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Review

Recent advances in endometrial angiogenesis research

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Abstract

This review summarises recent research into the mechanisms and regulation of endometrial angiogenesis. Understanding of when and by what mechanisms angiogenesis occurs during the menstrual cycle is limited, as is knowledge of how it is regulated. Significant endometrial endothelial cell proliferation occurs at all stages of the menstrual cycle in humans, unlike most animal models where a more precise spatial relationship exists between endothelial cell proliferation and circulating levels of oestrogen and progesterone. Recent stereological data has identified vessel elongation as a major endometrial angiogenic mechanism in the mid-late proliferative phase of the cycle. In contrast, the mechanisms that contribute to post-menstrual repair and secretory phase remodelling have not yet been determined. Both oestrogen and progesterone/progestins appear to have paradoxical actions, with recent studies showing that under different circumstances both can promote as well as inhibit endometrial angiogenesis. The relative contribution of direct versus indirect effects of these hormones on the vasculature may help to explain their pro- or anti-angiogenic activities. Recent work has also identified the hormone relaxin as a player in the regulation of endometrial angiogenesis. While vascular endothelial growth factor (VEGF) is fundamental to endometrial angiogenesis, details of how and when different endometrial cell types produce VEGF, and how production and activity is controlled by oestrogen and progesterone, remains to be elucidated. Evidence is emerging that the different splice variants of VEGF play a major role in regulating endometrial angiogenesis at a local level. Intravascular neutrophils containing VEGF have been identified as having a role in stimulating endometrial angiogenesis, although other currently unidentified mechanisms must also exist. Future studies to clarify how endometrial angiogenesis is regulated in the human, as well as in relevant animal models, will be important for a better understanding of diseases such as breakthrough bleeding, menorrhagia, endometriosis and endometrial cancer.

Abbreviations: bFGF – basic fibroblast growth factor; ER – oestrogen receptor; LNG – levonorgestrel; L_v – vessel length density; L_v/N_v – average vessel length per branch point; MMP – metalloproteinase; MPA – medroxyprogesterone acetate; MP-MMP – membrane type metalloproteinase; N_v – vessel branch point density; PA – plasminogen; PR – progesterone receptor; TNF-α – tumour necrosis factor-α; u-PA – urokinase-type plasminogen activator; VEGF – vascular endothelial growth factor; VEGFR – vascular endothelial growth factor receptor

Introduction

Angiogenesis is the formation of new blood vessels from pre-existing vasculature and is a process fundamental to the human menstrual cycle. The uterine endometrium is a dynamic tissue that undergoes regular cycles of growth and breakdown (Figure 1), and has long been recognised as one of the few adult tissues where significant angiogenesis occurs on a routine, physiological basis. Abnormal angiogenesis may contribute to several different endometrial-related pathologies including endometrial cancer, endometriosis, menorrhagia and breakthrough bleeding. Over recent years, a picture of increasing complexity and subtlety is emerging regarding the mechanisms and regulation of endometrial angiogenesis. Identification of multiple angiogenic mechanisms and regulatory factors establishes both the value of the endometrium as a model for studying angiogenesis, and the necessity to

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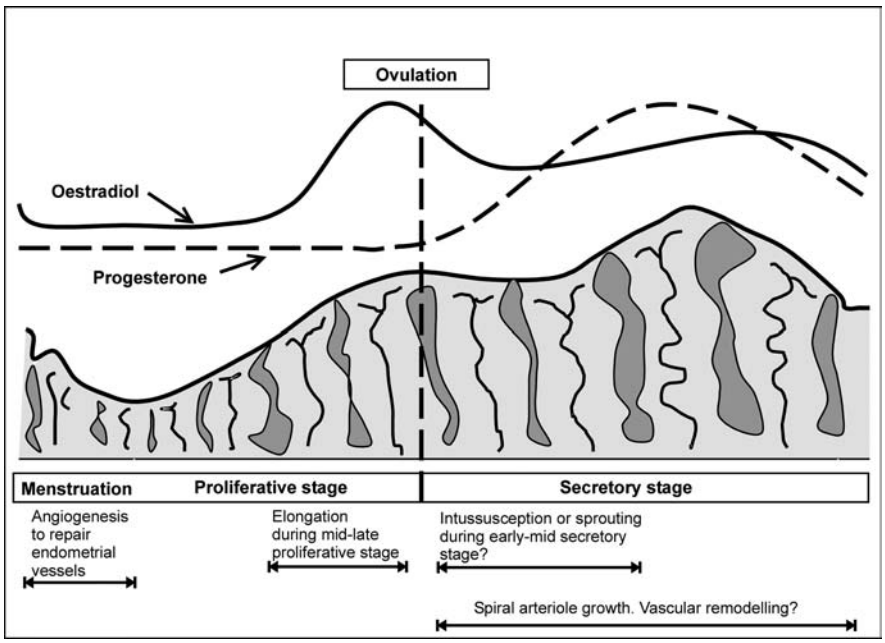

Figure 1. Schematic diagram of the human menstrual cycle illustrating changes in the endometrium and circulating oestrogen and progesterone concentrations. The proposed mechanism of endometrial angiogenesis for each stage of the menstrual cycle is indicated.

study these complex processes in *in vivo* systems. There have been a number of good reviews published on endometrial angiogenesis over the past few years [1–5]; the aim of this article is to build on these by synthesising the most recently published information and identifying areas for future work.

