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Review Article

Molecular regulation and role of angiogenesis in reproduction

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ABSTRACT

Angiogenesis is an essential process for proper functioning of the female reproductive system and for successful pregnancy realization. The multitude of factors required for physiological angiogenesis and the complexity of regulation of their temporal–spatial activities contribute to aberrations in human fertilization and pregnancy outcomes. In this study, we reviewed the current knowledge of the temporal expression patterns, functions, and regulatory mechanisms of angiogenic factors during foliculogenesis, early implantation/placentation and embryo development, as well as recurrent spontaneous abortions. Angiogenic factors including vascular endothelial growth factors and angiopoietins have documented roles in the development of primordial follicles into mature antral follicles. They also participate in decidualization, which is accompanied by the creation of an extensive network of vessels in the stromal bed that support the growth of the embryo and the placenta, and maintain early pregnancy. During placentation angiogenic and angiomodulatory cytokines, T and B lymphocytes and macrophages affect angiogenesis in a context-dependent manner. Defects in angiogenesis at the maternal–fetal interface contribute to miscarriage in humans. The establishment of more polymorphisms in the genes involved in angiogenesis/vasculogenesis, and their pathological phenotype and expression could give opportunities for prediction, creating a therapeutic strategy, and treatment of diseases related to female reproductive health and problematic conception.

Introduction

Angiogenesis is strongly connected to the female reproductive system and all its conditions—foliculogenesis, decidualization, implantation, and embryo development. Similar to these, angiogenesis is a highly organized process of growth (cell proliferation) and development (cell differentiation), whereby different molecules are involved, such as hormones, growth factors, receptors, cytokines, and immune cells, as well as stem and progenitor cells.

Angiogenesis and main angiogenic factors

Blood vessels formation consist of two processes: vasculogenesis and angiogenesis. In the process of vasculogenesis, angioblasts form primitive vessels during embryonic development. Capillary plexuses are the first primitive and uniform vascular structures, arising from mesoderm-derived endothelial precursors and finally developing into hierarchically organized arteries, capillaries, and veins. In angiogenesis, new vessels are produced from pre-existing ones [1]. Angiogenesis plays a crucial role in many physiological and pathological situations such as wound healing, tumor growth, adipositas, etc. [2].

Blood vessels supply all cells and tissues with oxygen (O_2), which is essential for their function, developmental processes, and homeostasis. Rapidly dividing cells increase their O_2 demand due to the increased metabolism, which results in local hypoxia [3]. Hypoxia is the primary regulator of neoangiogenesis through the activation of hypoxia-inducible factor (HIF)-1α, a highly conserved transcription factor that regulates a number of proangiogenic genes, including vascular endothelial growth factor (VEGF), angiopoietin (ANGPT)-1, ANGPT-2, Tunica interna endothelial cell kinase-2 (Tie-2), platelet-derived growth factor (PDGF), basic fibroblast growth factor, and monocyte chemoattractant protein-1. Stimulation of the HIF pathway induces localized angiogenesis, thus ensuring both short- and long-term adaptation to hypoxia.

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Various steps occur in angiogenesis from pre-existing blood vessels. The most important factors, which induce angiogenesis, are VEGF and basic fibroblast growth factor. VEGF induces nitric oxide and increases permeability of the pre-existing vessel, causing vasodilatation. VEGF also stimulates the motility and proliferation of endothelial cells, thus initiating the process of capillary sprouting. After their migration to areas of tissue injury, endothelial cells begin to proliferate. In the next step, endothelial cell proliferation is inhibited and the formation of capillary tubes starts. Finally, angiogenesis ends with the recruitment of periendothelial cells to build mature vessels—pericytes for small capillaries and smooth muscle cells for large vessels. The recruitment of pericytes, deposition of structural extracellular matrix (ECM) proteins into the subendothelial basement membrane and reduced endothelial cell proliferation promote vessel maturation—artery/vein differentiation, branching, and pruning [4]. PDGF, transforming growth factor beta, and ANGPT-1 and ANGPT-2 are needed for vascular stabilization. The differentiation pattern of the participating cells is strongly regulated both spatially and transitionally.

