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Signaling Circuitry in Vascular Morphogenesis

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Abstract

Purpose of review—In this mini-review, we have highlighted the recent breakthroughs in growth factor signaling that have made conceptual changes in our understanding of how blood vessels are formed.

Recent findings—Studies conducted over the past few years have focused on understanding the cell biology of vascular morphogenesis. The major themes include characterization of the different cell types that comprise a vascular sprout, as well as the regulatory influence of cell-cell and cell-matrix interactions on signaling outcomes. In addition, novel trends have emerged, including non-conventional ways in which VEGF contributes to cell survival and metabolic balance.

Summary—The growth of new capillary sprouts from a pre-existent vascular network requires a highly coordinated cellular response to both growth factors and morphogens. This response is sensed and triggered by cell surface receptors responsible for the activation of an intracellular cascade that efficiently initiates migration and proliferation programs. While the chief molecular players have been identified, great strides have been made in understanding their specific contributions to endothelial function during the angiogenic process.

Keywords

angiogenesis; capillaries; endothelial cells; VEGF; vascular system

1. Introduction

During the last two decades, the exploration of genetic targeting in mice has advanced our understanding of vascular morphogenesis. The resultant findings have identified a subset of signaling pathways that are essential for angiogenic growth during development and in the adult. These pathways include VEGF-VEGFR, Notch-DSL, Tie-Angiopoietin, VE-cadherin

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and Ephrin-Eph. Other prominent signaling pathways that contribute to further patterning and vascular remodeling also include plexins, TGF-beta, PDGF, and integrins.

This “genetic phase” of discovery has been recently followed by strategies that merge sophisticated cell biology with genetic modifications. The outcome has resulted in a plethora of publications that have refined the independent and combined contributions of these pathways in angiogenesis.

In this review we present breakthroughs in growth factor signaling during the last three years. Our emphasis focuses on the evidence that offers an in-depth and mechanistic understanding of how signaling pathways interact towards the organization of vascular sprouts. We have also made an effort to identify novel trends and highlight the unsuspected contribution of VEGF in metabolism.

2. The cellular anatomy of the vascular sprout

Information from cell biological studies and genetic deletion in mice has revealed that the cellular composition of a growing vascular sprout is molecularly and functionally heterogeneous. The endothelial cells at the invasive front exhibit unique properties and can be distinguished from their neighbors by the expression of a specific subset of molecules.

Growing vessel

The vascular sprout is composed of a **tip cell**, at the leading front followed by **stalk cells**. The later cells comprise the body of the sprout and are responsible for the elongation of the new vascular branch (Figure 1). Tip cells were first described as functionally specialized and distinct endothelial cells by Gerhardt and colleagues [1]. These investigators characterized tip cells as non-proliferative, with dynamic filopodial protrusions that express high levels of VEGFR2. Tip cells sense and respond to gradients of VEGF and maintain an active migratory phenotype. On the other hand, stalk cells proliferate in response to VEGF, promoting the physical extension of the vessel [1]. The concept of tip and stalk cells is fully accepted today, but has gained a more clear molecular identity. Studies published over the past three years have underscored the importance of crosstalk between the VEGF and Notch signaling pathways in determining the identity and number of tip and stalk cells.

One of the hallmark achievements in the field in recent years has been the identification of the Dll4-Notch1 pathway as the instructive regulator of tip versus stalk cell fate. The findings concurrently emerged from several groups using three distinct experimental models of angiogenesis including solid tumor growth in mice [2,3], postnatal mouse retinas [4-6], and zebrafish embryos [7,8]. In all these models, blockade of Dll4 activity, using pharmacological inhibitors or genetic inactivation of one allele, led to high vascular density due to an increase in the number of tip cells and excessive vascular sprouting. On the other hand, activation of Notch signaling led to a reduction in the number of tip cells and decreased vascular density [4,7]. Furthermore, VEGF-VEGFR2 signaling was shown to upregulate expression of Dll4 in tip cells, allowing Dll4 to activate Notch1 in the adjacent stalk cells, causing suppression of the tip cell phenotype. This coordinated regulation ensures the selection of a single cell as the leader (i.e., tip) while the adjacent cells become followers (stalk cells).