Mechanisms of endometrial angiogenesis

Angiogenesis may occur by at least four different mechanisms: sprouting, intussusception (internal division of the vessel by endothelial cells resulting in the vessel splitting in two), elongation/widening (lengthways growth of the vessel without formation of new vascular junctions), or incorporation of circulating endothelial cells into endometrial vessels [6–9]. Sprouting was the first angiogenic mechanism to be described and occurs during neovascularisation of avascular tissues, such as the rapidly growing corpus luteum, or vascular invasion of growing tumours [7]. Sprouting is the most commonly studied angiogenic mechanism, both because it is the easiest to visualise, and because most experimental models are based on one or more of the steps involved (endothelial cell migration, proliferation or tube formation).

However, there are several lines of evidence suggesting that sprouting is not the primary mechanism by which angiogenesis occurs in the endometrium. Structures that resemble sprouts were not identified in endometrial tissue sections, although there was limited evidence for endothelial sprouts in cytological preparations of endometrium [10]. Proliferating endothelial cells were always identified within vessel profiles, rather than associated with sprouts [11–13], and $\alpha_v\beta_3$ integrin, a putative marker of sprouting endothelium, has only been observed in existing vessel profiles [14].

Using recently developed stereological methods to measure anisotropic structures (structures that have a specific orientation rather than being randomly distributed) within endometrial tissue sections, it has been demonstrated that vessel elongation is a major angiogenic mechanism during the mid-late proliferative phase of the human menstrual cycle [15]. This study measured vessel branch point density (N_v) and vessel length density (L_v) at each stage of the menstrual cycle, and used these parameters to calculate average vessel length per branch point (L_v/N_v). The results showed that L_v/N_v remained constant at approximately 100 μm throughout the cycle, apart from the mid-late proliferative phase when it almost doubled to 190 μm. As well as proving that vessel elongation is a major angiogenic mechanism during the oestrogen driven proliferative phase of the cycle, these data also demonstrated that L_v/N_v returns to normal values, through an increase in N_v, in the progesterone dominated early-mid secretory stages of the cycle.

An increase in N_v can only occur through either sprouting (discussed above) or intussusception. Intussusception is known to play a role in the formation of vascular structures such as a capillary plexus or a vascular arcade [9]. Although ultrastructural techniques have been used to identify features characteristic of intussusception [16], definitive proof of endometrial intussusceptive angiogenesis will require

in vivo observations. Such observations have been made using video/digital recording of developing vessels in the chick chorioallantoic membrane using a shell-free culture technique [17]. This type of work will be difficult to replicate in endometrial tissue. Of more benefit would be specific markers that can be used to identify intussusceptive angiogenesis in tissue sections with immunohistochemical or similar techniques.

Despite stereological evidence for endometrial angiogenesis during the proliferative phase of the menstrual cycle, it has not been possible to correlate periods of endometrial endothelial cell proliferation with menstrual cycle stage. Using a double immunohistochemical staining procedure for CD34 and proliferating cell nuclear antigen, we have repeatedly shown that levels of endothelial cell proliferation within the human endometrium do not show any consistent pattern across the menstrual cycle [11–13, 18]. Samples taken from women at the same stage of the menstrual cycle may have endothelial cell proliferation rates that vary from 0 to 30%. Significant rates of endothelial cell proliferation may be seen at all cycle stages, including when endometrial growth is minimal. The lack of a cyclical pattern in endothelial cell proliferation is unexpected and may be due in part to the large variability in cycle length among women. If individual women could be tracked longitudinally through the menstrual cycle, a regular pattern of endothelial cell proliferation might be observed.

