Possible clinical interventions based on the current data*

An analysis of our findings suggests that an endocrine environment replete with oestrogen and progestin effects promotes high levels of VEGF mRNA and protein expression in normal endometrial stromal cells. This milieu exists in the normal secretory phase and may be mimicked in women using parenteral progestin contraceptives; according to correlative studies reviewed above, this milieu corresponds to maximal endometrial vascular capillary proliferation.

In summary, the data suggest that interference with the ovarian steroid milieu could be applied therapeutically to ameliorate irregular bleeding patterns encountered with DMPA or Norplant. Some studies indicate that the addition of oestradiol can be of benefit in cases of unpredictable bleeding (Diaz *et al.*, 1990; Witjaksono *et al.*, 1996); however, its effect may be short-lived (Said *et al.*, 1996). Alternatively, we propose that where these drugs are clinically available, low-dose anti-progestins (e.g. RU486 or onapristone) also may be of benefit. Future studies will be needed to better understand the myriad factors involved in human endometrial angiogenesis and abnormal vascular responses in certain predisposed women.

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