How might Notch signaling suppress the tip cell phenotype? Additional mechanistic insights have revealed that Notch inactivation through deletion of Dll4 results in a reduction of VEGFR1 levels [5,6]. This suggests that Notch signaling may block tip cell formation by regulating the levels of VEGFR1, a decoy receptor that antagonizes VEGFR2 function [9,10]. Another mechanism through which Notch signaling may suppress the tip cell

phenotype is through transcriptional downregulation of VEGFR3. VEGFR3 expression has been localized to the leading tip cells of endothelial sprouts in the zebrafish embryo [7], the postnatal retina, and in tumor vasculature [11], where VEGFR2 is also highly expressed. However, in the absence of Notch signaling, VEGFR3 becomes ectopically expressed throughout the sprout [7,11]. Consistent with these findings, inhibition of VEGFR3 signaling through genetic targeting, or with monoclonal antibody blockade, both result in decreased vessel density due to a reduction in the number of sprouts, branching points, and proliferating cells, suggesting that VEGFR3 is a positive regulator of tip cell selection. Importantly, the excessive sprouting that occurs upon genetic or pharmacological disruption of Dll4/Notch signaling is extinguished upon blocking VEGFR3 signaling [11].

More recently, the Notch ligand Jagged1 has been shown to act as an antagonist of Dll4-Notch signaling in sprouting angiogenesis [12]. While Dll4 is expressed highly in tip cells, Jagged1 expression is low. Instead, Jagged1 has increased expression in stalk cells [13]. Deletion of Jagged1 in endothelial cells led to a decrease in the number of tip cells and filopodia and reduced vessel density in postnatal mouse retinas. When Notch was glycosylated by the Fringe family of glycosyltransferases, Jagged1 was able to act as an antagonist of Dll4/Notch signaling. In addition, Jagged1 inactivation in postnatal endothelial cells led to reduced expression of VEGFR3 in tip cells in the retina, providing a mechanistic explanation for impaired sprouting in the absence of Jagged1 [12].

Quiescent vessel

Adding to the cellular heterogeneity of a vessel, recent work by Carmeliet and colleagues describes a third type of endothelial cell, restricted to quiescent vessels, which they refer to as the **phalanx cell** [14]. Using tumor blood vasculature as their experimental model, the authors showed that heterozygous deficiency of the prolyl-hydroxylase domain protein 2 (PHD2) normalized the endothelial lining without affecting tumor vessel density or lumen size. In wild type mice, the endothelial cells lining the tumor vessel walls were disorganized and irregular. However, the vessel wall in the PHD2 +/- mice formed a uniform “cobblestone” formation with clearly defined boundaries and branching points [14]. They described the endothelial cells that adopted this quiescent phenotype as a phalanx cell. Phalanx cells maintained lumen patency and formed a tightly aligned endothelial cell layer.

Mechanistically, maintenance of the quiescent endothelial cell phenotype depended on the HIF-mediated upregulation of soluble VEGFR1 and VE-cadherin. VE-cadherin is the main component of endothelial cell adherens junctions, and was recently shown to upregulate claudin-5, an endothelial specific tight junction protein [15]. Strengthening both adherens and tight junctions would serve as a mechanism for tightening the endothelial cell barrier, and promoting the quiescent, normalized phenotype.

Interestingly, PHD2 +/- phalanx cells showed a reduced migratory and mitogenic response, but an enhanced survival response to VEGF. This response may be regulated by an increase in the levels of VE-cadherin, which is required for VEGF-mediated survival of endothelial cells [16].

3. Cell-cell interactions as modulators of angiogenic signals

Studies on signaling in vascular cells have not deviated from similar analysis in other cell types, in that the focus has been on one growth factor at a time. In the last three years, however, a multitude of papers have dissected the intricacies of dual and/or tripartite interactions. Analysis of these associations has exposed an unsuspected and sophisticated hierarchy of cellular responses during angiogenesis. Furthermore, these studies have revealed that the same players might assume distinct roles depending upon the status of

endothelial cell confluency *versus* subconfluency, (perhaps analogous to phalanx *versus* tip cells) (Figure 2).

In quiescent endothelial cells, VE-cadherin promotes stabilization of the differentiated vascular wall, inhibits proliferation and decreases cell permeability [17,18]. Recent information has revealed that VE-cadherin binds to VEGFR2, and together with beta-catenin forms a multiprotein complex that promotes vascular stability and survival [19,20]. Interestingly, this tripartite association prevents phosphorylation and internalization of VEGFR2 in the presence of ligand [19]. The findings indicate that engagement of cell-cell interactions promotes a functional change in the response of VEGFR2 to its ligand. Furthermore, VE-cadherin, through the activation of Rho-kinase and myosin light chain 2, was shown to coordinate the stabilization of cell-cell junctions and suppression of a migratory phenotype [20]. Overall the combined information suggests that activation of cell-cell interactions inhibits the angiogenic response through the regulation of the cytoskeleton and by taming the response to VEGF (Figure 2A).