To overcome the inherent variability in menstrual cycle length, Nayak and Brenner [19] obtained endometrial samples from ovariectomised, artificially cycling rhesus macaques. The macaques received oestradiol-containing, and subsequently progesterone-containing, subcutaneous implants to simulate an artificial menstrual cycle. The progesterone implant was later removed (oestradiol implant remained) to stimulate menstruation and the subsequent proliferative phase. A large peak in endothelial cell proliferation was observed in animals classified as mid-proliferative (8–10 days after progesterone withdrawal [19]). This result is consistent with the timing of elongative angiogenesis observed in human endometrium, but gives no indication that angiogenesis or remodelling may occur during the secretory phase as suggested by the stereological analysis of human tissue [15]. This may be due to differences between the species (for instance, unlike human, the rhesus macaque does not show spontaneous decidualisation in the non-pregnant cycle) and/or the particular hormone regime used in the macaque studies. Unlike a natural menstrual cycle, the macaques received continuous oestrogen treatment with no variation in dosage.

Regardless of the macaque data, it seems unlikely that the variability in the endothelial cell proliferative indices among women is due solely to differences in menstrual cycle length. The challenge is to develop techniques that can differentiate between the mechanisms of endometrial angiogenesis and determine the relative contribution of endothelial cell proliferation to each. Several recent studies have developed techniques to produce high resolution three-dimensional reconstructions of endometrial microvasculature, either using digitised images of serial tissue sections or multiphoton excitation microscopy of thick sections ($\backsim 100\ \mu m$) [20–22]. These techniques will enable researchers to describe the distribution, shape and size of endometrial vessels, as well as their relationship to other endometrial structures such as glands or leucocytes. These techniques could be further developed to investigate blood vessel structural changes in conjunction with endothelial cell proliferation rates.

Hormonal control of endometrial angiogenesis

Endometrial growth and differentiation is under the overall control of oestrogen and progesterone. However, it is not clear how these steroids specifically regulate endometrial angiogenesis. Regression occurs following hormone withdrawal at the end of the secretory phase, suggesting that the angiogenesis required to repair the vascular bed occurs in the absence of steroid hormones. Detailed research is still required to determine not only the mechanism(s) of angiogenesis at different menstrual cycle stages, but also how each angiogenic event is regulated.

Most work on endometrial angiogenesis has focused on the effects of oestrogen. There is currently evidence that oestrogen stimulates angiogenesis by acting both directly on endothelial cells, and/or indirectly on other endometrial cell types via numerous potential promoters. Both oestrogen receptor (ER)-α and ERβ have been detected in endometrial vascular smooth muscle cells using immunohistochemistry [23, 24]. Only ERβ mRNA and protein were detected in endometrial endothelial cells, both *in vitro* and *in vivo* [23, 26]. ERα was only expressed at very low levels, if at all [25, 26]. Recently, an additional splice variant of ERβ (isoform initially described = ERβ1, new isoform = ERβcx/β2) was identified that is unable to bind to oestradiol, although it can form heterodimers with ERα and ERβ1 and potentially inhibits oestrogen-mediated action of the wildtype receptors [27, 28]. In agreement with their previous paper, Critchley et al. [29] revealed intense ERβ1 immunostaining in endothelial cells across the menstrual cycle in both functionalis and basalis layers. ERβcx/β2 displayed a heterogenous staining pattern with some endothelial cells negative and others positive [29]. The relative contribution of the different ERβ subtypes in regulating endometrial angiogenesis remains to be elucidated.

Conventionally, oestrogen is considered to be uterotrophic and a promoter of angiogenesis and vascular permeability. In *in vitro* assays, oestradiol treatment stimulated increased human endometrial endothelial cell proliferation within 48 h and the formation of angiogenic patterns (for instance, capillary formation

and branching) within 8 days [25]. Oestradiol also increased the proliferative response of human endometrial endothelial cells to VEGF [30]. Elongative angiogenesis has been demonstrated in the human endometrium during the oestrogen-dominant proliferative stage of the menstrual cycle [15]. In ovariectomised, artificially cycling macaques, a peak of endothelial cell proliferation was observed in animals classified as mid-proliferative (8–10 days after progesterone implant withdrawal, oestradiol implant remaining). A similar peak did not occur in those macaques in which both the progesterone and oestradiol implants were removed indicating that the endothelial cell proliferation was oestradiol dependent [19]. In ovariectomised ewes, oestrogen treatment caused an almost 2-fold increase in endometrial microvascular volume density (percentage of tissue volume occupied by microvessels) within 24 h [31]. This reflected an approximately 5-fold increase in total endometrial microvascular volume, while uterine tissue weight only increased 3-fold. It is well known that oestrogen stimulates an increase in uterine blood flow and vasodilation. In baboons, oestrogen treatment significantly increased the paracellular cleft width between endometrial endothelial cells within 6 h [32]. The increase in cleft width and apparent opening of tight junctions between endothelial cells was considered to result in the increased vascular permeability associated with oestrogen administration. Reynolds et al. [31] hypothesised that vasodilation accounted for a significant proportion of the increase in microvascular volume seen in the oestrogen-treated sheep, but that microvascular growth also occurred.

