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STEROID HORMONE REGULATION OF ANGIOGENESIS IN THE PRIMATE ENDOMETRIUM

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1. ABSTRACT

The human endometrium develops new capillaries from existing microvessels, i.e. angiogenesis, which then undergo maturation and remodeling, i.e. investment of microvessels with periendothelial mural cells, into a new vascular network during each menstrual cycle. Improper vascularization of the endometrium may cause implantation failure and infertility. Estrogen and progesterone have pivotal roles in establishing this vascular bed, but the cellular sites and mechanisms of action of these steroid hormones are incompletely understood. Vascular endothelial growth factor (VEGF), angiopoietin-1, and angiopoietin-2 and their receptors are expressed in the human and nonhuman primate endometrium and interact to control vascular development and remodeling. VEGF synthesis within and neovascularization of the endometrium seem to be sustained and ongoing processes designed to progressively promote growth and development of the endometrium with advancing stages of the menstrual cycle. However, estrogen rapidly upregulates VEGF expression by endometrial glandular epithelial and stromal cells *in vivo* in the nonhuman primate and *in vitro* in the human. Reports of the effects of progesterone on endometrial VEGF formation, however, are inconsistent and may reflect regulatory actions on particular isoforms of VEGF. In addition, estrogen has effects on vascular endothelial and smooth muscle cells, which may be direct or mediated by VEGF. Very little is known, however, about the steroid hormone regulation of other angiostimulatory and angioinhibitory factors, e.g. angiopoietin-1 and -2, in the endometrium. Moreover, the role of steroid hormones acting directly, or indirectly via VEGF and other angiogenic factors, on expression of integrins, cell adhesion and other molecules required for cell-cell and cell-extracellular matrix interactions important for angiogenesis in the human and nonhuman primate endometrium is largely unknown. Finally, further study is needed of cell-specific responsivity and function in the human endometrium with respect to steroid hormone regulation of angiogenesis.

2. INTRODUCTION

The endometrium undergoes extensive development of new capillaries from existing microvessels, i.e. angiogenesis, as well as vascular maturation and remodeling during each menstrual cycle (1-3). The newly developed vascular system in turn supplies nutrients and oxygen to support the growth and cellular differentiation of the endometrium for blastocyst implantation. Abnormal vessel development may cause implantation failure and

lead to dysfunctional bleeding, endometriosis, and menorrhagia, major health problems and causes of infertility in women. Vascular growth in the endometrium during each menstrual cycle is highly unique, because angiogenesis is typically a rare event in the adult, normally occurring only during fetal development and wound healing.

Although the steroid hormones, estrogen and progesterone, have pivotal roles in establishing the endometrial vascular bed during each menstrual cycle (4), the cellular sites and mechanisms of action of these hormones are incompletely understood. Vascular endothelial growth factor (VEGF) and other angiostimulatory, e.g. angiopoietin-1, and angioinhibitory, e.g. angiopoietin-2 and thrombospondin-1, factors interact in a coordinated manner to control development, maturation, and remodeling of blood vessels (5-7).

In this review, experimental evidence will be presented that estrogen and progesterone regulate VEGF expression in the endometrium and the concept developed that via the regulation of expression of VEGF, and possibly other angiostimulatory factors, these steroid hormones promote angiogenesis and consequently growth and development of the endometrium during the human and nonhuman primate menstrual cycle.

3. VASCULARIZATION OF THE PRIMATE ENDOMETRIUM

Changes in vascularization of the endometrium during the menstrual cycle were first described by Markee (8), in autologous transplants of rhesus monkey endometrium in the anterior chamber of the eye. During the menstrual cycle, endometrial angiogenesis occurs at the time of menstruation for repair of the vascular bed in the basalis zone, during the proliferative phase when spiral arteries lengthen and branch and there is rapid growth of the functionalis zone, and during the secretory phase when spiral arteries exhibit growth and coiling within the functionalis layer. Thus, upon initiation of a new menstrual cycle, preexisting vessels in the zona basalis give rise via angiogenesis to capillary sprouts. These sprouts become encapsulated with smooth muscle cells and elastic tissues, converting them into arterioles and arteries, which project almost to the luminal epithelium where they branch to form a rich subepithelial capillary plexus. With advancing stages of the menstrual cycle, these newly formed spiral arteries progressively lengthen, branch, and coil as arterial growth exceeds endometrial thickening (9). In the absence of implantation and demise of the corpus luteum, the spiral arteries close to the myometrial-endometrial junction exhibit vasoconstriction, resulting in necrosis and sloughing of most of the zona functionalis at the end of the cycle. In contrast, the basal arteries that ramify in the deepest layer of the endometrium do not undergo vasoconstriction and consequently remain intact to preserve the zona basalis as a bed for reconstruction after menstruation. The periodic proliferation and shedding of the endometrium during each menstrual cycle exemplifies a dynamic process of programmed angiogenesis.

