

Role of vascular endothelial growth factor in ovarian physiology – an overview

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SUMMARY

In the female reproductive system, as in a few adult tissues, angiogenesis occurs as a normal process and is essential for normal tissue growth and development. In the ovary, new blood vessel formation facilitates oxygen, nutrients, and hormone substrate delivery, and also secures transfer of different hormones to targeted cells. Ovarian follicle and the corpus luteum (CL) have been shown to produce several angiogenic factors, however, vascular endothelial growth factor (VEGF) is thought to play a paramount role in the regulation of normal and abnormal angiogenesis in the ovary. Expression of VEGF in ovarian follicles depends on follicular size. Inhibition of VEGF expression results in decreased follicle angiogenesis and the lack of the development of mature antral follicles. The permeabilizing activity of VEGF is thought to be involved in follicle antrum formation and in the ovulatory process. In the CL, VEGF expression corresponds to different patterns of angiogenesis during its lifespan. In most

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the species, higher VEGF expression in the early luteal phase is essential for the development of a high-density capillary network in the CL. However, high VEGF expression may be still maintained in the mid-luteal phase to increase vascular permeability that results in enhancement of luteal function. During gestation, VEGF is thought to be important for the persistence of the CL function for a longer than in the nonfertile cycle period of time. Further elucidation of specific roles of VEGF in ovarian physiology may help to understand the phenomenon of luteal insufficiency and reveal novel strategies of ovarian angiogenesis manipulation to alleviate infertility or to control fertility. *Reproductive Biology 2005 5 (2):111-136.*

Key words: ovary, follicle, corpus luteum, ovarian angiogenesis, VEGF, VEGF receptors

INTRODUCTION

The ovarian cycle is characterized by repeating patterns of cellular proliferation and differentiation that accompany follicular development as well as the formation and regression of the corpus luteum (CL). Follicular development begins when the granulosa cells start to proliferate. Under the appropriate gonadotropic stimulation, granulosa and theca cells of follicles continue to proliferate and differentiate until ovulation. Ovulation is the critical event that initiates the transformation of the fluid-filled preovulatory follicle into the solid CL. After ovulation, profound and radical changes occur in the theca and granulosa layers, which enable CL formation and maturation. During the luteal phase, the CL undergoes definitive structural and functional changes until regression and corpus albicans formation, or when pregnancy occurs its function is maintained until term. This rapid growth and regression of ovarian tissues are accompanied by equally rapid changes in their vascular beds [8, 13, 20]. The formation of a dense capillary network (angiogenesis) in the ovary enables the hormone-producing cells to obtain the oxygen, nutrients and also precursors necessary to synthesize and release different hormones essential for maintenance of the ovarian functions.

Angiogenesis refers to the formation of new blood vessels and is essential for normal tissue growth and development [37, 57]. The angiogenic process begins with capillary sprouting and culminates in formation of a new microcirculatory bed composed of arterioles, capillaries, and venules. The initiation of angiogenesis consists of at least three processes: 1) breakdown of the basement membrane of the existing vessel, 2) migration of endothelial cells from the existing vessel towards an angiogenic stimulus, and 3) proliferation of endothelial cells (fig. 1; [37, 57]). New blood vessel