In contrast to the evidence suggesting that oestrogen can be angiogenic, recent data has emerged suggesting that oestrogen inhibits angiogenesis. Ma et al. [33] concluded that oestrogen promotes vascular permeability but 'profoundly inhibits' endometrial angiogenesis. This conclusion was based partially on the reduction in the stromal area occupied by blood vessels 2 days after oestrogen treatment of ovariectomised mice. Measuring changes in blood vessel density, however, does not take into account the oedema and resulting tissue expansion triggered by oestrogen. Heryanto et al. [34] also observed a decrease in vascular density in ovariectomised mice following oestrogen treatment, as well as a reduction in the stromal cell density (cells/mm^2) illustrating that tissue oedema had also occurred. However, the ratio of vascular density to stromal cell density increased. This suggested that there was an increase in the number of vessels relative to the stromal compartment. In addition, a significant increase in endothelial cell proliferation was observed within 24 h of oestrogen treatment [34, 35]. In another study that also used ovariectomised mice, no change in the volume-fraction of total or proliferating endometrial endothelial cells was observed 24 h after oestrogen treatment despite the oedema and increase in uterine mass and endometrial area [36]. The discrepancy among these various studies is difficult to explain, particularly as all used similar oestrogen-treated ovariectomised mouse models. It may relate in part to the different approaches that were used to examine endometrial vasculature. Careful stereological studies identifying changes in vessel length and branching (such as those used in human studies outline earlier [15]) are required to determine how changes in vascular density, vascular volume fraction and endothelial cell proliferation relate to angiogenesis.

Few studies have investigated the effects of progesterone on normal endometrial angiogenesis, although there is a considerable body of work on the effects of long-term progestin contraceptive therapy on endometrial vasculature. Váquez et al. [37] identified functional nuclear progesterone receptors (PR) on approximately 25%–30% of endothelial cells from a wide variety of tissue types, including endometrium, although this work did not differentiate the progesterone receptor isoforms, PRA and PRB, which function in different ways. PRB is typically a strong activator of target genes, in contrast PRA represses PRB activity when both proteins are co-expressed in cultured cells [38]. Progesterone inhibited endothelial cell proliferation *in vitro*; and arrested the cell cycle in the G_0/G_1 phase in human dermal endothelial cells [37]. Iruela-Arispe et al. [30] also identified progesterone receptors (isoforms not differentiated) in human endometrial endothelial cells and found that progesterone inhibited VEGF-induced endothelial cell proliferation. In contrast, PR (A or B) were not identified on endothelial cells *in vitro* by Kayisli et al. [25] or Krikun et al. [26]. Despite a lack of receptors, progesterone treatment stimulated proliferation of human endometrial endothelial cells *in vitro* within 48 h of treatment and the formation of angiogenic patterns (for instance, capillary formation and branching) within 8 days of treatment [25].

Despite the varying *in vitro* results, endometrial angiogenesis has been observed in response to progesterone *in vivo*. During the secretory phase of the human menstrual cycle, there was an increase in the vessel branch point density, which may occur by sprouting or intussusceptive angiogenesis [15]. In both rat and mouse, considerable endothelial cell proliferation was observed during early pregnancy, correlating with increasing progesterone production by the corpora lutea [39, 40]. In ovariectomised mice, an increase in vascular density was observed following progesterone treatment [33]. Unlike oestrogen, the angiogenic effects of progesterone are believed to occur without concurrent vasodilation [33]. There was no change in endometrial endothelial paracellular cleft width 6 h after progesterone treatment in baboons [32]. Further research is needed to elucidate the mechanisms and pathways by which progesterone acts to promote endometrial angiogenesis, including its interaction with PRA and/or PRB in endometrial endothelial cells.

Long term progestin-only therapies (such as: injectable depot medroxyprogesterone acetate (MPA), levonorgestrel (LNG)-releasing implant and intrauterine systems) result in compromised vessel structure and integrity in some women. The resulting unpredictable and prolonged endometrial bleeding and spotting (breakthrough bleeding) is the most common reason why women discontinue use of these otherwise safe and effective contraceptive methods. Changes have been noted in vessel shape, amount of dilatation, components of the basement membrane and pericyte support (see [41, 42] and references therein). A relative increase in vessel density has been observed in the endometrium of women using the LNG implant Norplant in contrast to apparent atrophy of other endometrial tissues [43, 44]. At higher progestin doses than those delivered by Norplant, a reduction in vascular density has been observed [45]. An increase in vascular density has also been noted in normal cycling mice treated with long term progestin therapy (silastic implant containing MPA or LNG [46]). These mice will be a useful model to further investigate haemostatic and angiogenic factors that are linked with breakthrough bleeding including vascular endothelial growth factor (VEGF), angiopoietins, tissue factor and thrombin (for recent reviews see [47, 48]).