4. ANGIOGENESIS

Angiogenesis proceeds in at least two phases: (1) the activation phase, in which endothelial cells degrade their basement membrane via proteolytic enzymes, e.g. matrix metalloproteinases (MMPs) and enzymes of the plasminogen activator system, and undergo proliferation and migration in the surrounding stroma to form capillary sprouts; and (2) the resolution phase, in which proliferation and migration cease, a new basement membrane is deposited, and cells organize and mature into a new vessel. Both phases appear to be determined by a balance between stimulatory and inhibitory regulatory factors. Considering the complexity of vascular remodeling of the primate endometrium during the course of the menstrual cycle, it is likely that several growth factors are involved in this process. Although several pleiotropic growth factors described below are angiostimulatory, VEGF is the most prominent and well characterized angiogenic factor.

4.1. VEGF

VEGF is the prototype of a family of potent endothelial-specific mitogens which stimulates vascular endothelial cell proliferation, migration, organization into tubules and permeability (reviewed in 5, 10-12). Although other factors, e.g. epidermal growth factor, transforming growth factor, and platelet-derived growth factor, can also induce neovascular responses these agents are not specific for vascular endothelial cells. Alternative exon splicing of a single VEGF gene results in the synthesis of at least 5 polypeptide isoforms of 121, 145, 165, 189, and 206 amino acids (13). The active forms of these VEGF glycoproteins are homodimers linked via disulfide bridges (14, 15), and the various isoforms may be further processed by post-translational mechanisms, e.g. by plasmin. The existence of multiple VEGF species implies that they have different biological properties, distribution, and synthesis. The most widely expressed forms, VEGF 121 and VEGF 165, are freely soluble, while the 189 and 206 species are sequestered and thus remain in the extracellular matrix (5, 16). An important biological property that distinquishes the different VEGF isoforms, therefore, is their ability to bind to extracellular matrix components, such as heparin and heparan-sulfate. Thus, VEGF 121 lacks the amino acids encoded by exons 6 and 7 of the gene and binds weakly, if at all, to heparin (17). The 121 and 165 amino acid isoforms promote permeabilization of blood vessels and proliferation of vascular endothelial cells (18), while VEGF 189 uniquely induces endothelial cell proliferation (16).

Two structurally related vascular endothelial cell-specific tyrosine kinase receptors, fms-like tyrosine kinase (flt-1) and kinase domain region (KDR/flk-1), bind VEGF with high affinity (19). KDR/flk-1 appears essential for endothelial cell differentiation, while flt-1 may be involved in vascular assembly (20, 21). The physiological importance of the VEGF flt-1/KDR/flk-1 receptor system in blood vessel formation is based on several classical studies showing that: (1) the spatiotemporal expression of VEGF and its receptors correlates closely with angiogenesis in various sytems (5); (2) antibodies to VEGF

or flk-1 (22-24) or administration of truncated soluble flt-1 receptor to rats (25) or marmoset monkeys (26) block angiogenesis; (3) targeted inactivation of the VEGF gene in mice resulted in disruption of vasculogenesis and induced embryonic lethality (27, 28); and (4) mice lacking flt-1 or KDR/flk-1 die in early development because of the absence of vasculogenesis (20, 21).

4.2. Angiopoietin-1 and -2

Two other more recently discovered 75 kDal secreted proteins, angiopoietin-1 and angiopoietin-2, appear to work in concert with VEGF in signaling vascular morphogenesis by binding to the endothelial cell-specific transmembrane tyrosine kinase receptor Tie-2. The phase-specific expression of angiopoietin-1 and -2 and the results of Tie-2 gene knock-out studies (29, 30) have led to the proposal (6, 7) that VEGF, angiopoietin-1 and –2 interact to control angiogenesis, and vessel remodeling, maturation, and regression. Thus, it has been proposed that early in the process of vasculogenesis VEGF binds to the KDR flk-1 receptor to stimulate endothelial cell migration and proliferation, and simultaneously binds to the flt-1 receptor to promote endothelial cell-cell interactions and capillary tube formation. Angiopoietin-1 then binds to the Tie-2 receptor to recruit and stimulate the association of peri-endothelial support cells, e.g. pericytes and smooth muscle cells, with endothelial cells to mature and stabilize the newly-formed blood vessels and also promote endothelial cell survival. Angiopoietin-2, by exerting an antagonistic action on the angiopoietin-1/Tie-2 receptor signal, causes the vessel wall to loosen, reducing endothelial cell contacts with matrix and disassociating peri-endothelial support cells, rendering the endothelial cells more accessible and responsive to VEGF, presumably to further promote angiogenesis. However, in the relative absence of VEGF, angiopoietin-2 by loosening the endothelial cell-matrix interaction apparently elicits endothelial cell death via apoptosis. This sequential cascade of VEGF, angiopoietin-1 and -2 events may be the blueprint for the development, remodeling and regression of a new vascular system within the endometrium during each menstrual cycle.