ECM proteins participate in vessel sprouting, interacting with integrin receptors on endothelial cells. Nonstructural ECM proteins (e.g., plasminogen activator and matrix metalloproteinases) destabilize cell–ECM interactions to save continued cell migration, while other (e.g., thrombospondin and tenascin C) degrade the ECM to permit ingrowth and remodeling of vessels [2].

As it was mentioned above, the driver of the angiogenesis is hypoxia. It induces VEGF production, which then triggers a tyrosine kinase pathway leading to the first phases of angiogenesis [5]. This pathway induces endothelial cell differentiation, proliferation, and sprouting. Many further steps are required to direct endothelial sprouts into functional, blood-carrying vessels. The generation of the lumen and the beginning of blood flow are accompanied by the creation of new vascular connections and vessel stabilization [6]. Better oxygen delivery lowers local VEGF-A expression. Changes in the local balance of activating and suppressing angiogenic factors occur at this stage [7]. ANGPT-1 promotes stabilization of the immature endothelial cell network by upregulation of CD31, and vascular endothelial cadherin in endothelial cells. It also regulates pericytes and smooth muscle cell growth and differentiation.

In summary, VEGF-A is a strong mitogenic factor that stimulates the proliferation and migration of vascular endothelial cells for the creation and maintenance of vascular structures [8]. In contrast, ANGPTs are not mitogenic, but they are fundamental to the construction of vessels [9]. As an agonist to the Tie-2 receptor, ANGPT-1 helps in maturation and maintaining of blood vessels by recruiting periendothelial cells, whereas the endogenous antagonist ANGPT-2 assists in loosening the support cell framework to allow for further vascular expansion [10]. The angiolytic (degenerative) effects of ANGPT-2 may become pronounced in the relative absence of VEGF [9].

Angiogenesis and ovarian function

Vascular system of ovaries differs from that of many other organs by creating and degenerating blood vessels in response to physiological conditions, in particular the development of new blood vessels from pre-existing ones to ensure the development of growing follicles. Primordial follicles and early preantral follicles do not have their own blood supply, but rely on blood vessels from the surrounding stroma. In the process of development, growing follicles require formation of their individual blood supply. The process of angiogenesis is critical for the function of follicles, including steroidogenesis and maturation of the oocyte, and is locally regulated by angiogenic factors including VEGF (in particular VEGF-A) and ANGPTs [11].

Folliculogenesis is the process of producing a single dominant follicle from a pool of growing follicles in the cortex of the ovary and involves four major regulatory events: recruitment, preantral follicle development, selection, and atresia. The two main types of follicles—preantral (primordial, primary, secondary, and tertiary) and antral (Graafian, small, medium, large, and preovulatory)—are gonadotropin independent and gonadotropin dependent, respectively. Recruitment, the first major event in folliculogenesis, occurs in primordial follicles nearest the ovarian medulla where blood vessels are prominent. It seems that exposure to nutrients or blood-borne regulatory molecules plays a role in the control of recruitment. The cuboidal granulosa cells around the oocyte change their shape and begin to express follicle-stimulating hormone (FSH) receptors [12]. FSH-induced changes occur in response to abnormally high levels of plasma FSH, because there are blood vessels in the vicinity. The transition from a primary to a secondary follicle includes the acquisition of a second layer of granulosa cells and is made by the continuing division of the granulosa cells. Most importantly, the development of a secondary follicle is a characteristic of the acquisition of a theca layer. The tissue is consisting of a layer of stroma-like cells around the basal lamina, and is subsequently differentiated into the inner theca interna and outer theca externa. Theca development is accompanied by the neoformation of numerous small vessels, by means of angiogenesis. The outer layer of stroma cells in the theca externa is differentiated into smooth muscle cells innervated by the autonomic nervous system. The theca interna is highly vascularized and serves to deliver hormones (e.g., FSH and luteinizing hormone LH), nutrient molecules, vitamins, and cofactors required for the growth and differentiation of the oocyte and granulosa cells [13].

