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Review article

Molecular mechanism of angiogenesis

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Abstract

Angiogenesis is one of the chief events both in development and postnatal pathological conditions. Recent extensive studies revealed the key signaling and transcriptional regulators of sprouting angiogenesis. Sprouting angiogenesis is a multistep process composed of endothelial loosening, endothelial cell migration, stalk elongation, anastomosis, and stabilization. Although the initial process of sprouting is triggered by soluble proangiogenic factors such as VEGF and FGF, the rather complicated subsequent step recruits many signaling and adhesion molecules which are pivotal players in autonomous and conditional specification in development. There is a marked similarity at the molecular level between embryogenic and postnatal angiogenesis. By its physiological constraint, endothelium differentiation is closely linked with blood cell specification. Many transcription factors with a major role in hematopoietic stem cell generation are also involved in angiogenesis.

Key words: Sprouting angiogenesis, VEGF signal, Lmo2 complex

Introduction

Blood cell differentiation, blood vessel construction, and the initiation of circulation comprise one of the most interesting fields in developmental biology. This is especially true because the formation of all organs of the human body are closely linked with vascular development and blood supply. The vascular system is characterized by postnatal remodeling. Blood vessel remodeling and neovascularization are involved in many physiological process, such as endometrial thickening and formation of the placenta. Although the large vessels do not have the capacity for remodeling, capillaries are newly formed at the sites of oxygen deficiency. In addition, capillary growth is associated with a number of pathological conditions, such as the formation of granulation tissue in inflammatory disease and cancer growth. Tumor growth requires coordination between tumor cell proliferation and increased blood supply by newly formed vessels.

Postnatal remodeling of the vascular system might mimic the embryonic construction of the vascular system at a molecular level. In order to reveal the molecular mechanism of neovascularization, the optimal way is to look for common mechanisms in both embryonic and postnatal vascular system construction.

Embryonic construction of vascular system

The development of blood vessels occurs by two temporally separate process: vasculogenesis and angiogenesis. During vasculogenesis, the common precursors of blood and endothelial cells, hemangioblasts, are specified de novo from the lateral plate mesoderm to form the capillary network. During angiogenesis, this primary capillary network is remodeled to the mature vascular tree. The combination of BMP, Wnt, and Notch signals seems to be needed for the primary specification of hemangioblasts, and results in expression of hemangioblast specific transcription factor Etv2, the cell surface receptor VEGF2, and another receptor, FGFR. The final fate determination of the hemangioblasts seems to be based on the amount of Notch signal, in which its abundance makes them blood cells and its

scarcity endothelial cells. The major paracrine factors involved in vasculogenesis are FGF and VEGF signals. After the primary capillary plexus is completed, the stabilization of the endothelial network is accomplished using Angl/Tie2 signaling. In this process, pericytes which surround the endothelium produce Angl. Tie2 is the receptor for Angl expressed on the endothelial cell membrane. Upon the binding of Angl, its signal strengthens the endothelial cell-cell junction by preventing internalization of the junctional molecule VE-cadherin. During angiogenesis, the VEGF signal via VEGFR2 becomes the main signal for the growth of blood vessels, and the importance of the the VEGF signal is conserved in postnatal angiogenesis. In the final step of angiogenesis, the mature circulatory system is constructed by recruiting pericytes. TGF-β promotes the differentiation of precursor cells to pericytes, and PDGFR-expressing pericytes migrates in response to PDGF to surround the newly formed vessels.

The sites of vasculogenesis and hematopoiesis in embryogenesis

In mammalian development, both the blood cells and vascular system are derived from the posterior lateral mesoderm. Especially, blood and endothelial cells are considered to differentiate from common putative precursors called "hemangioblasts". The presence of hemangioblasts has been well established in culture system and yolk sac erythropoiesis. ES cells very efficiently differentiate into red blood cells in matrigel when LIF is deprived of ordinary ES cell culture medium. Hemangioblasts can be identified during this process as cells with bi-potential. In mouse embryogenesis, blood islands form in the wall of the yolk sac, where primary erythrocytes are produced in the inner portion of blood islands while peripheral flat cells of the islands become endothelial cells. The first hematopoietic stem cells (HSCs) are produced in the AGM region of the mouse embryo. Recent extensive studies revealed that HSCs are derived from hemogenic endothelial cells of the dorsal aorta. In extra-embryonic hematopoiesis (yolk sac erythropoiesis, primary hematopoiesis), hematopoiesis and angiogenesis proceed in parallel. On the other hand, hematopoiesis depends upon angiogenesis (formation of dorsal aorta) in intraembryonic hematopoiesis (definitive hematopoiesis), because the dorsal aorta is constructed before the specification of hematopoietic stem cells from its wall.