The subcellular signaling mechanisms and pathways underlying VEGF and angiopoietin action are incompletely understood. However, it appears that adapter proteins Grb, protein tyrosine phosphatase SHP2, and STAT, which have been implicated in cell survival, migration, proliferation and differentiation (31), may modulate angiopoietin-Tie receptor activation. Moreover, endothelial cell-cell and endothelial cell-extracellular matrix interactions are intricately involved in cell proliferation, survival, migration and remodeling processes essential for vascularization of the endometrium. VEGF and angiopoietin-1/-2 seem to modulate the latter processes by controlling expression of integrins, extracellular matrix proteins and cell adhesion molecules (reviewed in 32). For example, VEGF induces expression of $\alpha v\beta 3$, $\alpha 1\beta 1$ and $\alpha 2\beta 1$ integrins which function as vascular cell adhesion molecules and as receptors for interstitial collagen in microvascular endothelial cells (33).

4.3. Basic Fibroblast Growth Factor (bFGF)

Other than VEGF, bFGF is the most widely recognized growth factor thought to be important to angiogenesis. bFGF, a member of the FGF superfamily, is comprised of nine distinct gene products (reviewed in 34, 35), is a cationic 18 kDal polypeptide which lacks a signal peptide and therefore fails to enter the classical secretory pathway, and induces angiogenesis *in vivo* and in three-dimensional invasion models *in vitro* (36-38). However, unlike VEGF, the stimulatory effects of bFGF are not restricted to endothelial cells, but also involve proliferation, migration, and differentiation of smooth muscle cells and fibroblasts, all of which express FGF receptors (39). For example, suppression of bFGF expression by adenovirus transfer of an antisense bFGF to cultured vascular smooth muscle cells decreased proliferation of and induced apoptosis in these cells (40). Although the level of angiogenesis in microvascular endothelial cell monolayers was five-fold greater in the presence of bFGF and VEGF than with either one alone, suggesting that these factors act synergistically (38, 41), VEGF was much more effective than bFGF in stimulating endometrial microvascular endothelial cell proliferation (42). Recently, KDR/flk-1 tyrosine kinase anilinophthalazine antagonists were shown to inhibit bFGF as well as VEGF-induced angiogenesis *in vitro*. However, because endogenous VEGF was required for this effect, it appears that these antagonists act on bFGF by interrupting an autocrine bFGF-VEGF loop (43).

The bFGF-induced endothelial responses are mediated via a high affinity type 1-C receptor (44), however, three other transmembrane tyrosine kinase receptors and a large number of FGF receptor variants generated by alternative mRNA splicing and differential polyadenylation also exist. This diversity in receptor expression results in a complex and incompletely understood pattern of overlapping binding specificities and actions for the various FGF's.

4.4. Other Angiostimulatory and Angioinhibitory Factors

Four closely related genes which show homology with VEGF, i.e. VEGF-B, VEGF-C, VEGF-D, and placenta growth factor, also may be involved in blood vessel development, although the roles for these factors are poorly understood. VEGF-C and –D bind to KDR/flk-1 and flt-4 receptors, while the receptor for VEGF-B is unknown. VEGF-C induces angiogenesis *in vivo* (45). Placenta growth factor forms heterodimers with VEGF and binds to flt-1, however, it is very weakly mitogenic when compared to VEGF (46). Angiogenin, a heparin-binding 14.1-kDal single-chain polypeptide and member of the pancreatic ribonuclease superfamily, also is a potent inducer of angiogenesis (47). Other factors, e.g. epidermal growth factor (48), platelet-derived growth factor (49) and transforming growth factor α and β (50), also promote certain aspects of angiogenesis.

Angiogenesis and vascular remodeling (i.e. investment of microvessels with periendothelial support cells such as vascular smooth muscle cells and pericytes) are orchestrated by coordinated interactions of stimulatory and inhibitory factors. One of the most widely studied angioinhibitory molecules is thrombospondin-1, a 450-kDal glycoprotein released from platelet granules by thrombin,

which is deposited in and interacts with extracellular matrix and cell surface integrins (51, 52). Thrombospondin-1 exerts antagonistic effects on vascular endothelial cell proliferation, migration, and assembly into microvessels and induces vascular endothelial cell apoptosis (53). Thrombospondin-1 is expressed in human endometrial stromal cells and is increased by progesterone (52).