In addition to progesterone, the corpus luteum also secretes relaxin, a peptide with structural similarities to insulin [49]. There are considerable differences in relaxin's functions among species. In the pig and rodent, relaxin is required during late pregnancy to prepare the birth canal for parturition. In contrast, in primates, relaxin is first detected in the maternal circulation during the late secretory phase of the menstrual cycle, peaking during the first trimester of pregnancy, and is hypothesised to play a role in decidualisation of stromal cells [50]. In the primate endometrium, a significant increase in relaxin binding and the relaxin G-protein coupled receptor LGR7 has been identified in the secretory phase of the menstrual cycle in comparison to the proliferative phase [51–53].

As well as decidualisation, relaxin is thought to have an important role in endometrial angiogenesis. Relaxin is also produced by the endometrium and is known to stimulate production of VEGF by endometrial cells *in vitro* [54, 55]. Goldsmith et al. [56] used ovariectomised rhesus monkeys given exogenous relaxin in addition to oestrogen and progesterone to simulate the human menstrual cycle. The effects of relaxin included stimulating a pronounced increase in uterine weight, arteriole number per unit area, and the number of endometrial leucocytes. Based on these findings, they hypothesised that relaxin has an important role in establishment and/or maintenance of pregnancy, including the growth of new blood vessels [56]. In a clinical trial in which relaxin was tested as a treatment for progressive systemic sclerosis, the most frequently reported adverse event was menorrhagia or irregular menstrual bleeding [54]. These observations are consistent with a role for relaxin in regulating the endometrial vasculature. It is clear that relaxin's role in controlling endometrial angiogenesis warrants further investigation. The value of the relaxin-1 knockout mouse is likely to be limited due to the differing functions of relaxin in the endometrium between primates and mice [49, 57].

Another factor receiving increasing attention is adrenomedullin, a 52-amino acid peptide hormone with similarities to the calcitonin gene-related peptide and links with various physiological and pathological pathways [58]. It is expressed by many tissues in the body and is mediated by paracrine, autocrine and potentially endocrine mechanisms. The peptide has a wide range of effects including regulation of cell growth and differentiation, and of interest to this review, vasodilation and angiogenesis. Adrenomedullin has been shown to stimulate angiogenesis *in vivo* in the chick chorioallantoic membrane assay [59].

Adrenomedullin immunoreactivity has been demonstrated in endometrial epithelium and stroma with the highest degree of immunostaining in stromal macrophages [59, 60]. Endometrial endothelial cells also express adrenomedullin both *in vitro* and *in vivo* [61]. It promotes endometrial endothelial cell proliferation *in vitro*, as well as inducing cyclic AMP production, illustrating that adrenomedullin can act as an autocrine growth factor for human endometrial endothelial cells [61].

Role of VEGF in endometrial angiogenesis

Although growth and differentiation of the endometrium and its vasculature is ultimately under the control of the hormones oestrogen and progesterone, the sex steroids are thought to exert the majority of their effects indirectly via a variety of growth factors. In the case of angiogenesis, one of the most important factors is vascular endothelial growth factor (VEGF), a potent endothelial cell mitogen that also increases vascular permeability and plays a central role in inflammation and other pathologies [7, 62, 63]. VEGF is expressed in a wide variety of cells and tissues, including primate and rodent endometrium [3, 64–66]. It acts via the tyrosine kinase receptors VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1/KDR), which have been identified in endometrial endothelial cells using immunohistochemistry, with variations in the intensity of staining and the proportion of stained capillaries depending on the stage of the menstrual cycle [67]. How hormonal changes associated with a cycling endometrium influence VEGF-A distribution and functioning is not yet fully understood.

Ovarian steroids are known to influence endometrial VEGF mRNA and protein levels *in vitro* [68–73] and *in vivo* [32, 74–78], although it is apparent that total endometrial VEGF production does not necessarily correlate with endometrial angiogenesis. To better understand this issue, it is important to consider the