The construction of blood vessels has a physiological constraint: in the developmental process, the vascular system should supply oxygen and nutrients to the developing organs even during its construction. This constraint becomes more crucial in a closed circulatory system. In organisms with a close circulation, the blood cells, the oxygen carriers, should arise in parallel with vascular development. The inevitably common mechanism and close relationship between the two will be discussed later.

Sprouting angiogenesis and the key cell types

Angiogenesis is the physiological process through which new blood vessels form from pre-existing ones. Angiogenesis is classified into two types: sprouting angiogenesis and intussusceptive angiogenesis (also known as splitting angiogenesis). Sprouting angiogenesis is the more common form, its molecular mechanism has recently been studied extensively, and it is discussed mainly in this article.

In the process of vessel sprouting of the existing blood vessels, the endothelial cells of the vessels are specified into three types of endothelium. The tip cells form the migrating front of the vascular buds. They have filopodias protruding from their cell membrane, which play a role in determining the direction of migration and also in capillary anastomosis. The stalk cells are just neighboring the tip cells and markedly proliferates during the growth of the vascular stalks. These two types of cells characterize the "activated endothelium", because they are specified by the influence of the proangiogenic factors at the site of sprouting. The third cell type of the endothelium, called "phalanx cells" is not activated during the process of angiogenesis and does not proliferate during sprouting.

Tip cell selection and the lateral inhibition

The mouse retina presents the best model system to study the molecular mechanism of angiogenesis because angiogenesis occurs postnatally there. The vascular system of the mouse retina is mainly constructed by the process of sprouting angiogenesis, where the sprouts migrate into the extracellular matrix from the existing blood vessels. Other in vivo and in vitro angiogenesis models also contribute greatly in order to clarify its molecular mechanism. Among these, the zebra fish is particularly useful for the

time-lapse observation of vascular development. The genetically modified Fli1-eGFP fish, expressing GFP on the endothelial cells, provides an extremely informative model for the study of sprouting angiogenesis.

By the influence of proangiogenic factors (VEGF or FGF), which are usually secreted on oxygen and nutrient deficiency, the part of endothelial cells of the existing blood vessels differentiate into tip cells which form the single apex cell of the sprouting buds. They are selected at the site where the VEGF concentration is the highest. All parts of the existing capillaries' endothelium have the capacity to respond to the inductive signal of proangiogenic factors. In the case of VEGF signals, the endothelial cells at the highest concentration of VEGF receive the signal via membrane-bound VEGF receptors (VEGFR). The main VEGFR is VEGFR2 and the signal through VEGFR2 stimulates tip cell differentiation. It is enhanced by the co-receptor neuropilin1. Vessel sprouting requires coordination between migrating tip cells and proliferative stalk cells, and the specification between tip and stalk cells is regulated by lateral inhibition using a signal through Notch. On receiving the VEGF signal, the tip cells express DLL4 on their membrane, which binds Notch on the neighboring endothelium. This DLL4-Notch signal results in the downregulation of VEGFR2/neuropilin1 in the neighboring endothelial cells and makes them the less VEGF- responsive stalk cells. Thus, using both VEGF and the Notch signal, the specification of the activated endothelium into the tip and stalk cells is achieved. Once luminized connections are completed, the activated endothelial cells become quiescent endothelium, the phalanx cells (discussed later).

Preparation for tip cell migration

Tip cell migration requires the following processes: basement membrane (BM) degradation, endothelial cell (EC) junction loosening (VE-cadherin), and pericyte detachment. Angiopoietin1 (Ang1) and Ang2 bind Tie2, a tyrosine kinase receptor expressed in the endothelium. Perivascular cell (pericyte) expression of Ang1 stabilizes and tightens the inter-endothelial junction by increasing VE-cadherin expression on the endothelial cell membrane. Ang2 functions as a competitive antagonist of Ang1 for endothelial junction loosening.

Sprout guidance and progression

In neuronal guidance, a number of ligand-receptor interactions (Eph-ephrin, netrins-Unc, Robo-Slit, semaphorins-plexins, and semaphorines-neuropilins) are involved. Taking the marked alignment of neurons and blood vessels into consideration, it is not surprising that they share the same soluble signal for their guidance. How common their guidance mechanism is and their mutual interaction are questions attracting attention. VEGF again seems to be the main soluble factor for tip cell migration and guidance, and, interestingly, neuropilins are also receptors for VEGF in addition to semaphorins. The neighboring cells of tip cells (i.e., the stalk cells) express VEGFR1 which trap the VEGF, thus resulting in a VEGF- rich corridor in front of the tip cells. This enables the almost perpendicular growth of the tip cells from the pre-existing blood vessel walls.