5. EXPRESSION OF ANGIOGENIC FACTORS IN THE ENDOMETRIUM DURING THE MENSTRUAL CYCLE

In the human, VEGF and angiopoietin-1 and -2 mRNA and protein have been localized by *in situ* hybridization and immunocytochemistry in glandular epithelium and stroma (54-57). Each of the VEGF isoforms is expressed in the human endometrium, although the 121 and 165 species seem to be the most important physiologically (54). Endometrial microvascular endothelial cells also express the flt-1 and KDR/flk-1 receptors (58-60) and pericytes/smooth muscle precursor cells express the Tie-2 receptor (7). Levels of VEGF mRNA and protein in whole endometrium (55, 56) and glandular epithelium (61, 62), and VEGF formation by endometrial cells in culture (62), appeared somewhat greater in the secretory than early proliferative stages of the human menstrual cycle, although VEGF mRNA expression appeared to decrease in the stroma during the secretory phase (54). Others have reported that VEGF immunostaining of endometrial glandular and stromal cells (60, 63, 64), VEGF protein formation by explants of glandular epithelial and stromal cells (65) and endometrial flt-1 and KDR/flk-1 receptor expression (58) did not change during the course of the human menstrual cycle, despite the surges in estrogen and progesterone levels.

Steady-state VEGF mRNA levels in glandular epithelial and stromal cells, isolated from the baboon endometrium by laser capture microdissection, also were relatively similar in the proliferative, midcycle estradiol surge and secretory phases of the menstrual cycle (66), although the levels of VEGF mRNA and protein within the stromal compartment appeared to decline somewhat in the secretory phase. Thus, VEGF expression by glandular epithelial and stromal cells, and flt-1 and KDR/flk-1 receptor expression within the respective vascular endothelium are available throughout the menstrual cycle as components of the angiogenic system to promote vascular reconstruction of and angiogenesis within the endometrium. Indeed vessel density and percent vascularized area of the baboon (66) and human (60, 67, 68) endometrium, and endothelial cell mitosis in the human endometrium (65, 69), remain relatively constant throughout the menstrual cycle. Therefore, VEGF synthesis within and neovascularization of the endometrium seem to be sustained and ongoing processes designed to progressively promote growth and development of the endometrium with advancing stages of the menstrual cycle.

Because 80% of glandular epithelial VEGF is thought to be secreted from the luminal surface (70), it has been suggested (65) that most of the VEGF produced in the glands does not have a role in endometrial angiogenesis. It is possible, therefore, that VEGF synthesized locally within the stroma and/or vascular endothelial cells (63), pericytes (71, 72), and/or vascular smooth muscle cells (73) has the more important role in regulating, in a paracrine/autocrine manner, vasculogenesis within the endometrium. Moreover, since neutrophils constitutively express VEGF focally in association with microvessel endothelial cells (74), it has been proposed that cells within the vasculature are the principal source of angiogenic factors for non-sprouting angiogenesis, i.e. intussusception and elongation, within the endometrium.

bFGF and EGF, and their respective receptors, are also expressed in glandular and luminal epithelial cells, basal lamina of blood vessels, stroma, and extracellular matrix of the human endometrium (60, 75-77). Although endometrial stromal cell bFGF and FGF receptor expression was higher during the proliferative phase of the menstrual cycle (77), others reported that bFGF expression did not change during the menstrual cycle (60, 75). Because FGF receptor was markedly reduced in women with menorrhagia, Sangha *et al* (77) have suggested that FGF is critical for endometrial regeneration. In contrast, because bFGF was increased in atrophic endometrium of postmenopausal women (75), and FGF type-1 and -2 receptors were only sparsely expressed in human endometrial blood vessels, it has been suggested that bFGF may be of lesser importance in regulating angiogenesis in the uterus (60).

Finally, angiogenin mRNA and protein are expressed by endometrial glandular epithelial and stromal cells and increase during the mid to late secretory phase of the human menstrual cycle (78). Since vascular smooth muscle cells around uterine spiral arteries show increased proliferative activity during this interval (79), and angiogenin stimulates proliferation of vascular smooth muscle cells and endothelial cells (80), Koga *et al.*, (78) have suggested that angiogenin may participate in the process of thickening and convolution of the arterioles during the secretory phase of the menstrual cycle.