Not only cell-cell, but also cell-matrix interactions contribute to regulate the responses mediated by VEGFR2. A series of recent experiments have found that activation of VEGFR2 by matrix-bound VEGF results in prolonged phosphorylation of this receptor and preferential p38, rather than Akt, activation [21] (Figure 2B). Association of VEGF with matrix also induced the binding of VEGFR2 to beta1 integrins and disfavored its interaction with Neuropilin1 [21]. In contrast, activation of VEGFR2 by VEGF when in its soluble form, i.e., not associated with matrix proteins, triggers the formation of a complex that includes Neuropilin1 and beta3 integrins [22,23]. This complex is functionally and molecularly distinct from the one that is activated by matrix-bound VEGF and results in two distinct forms of vascular expansion: sprouting growth (matrix-bound VEGF) or vascular hyperplasia (soluble VEGF) [24].

Cell-cell and cell-matrix interactions have also been noted to distinctively modulate responses to Angiopoietin1 through the Tie2 receptor [25,26] (Figure 2). Two elegant publications convincingly showed that in confluent endothelial cells, Angiopoietin1 bridges Tie2 at cell-cell contacts. This interaction results in trans-association of Tie2 and downstream activation of Foxo1 and eNOS [25]. Clustering of Tie2 at the cell-cell interface also results in binding to VE-PTP [26]. The interaction results in a marked decrease in endothelial permeability and provides a mechanistic explanation for the phenotype of the Tie2 and Angiopoietin null mice [27-29]. In the absence of cell contacts, Angiopoietin1 induces a translocation of Tie2 to the rear of the cell in clusters similar to, but distinct from, focal adhesions, without the association of VE-PTP [26].

4. Regulation of endothelial cell survival

VEGF signaling has long been regarded as a necessary factor for endothelial cell survival. In recent years, several new aspects through which the VEGF family of ligands mediates the survival of endothelial cells, independent of classical VEGF signaling, have come to light.

It was recently demonstrated that endothelial cells produce VEGF, and that autocrine VEGF signaling is required for endothelial cell survival in a cell autonomous manner [30]. Deletion of VEGF specifically from endothelial cells resulted in progressive endothelial degeneration. While autocrine VEGF signaling is dispensable angiogenesis, it is crucial for the maintenance and homeostasis of blood vessels. Furthermore, circulating and exogenous VEGF was unable to compensate for the loss of VEGF in endothelial cells [30]. Endogenous VEGF signaling has also been shown to mediate survival in other cell types, in particular müller cells and photoreceptors [31].

Another growth factor with significance to endothelial cell survival is VEGF-B. VEGF-B is not required for blood vessel growth, but is necessary for the survival of not only endothelial cells, but also vascular smooth muscle cells and pericytes. The effect of VEGF-B on survival was mediated, in part, by an upregulation of VEGFR1 and Neuropilin1 [32].

5. Contribution of VEGF signaling to metabolic balance

The response of VEGF to hypoxia has been acknowledged and well supported in the literature. VEGF is transcriptionally regulated by HIFs, and changes in oxygen tension result in a concurrent increase in VEGF expression and vascular density [33]. During the past few years, we have expanded our understanding of the regulatory effects of oxygen sensing proteins on VEGF in both the endothelium and other cell types. Combined, the information seems to reveal that the VEGF family might act as a global sensor of metabolic stress in a large variety of tissues.

Recent advances have linked the regulation of oxygen consumption by other cell types to the activity of VEGF in the vasculature. Intracellular levels of oxygen directly control metabolic pathways and regulate a cohort of transcription factors that provide further homeostatic support favoring aerobic metabolism. Amongst the transcription factors, HIFs and ARNT are constitutively expressed, but are kept separate and therefore inactive by prolyl hydroxylase domain proteins (PHD1-3) under normoxic conditions. PHDs target HIFs for proteasomal degradation under normal oxygen tension. Hypoxia downregulates PHD activity, enabling accumulation of HIFs [34]. Genetic studies in mice have recently highlighted additional functions for PHD proteins in both the endothelium and surrounding tissues.