particular cell types expressing the VEGF mRNA and protein, and how they interact with other cell types in the endometrium. In ovariectomised mice, VEGF mRNA increased primarily in stromal cells within 2 h, and VEGFR-2 mRNA within 6 h, of oestrogen administration and was thought to be responsible for the increase in vasodilation [33]. Interestingly, there was no stromal VEGF mRNA (or VEGFR-2) 24 h after oestrogen, and VEGF expression was primarily limited to the luminal epithelium. In primate species (baboons and rhesus monkeys), VEGF mRNA levels were markedly suppressed in endometrial glandular epithelial and stromal cells and levels were then restored following oestradiol administration [19, 78, 79]. Oestrogen also stimulates VEGF expression in cultures of human endometrial cells [64, 69, 80]. In both primates and rodents, endometrial VEGF is predominantly epithelial and it has been shown *in vitro* that most epithelial VEGF is secreted apically into the uterine lumen. This suggests that epithelial VEGF is unlikely to have a role in endometrial angiogenesis [81]. However, research by Albrecht et al. [82], who established methodology to co-culture endometrial epithelial and stromal cells with human myometrial microvascular endothelial cells, does not support this hypothesis. Human recombinant VEGF increased myometrial microvascular endothelial cell tube formation by approximately 65% over formation in media only. A similar increase was observed when the endothelial cells were co-cultured with endometrial epithelial cells. A further increase was observed when endothelial cells were co-cultured with epithelial cells and oestrogen. Tube formation was similar to that in media only when endothelial cells were co-cultured with endometrial stromal cells, with and without oestrogen [82]. This research suggests that oestrogen regulates angiogenesis by controlling the expression and secretion of angiogenic factors such as VEGF by endometrial epithelial cells. As endothelial cells exhibit organ and tissue specific gene expression and activity, it will now be necessary to address this hypothesis using endometrial endothelial cells.

In contrast to the rapid actions of oestrogen on VEGF and endothelial cell proliferation, the onset of progesterone effects on VEGF production are slower and of a lower magnitude. In mice given a single injection of progesterone, a modest but steady increase in VEGF and VEGFR-2 mRNA occurred over 24 h. The VEGF mRNA accumulation was prominent and primarily stromal [33, 77]. Future research will need to determine how the different patterns of VEGF expression relate to endometrial angiogenesis.

Consideration of the effects of VEGF on endometrial angiogenesis is further complicated by the presence of alternatively spliced VEGF isoforms in endometrium ($VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$ and $VEGF_{189}$ [64, 70, 71]). These splice variants have different solubilities, binding affinities for heparin and extracellular matrix, and interactions with VEGF receptors [63]. For instance, $VEGF_{121}$ is a freely diffusable protein, whereas $VEGF_{189}$ is almost completely sequestered in the extracellular matrix. Thus, some isoforms may provide a reservoir for locally controlled release by heparinases or plasmin [63].

To date, few studies have considered in detail where and when these different VEGF isoforms are located and what implications this may have on endometrial angiogenesis control. Ancelin et al. [83] used a variety of approaches to illustrate that $VEGF_{189}$ is upregulated by progesterone in decidualised stromal cells *in vivo* and *in vitro*. Immunohistochemistry and *in situ* hybridisation have been used to localise $VEGF_{189}$ to a subset of perivascular decidual cells in both mid-late secretory and pregnant endometrium. Using the Miles assay, it was shown that $VEGF_{189}$ is a potent permeability factor *in vivo* and it was hypothesised that progesterone may modulate vascular permeability changes necessary for implantation by its actions on decidualised cells and $VEGF_{189}$ [83]. The specific role of $VEGF_{189}$ and the other isoforms in endometrial angiogenesis remains to be described.

It has traditionally been thought that the tissue shedding occurring at menstruation is due to tissue necrosis resulting from hypoxia. This was based on the observations by Markee [84] who transplanted endometrial explants into the anterior chamber of the eye in rhesus macaques and observed vasoconstriction and then vasodilation of the spiral arterioles after progesterone withdrawal. If vasoconstriction occurs, this could provide a mechanism for subsequent vascular repair through hypoxia-induced upregulation of VEGF. In the few studies analysing menstrual stage endometrium, considerably more VEGF mRNA and GLUT-1, a glucose transporter and marker of anaerobic metabolism that is induced by ischaemia, have been observed in the endometrium of menstruating women when compared to proliferative and secretory samples [73]. Charnock-Jones et al. [64] observed high levels of VEGF mRNA in glands and in necrotic areas of menstrual stage endometrial tissue using *in situ* hybridisation. VEGF mRNA was upregulated in glands and stroma of the superficial endometrial zones in rhesus macaque 1–2 days after progesterone withdrawal in artificially induced menstrual cycles [19].

Despite the above observations, there is no direct evidence for menstrual vasoconstriction or hypoxia [85, 86]. Overall blood flow in the endometrium was not significantly reduced peri-menstrually [87] and no endometrial ischaemia/reperfusion episodes have been detected peri-menstrually by a Doppler technique [88]. In addition, only very low levels of the early markers of hypoxia HIF-1α and HIF-2α have been observed in the human endometrium [89]. Currently, menstruation is believed to be a result of an inflammatory process [85, 86]. Further research is required to determine how repair of endometrial vessels is regulated following menstruation.