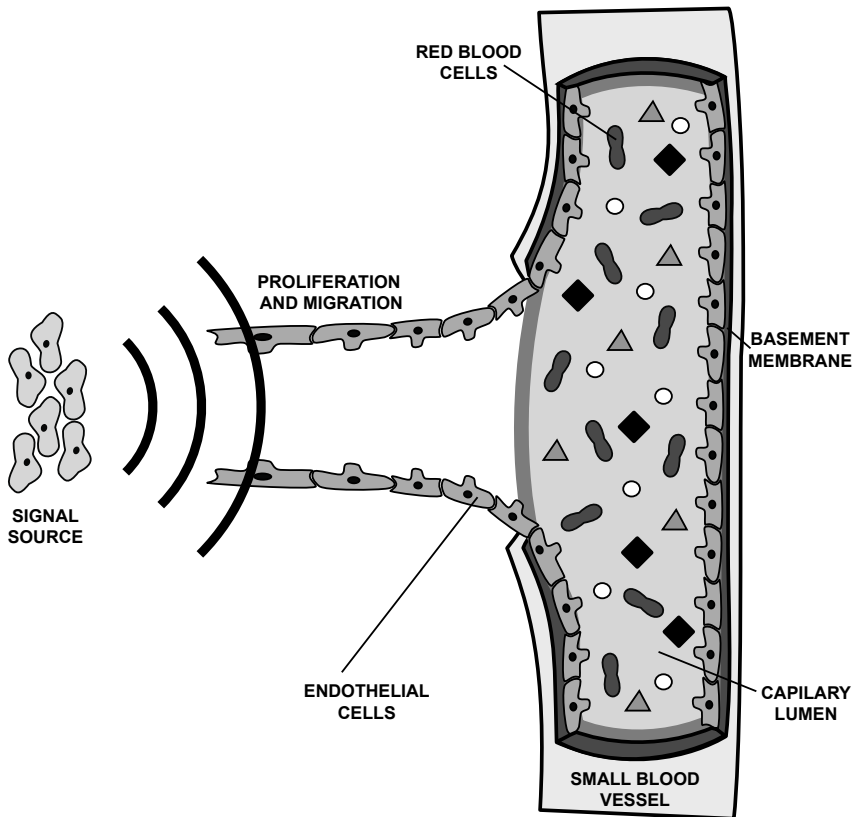


Figure 1. Initial cellular events observed during angiogenesis. The secretion of enzymes that digest the collagen fibers present in the basement membrane is a response of endothelial cells to the pro-angiogenic signal. This leads to breakdown of the basement membrane of existing vessel and creates a breach through which proliferating endothelial cells can migrate towards the signal source.

development is completed by formation of capillary basal lumina and differentiation of new capillaries into arterioles and venules.

Under physiological conditions, in most adult tissues, capillary growth is rather limited and vascular endothelium represents a stable population of cells with a low mitotic rate [25]. However, during periodic and dynamic changes in the ovary, uterus, and placenta endothelial cells are able to proliferate [36]. In contrary to that observed during pathological tissue growth (e.g. tumor growth), the angiogenic process in female reproductive tissues is limited and, therefore, must be tightly regulated [83]. Several potential regulators of angiogenesis have been identified including acidic fibroblast growth factor (aFGF or FGF-1), basic fibroblast growth factor (bFGF or FGF-2), angiopoietins, insulin-like growth factors (IGFs), transforming growth factor- α (TNF- α), interleukin-8 (IL-8; [38, 57]). Work done by several laboratories over the last several years has elucidated the pivotal role of vascular endothelial growth factor (VEGF) in the regulation of normal and abnormal angiogenesis [33].

VEGF LIGANDS AND RECEPTORS

The VEGF family currently comprises several members, including the first identified molecule, VEGF-A, placental growth factor (PlGF), VEGF-B, VEGF-C, VEGF-D, and two VEGF-like proteins [35]. In addition, at least five molecular isoforms of VEGF-A (also referred to as VEGF) that differ in total amino acid number, are produced as a result of alternative splicing of its gene. In humans, these isoforms correspond to VEGF121, VEGF145, VEGF165, VEGF189, and VEGF206 (fig. 2).

VEGF121 and VEGF165 are usually the predominant molecular species produced by a variety of normal and transformed cells. Both of them are diffusible, but VEGF165 secreted protein can be bound to the cell surface and extracellular matrix [35]. VEGF189 is detected in the majority of cells and tissues expressing the VEGF gene. In contrast, VEGF206 is a very rare form, almost completely sequestered in the extracellular matrix [35]. VEGF145 is another secreted isoform binding to the endothelial cells and

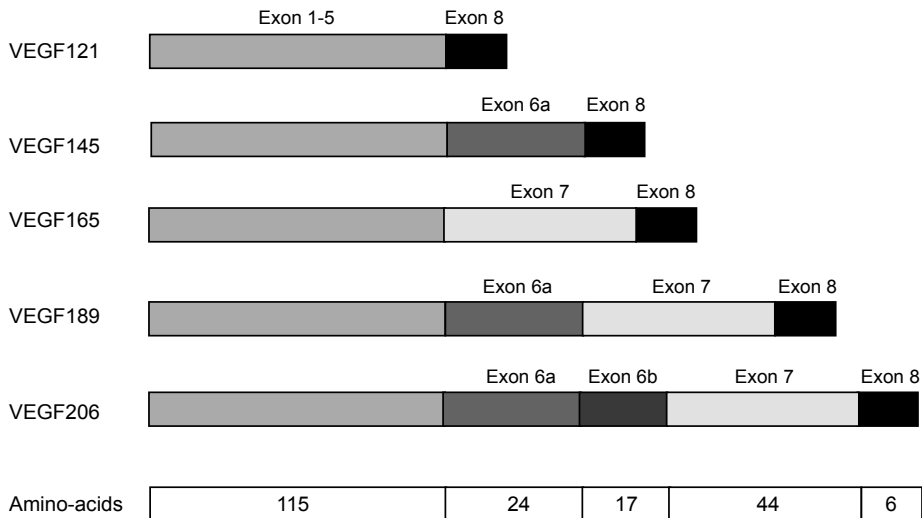


Figure 2. Structure of the VEGF splice variants. The peptides encoded by various exons of human VEGF gene are shown schematically as boxes. The number of amino acids in each of the exon-encoded peptides is shown in the bottom.

its expression seems to be more restricted compared with other VEGF forms [78]. VEGF145 expression was thought to be limited to reproductive tissues where the expression level was relatively low with comparison to VEGF121 and VEGF165 [16, 17, 60]. Further experiments demonstrated that VEGF145 expression is not restricted to reproductive tissue, since its level is detectable in human hair follicular cells [59] and several breast cancer specimens [94].

