Angiogenesis in female reproductive system

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Neovascularization, i.e. new blood vessels formation, can be divided into two different processes: vasculogenesis, whereby a primitive vascular network is established during embryogenesis from multipotential mesenchymal progenitors; and angiogenesis, which refers to the new blood vessels formation from pre-existing vessels[1,2]. Angiogenesis contributes to the most process throughout the whole life span from embryonic development to adult growth[2]. In this meaning, neovascularization is usually used to imply angiogenesis. Under physiological conditions, angiogenesis is a strictly regulated event and rarely happens in most adult tissues except for fracture or healing of wounds[2,3]. However, a notable phenomenon is that the tissues of ovary and uterine endometrium are unique in the cycle-specific changes in vascularity that occur in each estrous/menstrual cycle. Active angiogenesis occurs in placenta to satisfy the needs of embryonic implantation and development. Defects in angiogenesis are associated with some gynecopathies including luteal phase defect, endometriosis, pregnancy loss and preeclampsia[4].

1 Process of angiogenesis

Generally, angiogenesis can be organized as a continuum of three stages: (ⅰ) Initiation phase. Normally quiescent endothelial cells (EC, the primary cell type comprising capillaries) are activated by a variety of angiogenic stimulus including vascular injury, vascular endothelial cell growth factor/vascular permeability factor (VEGF/VPF) and fibroblast growth factor (FGF); (ⅱ) invasive/proliferative phase. EC produces proteolytic enzymes, facilitate EC to escape via the local degradation of the basement membrane of the vessel, and invade and migrate into the perivascular extracellular matrix (ECM) towards the angiogenic stimulus and simultaneously ECs proliferate; (ⅲ) differentiation/maturation phase. Migrating ECs elongate and align with one another to form a capillary sprout, and EC division, which occurs proximally to the migrating tip, further increases the length of the sprout. The solid sprout gradually develops a lumen proximal to the region of proliferation. Contiguous tubular sprouts anastomose at their tips to form a functional capillary loop in which blood flow is established. Peri-endothelial support cells (pericytes) are recruited to encase the loop. New sprouts continuously grow at the tip of the capillary loop, make the capillaries extend around to form a network structure. Angiogenesis is accomplished by the formation of capillary lumen and differentiation of some of the newly formed capillaries into arterioles and venules and EC recurs to quiescent state[4, 5].

2 Models used for studying angiogenesis

Several different models have been developed to study the angiogenesis. The most commonly used *in vivo* models are as follows: (ⅰ) corneal micropocket technique[5,6]; that is, to implant an interested substance into avascular cornea of rabbit, mouse or rat to evaluate its effect; (ⅱ) chicken embryo chorioallantoic membrane (CAM), which can be used to determine the stimulative or inhibitory capability of a substance placed in chorioallantoic membrane of hatched egg around day 5[5,7]; (ⅲ) syrian hamster cheek pouch assay[5,8]. The authors have transferred the human endometrium into hamster cheek pouch to study the hemorrhage mechanism and vascular changes[8]. Different *in vitro* models have been used to culture vascular EC from the sources of umbilical vein, aorta and capillaries and study: (ⅰ) migration of EC by the ingrowth of EC into matrix-containing gels and production of proteases by EC, (ⅱ) EC proliferation by determinating the ^{3}H thymine marked DNA, and (ⅲ) tube-like structures formation in two- or three-dimensional collagen gels[7].

3 Angiogenic regulators

(ⅰ) Growth factor/cytokines. Angiogenesis is initiated by angiogenic factors including cytokines/growth factors and other angiogenic molecules, such as integrins, selectins[7,9], which can be released from a number of sources including macrophages, mast cells, inflammatory cells as well as tumor cells. The cytokines that have been most extensively studied in the context of angiogenesis are VEGF and bFGF[1,3]. VEGF and bFGF positively regulate many EC functions, including proliferation, migration, extracellular proteolytic activity and tube formation, which implies that these factors are direct-acting regulators[3,7]. VEGF activates the normally quiescent EC by binding to EC surface tyrosine kinase receptors, and two of VEGF receptors termed Flt-1 and KDR/Flk-1. VEGF is a specific mitogen for EC and stimulates angiogenesis and increases the vascular permeability[10]. Studies of the gene knock-out mice have implicated that VEGF and the two receptors, genes flk-1 and flt-1/kdr, are necessary for angiogenesis and vascular development. Loss of these genes can lead to development defect and embryonic lethality at days 8.5—9.5[1,11,12]. Unlike VEGF which is a secretion type peptide, FGF has no signal peptide and is believed to secrete in an unconventional way. It is stored in cells and releases only if needed[13,14]. In contrast to VEGF and FGF, TNF-α and TGF-β inhibit EC growth *in vitro* but are angiogenic *in vivo*, and it has been demonstrated that the two cytokines induce angiogenesis