In recent years, techniques to culture endometrial endothelial cells have been developed and are being used to characterise their activity [for instance: 90–92]. The ability to specifically isolate endometrial endothelial cells is important as endothelial cells exhibit organ-specific characteristics. For instance, human endometrial microvascular endothelial cells are much more sensitive to VEGF stimulation than human foreskin microvascular endothelial cells, which respond better to basic fibroblast growth factor (bFGF) [90]. Human endometrial microvascular endothelial cells formed capillary-like structures when they were cultured in 2% human serum on top of 3D fibrin matices; VEGF or bFGF increased this tube formation. This can be contrasted with human foreskin microvascular endothelial cells, which only formed tubes when stimulated with both tumour necrosis factor-α (TNF-α) and a growth factor. The difference in response was hypothesised to be due to the enhanced VEGFR-2 expression on the human endometrial microvascular endothelial cells in comparison to human foreskin microvascular endothelial cells [90].

Human endometrial microvascular endothelial cells also display enhanced expression of urokinase-type plasminogen activator (u-PA) compared with human foreskin microvascular endothelial cells, but there were no differences in tissue-type PA, PA inhibitor-1 or u-PA receptor expression [90]. Extracellular proteolysis is implicated in the initial stages of angiogenesis, including matrix remodelling, basement membrane degradation, migration of endothelial cells, and activation of cytokines and release of growth factors [93]. The group of proteinases involved includes the plasminogen system and metalloproteinases (MMPs). Urokinase plasminogen (u-PA) and tissue-type PA convert the inactive proenzyme PA into the serine protease plasmin, which is able to degrade matrix proteins as well as activate several MMPs [93]. u-PA production by human endometrial microvascular endothelial cells increased after the addition of VEGF, bFGF, or a combination of these factors with TNF-α [90]. The enhanced u-PA/plasmin activity, as well as the increased VEGFR-2 expression, may enhance the angiogenic capacity of endometrial endothelial cells [90]. In human endometrial endothelial cells, the protein kinase C agonist phorbol myristate acetate transiently enhanced tissue factor mRNA levels and elicited a more prolonged elevation in tissue factor protein levels. In contrast, phorbol myristate acetate did not affect PA inhibitor-1 mRNA and protein levels [91].

Endometrial endothelial cells express various MMPs including MMP-1, MMP-2, membrane type (MP) 1-MMP, MT3-MMP and MT4-MMP mRNA under basal as well as VEGF stimulated conditions [92]. The VEGF-enhanced capillary tube formation in fibrin and/or collagen matrices was reduced when MMPs were inhibited using the MMP inhibitor BB94 or u-PA blocking antibodies. Overexpression of the tissue inhibitor of MMP (TIMP)-1 or TIMP-3 also reduced tube formation by endometrial endothelial microvascular cells [92].

An alternative *in vitro* system was used by Print et al. [94]. Supernatants were collected from cultured endometrium and were found to contain soluble factors, which were able to stimulate proliferation and angiogenesis in cultures of human umbilical endothelial cells *in vitro*. A component of the supernatant was VEGF and the proliferative effects of endometrial supernatants could be partially blocked by an antibody against VEGF. Gene array technology was used to examine the effect of the supernatants on gene expression in human umbilical vein endothelial cells. The supernatants obtained from cultured endometrium (proliferative and, to a lesser extent, secretory) induced significant pro-angiogenic changes including increased expression of several angiogenic promoters (for example: CXC receptor-4, VE-cadherin, endoglin, PECAM-1) [94]. Gene arrays have also been used to examine gene expression in cultured human endometrial endothelial cells following oestrogen or progestin (MPA) treatment [26]. The hormones had a differential effect on a wide variety of genes with cluster analysis indicating that many were involved with intracellular signalling pathways. Several of the classic angiogenic factors were detected (for instance: VEGF-A, VEGFR-2, angiopoietin-2), however, and of interest, no steroidal regulation of these factors were observed.

Although factors other than VEGF have not been discussed in detail in this review, it is obvious that endometrial angiogenesis is a multifactorial process that will require the use of both *in vivo* and *in vitro* experimentation with a variety of human and animal models to tease out the various relationships.

Neutrophils and endometrial angiogenesis

Although there is no consistency in the results obtained from the various studies attempting to correlate epithelial, stromal or total endometrial levels of VEGF mRNA and protein with phases of the menstrual cycle or endothelial cell proliferation [66, 69–73, 95–99], foci of intense VEGF immunostaining have been observed within the human endometrium [99]. Many of these foci were within blood vessels. Using immunohistochemical techniques on full thickness endometrial sections, focal VEGF was found to correlate both temporally and spatially with endometrial endothelial cell proliferation [100]. A significantly greater percentage of focal VEGF-expressing microvessels were found during the proliferative phase in comparison to the secretory phase, with the greatest numbers of immunopositive vessels within the subepithelial capillary plexus. There was also a significant correlation between focal VEGF-expressing microvessels and proliferating vessels in all three regions of the endometrium (subepithelial capillary plexus, functionalis and basalis). The focal VEGF associated with microvessels was localised within marginating and adherent neutrophils and it was hypothesised that these leucocytes

provide a source of VEGF to stimulate endometrial endothelial cell proliferation [2, 100, 101].