The biological effects of VEGFs are almost exclusively mediated *via* two high affinity binding sites belonging to the tyrosine kinases receptor family, *fms*-like tyrosine kinase (Flt-1 or VEGFR-1) and fetal liver kinase-1/kinase insert domain-containing receptor (Flk-1/KDR or VEGFR-2; [35]). These receptors contain an extracellular region with seven immunoglobulin (Ig)-like loops as well as a single transmembrane region and a split tyrosine kinase domain (fig. 3). The Flt-1 receptor mRNA can be spliced to generate forms encoding either the full-length membrane-spanning receptor or a soluble form, sFlt-1 that is truncated on the C-terminus (fig. 3; [55]). Like other tyrosine kinase receptors, VEGF receptors undergo ligand-induced

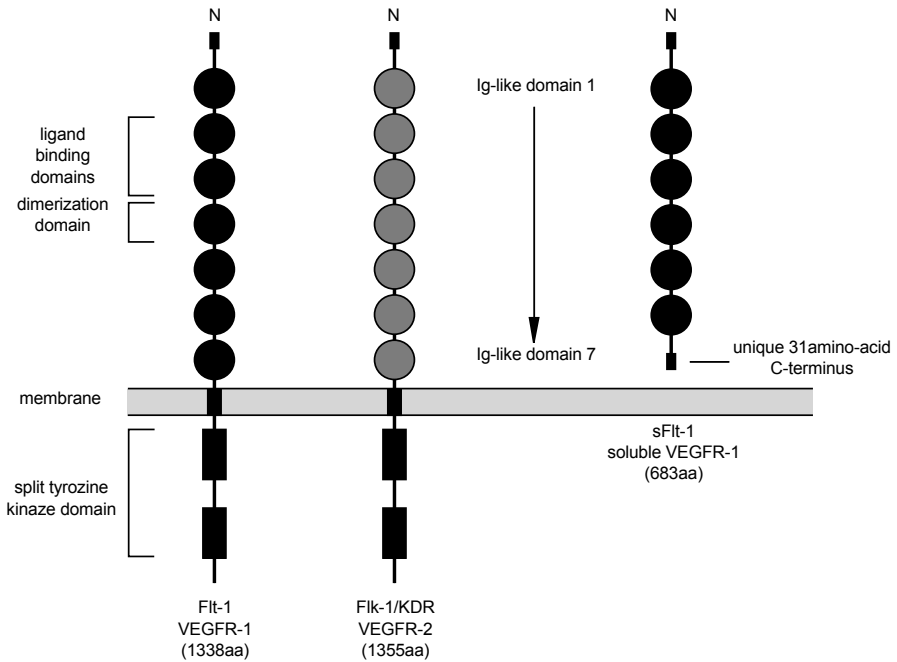


Figure 3. Structure of the VEGF high-affinity receptors. The figure presents two tyrosine kinase receptors, Flt-1 and Flk-1/KDR with major structural motifs: 7 extracellular Ig-like domains containing the dimerization and ligand-binding region, a single plasma membrane-spanning sequence, and intracellular split tyrosine kinase domain. An alternatively expressed soluble truncated form of Flt-1, sFlt-1, containing 6 Ig-like domains and unique 31-amino acid C-terminal sequence is presented. Human amino acid (aa) sequence lengths are given in parentheses.

dimerization to activate transduction pathways and dimers between sFlt-1 and full-length VEGF receptor block signal pathways dependent on intracellular tyrosine kinase dimerization [55].

VEGF receptor-signaling pathways remain poorly understood. Several studies have suggested that Flt-1 and Flk-1/KDR may have distinct signal transduction pathways; therefore the biological response mediated by their activation may be different [23]. It is thought that VEGF-dependent activation of Flk-1/KDR is involved in mediating endothelial cell proliferation, survival and vascular permeability, whereas Flt-1 might play an inhibitory role by sequestering VEGF, and preventing its interaction

with Flk-1/KDR [34]. Several phosphorylation sites and potential binding molecules have been identified [90], but their role in VEGF-stimulated cellular response remains to be elucidated.

BIOLOGICAL ACTIONS OF VEGF

VEGF is a strong mitogen for vascular endothelial cells derived from arteries, veins and lymphatics [35]. Several studies have also reported a mitogenic effect of VEGF on a few non-vascular endothelial cell types including cultured human retinal pigment epithelial cells, rat pancreatic ductal epithelial cells and several cell types in the mouse peripheral nervous system [48, 72, 93]. By stimulation of some proteolytic enzymes (e.g. proteases, collagenases) and tissue-type plasminogen activators, VEGF creates a prodegradative environment that facilitates migration and sprouting of endothelial cells [77, 100].

There is also strong evidence that VEGF may be a key survival factor for endothelial cells of at least some mammals [3, 9, 42]. Acting as a survival factor, VEGF induces expression of antiapoptotic proteins Bcl-2 and A1 (homolog of the Bcl-2 family) in human endothelial cells [42]. It was shown that Flk-1/KDR and phosphatidylinositol 3'-kinase (PI3-kinase)/nonreceptor tyrosine kinase (Akt) are crucial mediators in the processes leading to endothelial cell survival induced by VEGF [43]. Benjamin and co-workers [9] suggested that pericyte coverage is critical for endothelial cell dependency on VEGF survival activity.