Stalk cell proliferation

The marked proliferation of stalk cells is needed for stalk elongation. However, the tip cells do not progress into ECM by being pushed by the proliferating stalk cells. On the contrary, the tip cells pull the stalk cells by their progress. Some mechanical pulling force should be transformed to the chemical signal on the stalk cells. The Wnt signal stimulates the proliferation of the stalk cells and the Notch signal inhibits it. In the process of stalk elongation, the proliferation-stimulating Wnt signal should override the inhibitory Notch signal. For that purpose, stalk cells begin to express the Notch target Nrarp (Notch-regulated Ankyrin repeat protein), which limits Notch signaling. As the vessels elongate, the stalk cells form a lumen within the cells, which eventually facilitates blood flow.

Vessel fusion aided by macrophages

When tip cells of adjacent vessels meet via filopodia, they anastomose. The cell junction at the site of cell contact expands into rings, generating an interface of apical membrane compartments. In this anastomosis, macrophages play the connecting role for the two tip cells by excreting several soluble proangiogenic factors. The macrophages express Tie2, Notch, and CXCR4, which accept the soluble molecule presumably secreted by the anastomosing tip cell and function as bridging molecules for connecting

the tip cells with VE-cadherin on their filopodias.

Tip/stalk cell balance

Alk1 (Activin receptor-like kinase), an EC-specific member of the TGF-β superfamily, is inactivated genetic disease hereditary hemorrhagic telangiectasia (HHT). Smad1/5, an Alk1 downstream transduction molecule, plays the most important roles in the Tip/Stalk balance. The resulting Smad1/5 loss impairs Notch signaling, which results in the increased tip cell selections and, therefore, over-branching blood vessels. The appropriate distribution of the tip cells is one of the most important mechanisms for effective post-natal angiogenesis by determining the number of branches of the vascular tree and also the diameter of each blood vessels.

Lumen formation

A second important role of the stalk cells within a newly forming blood vessel is to establish a vascular lumen. The unique mechanism of lumen formation was observed in zebra fish ISV (intersomitic vessel) development, in which the vascular lumen of ISVs is formed by the fusion of intercellular vacuoles. These vacuoles coalesce to eventually form the continuous route for the blood stream.

Vessel maturation

Mural pericyte attachment surrounding the nascent blood vessels is necessary for blood vessel maturation. As discussed previously, TGF-β promotes the mesenchymal precursor cells to differentiate into pericytes, and PDGFR-expressing pericytes migrate in response to PDGF to surround the newly formed vessels. Angl is secreted from the pericytes to strengthen the inter-endothelial junction and, thus, contribute to the stabilization of the nascent blood vessels.

Resuming quiescence in endothelial cells

Phalanx cells represent the quiescent cells in angiogenesis. After oxygen and nutrient saturation provided by the newly formed blood vessel, the level of the proangiogenic factors is decreased. The withdrawal of the proangiogenic factors and increased pro-quiescent molecules lead to stabilization of the nascent endothelium. Autocrine signals of VEGF, Angl, FGF, and Notch maintain the endothelium in quiescence. The basement membrane which expresses laminin-α4 in tip cells limits their number by inducing Notch signals.

The transcriptional regulation of angiogenesis and hematopoiesis by Lmo2

As mentioned before, in development, angiogenesis is closely linked with hematopoiesis. FGF inductive signal results in the hemangioblast-specific transcription factor Etv2. The expression of Etv2 then triggers the hematopoiesis/angiogenesis transcription factor Lmo2. The oncogenic LIM domain transcription factor Lmo2 is necessary for definitive hematopoiesis in addition to yolk sac erythropoiesis (Yamada et al. 1998). Lmo2 has no DNA binding capacity but forms a transcription factor complex through its two tandemly arranged LIM domains. It has been shown to bind to other DNA binding protein such as GATA1/2/3, Tal1, and Lyl1 and form a transcription factor complex. Lmo2 also plays a pivotal role in embryonic angiogenesis (Yamada et al., 2000). In the event of the first specification of HSCs, the transcription factor complex including Lmo2 (the Lmo2 complex) promotes the angiogenic process in the mouse AGM region and is involved in the specification of blood cells from the dorsal aorta endothelium by triggering the upregulation of the appropriate downstream genes. The mouse vascular system is also constructed by two consecutive processes: vasculogenesis and angiogenesis. Without the Lmo2 transcriptional signal, this sprouting angiogenesis process is completely abolished, although vasculogenesis, the specification of hemangioblasts is not affected (Yamada et al., 2000; Yamada et al., 2002).