6. STEROID HORMONE REGULATION OF VEGF EXPRESSION AND VASCULARIZATION IN THE ENDOMETRIUM

6.1. Estrogen and Progesterone Receptors

The receptors for both estrogen α and β and progesterone are present within the nuclei of glandular epithelial and stromal cells of the human (81-84) and nonhuman primate (84, 85) endometrium during the menstrual cycle. In elegant studies, estradiol was shown to increase estrogen and progesterone receptor mRNA and protein expression in glandular epithelial, stromal and vascular smooth muscle cells in ovariectomized rhesus monkeys (4, 85-87). As estrogen was withdrawn and progesterone elevated to mimic the peripheral serum levels characteristic of midsecretory phase, estrogen (4, 85, 87, 88) and progesterone (4, 82, 85, 88) receptors were markedly suppressed in glandular epithelial and stromal cells of the functionalis zones I-III, but not basalis zone IV, or in vascular smooth muscle or perivascular stromal cells of the spiral arteries. These results are consistent with observations in the

human, where immunocytochemical staining for progesterone receptor protein was significantly reduced during the mid and late secretory phase compared to the proliferative phase of the menstrual cycle (61, 84, 89). It is apparent, therefore, that estrogen generates the receptors for estrogen and progesterone during the proliferative phase, and that the surge in progesterone during the secretory phase is responsible for the decline in receptor expression. Thus, the receptors for both estrogen and progesterone are expressed in a cell and zone-specific manner to mediate the action of these hormones on cell proliferation, differentiation (90) and angiogenesis within the human and nonhuman primate endometrium.

6.2. Estrogen and Progesterone Action

6.2.1. Estrogen

It has been generally concluded that estrogen has a pivotal role in establishing the new vascular bed and promoting growth and cellular differentiation within the endometrium during each menstrual cycle (1, 2, 4, 91). The uterotropic effects of estrogen are both early and long-term. The early events, e.g. increased vascular permeability (92, 93), create an environment optimal for cellular hypertrophy and hyperplasia and consequently angiogenesis. Thus, in the rat a single injection of estradiol enhanced vascular permeability and water inhibition within 3-6 h (94-96) and endothelial cell mitosis indicative of basal sprouting of capillaries within 24 hours (97).

There is compelling evidence in various species to support the concept that the tropic effects of estrogen on vascularization of the uterus are mediated by VEGF. Thus, estradiol rapidly elevated VEGF mRNA levels and vascular endothelial cell proliferation *in vivo* in the mouse and rat (97-101) and sheep (102-103) uterus. The estrogen induction of VEGF in the rat uterus occurred primarily in the stroma (104), involved upregulation of the 120 and 164 VEGF rodent species (100), and was blocked by estrogen receptor antagonist ICI 182,780 (105) suggesting regulation via the classical estrogen receptor pathway. Consistent with this concept, estrogen had no effect on angiogenesis in transgenic mice with targeted disruption of the estrogen receptor gene (106).

Although it is difficult to study the potential effect of ovarian steroid hormones on endometrial angiogenesis *in vivo* in the human, in cultures of isolated human endometrial epithelial and stromal cells, estrogen rapidly upregulated VEGF mRNA levels and protein (54, 56, 107, 108), an effect blocked by ICI 182,780 (109). Estradiol also stimulated DNA synthesis within and proliferation of human endometrial vascular endothelial cells *in vitro* (110).

In the baboon, used as a nonhuman primate model to study human reproductive endocrinology (111), VEGF mRNA levels in glandular epithelial and stromal cells isolated by laser capture microdissection and VEGF immunoreactivity in the endometrium were decreased to very low values by ovariectomy which suppressed serum estrogen and progesterone to nondetectable levels (66). Chronic administration of estradiol to ovariectomized baboons via silastic implants in levels which replicated the hormonal pattern of the proliferative phase of the menstrual cycle, returned endometrial glandular epithelial and stromal cell VEGF mRNA and protein expression to normal (112). The estrogen-dependent stimulation of endometrial VEGF expression observed in ovariectomized baboons is similar to that shown in the rhesus monkey by Nayak and Brenner (113). Acute administration of estradiol to ovariectomized baboons significantly increased glandular epithelial and stromal VEGF mRNA levels within 2 hours and the width of paracellular clefts between adjacent endometrial microvascular endothelial cells, indicative of increased microvascular permeability, within 6 hours (114). Therefore, it appears that ovarian estrogen has a major role in regulating and maintaining VEGF synthesis within the glandular epithelial and stromal cells during the primate menstrual cycle. Moreover, the rapid estrogen-induced up regulation of endometrial VEGF expression precedes, and therefore may mediate, the early action of estrogen on microvascular permeability, an early event in angiogenesis (92, 93).

In addition to effects on VEGF, estrogen also stimulated bFGF synthesis in human endometrial adenocarcinoma HEC-1 cells (115), however, there are conflicting reports on the effects of and mechanisms underlying the steroid hormone regulation of this growth factor (116).