VEGF is also known as vascular permeability factor (VPF). This activity was first demonstrated *in vivo* in the skin of a guinea pig, where the administration of VEGF/VPF caused blood vessel hyperpermeability [22]. The possible mechanism underlying the VEGF-induced permeability is based on a rapid formation of fenestrations, which were observed in the rodent endothelium of small vessels after treatment with recombinant human VEGF165 [85]. Several other effects mediated by VEGF include regulatory effect on blood cells, stimulation of vascular cell adhesion molecule-1 (VCMA-1) and intercellular adhesion molecule-1 (ICMA-1)

in endothelial cells, regulation of differentiation of hemangioblast (the precursor of endothelial and hemopoietic cells), induction of vasodilation *in vitro* [33-35].

OVARIAN PHYSIOLOGY

Follicular phase

During folliculogenesis, primordial follicles composed of single layer of granulosa cells, develop to form secondary follicles, in which the outer theca layer has its own vascular network and the granulosa inner layer remains avascular. Follicular angiogenesis is initiated early during follicular development and continues throughout follicle growth [97]. Pre-antral follicles have no vascular supply of their own. However, during antrum development, follicles acquire vascular sheath in the theca layer which, when fully established, consists of two capillary networks, located in the theca interna and externa. These newly formed ovarian blood vessels secure an increasing supply of gonadotropins, growth factors, oxygen, steroid precursors, as well as other substances to the growing follicle. In the theca layer, blood vessels increase in number and size as the follicle develops but do not penetrate the basal membrane separating theca interna from the granulosa cell layer. Increased vascularization of individual follicle results in preferential delivery of gonadotropins and, therefore, may play an instrumental role in selective maturation of the preovulatory follicles [108]. On the other hand, degeneration of the capillary bed in follicles that fail to develop is a relevant factor causing follicular atresia. However, microvascular changes of atretic follicles seem to be a consequence rather than the origin of atresia as hypothesized for rabbits [64].

VEGF production is regulated differently in follicles according to their size. VEGF mRNA and protein in the primate ovary are expressed in the theca cells of antral follicles and in the granulosa cells nearest the oocyte in the preovulatory follicle but not in granulosa cells of primordial and preantral follicles [95]. In bovine and porcine follicles VEGF is weakly

expressed during early ovarian follicular development and becomes more pronounced in granulosa and theca cells along with dominant follicle development [5, 47]. Similar results were found in the rat ovary, while some secondary follicles showed extremely strong VEGF immunoreactivity in zona pellucida (ZP). The authors suggested that this might be an important factor for the selection of dominant follicles in rats [15].

Expression of VEGF mRNA and/or protein in granulosa cells of various species is stimulated by LH surge (natural cycles) or human chorionic gonadotropin (hCG; artificial cycles) bolus [5, 19, 58]. Recently, it has been shown that exposure to elevated levels of gonadotropins increases not only the expression of VEGF but also Flt-1 and Flk-1/KDR receptors in porcine and rat ovarian follicles [45, 91]. Studies performed in the marmoset revealed that VEGF expression in the preovulatory follicles is under gonadotropic control. In contrast, the expression of VEGF in tertiary follicles was not dependent on gonadotropin secretion. It is possible that other paracrine factors are involved in the regulation of VEGF expression in the developing ovarian follicles [99].

Available data indicate that enhancing VEGF expression during the follicular phase could be useful in increasing the number of predominant follicles destined for ovulation. The injection of VEGF gene fragments into ovaries of gilts treated with equine chorionic gonadotrophin (eCG) increases the number of large follicles and development of the vascular network in the theca layer [92]. Moreover, direct ovarian administration of VEGF increases the number of preantral follicles in the rat ovary, similar to the well-known effect of estrogen [24]. Additionally, Quintana et al. [79] suggested that direct ovarian administration of VEGF diminishes ovarian apoptosis in mice. Intraperitoneal administration of VEGF120 and VEGF164 markedly stimulates follicular angiogenesis in the theca interna layer, and increases the number of healthy preovulatory follicles and oocytes ovulated in rats [50]. In contrast, administration of an anti-Flk-1/KDR antibody inhibits gonadotropin-dependent follicular angiogenesis in mice which, in turn, blocks development of mature antral follicles [109]. Inhibition of VEGF with a VEGF Trap antibody results in decreased follicle angiogenesis, reduced recruitment and growth of antral follicles, as well as

decreased Flt-1 and Flk-1/KDR expression in the primate [105]. The elevated vascularity or vascular permeability of developing follicles could facilitate the delivery of folliculotropic agents (e.g. follicle stimulating hormone, estrogen, androgen) that results in increase of follicular recruitment from the primordial pool and/or an inhibition of follicular atresia [108].