It was shown that VEGF is expressed in the theca cells of antral follicles and in the granulosa cells nearest the oocyte in preantral follicle, but not in granulosa cells of primordial and primary follicles. After *in vitro* culture, VEGF caused a decrease in the number of primordial follicles and a concomitant increase in the number of primary follicles that showed growth initiation and reached the secondary and preantral stages of development after 7 days and 14 days, respectively. Follicular viability was also improved in the presence of VEGF after 7 days and 14 days in culture [13]. The growing follicles produce significant quantities of VEGF-A and ANGPT-2, but not ANGPT-1, especially after antrum formation. VEGF production by slow-growing follicles was influenced by FSH concentration and O_2 tension [14]. The production of VEGF is related to the stage of follicle growth (antrum formation), but the magnitude of VEGF production is influenced by follicle size and activity [15]. In slow-growing follicles, it increased with the decrease of O_2 concentration, from the typical tissue culture milieu (20%) to concentrations commonly found in vascularized tissues (5%), in the presence of a high/mid dose of FSH. The current data extend the evidence that hypoxic-to-normoxic (0–5%) O_2 concentrations promote VEGF production by luteinizing granulosa cells collected from women during controlled ovarian stimulation cycles or from dispersed luteal cells from monkeys or women [16]. In contrast to slow-growing follicles, VEGF production by fast-growing follicles (the tertiary or large preovulatory follicle) is independent of gonadotropins [17] and O_2 [18].

VEGF promotes follicular development and steroidogenic function by an angiogenic or angiotropic action, as is evident from *in vivo* studies [19–23]. There is also increasing evidence that VEGF has extravascular effects in the ovary [8,23,24]. It is worthy to investigate other than vascular (extravascular) actions of VEGF in the growing follicle, to provide information about VEGF as a possible marker for high-quality follicles destined to provide a

mature oocyte capable of fertilization. Except for VEGF, the other important angiogenic factor in ovarian folliculogenesis, suggested from the studies in rodent [25], bovine [26], and monkey [27], is ANGPT-1 since its mRNA and protein expression increase in antral follicles. The high ANGPT-2:ANGPT-1 ratio in developing follicles *in vitro* was found in a granulosa cell study, where nonluteinized cells produced more ANGPT-2 than ANGPT-1 during a short-term culture. There is also evidence of similar characteristics in the follicular fluid of "lead" follicles from controlled ovarian stimulation protocols in women [28,29].

Another important angiogenic factor for human follicle development is PDGF. Pinkas et al [30]detected PDGF-A and PDGF-B protein expression in oocytes and granulosa cells and PDGFR-β in granulosa cells from primary follicles. They assumed that binding of PDGF ligands to their receptors acts as a signaling factor that triggers the activation of primordial follicles. The study of Pascuali et al [31] demonstrated that inhibition of PDGF signaling by local injection of a selective PDGFR inhibitor under the bursa of the rat ovary affects follicular development and steroid hormone concentrations by decreasing blood vessel formation and stability in the ovaries.

In summary, folliculogenesis, i.e., the development of primordial follicles into mature, antral follicles, requires the creation of a vascular network in the follicle wall. Angiogenic factors including VEGFs, PDGFs, and ANGPTs have documented roles in this process. The concentration of angiogenic factors, similar to other local factors [e.g., anti-Müllerian hormone and steroid hormones (progesterone, androstenedione, and estradiol)] [15,32,33] is influenced by the follicle growth rate.

