Vascular endothelial growth factor and the endometrium

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Introduction
Endometrium in adult female primates undergoes cyclical regeneration during the menstrual cycle. A key feature of this remarkable tissue remodelling is the growth and development of the vasculature. At the end of menstruation, when two-thirds of the endometrium has been shed, the basalis contains ruptured arterioles and venules. Yet by day 5 of menstruation these vessels have been repaired and continue to undergo proliferation and branching during the rest of the cycle. Whilst intermenstrual bleeding may arise in some women, this is not usually the case, and presumably reflects the ability of endometrial vessels to maintain their integrity during the menstrual cycle. The process of new vessel growth is called angiogenesis.

Angiogenesis
New blood vessels arise (Folkman and Shing, 1992) by the sprouting of capillaries from pre-existing blood vessels (Risau, 1991). In most circumstances in adult life, angiogenesis occurs infrequently and the turnover of endothelial cells in blood vessels is in the order of years (Denekamp, 1984). Normal angiogenesis is part of the body's repair system involved in wound healing and bone repair. In addition, pathological angiogenesis arises in a wide range of diseases, including the growth of solid tumours (Folkman, 1990; Bicknell and Harris, 1991), diabetic retinopathy, arthritis and some autoimmune diseases (Folkman and Klagsbrun, 1987). However, by far the most common exception to this rule is in the female reproductive tract. Here angiogenesis occurs on a monthly basis.

This process of angiogenesis is a closely regulated mechanism. Endothelial cells undergo proliferation, differentiation, migration and morphogenetic changes which form new tubular structures (Folkman and Shing, 1992). The endothelial cells and pericytes in capillaries contain all the genetic material necessary for these changes. In addition, the endothelium can regulate the development of vascular smooth muscle and is involved in the formation of larger blood vessels (De Mey and Schiffers, 1993).

At menstruation, approximately the upper two-thirds of endometrium is shed, leaving the desquamated basal endometrium. Blood vessels are ruptured, leaving open damaged vessels (Ludwig and Metzger, 1976). Blood does not pass through these vessels firstly because of the weak platelet fibrin thrombi which form in endometrium during the first 24 h of menstruation, and secondly because of the intense vasoconstriction that arises in the basal parts of the vessels (Markee, 1940). By day 5 after menses, the blood vessels have been repaired and healthy tubular structures re-established. Growth of the endometrium during the proliferative phase, including the increased thickening that arises from the action of 17β-oestradiol, is associated with growth of the spiral arterioles and development of a capillary subepithelial plexus. However, endometrial microvascular density does not alter throughout the cycle (Hourihan et al., 1986; Rogers et al., 1993).

Uterine bleeding from women taking long-acting gestagens is a significant problem, but the mechanism of this bleeding is poorly understood. There is a poor correlation between peripheral steroid concentrations and the onset of endometrial bleed-
ing, and bleeding is not thought to follow steroid withdrawal which occurs at the end of a normal menstrual cycle. There is confusion as to whether or not there is an increase in microvascular density. Rogers et al. (1993) demonstrated an increase in microvascular density in women exposed to long-term levonorgestrel use. However, Song et al. (1995) found decreased microvascular density in endometrium from such women. Both groups used CD34, an endothelial cell marker. In the latter study, the number of dilated venules was increased. Similarly, endothelial cell migratory response was decreased in Norplant users (Subakir et al., 1995).

This is a surprising finding because progesterone increases endothelial cell migratory response in the rat endometrium (Abberton and Rogers, 1995). The increased microvascular density reported by Rogers et al. (1993) is not associated with increased endothelial cell proliferation (Goodger et al., 1994). These authors suggest that their findings could be explained by a reduced turnover of blood vessels or reduced endothelial cell death. In any event, it is clear that the mechanisms regulating the growth and development of blood vessels in the endometrium are in some way deranged in women exposed to long-acting progesterones. As yet unpublished findings (Fraser, personal communication) suggest that blood vessels in the endometrium of women taking long-acting progesterones are more fragile than those in normal endometrium. This suggests that the normal mechanisms of endothelial cell integrity, including its relationship to the basement membrane, the expression of its intercellular adhesion molecules and the expression of adhesion molecules on its intimal surface, are altered in women using long-acting progesterone. This altered endothelial cell integrity appears to be confined principally to the endometrium; systemic abnormalities of endothelial cell dysfunction, as found in pre-eclampsia, are not found.

**Angiogenic growth factors**

Over the past 20 years an increasing number of angiogenic growth factors have been identified. In vitro these agents induce endothelial cells to undergo proliferation, migration, differentiation and tube formation in soft agar gels (Pepper et al., 1992), and to form blood vessels in the chick chorio-allantoic membrane and rabbit corneal preparation (Auszprunk et al., 1974; Gimbrone et al., 1974). These factors may be broadly divided into peptides and non-peptides, the latter including L-butyryl glycerol (Dobson et al., 1990), prostaglandin E1 (Ziche et al., 1982) and prostaglandin E2 (Form and Auerbach, 1983), nicotinamide (Morris et al., 1989), adenosine (Dusseau et al., 1986) and okadaic acid (Oikawa et al., 1992).