Expression of mRNA for both VEGF receptors increases in theca layer of medium and large porcine follicles after eCG treatment. Moreover, parallel expression of VEGF120 and VEGF164 (porcine mature VEGF isoforms are one amino acid shorter than those of humans) mRNA in the granulosa layer suggests that both receptors may be activated by VEGF [91]. Further experiments revealed that only Flt-1 mRNA level has a tendency to increase in theca tissues of antral follicles of gonadotropin-treated gilts injected with VEGF gene fragments [92]. This indicates that Flt-1 is predominantly involved in the regulation of the capillary network in theca interna during the folliculogenesis in pigs.

Capillaries of the theca layer become hyperpermeable around the time of ovulation in several mammalian species [67, 76]. In bovine and porcine follicles, this is accompanied by increased granulosa VEGF expression and very high accumulation of VEGF protein in follicular fluid [5, 10]. The high levels of VEGF present in the follicular fluid of preovulatory follicles seem to diffuse toward the outer layers and create an angiogenic gradient in the theca layer that attracts blood vessels towards the granulosa layer [5]. Because vessels cannot cross the barrier of the basal membrane, they develop within the theca layer in close vicinity to the membrane, representing the main source of nutrients and gasses for the granulosa and germinal cells. Early signs of atresia include disappearance of these inner vessels without substantial modification of vascularization in other regions of theca layer [67]. Therefore, the persistence of this inner capillary network appears to depend directly on VEGF accumulation in follicular fluid and when such a store is no longer available as it occurs in early atretic follicles, the capillary network undergoes intensive degeneration. It was demonstrated that among porcine follicles of the same size, those accumulating high levels of VEGF in follicular fluid have a significantly denser vascularization of the follicle wall [65].

Correlation between increasing levels of VEGF and estradiol-17 β (E₂) in follicular fluid strongly supports the hypothesis that those follicles will effectively grow and reach preovulatory stage of development [65]. The results of the intrabursal estrogen administration experiments in rats clearly demonstrate that estrogen can directly stimulate VEGF expression at the level of the ovary [24]. In pigs, however, culture of follicles *in toto* did not show the stimulatory effect of estrogen on VEGF production [65]. On the other hand, both VEGF120 and VEGF164 have been shown to indirectly stimulate the production of E₂ by granulosa cells in rodents [50]. Therefore, demonstration of causal relationships between estrogen-stimulated follicular growth and increased VEGF expression requires additional studies.

VEGF may be involved in the process of antrum formation [109]. It was demonstrated that cavity development mediated by gonadotropins in mice is blocked by anti-Flk-1/KDR antibody treatment. These data support the concept that VEGF/VPF may increase vascular permeability that contributes to the physiologic process of antrum formation. Since the mechanism of development of the fluid-filled antrum is not fully understood, further research is needed to address the question of whether the VEGF/VEGFRs are involved in the regulation of follicular antrum formation.

The earliest results showed that VEGF is a key survival factor for endothelial cells [3, 9] and induces the expression of antiapoptotic proteins Bcl-2 and A1 in human endothelial cells [42]. Recently, Greenaway et al. [46] have indicated that VEGF has also a cytoprotective role in the bovine extravascular granulosa cell compartment. Co-expression of VEGF and Flk-1/KDR in bovine ovarian granulosa cells protect these cells against apoptotic cell death and follicle atresia. Healthy follicles exhibit a very low incidence of apoptosis and high Flk-1/KDR expression in the granulosa cells. Early atretic follicles, on the other hand, have significantly more apoptotic granulosa cells and reduced Flk-1/KDR staining. Furthermore, blockage of Flk-1/KDR significantly inhibits the ability of cells to respond to endogenous and exogenous VEGF, reducing protection against caspase-3 activation and apoptosis [46]. The protective effect of VEGF in the granulosa cells appears to occur *via* interaction with Flk-1/KDR.

Therefore, additional studies should be undertaken to elucidate whether the examination of VEGF/Flk-1/KDR expression could be a sufficient method for the recognition of follicular atresia.

VEGF is thought to be involved in the ovulatory process. Increased expression of VEGF after administration of an ovulatory dose of gonadotropins is correlated with prostaglandin levels, which have long been known to play a role in ovulation [31]. A rapid increase in permeability around the time of ovulation, attributable to the interaction of VEGF and prostaglandins, could facilitate the formation of the follicular fluid and the rapid swelling of the follicle that occurs in response to the gonadotropin surge. Prolonged edema in a dominant follicle may provide the force that derives its enlargement and protrusion from the ovarian surface, the steady thinning of its weakened wall, the formation of the stigma, and finally, rupture and cumulus expulsion [58]. Moreover, VEGF as a stimulator of some proteolytic enzymes and plasminogen activators in endothelial cells [77, 100] may structurally weaken the follicle wall prior to rupture. It is believed that these enzymes are involved in ovulation and that they originate from the granulosa cells and/or the fibroblast of the theca layer [31]. These results demonstrate possible role of VEGF in the ovulatory process, but further studies are needed to prove that hypothesis.