indirectly by stimulating the production of direct-acting positive regulators from stromal and chemoattracted inflammatory cells[3]. Thus TNF-α and TGF-β are considered to be indirect positive regulators. Other cytokines have been reported to stimulate angiogenesis *in vivo* including insulin-like growth factor (IGF), platelet-dirived endothelial cells growth factor (PDEGF), epidermal growth factor (EGF), interleukines (IL-1, -6, -8), and placenta growth factor[3].

(ii) Proteolytic enzymes. Basement membrane degradation, extracellular matrix invasion and blood vessel differentiation and maturation are essential components of the angiogenic process, all of which are dependent on a cohort of proteolytic enzymes and their inhibitors produced by EC and other cell types. Increased attention has been focused on the PA/plasmin-PAI and MMP-TIMP systems[3,15].

Urokinase-type plasminogen activator (uPA) and plasmin (derived from plasminogen by the action of uPA) are the most important proteinases in serine proteinase family. These enzymes exert their matrix degradation via their receptors located on cell surface, and this degradation can be suppressed by PA inhibitor named plasminogen activator inhibitor (PAI)[16,17]. It has been abundantly demonstrated that uPA, uPA receptor, and PAI-1 are expressed by EC during angiogenesis *in vivo* and *in vitro*, and all of these components are induced by VEGF and bFGF[16,17]. Also, it has been proved that the proteolytic effect of PA system is partly mediated by activating MMPs and TGF-β, which are secreted as zymogens[17].

Matrix metalloproteinases are a family of highly homologous zinc-dependent endopeptidases, so far more than 26 MMP members have been cloned and characterized. MMP is extensively distributed in female reproductive tract and the levels vary with estrous (menstrual) cycle and pregnancy[18,19]. MMP plays an essential role in the angiogenesis process from initial to maturation stage. A body of data demonstrated that the focalized degradation of basement membrane during the initial stage of angiogenesis was carried out by MMP members. One of the evidence is that EC from microvascular endothelial cells can express MMP, while macrovascular endothelial cells cannot[20], showing the fact that angiogenesis develops from microvessel. The most important MMP members during angiogenesis may be MMP-2 (72-ku type Ⅳ collagenase; gelatinase A) and MMP-9 (92-ku type Ⅳ collagenase; gelatinase B), both of which cleave the helical domains of type Ⅳ collagen, the principal structural component of BMs[21], and are produced by a variety of cell types including endothelial cells[22]. The regulation of MMP activity occurs at several levels, including gene transcription control, the proenzyme activation and inhibition of activated MMP by endogenous specific inhibitors termed tissue inhibitor of metalloproteinase (TIMP-1—4). A series of studies showed that the blocking of MMP activity in both *in vivo* and *in vitro* models by TIMP and synthetic MMP specific inhibitors resulted in the inhibition of angiogenesis[23]. The authors also observed that the treatment of animals with injection of two MMP inhibitors, doxycycline and BB-94, can reduce the density of vascularity of human endometrium implanted into hamster cheek pouch (unpublished data).

(iii) Hormones. Some endogenous hormones have been proved to influence vascular changes and angiogenesis. For example, estrogen has potent vasodilation effect on endometrium and can stimulate angiogenesis while progesterogenic steroids can down-regulate angiogenesis both *in vivo* and *in vitro*[17]. Some prostaglandins such as PGE-1 and PGE-2 have angiogenic effects but some not, for example, PGA and PGF[13]. Glucocorticoid hormone prednisone and its analogues are angiogenesis antagonists. The mechanism that hormones influence angiogenesis may exert through other angiogenic regulators.