Angiogenesis is a critical process not only for follicular growth, but also for ovulation and corpus luteum development and function [11]. During the follicular–luteal transition, there is a dramatic increase in the expression of fibroblast growth factor 2 (FGF2), which occurs in the context of already high levels of VEGF-A. It is a period of intense angiogenesis, since the rupture of basement membrane after ovulation allows endothelial cells and pericytes to migrate and vascularize the luteinizing granulosa cells. Increased blood flow and vasodilation are characteristic events in periovulatory follicle, but in the same time upregulation of HIF-1α was detected. It was assumed that luteinizing hormone surge and human chorionic gonadotropin (hCG) are stimulators of the HIF pathway in this situation instead of hypoxia [34]. Matrix metalloprotease (MMP) family members are highly activated after basement membrane breakdown in ovulation, and it was shown that the administration of an anti-MMP2 antibody to preovulatory follicles disrupted the luteal tissue making it vascular deficient [35]. The most prominent feature of luteal angiogenesis is extensive tissue remodeling with a valuable role of pericytes in vessel stabilization.

Angiogenesis and decidualization

In the initiation of pregnancy, maternal uterine blood vessel formation is a basic event for developing embryo. The process is preceded by decidualization—around the attached blastocyst it is a formation of a primary and a secondary decidual zone, important for providing a source of growth factors and cytokines for embryo development up to Day 10.5 of gestation. Decidualization is a process of differentiation and tissue remodeling. It is determined in the late secretory phase of each menstrual cycle following ovulation. Decidual cells are modified stromal cells harboring unique biochemical and cellular properties that allow them to support embryo implantation. The steroid hormones estrogen and progesterone are critical for the regulation of processes in human endometrium. The action of the steroid hormones are mediated by intracellular estrogen receptor and progesterone receptor proteins, which are nuclear transcription factors binding to specific DNA response elements in the promoters of target genes. Critical to this is the endometrial expression of several genes closely related to angiogenesis. Decidualization is accompanied by the creation of an extensive network of vessels in the stromal bed that supports the growth of the embryo and the placenta, and maintains early pregnancy. In the study of Laws et al, the expression of connexin 43 (Cx43), which is a major gap junction protein, was enhanced in response to estrogen in the uterine stromal cells that surround the implanted embryo during the early stages of pregnancy [36]. Once the gap junctions are formed, signaling molecules may pass from the donor to the recipient stromal cells via these connections. These regulatory molecules, which may include second messengers such as cyclic nucleotides, calcium ions, or prostaglandins, are likely to impact the gene expression of recipient stromal cells, changing their ability to produce VEGF, ANGPT-1, ANGPT-2, and possibly other paracrine angiogenic effectors. Lack of the *Cx43* gene in the stromal cells led to a striking impairment in the development of new blood vessels within the stromal compartment, resulting in the arrest of embryo growth and early pregnancy loss. Further analysis of this phenotypical defect revealed that loss of Cx43 expression resulted in aberrant differentiation of uterine stromal cells and impaired production of several key angiogenic factors, including the VEGF. One of the earliest signs of the uterine response to its angiogenic stimulus is an increase in microvascular permeability at the sites of implantation [37]. VEGF influences the proliferation and function of uterine endothelial cells in the mesometrial region of the pregnant uterus where neovascularization mostly occurs.

The hCG (choriongonadotropin) is another important hormone during pregnancy, and it has a large number of effects on uterine receptivity [38]. Apart from the classic effects of hCG in the female reproductive system, there is growing evidence that hCG can induce angiogenesis [39,3]. These studies have shown that hCG is able to induce neovascular activity in several *in vivo* assays [38,39]. Uterine vascular endothelial cells express functional luteinizing hormone/hCG receptors. The hCG can have direct effect on endothelial cells to induce proliferation in some [38], but not all [39], *in vitro* assays and can promote migration and *in vitro* tube formation [39]. *In vivo*, the mechanism of hCG-induced angiogenesis increased by inducing VEGF expression in endometrial epithelial cells and/or directly from trophoblast.