**Vascular endothelial growth factor (VEGF)**

**Structure and function**

After the identification of follicular growth factors (FGF) as potent angiogenic growth factors, another group of heparin-binding growth factors has been identified whose specificity of action is less diffuse than that of FGF. VEGF is an ~46 kDa protein consisting of two identical 23 kDa subunits (Connolly et al., 1989a,b; Ferrara and Henzel, 1989; Gospodorowicz et al., 1989; Tischer et al., 1991) which arise by alternate splicing of a single gene product. Characterization of the human gene shows it to comprise eight exons (Tischer et al., 1991). At least five species have been identified in the human arising from alternate splicing. The first four exons are common to all species, with the rest arising by alternate splicing of the exons encoding the C-terminus of the peptides (Leung et al., 1993; Houck et al., 1991; Charnock-Jones et al., 1993). Exon 1 contains a 26 amino acid hydrophobic consensus secretory signal common to all peptides (Tischer et al., 1991). VEGF165 is transcribed from all eight exons. VEGF165 does not transcribe exon 6 and contains a predicted 24 amino acid deletion (the splice site being at position 116), and is accompanied by a lysine → asparagine change at residue 115. VEGF145 is predicted to transcribe exon 6 but lacks exon 7. VEGF121 lacks the products of exons 6 and 7. A further VEGF206 has been described which contains an additional 17 codons after the 24 amino acid insertion in VEGF165 (Houck et al., 1991). This peptide has only been described in fetal liver and is not present in the reproductive tract.

The different species of VEGF have alternate patterns of secretion and significant structural and isoelectric point differences. Initial studies suggested that VEGF165 and VEGF121 were secreted
peptides, with biological activity being present in the supernatant of cells transfected with the gene (Houck et al., 1991). More recently, evidence has suggested that VEGF$_{189}$ is released from the cell but is tightly bound to proteoglycans in the cell membrane or in the extracellular matrix. This is indicated by the release of VEGF$_{189}$ into culture medium with treatment by suramin and heparinases I and III (Houck et al., 1992). VEGF$_{121}$ is secreted and is freely soluble. This binding of VEGF to heparin is similar to that with the FGF described above.

It is now clear that an alternatively described peptide, vascular permeability factor (Senger et al., 1987, 1990; Connolly et al., 1989a,b) is analogous to VEGF$_{189}$ (Keck et al., 1989), indicating that at least the larger molecular weight species of VEGF do not just promote angiogenesis but also increase vascular permeability. All species have direct effects on endothelial cells, stimulating the proliferation of cloned bovine microvascular endothelial (BME) cells (Pepper et al., 1991) and human umbilical vein endothelial (HUVE) cells (Bikfalvi et al., 1991), and are angiogenic in the chick chorio-allantoic membrane assay (Leung et al., 1989; Plouet et al., 1989). In the cases of VEGF$_{189}$ and VEGF$_{165}$, this action is only present or enhanced respectively when the peptides are released from their bound states (Houck et al., 1991, 1992). They also exert actions consistent with their in-vivo role of angiogenesis. VEGF$_{165}$ induces plasminogen activator (PA) activity in BME cells (Pepper et al., 1991). Urokinase and tissue PA mRNA levels in these endothelial cells are increased 7.5- and 8.0-fold after 15 h in culture, and PA inhibitor (PAI-1) mRNA levels increased 4.5-fold after 4 h. VEGF exert angiogenic actions in vitro, causing endothelial cells to invade three-dimensional collagen gels and forming tubules (Bikfalvi et al., 1991; Pepper et al., 1992). VEGF stimulates von Willebrand factor release from endothelial cells (Brock et al., 1991) and stimulates the release of tissue factor from HUVE cells (Clauss et al., 1990). VEGF was found to be the only growth factor which stimulated the growth of human decidual endothelial cells maintained in culture (Grimwood et al., 1995).

**Expression of VEGF in the endometrium**

Four transcripts of VEGF are found in human endometrium at all stages of the menstrual cycle (Charnock-Jones et al., 1993). Three of these transcripts predict the previously described VEGF$_{189}$, VEGF$_{165}$ and VEGF$_{121}$. A fourth product predicts a new splice variant encoding a peptide of 145 amino acids, transcribed by mRNA lacking exon 7 but incorporating exon 6. This region of VEGF encodes a sequence of the peptide which increases its binding to heparin and suggests that this 'new' peptide would have the same extracellular distribution as VEGF$_{189}$ and VEGF$_{165}$. Interestingly, peripheral monocytes show two polymerase chain reaction products, consistent with VEGF$_{165}$ and VEGF$_{121}$. In-situ hybridization identifies the specific cellular localization of VEGF.