VEGF is known to be the primary factor mediating endothelial cell proliferation in response to oestrogen within the mouse endometrium [35]. However, the source(s) of this VEGF, or the mechanisms leading to its release, are still unknown. Based on the observations in human endometrium, it was hypothesised that neutrophils may play a role in oestrogen-induced endothelial cell proliferation in mice. In ovariectomised mice, oestrogen treatment stimulated a significant increase in VEGF positive, intravascular, endometrial neutrophils, coincident with an increase in endothelial cell proliferation [102]. Concurrent induction of neutropenia with two different anti-neutrophil antibodies reduced endometrial neutrophils to minimal levels, and reduced endothelial cell proliferation by 30–40%. These experiments also identified a sub-group of non-neutrophil, VEGF positive intravascular leucocytes that were not reduced by neutropenia. One candidate for these cells is monocytes/macrophages as they constitute around 10% of all endometrial cells. Ovariectomy results in a reduction of endometrial macrophages in the mouse, while treatment with oestrogen or progesterone significantly increases this number [103]. Detailed studies of transmigrating monocytes in the endometrium, or the potential role of macrophages in endometrial angiogenesis, are yet to be undertaken.

Ancelin et al. [104] hypothesised that neutrophil-derived VEGF can modulate neutrophil migration into the endometrium and that such an autocrine amplification mechanism may play a role in VEGF-induced angiogenesis and inflammation. They found that $VEGF_{189}$, which was upregulated in the endometrium during the secretory phase, was chemotactic and chemokinetic for neutrophils, while $VEGF_{165}$ was only chemokinetic. These effects of $VEGF_{189}$ were blocked by anti-VEGF antibodies, but not by anti-VEGFR-2 antibodies, suggesting that VEGFR-1 expressed by neutrophils mediate these effects. In neutrophils, $VEGF_{165}$ mRNA expression was stronger than that of $VEGF_{189}$. In contrast, the major protein isoform released by neutrophil degranulation was $VEGF_{189}$ (located in both azurophilic and specific granules). It is interesting to note that neutrophils are currently thought to play a role in endometrial angiogenesis during the oestrogen-driven proliferative phase, not during the secretory phase when $VEGF_{189}$ levels increased [104]. The role of neutrophils in endometrial angiogenesis requires further investigation.

Conclusions

Angiogenesis occurs on a regular basis as part of the growth and regression of the human endometrium during the menstrual cycle. There is evidence for multiple mechanisms of angiogenesis, depending on the stage of menstrual cycle. Elongative angiogenesis has been described during the proliferative stage of the cycle, and intussusception is hypothesised to occur during the secretory phase. It has not been possible to correlate endometrial endothelial cell proliferation with menstrual cycle stage. Techniques are required to differentiate between the mechanisms of endometrial angiogenesis and determine the relative contribution of endothelial cell proliferation to each.

There are also multiple regulators of endometrial angiogenesis, although changes in the endometrial vasculature are under the overall control of oestrogen and progesterone. It is still unknown whether these steroids act directly and/or indirectly on endometrial endothelial cells. Oestrogen is usually thought to be uterotrophic and a promoter of angiogenesis. However, recent data have provided conflicting results with some suggesting that oestrogen may inhibit angiogenesis. The conflicting results may be due in part to the different approaches used to examine endometrial vasculature. Careful stereological analysis is required to determine how changes in vascular density, vascular volume fraction and endothelial cell proliferation relate to angiogenesis. Relaxin concentrations also vary during the menstrual cycle and additional studies are required to determine exactly how this hormone is involved in endometrial angiogenesis. VEGF is fundamental to endometrial angiogenesis. Further research is needed, however, to determine the source of the VEGF needed for angiogenesis and to clarify how the different VEGF splice variants are involved.

The research is complicated by major species differences between the menstrual cycle in humans and primates and the oestrus cycle in commonly studied rodent models. Although rodent models will continue to provide invaluable information, caution is required when translating information to the human menstrual cycle. Despite the difficulties, the endometrium is an important model for studying physiological angiogenesis in adults. Continuing research will not only increase our understanding of angiogenesis in general and angiogenesis during the normal menstrual cycle, but may lead to knowledge relevant to the various blood-vessel associated disorders and pathologies of the endometrium.

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