Angiogenesis in placentation and embryo development

Many molecules—proteases, metabolites, ions, growth factors, matrix proteins, cytokines—as well as mechanical forces are involved in positive or negative regulation of the processes of angiogenesis/vasculogenesis. Trophoblast forms the interface between fetal and maternal tissues, and it is a rich source of angiogenic growth factors [26]. At approximately Day 21, in human placentae, chorionic villi are developed and subsequently become vascularized via pluripotent mesenchymal precursor cells (vasculogenesis) [10]. Other cells contribute to the vascularization in early pregnancy as well. These are uterine natural killer cells. They are recruited to the endometrium during the transition of endometrial secretory phase and support the deciduas [24]. The main function of uterine natural killer cells is the secretion of cytokines that assist in successful implantation and placental development [29]. Many of those cytokines direct angiogenesis during early pregnancy and influence spiral arteriole modifications later in pregnancy [29]. Such angiogenic and angiomodulatory cytokines are ANGPT-1 and ANGPT-2, placental growth factor, VEGF-C, interleukin (IL)-18, and interferon-γ [29]. T and B lymphocytes (and macrophages) surround the embryo after implantation and secrete cytokines [21], such as tumor necrosis factor-α, a proinflammatory cytokine that affects angiogenesis in a context-dependent manner [21]. Tumor

necrosis factor-α upregulates VEGF production in first-trimester trophoblast, and therefore may indirectly modulate placental vascular permeability and angiogenesis [33]. The secretion of Th2 cytokines (e.g., IL-4, IL-5, IL-6, IL-10, and IL-13) by T lymphocytes leads to the release of human placental lactogen and hCG from trophoblast [19]. IL-6 is a proangiogenic cytokine, while IL-4 may have a positive or negative effect on endothelial cell function [40]. During implantation, the balance of immune cytokine production between mother and fetus determines successful pregnancy, the so-called decidual cytokine profile. Macrophages are normal components of the endometrium, and their numbers increase in response to pregnancy. A wide variety of macrophages, known as Hofbauer cells, are found in the placenta. These cells express angiogenic growth factors such as VEGF [3] and IL-17 [41], among others. Macrophages can also inhibit angiogenesis through secretion of antiangiogenic mediators, such as a soluble variant of the VEGF receptor-1 (sVEGFR-1 or sflt-1), which may play an important role in pregnancy loss [42].

It is known that defects in angiogenesis at the maternal–fetal interface contribute to miscarriage in humans. The expression of trophoblastic VEGF was found lower in 8–9 weeks of gestation in samples from idiopathic recurrent spontaneous abortions compared with samples of gestational age-matched elective terminations [1]. In addition, decidual endothelial cells of recurrent abortion samples expressed quantitatively fewer receptors for VEGF and ANGPTs [1]. One of the roles for FGF2 in vascular development of the human placenta is also likely. FGF2 mRNA expression is high in syncytiotrophoblast and cytotrophoblast of first-trimester human placenta, and *FGF2* gene expression is greater in the first trimester than in the term placenta, suggesting a developmental control of its expression. Examples have been shown that FGF2 is released by human embryos as well as in gilts. During early human pregnancy, ANGPT-1 mRNA and ANGPT-2 mRNA/protein are also expressed in the syncytiotrophoblast [28], and there is evidence that ANGPT-2 mRNA and protein expression also occur in invasive cytotrophoblast [43]. The ANGPT receptors, Tie-1 and Tie-2, have almost exclusive expression on endothelial cells in humans and other primate species [44]. ANGPT-1 and ANGPT-2 serve as functional antagonists, because they compete for Tie-2 receptor binding. ANGPT-1 binding to Tie-2 promotes vascular maturation by recruiting periendothelial supportive cells, while ANGPT-2 binding leads to destabilization of blood vessels, allowing initiation of neovascularization [45]. Fluctuations in ANGPT-1/ANGPT-2 protein ratios can change the angiogenic response. The importance of the ANGPTs and their receptors in vascularization in general has prompted studies into their aberrant expression levels in human miscarriage. Compared with gestational age-matched control tissue, there is a reduced expression of the receptors Tie-1 and Tie-2 from endometrial vascular endothelia in recurrent miscarriage and a low expression of Tie-1 in the trophoblast. There are no data for the correlation between *ANGPT-2* gene polymorphisms and recurrent miscarriages in humans [44]. With recent advances in the placental/uterine localization of the ANGPT/Tie-2 receptor axis, further clinical studies are necessary to determine its potential role to modulate vascularity associated with early implantation.