Although sequences corresponding to classical estrogen response elements have not been identified in the 5′-flanking region of the VEGF gene, consensus half-palindromic sequences which bound estrogen receptor alpha in band shift assays and which confer estrogen inducibility to reporter constructs have been identified in two regions of the gene, one in the 5′-untranslated region (117). In primary human endometrial epithelial and stromal cells, as well as Ishikawa endometrial adenocarcinoma cells, estradiol stimulated VEGF gene transcription through a functional single variant estrogen response element located 1.5-kb upstream from the transcriptional start site (107). Considering these observations, plus findings that VEGF induction in the uterus by estrogen is rapid, blocked by antiestrogens and inhibited by actinomycin D but not puromycin or cycloheximide (100, 101), it is likely that the regulatory effects of estrogen on VEGF expression in the uterus are mediated by the estrogen receptor.

6.2.2. Progesterone

Progesterone also regulates endometrial angiogenesis and VEGF expression, however, there is considerable inconsistency in the literature on its specific role. For example, in nonhuman primates, the progesterone antagonist ZK137316 inhibited spiral artery development and endometrial proliferation (118), suggesting a stimulatory role for progesterone in these events. Moreover, Norplant which contains the synthetic progestin levonorgestrel increased endometrial microvascular endothelial cell density in women (67, 119). Therefore, it has been suggested that the significant growth of the coiled arteriolar system in the endometrium during the secretory phase of the menstrual cycle may reflect the influence of luteal progesterone (120). These effects of progesterone on endometrial vascular development may be mediated by VEGF, because chronic administration of progesterone to cynomolgus monkeys in which ovarian

function was suppressed by GnRH administration, increased VEGF immunoexpression in the endometrial stroma (121). Progesterone also induced endometrial VEGF expression *in vivo* in the rat (98, 101), and *in vitro* in the human (56), however, the magnitude of increase was less and the onset of induction slower than observed by estrogen.

In contrast to the stimulatory effects shown for progesterone on endometrial angiogenesis, a negative correlation has been reported between progesterone receptor and VEGF expression in endometrial glandular epithelium during the normal human menstrual cycle, results consistent with the notion that progesterone suppresses VEGF expression (61). Indeed, administration of oral contraceptives, containing a progestin in combination with ethinyl estradiol, to normal women decreased glandular epithelial VEGF immunostaining (61). Moreover, progesterone inhibited VEGF secretion by human decidualized stromal cells (122) and endometrial angiogenesis (10) when examined by *in vitro* assay systems. Consistent with the latter findings, acute simultaneous administration of estradiol and progesterone to ovariectomized baboons diminished the stimulatory effect of estrogen alone on endometrial glandular epithelial and stromal cell VEGF mRNA and protein expression (114). Several studies have shown that endometrial arteriole development is decreased by long-term progestin administration (123, 124). Moreover, there is substantial evidence that chronic use of progestin-containing oral contraceptive pills leads to abnormalities in endometrial microvessel structure (125), via a reduction in basal lamina collagen, laminin, and heparan sulfate proteoglycan. The latter changes may contribute to vascular fragility, dilation of superficial vessels (126), a reduction in vascular perfusion, and a decrease in endometrial vascular density (124), and consequently breakthrough bleeding. Brenner and colleagues (127) have shown both in the human and macaque that the KDR/flk-1 receptor, normally only expressed in the vascular endothelium, was markedly upregulated in stromal cells of the superficial endometrial zones upon progesterone withdrawal during the premenstrual phase. The increase in receptor expression was cell and zone specific and because promatrix metalloproteinase (MMP-1) was coordinately upregulated in the same stromal cells, it was suggested that VEGF-KDR/flk-1 interaction may influence MMP expression and thus play a role in the induction of menstruation (127).

Therefore, apparently conflicting studies exist on whether progesterone stimulates, inhibits or has no effect on VEGF expression and angiogenesis in the endometrium. Although the VEGF gene does not contain a classical progesterone response element, the 5′-flanking region contains sequences that confer progestin inducibility to reporter constructs in transfection studies (101). Some of the apparently disparate results reported for progesterone may reflect the need to consider particular VEGF isoforms. For example, considerable expression of VEGF 189 occurs in the human endometrium during the mid-late secretory phase, and estradiol plus progesterone stimulated VEGF 189 expression upon the differentiation of isolated human endometrial stromal into decidual cells (128). Moreover, the effect of progesterone may be cell-specific, because progesterone increased VEGF secretion by human endometrial epithelial cells but decreased expression by endometrial stromal fibroblasts (122).

Thus, regulation of uterine VEGF and angiogenesis very likely results from an interplay of estrogen and progesterone, however, the specific role(s) and site(s) of action of progesterone remain unclear.