It has been suggested that VEGF expression associated with high vascularization and oxygenation of follicles results in oocytes with superior pregnancy potential [101, 102]. Einspanier et al. [29] showed that bovine oocyte maturation in VEGF-supplemented medium results in increased extrusion of the first polar body and developmental potential of oocytes. Therefore, the observed decrease of VEGF level during the *in vitro* culture may lead to delayed oocyte ripening. VEGF may also be essential for nuclear and cytoplasmic *in vitro* maturation of bovine oocytes [62]. Results of Luo et al. [63] imply that the promoting effect of VEGF on *in vitro* development of bovine embryos requires cumulus cells. Abbas et al. [1] reported responsiveness of human cumulus cells to gonadotropins that results in a dose- and time-dependent stimulation of VEGF secretion by cumulus cells *in vitro*. Recently, Iijima et al. [50] have demonstrated in rats, an increase in ovulation rate after intraperitoneal injections of VEGF164

and VEGF120. However, developmental competence to term of ovulated oocytes was not affected. In contrast, elevated levels of VEGF in follicular fluid after controlled ovarian stimulation for *in vitro* fertilization (IVF) in women are associated with fewer retrieved oocytes, fewer mature oocytes, and fewer embryos and then reduced pregnancy rates [73]. Therefore, it was suggested that VEGF levels in follicular fluid can not be a relevant marker of embryo quality in IVF patients [7, 56]. VEGF participation in oocyte development must be further analyzed, since most of the available data concern *in vitro* maturation/fertilization.

Luteal phase

Following ovulation, the follicle undergoes remarkable changes and is converted into CL, a transient endocrine structure. The granulosa cells luteinizes, begins progesterone (P_4) secretion, and newly formed luteal tissue becomes highly vascularized. The angiogenic process in the developing CL begins with dissolution of the basal membrane between granulosa and theca interna layers. Following this, the expansion of theca capillaries is initiated by sprouting into the avascular granulosa layer to form a dense network of capillaries surrounding the granulosa cells. The duration of the intense angiogenic phase in the CL varies among species, and is characterized by the development of a high-density capillary network, where microvascular endothelial cells are the most abundant and mostly proliferating cells in the CL [18, 32, 61, 84]. During the maturation of the newly formed vascular bed, endothelial cells of arterioles and venules recruit smooth muscle cells to stabilize them and control their vasotonia. Endothelial cells in microvessels attract the pericytes to ensheath the capillaries and to influence vessel function [14]. Intensive blood vessel formation in the newly forming CL, often compared with angiogenesis in rapidly growing and aggressive tumors, enable mature CL to receive one of the greatest rates of blood flow of any tissue in the body [71]. If pregnancy does not occur, the fully developed CL starts to regress. Luteal regression includes structural and functional changes within luteal tissue, where vasculature as well as steroidogenic cells degenerate. Total volume

density of blood vessels, determined in several species, decreases during early luteolysis. Nevertheless, some of the large microvessels are still maintained, perhaps to assist the resorption of luteal mass, and ultimately, corpus albicans [95].

Very early CL is characterized by hemorrhaging in the ovulatory cavity which is accompanied by intense vascular sprouting. It was suggested that VEGF-induced vascular permeability around the time of ovulation, may be further essential for remodeling that transform the ruptured follicle in a CL. Hyperpermeability is believed to play a fundamental role in both normal and abnormal tissue growth and remodeling. It allows fibrin and other blood components to enter the extravascular compartment, thereby creating a temporary environment for optimal cell growth and migration [89]. Fibrin deposits implanted in the subcutaneous space of guinea pigs stimulated the growth of new blood vessels and endothelial cells [28]. Therefore, the fibrin deposited in the residual cavity after ovulation likely contributes to the induction of angiogenesis in newly forming CL.

VEGF as a mitogen for endothelial cells was also shown to be hemotactic for leukocytes [6, 21] and leukocyte number increases in/ around the follicle prior to ovulation [31]. Serum, platelets and leukocytes could all be a source of additional growth factors essential for rapid tissue repair and remodeling in developing CL. Redmer et al. [82] showed that in early ovine CL, pericytes which express VEGF, represent a population of cells first migrating into the hypoxic granulosa layer after ovulation and, therefore, may play critical role in angiogenesis during luteinization.

In the newly forming CL, VEGF mRNA and protein expression are observed in the granulosa- and theca-derived luteal cells. In several species, however, expression levels are higher in granulosa-derived than theca-derived luteal cells [12, 30, 52]. Highly expressed VEGF in granulosa-derived luteal cells may act as a chemoattractant for endothelial cells in order to initiate the invasion of avascular granulosa layer establishing an extensive capillary network that nourishes the developing CL and assists in the maintenance of luteal function throughout its' lifespan.

Gonadotropin regulation of VEGF production does not end with ovulation. It was demonstrated that VEGF expression is up regulated

by gonadotropins in human luteinized granulosa cells [69, 106]. In the marmoset, deprivation of gonadotropin support to luteal steroidogenic cells in the early luteal phase by GnRH antagonist administration reduces endothelial cell proliferation by 90% and plasma P_4 concentration by 95%. This demonstrates the dependence of early luteal angiogenesis and function on gonadotropin support [26]. Furthermore, neutralization of VEGF in the marmoset, over the same time period, reduces endothelial cell proliferation by 80%, as compared with control levels [41]. Therefore, it was suggested that the adverse effect of GnRH antagonist treatment on early luteal angiogenesis is predominantly a consequence of decreased VEGF production by steroidogenic cells of the developing CL [26]. The influence of gonadotropins on VEGF expression in the CL during the early luteal phase must be further examined, since Dickson and Fraser [26] did not measure VEGF expression after treatment with GnRH antagonist during the early luteal phase in marmosets.