In the healthy adult organism, it is usually stated that the mitogenic activity of EC is very low with the exception of angiogenesis situation. The maintenance of endothelial quiescence is considered to be due to the presence of endogenous anti-angiogenic regulators. Some cytokines have been reported to down-regulate angiogenesis *in vivo* including interferones (IFN), IL-12[13]. In this meaning, angiogenesis is a dynamic process and reflects a balance between stimulation and inhibiton. So coexistence of angiogenic factors and anti-angiogenic factors is necessary to maintain normal physiological blood vessel growth and prevent from abnormal vascularization[13]. Although the definitive proof awaits further clarification, it is generally assumed that the "switch" to the angiogenic state may involve the loss of a negative regulator, the induction of a positive regulator, or both[3].

4 Angiogenesis in female reproductive tract

Angiogenesis is essential for the selection of dominant follicle, ovulation, corpus luteum development endometrium growth and differentiation and placentation preparation for embryo development.

(i) Ovary. Vasculary changes in ovary occur on a cyclic basis[24,25]. Without an independent capillary network, primordial follicles depend on ambient stromal vessels for delivery of nutrients and hormones. Primary follicles develop an initial vascularity consisting of one to two arterioles. Basement membrane separates the avascular granulose cells from a vascular theca layer, in which the vascularity develops into a complex wreathlike network as the follicle continues to grow[25]. At the time of ovulation, the basement membrane degeneration and the rapid invasion of capillaries into previously avascular granulosa cell layer happen. It is well recognized that a majority of growing follicles in the ovary of most mammals never reach ovulation, but rather undergoing a process of regression known as atresia. Only the follicle destined to ovulate (dominant follicles) develops to ovulation

and produces estrogens. The early scholars noticed that the capillary network of preovulatory follicles was more extensive than that of other follicle, and proposed that initiation and maintenance of follicular growth were dependent on the development of follicular microvessels. Increasing evidence implied that angiogenesis is the key regulator of follicular growth and atresia. Zeleznick et al.[26] found that all preovulatory follicles of monkeys had similar concentrations of gonadotropin binding sites, but only the dominant follicles were markedly labeled after intravenous injection of radio-labeled gonadotropin. This selective *in vivo* uptake of gonadotropin was associated with increased vascularity of the dominant follicle. One of the earliest signs of atresia was the reduced DNA synthesis of follicular EC in association with reduced follicular vascularity, and the atretic follicles regenerated when placed into culture[13]. These facts suggested that the vascurity determines the fate of follicles: poor vascularity limits access of atretic follicles to nutrients and hormones and thereby maintaining these follicles in an atretic state while the rich vascularity provides enough nutrients to the "lucky follicles" and assures it to ovulation.

The remaining cells of the ovulated follicles form a transient endocrine gland known as the corpus luteum (CL), which is critical for successful maintenance of pregnancy by the producing progesterone. Approximately 50% of the cells of the mature CL are EC, and the majority of parenchymal cells are adjacent to one or more capillaries[13]. Mature CL also receives most of the ovarian blood supply. Reduced ovarian blood flow may cause inadequate luteal progesterone production, i. e. luteal phase defect and even luteal regression[25].

(ii) Uterine endometrium. The human edometrium demonstrates a remarkable growth each cycle in response to estrogen and a subsequent rapid switch to differentiation in response to progesterone. The cyclic growth of the endometrium is tightly associated with the dynamic vascular remodeling. Regulated by ovarian steroids hormones, a complex process of vascular and glandular proliferation, differentiation, sloughing and regression occurs each month[24,27].

Endometrium is supplied by uterine artery that gives rise to arcuate arteries in the myometrium. Radial arteries penetrate the endometrium where basal arteries are divided into horizontal branches supplying the basal layer and the vertical branches supplying the functional layer. These vessels give rise to spiral arteries and end-arterioles, which are very hormone-responsive and contribute much to the uterine bleeding during menstrual cycle. The endometrial vascular architecture changes throughout the menstrual cycle in parallel to the changes in the epithelium and stroma. From cycle days 0—25, there is a gradual increase in branching and coiling of spiral arterioles, corresponding to an increase in the length and coiling of endometrium glands. During the late proliferative phase and throughout the secretory phase, a complex subepithelial capillary plexus develops. On days 25—28 the perivascular and stromal decidualization happens. In response to the withdrawal, the distal segments of the spiral aeteries start vasoconstriction. On cycle days 1—4, menstruation leads to diffuse necrosis, inflammation and vascular thrombosis. Then the next proliferative phase occurs. Active angiogenesis occurs from this phase and persists throughout the whole secretory phase (luteal phase) to compensate the loss of the blood vessels in menstruation[24,27]. Although the mechanism that regulates endometrial angiogenesis after menstruation is not clearly demonstrated, it was believed that VEGF plays an important role in cyclic endometrial angiogenesis[4,24].