6.2.3. Correlation of Steroid Hormones, VEGF Expression and Angiogenesis During the Menstrual Cycle

Despite the cyclical surges in estrogen and progesterone, human endometrial microvascular density (60, 67) and vascular endothelial cell proliferation (65, 129) and density (61), as well as VEGF expression, remain relatively constant throughout the menstrual cycle. Consequently, some investigators (61, 65, 130) have concluded that there is no relationship between, and/or steroid hormones are not the main regulators of, these processes in the human endometrium.

The limitation of studying endometrial angiogenesis during the normal human menstrual cycle, and difficulty in conducting invasive *in vivo* studies in humans, have led to apparently conflicting information on the effect of estrogen and progesterone on endometrial VEGF expression and vascularization. However, although endometrial VEGF mRNA levels and vessel density also remain relatively constant during the baboon (66) and rhesus monkey (113) menstrual cycle, there was a striking decline in endometrial VEGF expression and size after ovariectomy, effects which were completely reversed by estradiol or estradiol and progesterone (66, 113). It appears, therefore, that endometrial VEGF synthesis is dependent upon estrogen and that the levels of estrogen preceding and following the midcycle surge, albeit low, are nevertheless sufficient to bind to estrogen receptor to maintain VEGF synthesis and thus promote endometrial angiogenesis throughout the course of the advancing menstrual cycle.

6.3. Direct Actions of Estrogen on Vascular Cells

Although estrogen and progesterone may promote endometrial angiogenesis by upregulating the expression of angiogenic factors such as VEGF by glandular epithelial and stromal cells during the menstrual cycle, these steroid hormones may also exert direct actions upon vascular endothelial and smooth muscle cells. Thus, estrogen and progesterone receptors have been detected in vascular smooth muscle cells (84, 131) and vascular endothelial cells (132, 133) within the endometrium, although others have not found estrogen receptors in endothelial cells (134, 135). In cell culture, estrogen promoted vascular endothelial cell proliferation (136), migration (137), and survival (138). The stimulatory effect of estradiol on myometrial microvascular endothelial cell proliferation may reflect an estrogen-dependent increase in expression of the KDR/flk-1 receptor on these cells (139). In ovariectomized mice, estrogen stimulated endometrial vascular endothelial cell proliferation within 24 hours and glandular epithelial cell proliferation after 48 hours (140), raising the possibility that endometrial tissue growth and mass are regulated by growth of endothelial cells (141). Although the effects of estrogen on microvessel cell function may be direct, it is also possible they are modulated

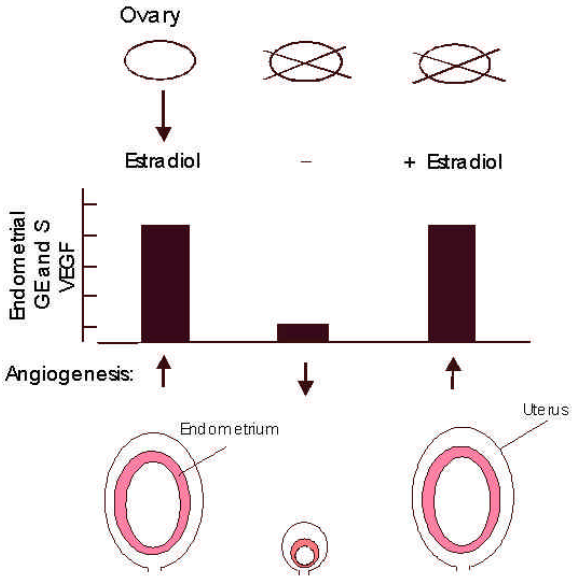

Figure 1. Proposed role of ovarian estrogen on endometrial glandular epithelial (GE) and stromal (S) VEGF expression, angiogenesis, and growth in the primate.

in an autocrine and/or paracrine manner via VEGF. Thus, estrogen stimulated VEGF expression by vascular smooth muscle cells (5, 131, 142), and anti-VEGF antibody blocked the increase in vascular endothelial cell proliferation elicited by estradiol (143).

6.4. Other Regulatory Elements

Factors in addition to estrogen and progesterone, notably hypoxia, cytokines, and thrombin are extremely potent in their capacity to upregulate VEGF expression in various tissues including the human endometrium (5, 62, 144, 145). Hypoxia also upregulates (146) and TGFβ down regulates (147) expression of the VEGF flk-1 receptor in vascular endothelial cells. However, the physiological role of hypoxia upon endometrial VEGF expression may be most important at the time of menstruation when both oxygen tension and steroid hormone levels become very low (130). Indeed, shortly preceding menstruation there is a striking increase in expression of VEGF mRNA in the baboon (Albrecht ED, Pepe GJ, unpublished observations) and VEGF and KDR/flk-1 receptor in the human and rhesus monkey endometrium (113, 127). Under these circumstances, hypoxia and/or other factors such as cytokines important to wound healing may overcome the absence of other regulatory factors, e.g. estrogen, resulting in the induction of VEGF synthesis, which may be important for vessel repair and reconstruction.