The downstream molecules activated by Lmo2

Recently, Lmo2 transcriptional signals have been examined in detail, and several Lmo2 downstream genes which are considered to have pivotal roles in sprouting angiogenesis have been clarified. VE-cadherin, angiopoietin2, neuropilin2 (VEGF receptor), and TGF-β are direct Lmo2 target genes, and most VEGF receptors are upregulated by the expression of Lmo2. The Lmo2 complex (discussed in detail later) regulates sprouting angiogenesis via these soluble factors, its receptors, and adhesion molecules.

In summary, the FGF signal mainly specifies the hemato-vasculogenic mesoderm by transcriptionally activating Etv2. Then, this transcription factor activates Lmo2, Tal1, and Gata2 which have

key roles both in hematopoiesis and angiogenesis. The Lmo2 complex formed by Lmo2, Tal1, and Gata2 determines the hemogenic endothelium in the dorsal aorta endothelial cells. In embryogenesis, this hemogenic endothelium differentiates the hematopoietic stem cells by expressing Runx1 transcription factor and c-kit membrane bound receptor. Another important role of this Lmo2 transcription factor complex is angiogenesis, although the Lmo2 binding partners have not been fully identified. Of course, angiogenesis is necessary for the construction of large blood vessels such as the dorsal aorta, which is the first intraembryonic hemogenic site. The angiogenic role of the Lmo2 complex should be performed immediately after the specification of hemangioblasts. It could be either before or after the specification of the hemogenic endothelium in the dorsal aorta.

Multistep angiogenesis by Lmo2 downstream molecules

The process of sprouting angiogenesis from the existing blood vessels can be divided into several steps, as mentioned. In each step, Lmo2 downstream genes are involved, as shown below. Key players are indicated in each step and Lmo2 complex target molecules are underlined.

(1) ECM remodeling and pericyte detachment : angiopoitin-2
(2) Loosening endothelial junction : VE-cadherin
(3) Tip cell formation and migration : VEGF and its receptors
(4) Stalk elongation and tip cell guidance : Semaphorines and neoropilin
(5) Endothelial junction formation : VE-cadherin
(6) Pericyte recruitment and its maturation : angiopoitin-1 and TGF-β

Obviously, the Lmo2 complex transcriptional signal orchestrates the whole sprouting process.

Gata2 and Lmo2 regulate angiogenesis and lymphangiogenesis cooperatively

Thomas Graf presented the "cocktail party model" for transcription factor complex transition in the progression of blood cell specification. According to the model, the hematopoietic transcription factor complex changes gradually, by one joining and another leaving, in each stage of blood cell differentiation to construct the entire blood cell system. At that time, the Lmo2 complex itself was one of the strong candidates for this complex, especially in hematopoietic stem cell differentiation into progenitor blood cells.

Now, there is strong evidence that the Lmo2 complex also plays key roles in the process of angiogenesis. As mentioned, Gata2 and Lmo2 form common transcription complexes during hematopoietic differentiation by direct interaction between the two molecules. These two transcription factors also play a key role in endothelial cells and lymphatic endothelial cell function cooperatively. Silencing of Lmo2 and Gata2 expression by siRNA inhibited VEGF-induced angiogenic activity including endothelial migration and sprouting in vitro (Coma et al., 2013). Interestingly, the knockdown of both Lmo2 and Gata2 completely blocks the vessel sprouting and all the VEGF receptors including VEGFR2 are downregulated. This inhibition of the endothelium is also associated with the downregulation of another VEGF receptor, neuropilin2. A Chip study clearly showed that neuropilin2 is the downstream molecule of the transcription factor complex composed of Lmo2 and Gata2. In a previous section, the phenotypes of the Lmo2-KO mouse were discussed. Those of the Gata2-KO mouse are markedly similar to the defects of the Lmo2-KO mouse and its defects in hematopoiesis (lack of hematopoietic stem cells), angiogenesis, and lymphangiogenesis. Both transcription factors are strongly expressed in the endothelial cells.

Conclusion : Cocktail party model for sprouting angiogenesis

The first hematopoietic stem cell (HSC) emerges from the wall of the dorsal aorta. The site of emergence of HSC is the hemogenic endothelium, which constitutes a part of the arterial wall endothelium. The key transcription factor complex characterizing the hemogenic endothelium is the Lmo2 complex : Tal1-Lmo2-Gata2. The basic helix-loop-helix transcription factor Tal1 is expressed in the static (quiescent, not activated) endothelium. In the process of vessel sprout, Tal1 is downregulated in the activated endothelium. On the other hand, Lmo2 and Gata2 are slightly upregulated in the activated endothelium. Therefore, the putative Lmo2 transcription factor complex composition in the activated endothelium is Gata2-Lmo2-Lmo2-Gata2. Further studies are needed for the identification of the tip and stalk cell-specific transcription factors. The Lmo2 complex is, of

course, one of the candidates.

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