Non-primate species such as rodents also show cyclic angiogenesis in the endometrium associated with estrous cycle.

During the early pregnancy dramatic angiogenesis also happens in endometrium to prepare for the successful embryonic implantation. Consistent with the changes of ovary steroids, the angiogenesis occurs just before implantation reaction. It is well known that the implantation sites in rodents can be recognized as clear bands through injecting a dye named trypan blue from tail vein because of the increased focal vascular permeability at implantation sites.

(iii) Placenta. Successful pregnancy requires the development of a vascular network that facilitates the maternal-fetal exchange of oxygen, nutrients and metabolic waste, which is carried out by placenta. Most mammal placenta consists of maternal (endometrium) and fetal (chorioallantoic) tissues[4,13] and represents a balance between the concurrent degradation of the maternal decidual vasculature and the highly coordinated angiogenesis directed by the developing embryo. At the initiation of pregnancy, the secretory endometrium undergoes deciduaslization accompanied by widespread vascular expansion. After implantation, cytotrophoblasts invade and migrate through endometrium and reach the inner third of the myometrium. The invasion and migration of cytotrophoblasts through the endometrium result in the formation of physical connections, feto-placenta tissues. The blood flow to the feto-placenta tissues is dramatically increased, in fact 85% of the uterine blood flow are directed to the feta despites a 15-fold increase in uterine volume and weight[4]. The vasculogenesis in feto-placenta tissues is initially observed around day 19 postconception (PC), and accounts for the majority of neovascularization throughout the first and second trimesters. The ECs are derived from villous mesenchyme and develop into tubular capillaries. The mechanism of new blood vessels formation is different from angiogenesis. Complete fetal capillaries appear at about day 22 PC and begin transporting fetal-erythrocytes by day 28. In the last trimester it is angiogenesis that accounts for the most new vessel formation[2,4,24].

5 Clinic applications of angiogenesis research

The research on angiogenesis and its mechanism provides a potential prosperity in clinic therapy. Presently

the most valuable applications of angiogenesis are the treatment of tumors and some angiogenic diseases, including retinopathies, rheumatoid arthritis, hemangioma, atherosclerotic plaque neovascurization[3,28,29]. Increasing agents have been developed and characterized to be the inhibitors of angiogenesis. Some of these inhibitors are currently used in the clinic or in the early phases of clinical trials, including polypeptides such as peptides, IFN-α, IL-12, platelet factor Ⅳ, antibiotics derivatives, AGM 1470/TNP-470, an analogue of fumagillin[3]. Besides, TIMPs and PAIs, angiostatin, antagonists to VEGF, VEGF receptors and α v β3 integrin, minocycline, synthetic metalloproteinase inhibitors[3,29,30] and some biological preparations, for example, shark cartilage preparation (SCP), have been reported as the potent inhibitors of angiogenesis and have prosperous application value.

6 Anti-angiogenesis: a way for birth control?

It was reported that adminstration of AGM-1470, an angiogenesis inhibitor, resulted in complete failure of embryonic growth due to interference with decidualization, placental and yolk sac formation, and embryonic vascular development[31]. The author observed that endometrial neovascularization implanted into hamster cheek pouch was inhibited by 6-α- methylprednisolone (an analog of prednisone), and the treated pregnant rats had fewer implantation sites than control (unpublished data). Traditional clinic contraption or abortion medicine is RU486 (mifepristone), the progesterone receptor inhibitor. Since angiogenesis is essential for ovulation, corpus luteum, development and embryonic implantation and thereafter development, a possible future application of angiogenesis will be birth control, although there is a long way to go.

In general, studying on angiogenesis in female reproductive system is a broad and valuable field. Further research will not only deepen our understanding of the mechanism of reproduction, provide a prospective way of therapy of the female disorders and other angiogenic diseases, but also offer a possible way for birth control.

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