Differences between species in VEGF expression in luteal cells mirror different patterns of angiogenesis observed during the CL lifespan. In some species VEGF mRNA levels are higher during the early luteal phase when the angiogenic process is more intensive [11, 81]. However, in the human [30, 74] and equine CL [4], increased VEGF mRNA and protein levels are still maintained in the mid-luteal phase. Furthermore, in the macaque [49] and caprine CL [54], VEGF mRNA expression is even higher during the mid-luteal than early luteal phase. Our recent results demonstrated similar patterns of VEGF expression in the porcine CL. The highest VEGF164 protein levels were observed on Days 8-10 of the estrous cycle¹. Otani et al. [74] hypothesized that during the less prolific mid-luteal phase, VEGF increases the vascular permeability and the uptake of cholesterol to luteal cells that results in the enhancement of luteal function. Administration of anti-VEGF antibody during the mid-luteal phase suppresses the function of

¹Kaczmarek M, Kowalczyk AE, Waclawik A, Blitek A, Ziecik AJ **2004** Expression and localization of vascular endothelial growth factor (VEGF) and its receptors, Flt-1 and Flk-1/KDR, in the porcine corpus luteum during the estrous cycle. The European Society of Domestic Animals Reproduction 8th Annual Meeting, Warsaw, Poland. *Reproduction in Domestic Animals* 39; 262, #OC4.3, abstract.

the CL in the marmoset [27]. The rapid decline in plasma P_4 concentration after anti-VEGF treatment supports the concept that beside mitogenic activity, VEGF is also a modulator of the vascular permeability in the CL. The decrease in permeability of capillaries can deprive the luteal cells of both the necessary precursors for P_4 production and the efficient spreading of their products into the bloodstream, which may result in a marked reduction in plasma P_4 concentration [27].

Regulation of expression of VEGF and its receptors during CL maturation has not been fully elucidated. Experiments performed on primates showed that administration of GnRH antagonist during the mid-luteal phase reduces VEGF mRNA expression in the CL suggesting that LH influence the expression of VEGF [80]. However, recent studies have demonstrated that suppression of LH release does not markedly affect the expression of VEGF and its receptors in the caprine CL [54]. This discrepancy may be due to the species-specific mechanisms regulating CL development. In the caprine CL, the administration of GnRH antagonist inhibits CL development only partially [53]. In primates, LH withdrawal leads to irreversible luteal regression [40] indicating that different factors participate in the regulation of development and maintenance of the CL in these species.

In regressing CL, VEGF expression decreases along with gradual dissolution of small blood vessels and decline of blood flow. However, VEGF expression during luteolysis of the CL might be regulated differentially depending on species-specific mechanisms involved in luteal regression. Stouffer et al. [95] suggested that the reduction of the primate CL sensitivity to LH in the late luteal phase is casually linked to the decline in VEGF expression near the end of a nonfertile cycle. They also suggested that hCG promotes VEGF expression which is essential for the maintenance of luteal function during pregnancy. On the other hand, in domestic animals such as the sheep, cow and horse, decline of VEGF expression in the regressing CL seems to be rather associated with prostaglandin (PG) $F_{2\alpha}$ secretion, a luteolysin [2]. It was shown that VEGF mRNA and protein levels sharply decrease after $PGF_{2\alpha}$ -induced luteolysis in mares which is accompanied by low endothelial cell proliferation rates [4]. Recently, Neuvians et al. [70] demonstrated that mRNA expression of

VEGF and its two receptors is significantly down-regulated 12 h after i.m. injection of the $\text{PGF}_{2\alpha}$ analogue in cows. Therefore, it was suggested that the cessation of VEGF support for the CL plays a role during structural luteolysis. Since regression of the vasculature may be involved in the functional and structural regression of the parenchymal-steroidogenic cells [39] we can speculate that VEGF may be involved in luteolysis.

Nevertheless, a uniform role of degeneration of vasculature during luteolysis cannot be easily established since mechanisms regulating the cascade of luteolytic events vary between species. Results of experiments performed in sheep suggest that endothelial cell apoptosis in the CL is followed by apoptosis of parenchymal cells [88]. However, Modlich et al. [66] suggested alternative to apoptosis mechanism of endothelial cell regression during physiological luteolysis based on spontaneous detachment of endothelial cells from the basement membrane. These findings and the fact that VEGF is a key survival factor for endothelial [3, 9, 42] and granulosa [46] cells suggest that decline of VEGF expression is one of the mechanisms involved in vascular dissolution as well as in functional and structural luteolysis of luteal cells. However, there is little evidence that luteal vascular degeneration is a trigger for functional and structural luteolysis in the primate CL [107]. Therefore, the precise relationship between regression of the vasculature, functional and structural integrity of the hormone-producing cells and VEGF expression awaits further study.