7. PERSPECTIVE

A complex interplay of different cell types which express angiostimulatory agents such as VEGF, angiopoietin-1, and bFGF, and angioinhibitory factors such as thrombospondin-1, and which respond in a coordinated manner to regulatory factors such as estrogen and progesterone, exist to promote and maintain a balance in the level of vessel growth within the human endometrium during each menstrual cycle. The physiological roles and sites of action of VEGF and angiopoietin -1 and -2 in vascular development have primarily been elucidated by transgenic approaches in mice, while the majority of the studies of the regulatory effects of estrogen and progesterone on the expression of angiogenic factors in the endometrium have been conducted *in vivo* in laboratory rodents and *in vitro* with human cells. However, because of the difficulty for ethical reasons in conducting invasive *in vivo* experimental studies in the human, investigation of the regulatory actions of steroid hormones on endometrial expression of VEG/PF, angiopoietin-1, and angiopoietin-2 and consequently vessel development and remodeling in the human endometrium has been limited. Thus, many of the *in vivo* studies on angiogenesis in the human have been conducted during the normal menstrual cycle where relatively little change in endometrial VEGF expression and microvessel density were observed despite the cyclical surges in estrogen and progesterone. Consequently, these observations have led to the conclusion that steroid hormones do not have a role in regulating VEGF or angiogenesis in the endometrium. However, studies conducted *in vivo* in nonhuman primates, in which the hormonal *milieu* can be experimentally manipulated, show that endometrial glandular epithelial and stromal VEGF expression was decreased to baseline levels by ovariectomy and restored to normal by chronic administration of estrogen in levels which replicated those observed during the normal menstrual cycle (Figure 1). Therefore, the relatively low levels of estrogen to which the uterus is exposed preceding and following the midcycle surge in estrogen apparently are sufficient to sustain VEGF expression throughout the menstrual cycle and only when estrogen is decreased to nondetectable values, e.g. after ovariectomy, does VEGF formation substantially decline. Thus, VEGF and its receptors are expressed and available as components of the angiogenic system throughout the menstrual cycle to promote vascular reconstruction of the endometrium. Collectively, the studies conducted *in vivo* in the laboratory rodent, *in vitro* with isolated human endometrial cells, and *in vivo* in nonhuman primates are consistent with the proposal that ovarian estrogen, potentially acting in conjunction with autocrine/paracrine factors such as hypoxia, has an essential physiologic role in stimulating VEGF expression by endometrial glandular epithelial and stromal cells (Fig. 1), thereby promoting angiogenesis and the progressive development of a new vascular system necessary to support growth and differentiation of the endometrium during the menstrual cycle.

It is also apparent that studies to this point have focused primarily on VEGF expression in the uterus. Thus, very little is known about the regulation of other angiostimulatory components, e.g. angiopoietin-1 and -2, required for vessel maturation and development. Moreover, although cell culture studies show that angiogenesis involves a balance between stimulatory and inhibitory factors, very little is known about the timing of expression and regulation of angioinhibitory factors such as thrombospondin, and how they interact with angiostimulators, such as VEG/PF and angiopoietin-1 and –2, in promoting vessel development in the human and nonhuman primate endometrium.

Experimental evidence also shows that development and remodeling of the vascular bed in the endometrium involves complex cell-cell and cell-extracellular matrix interactions, including the mural investment of vascular endothelial cells with pericytes and vascular smooth muscle cells. However, very little is known about the particular roles which estrogen and progesterone, acting directly or indirectly via VEGF, angiopoietins-1/-2, and other stimulatory and inhibitory growth factors, play in the latter architectural processes. Moreover, the actions which estrogen and progesterone have in regulating cell adhesion molecules, integrins, and proteinases integral to these cellular remodeling processes for endometrial angiogenesis during the menstrual cycle are largely unknown.

Finally, the uterine endometrium is a heterogenous organ comprised of vascular components, secretory glandular and luminal epithelial cells, and stromal elements including immunomodulatory macrophages and fibroblasts, each of which may display a very different repertoire of steroid hormone responsivity, expression of cell-cell matrix molecules, and autocrine/paracrine molecular interaction. Relatively little attention has been directed, however, to cell-specific responsivity and function in the human endometrium with respect to the steroid hormone regulation of angiogenesis.

8. ACKNOWLEDGMENT

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