Clinical perspectives

The establishment of more polymorphisms in the genes involved in angiogenesis/vasculogenesis, and their pathological phenotype and expression could give opportunities for prediction, creating a therapeutic strategy and treatment of diseases related to female reproductive health and problematic conception.

There are numerous studies on the role of several genetic polymorphisms in *VEGF-A*, relating to various cancer diseases, diabetic retinopathy [46], lateral sclerosis [47], and others. Polymorphisms in VEGF genes are often associated with tumor angiogenesis. Several single-nucleotide polymorphisms, such as +405 C/G, −1154 G/A, −634 G/C in 5′ untranslated region and +936 C/T, located in the 3′ untranslated region of the VEGF-A gene are often associated with different expression of the VEGF gene. This can lead to pathological angiogenesis in progressively growing tumor formations, diseases of the cardiovascular system, psoriasis, and others [48,49].

Various studies have been conducted on polymorphisms (+405 G/C, +936 C/T) in the gene for VEGF and their association with endometriosis, recurrent implantation failure, spontaneous abortion in assisted reproduction, and pathologies associated with the female reproductive health [50–52]. Modern methods of analysis of known point mutations in the human genome, called single-nucleotide polymorphisms, are based on the specific amplification of a region of the genome to be tested by classic polymerase chain reaction, followed by restriction analysis (restriction fragment length polymorphism RFLP) or sequencing, or by real-time polymerase chain reaction, expression analyses, and next generation sequencing.

Ovarian hyperstimulation syndrome (OHSS) is a severe and potentially life-threatening iatrogenic complication due to controlled ovarian stimulation during assisted reproductive technology. This condition is characterized by a broad spectrum of clinical manifestations. The levels of VEGF and its receptor VEGFR2 correlate with the severity of OHSS. The severe forms registered tense ascites, hemodynamic instability, renal failure, respiratory distress syndrome, hemorrhage from ovarian rupture, and thromboembolism. Recent data also suggest that hCG increases the expression of VEGF/VEGFR2 in human granulosa cells and raises serum VEGF-A [53]. Pregnant patients with OHSS must be monitored very closely because of the increased risk of deterioration due to the rapidly rising serum concentrations of hCG. Many authors globally indicate VEGF-A +405 G/C as well as other polymorphisms in the gene as a key factor for the development of OHSS. Serum levels of VEGF-A are regarded as one of the main predictors of OHSS. Probably many other factors take part, directly or indirectly through VEGF, including angiotensin II, insulin-like growth factor 1, transforming growth factor, PDGF, IL-1, IL-6, and others [54].

Different polymorphisms and mutations in the VEGF gene itself would be reliable biological markers for an ovarian stimulation approach. In-depth studies would provide clarity and predictability for the opportunity to develop its adequate therapeutic management without endangering the patient's life.

Conclusion

It remains to be characterized whether single gene defects contribute to aberrations in human pregnancy outcomes. The multitude of factors required for physiological angiogenesis and the complexity of regulation of their temporal–spatial activities suggest that more than one factor may be required for the robust angiogenesis associated with successful early pregnancy. Many of the factors could also play critical roles in developmental angiogenesis/vasculogenesis within the embryo proper. Clearly, the complexities of both of these systems are likely to contribute to the high embryo mortality that occurs in human pregnancy. Increased knowledge of the temporal expression patterns, functions, and regulatory mechanisms of angiogenic factors during early implantation/placentation will enable novel therapeutic advances to be made for some forms of human implantation failure and recurrent spontaneous abortion.

Conflict of Interest

None.

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