In the proliferative phase of the menstrual cycle hybridization is found in both glandular and luminal epithelial cells and most cells of the stroma. These studies cannot discriminate between each splice variant nor do they identify the specific cells of the stroma. Immunoreactivity for VEGF mirrors the findings for the distribution of the mRNA. However, in the luteal phase of the cycle hybridization is reduced in the stromal cells, being present predominantly in the epithelial cells. Once again this pattern is reflected in the distribution of immunoreactive VEGF. Expression appears to occur in both luminal and basal epithelium, and there is no regional variation in VEGF expression. As with the in-situ hybridization, the antibody used cannot discriminate between the different splice variables. Individual cells demonstrate immunoreactivity in the stromal compartment at this time, and these cells are CD68$^+$, indicating them as macrophages. However, studies to characterize other cells staining for the growth factor in the stroma have not been undertaken, and it is not clear if large granular lymphocytes also express VEGF.

The most intense hybridization is found in the glandular cells of menstruating endometrium. The reason for this is not clear, although hypoxia is known to stimulate VEGF synthesis, and it is assumed that the endometrium is responding to the ischaemia which precedes menstruation. It would seem appropriate to have up-regulation of growth factor expression at menstruation to facilitate tissue repair at this time.

**Regulation of VEGF expression in the endometrium**

No significant changes in mRNA levels for VEGF could be found throughout the menstrual cycle.
There was a trend for higher levels at menstruation, but this did not achieve statistical significance. Because of the changes in the distribution of VEGF expression during the cycle, this does not mean that steroids have no effect on VEGF expression. To investigate this more closely, steroidal regulation of VEGF expression in endometrial cell lines was undertaken. Northern blot and RNase protection assays showed that 17β-oestradiol increased the steady state levels of mRNA in endometrial carcinoma cell lines HEC-1a and HEC-1b, shown previously by the reverse transcription polymerase chain reaction to contain mRNA encoding the 17β-oestradiol receptor. Greatest induction occurred at ~20 h. Further studies using the cell line RL-95, also shown to have mRNA encoding both 17β-oestradiol and progesterone receptor, failed to demonstrate the induction of VEGF mRNA by 17β-oestradiol but did show a significant 2-fold increase in mRNA levels in response to progesterone. 17β-oestradiol did not increase this effect. In the same studies, hypoxia was found to increase mRNA encoding VEGF 6-fold.

**Immunoreactive VEGF in the endometrium of women exposed to Norplant**

Immunoreactivity for VEGF was found in samples of endometrium removed from women at 6 and 12 months of Norplant treatment. As with normal endometrium, consistent staining was found in the epithelial cells. Diffuse staining was also found throughout the stromal compartment of the small pieces of endometrium. This was most pronounced in the large decidualized stromal cells, which constituted a significant part of the stromal compartment. In addition, the stroma contained aggregates of cells which were CD68+, i.e. macrophages. These cells stained intensely for VEGF. In addition to the macrophages infiltrating into the stroma, large aggregates of these cells were found in the uterine lumen. Superficial to the epithelium there appears an amorphous substance in the uterine lumen in which can be found the macrophage aggregates. These cells also stain intensely for VEGF. It is possible that these cells migrate into the uterine cavity with the degradation of the atrophic endometrium found in Norplant users.

Direct measurement of mRNA or protein levels of VEGF have yet to be undertaken in this tissue. However, as progesterone increases VEGF levels and immunoreactivity is found throughout the specimen, it is possible that VEGF levels are increased in these women.

**VEGF and the vascular integrity of endometrium in women using Norplant**

VEGF acts by binding to specific receptors, the fms-like tyrosine kinase receptor (De Vreis et al., 1992) and the kinase domain receptor (Terman et al., 1992). Both of these receptors are expressed by endothelial cells in endometrium, as determined by in-situ hybridization. It is difficult at this stage to be certain as to how the vasculature of Norplant users is being regulated. The uncertainty as to whether there is an increase in microvascular density needs to be resolved. Certainly there does not seem to be increased proliferation in the endothelial cells, at least when tested several months after the initiation of treatment. This does not discount the possibility that proliferation occurs in the early stages of Norplant use. Similarly, VEGF may be acting to inhibit apoptosis in endothelial cells. However, it is clear that a simple relationship between VEGF levels and endothelial cell proliferation does not exist. Further studies are needed to resolve these issues.

**Conclusions**

Despite the inconsistencies in current studies, there appears to be a consensus that there is deranged angiogenesis in the endometrium of women using long-acting progesterone. Angiogenic growth factors including VEGF are expressed in endometrium, and this expression is regulated by steroids. Further studies are needed to determine more precisely the relationship between VEGF levels and endothelial cell proliferation does not exist. Further studies are needed to resolve these issues.

**References**