Pregnancy

When pregnancy occurs, the lifespan of the CL must be extended to support embryonic and fetal development. In the CL of pregnancy, vascularization seems to be necessary for the enhancement of luteal function [39, 44]. Luteal rescue does not appear to be associated with a further burst of angiogenesis in primates [18, 86] and ovine CL [51] suggesting that the vascular bed required for the pregnant CL is already established during the luteal phase. In contrast, intensive proliferation of endothelial cells was observed in the rodent CL during early pregnancy [98]. Therefore, there is a possibility that the rescue of the CL may be associated with a second burst of angiogenesis

in some species. Certainly, survival of the CL during pregnancy requires a stable vasculature with increased requirement of pericytes and prolonged endothelial cell survival in addition to prolongation of the lifespan of hormone-producing cells [87, 104].

In the CL obtained from pregnant women (6-8 weeks of pregnancy), VEGF mRNA is higher than during the mid-luteal phase [96]. Moreover, VEGF mRNA and protein is up-regulated in the CL during stimulated pregnancy [103, 104]. The elevated expression of VEGF suggests that the initial angiogenic process during the early luteal phase may be renewed after hCG treatment in human CL. In contrary, VEGF expression is not elevated in marmoset [87] and bovine CL [11] during early pregnancy. Similar results were recently observed in our laboratory. VEGF mRNA and protein levels in porcine CL during early pregnancy¹ were comparable to those observed during mid-luteal phase². It seems that in mature, cyclic CLs of latter species, molecular and cellular mechanisms responsible for vasculature maintenance are already activated. However, it was suggested that early pregnancy is associated with an increase in vessel stability achieved by recruiting periendothelial support cells such as pericytes. This hypothesis was proven in humans, where hCG-induced rescue of the CL is associated with high coverage of the vasculature by pericytes [104]. These changes were not observed, however, in the marmoset model [87]. It is currently unclear if prolongation of the CL lifespan involves further vessel formation during early pregnancy or only requires stabilization of the vascular network already developed during initial angiogenesis.

The VEGF/Flk-1/KDR pathway has been shown to play a critical role in the regulation of angiogenic events in the CL of pregnancy. Pauli et al. [75] reported that administration of anti-Flk-1/KDR antibody, during the

¹ Kowalczyk AE, Kaczmarek M, Waclawik A, Blitek A, Ziecik AJ **2004** Expression and localization of vascular endothelial growth factor (VEGF) and its receptors (Flt-1 and Flk-1/KDR) in porcine corpus luteum during early pregnancy. 8th SPIN Symposium and Society for Biology of Reproduction 4th National Meeting, Białowieża, Poland, #186, abstract.

² Kaczmarek M, Kowalczyk AE, Waclawik A, Blitek A, Ziecik AJ **2004** Expression and localization of vascular endothelial growth factor (VEGF) and its receptors, Flt-1 and Flk-1/KDR, in the porcine corpus luteum during the estrous cycle. The European Society of Domestic Animals Reproduction 8th Annual Meeting, Warsaw, Poland. *Reproduction in Domestic Animals* 39; 262, #OC4.3, abstract.

pre- and post-implantation periods in rodents, disrupted maternal ovarian function eliminating preexisting luteal blood vessels. The decrease in luteal size was reflected by a significant decline in ovarian P_4 secretion. There were also secondary effects of anti-Flk-1/KDR antibody treatment on pregnancy development. Uterine weight and number of implantation sites decreased dramatically, probably as a result of cessation of P_4 support. Other researchers concluded that the VEGF/Flk-1/KDR pathway in pregnant rodent CL is necessary for maintenance and stabilization of preexisting vessels. It also seems that the pathway ensures sufficient vascular permeability [68].

CONCLUSIONS AND FUTURE DIRECTIONS

Knowledge on the importance of ovarian angiogenesis and its regulation including the apparent role of VEGF has increased greatly over past decades. The current data demonstrate that VEGF is expressed and secreted by normal ovary in a cyclic manner. In addition, it appears that the VEGF ovarian expression might be regulated by gonadotropins during the cycle. VEGF seems to play an important role in several reproductive processes in the ovary e.g. formation of follicle antrum, selection of predominant follicles, oocyte maturation, ovulation and CL formation. Mitogenic, permeabilizing and “survival” actions of VEGF are fundamental for appropriate development and functioning of follicle and CL during the estrous cycle or pregnancy. However, our understanding of the distinct roles of VEGF types and VEGF-A isoforms in ovarian angiogenesis is still limited. Further information is needed to elucidate the relevance of changes observed in the vasculature during folliculogenesis and luteal development, regression or CL rescue during pregnancy. Further studies are also required to define regulations and clarify interactions between VEGF and hormones (LH, steroids) essential for the maintenance of normal ovarian function. New information may help in understanding the phenomenon of luteal insufficiency and reveal novel strategies of manipulation ovarian angiogenesis to alleviate infertility as well as control fertility.

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