In the absence of PHD2, the oxygen sensing machinery of endothelial cells is altered in a way that appears to precondition the cells to better adapt to hypoxia. In particular, inactivation of the oxygen sensor PHD2 caused endothelial cells to readjust their shape and molecular signature to restore oxygen supply to the vasculature. Haploinsufficiency of PHD2 resulted in normalization of tumor endothelial cells into a quiescent phalanx cell phenotype [14]. Tumor blood vessels are considered “abnormal” in that they are leaky and are inefficient in oxygenation, resulting in hypoxia, which induces the production of growth factors and cytokines by the surrounding tumor cells. This in turn promotes tumor invasion and metastasis. Therefore, the concept of vessel “normalization” has gained significant interest in the field as a means of controlling the tumor vasculature to improve drug delivery [35]. One would hypothesize that reducing the levels of endothelial PHD2 would lead to an increase in tumor metastasis due to the upregulation of the HIF signaling pathways. In contrast, the tumors in PHD2 +/- mice were less invasive and metastatic due to blood vessel normalization. In addition, the response of phalanx cells to VEGF induced endothelial survival, rather than migration or proliferation [14].

In contrast to PHD2, loss of PHD1 does not cause abnormal angiogenesis, but instead has an effect on the metabolic program of skeletal muscle cells [36]. PHD1 deficiency lowers oxygen consumption in skeletal muscle through a shift from oxidative to a more anaerobic metabolic program. While under normal conditions the loss of PHD1 impairs oxidative muscle performance, under ischemic conditions the enhanced oxygen conservation in myofibers lacking PHD1 prevents excess oxygen damage by reducing necrosis. Overall it seems that the regulation of HIF, by PHD1 and 2 are cell-specific and that each protein offers alternative outcomes depending on the cell type.

Recent work has further linked the regulation of oxygen consumption by other cell types to the activity of VEGF in the vasculature. The metabolic regulator PGC-1 α is increased in various cell types under nutrient and oxygen deprivation. Moreover, PGC-1 α has been

shown to regulate angiogenesis by inducing the expression of VEGF, PDGF-BB and Angiopoietin2 in skeletal muscle [37]. Interestingly, induction of VEGF by PGC-1alpha is independent of the HIF pathway, but instead requires PGC-1alpha interaction with estrogen-related receptor-alpha, a protein involved in fatty acid oxidation and oxidative phosphorylation. The emerging interactions between metabolic pathways and VEGF uncover an important and unsuspected contribution of this growth factor in the overall regulation of energy consumption by the cell.

6. Concluding remarks

Our understanding of vascular morphogenesis has continued to grow at an exponential rate. While the major focus of endothelial cell biology in the last decade has been on the identification of the central signaling cascades and characterization of their main functions, the last three years have been marked by an increased emphasis on the cell biology of vascular morphogenesis. In particular, the cellular dissection of the nascent vascular sprout into tip and stalk cells has provided a critical conceptual advance that permeates the field. This has been coupled with the critical and recent advances on how cell-cell and cell-matrix contacts modulate receptor tyrosine kinase signaling in endothelial cells. Finally, publications in the last three years have contributed to an emerging notion that the VEGF family of signaling molecules might contribute to metabolic sensing.

Where do we go from here? Clearly the cell-biological phase of vascular biology will continue to expand. The intricacies of binding partners and their biological effects will most certainly fill the framework of information on vascular network formation and remodeling. Additional trends, already appearing in the literature, are likely to include how organ-specific microenvironments impact endothelial differentiation and heterogeneity. Along these lines, the cell biological consequences of paracrine interactions in a growing vascular bed are likely to be critical in further completing the picture of signaling circuitry in the vascular endothelium.

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Abbreviations

ARNT	aryl hydrocarbon receptor nuclear translocator
Dll	delta-like ligand
DSL	delta, serrate, lag2
eNOS	endothelial nitric oxide synthase
HIF	hypoxia inducible factor
PDGF	platelet derived growth factor
PGC	peroxisome-proliferator-activated receptor-gamma coactivator
PHD	prolyl hydroxylase domain
TGF	transforming growth factor
VEGF	vascular endothelial growth factor

VEGFR	vascular endothelial growth factor receptor
VE-PTP	vascular endothelial protein tyrosine phosphatase

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Figure 1. Endothelial cells in a growing vascular sprout are functionally and molecularly distinct. A growing sprout is composed of tip cells (green), stalk cells (red), and phalanx cells (blue). Each cell type is characterized by a unique molecular signature, resulting in a differential response to VEGF. Tip cells exhibit a migratory response to VEGF, and show an upregulation of Dll4, VEGFR3, and VEGFR2. Stalk cells undergo proliferation and show upregulation of Notch1 and Jagged1. VEGF signaling in phalanx cells leads to a survival response mediated by increased levels of VE-cadherin and VEGFR1.

Figure 2.

Location and confluency status dictate the distribution of signaling mediators during vascular morphogenesis. The maintenance of vascular integrity is partially mediated through the tripartite interaction of VEGFR2, VE-cadherin, and beta-catenin (A). Interactions between Tie2 and Angiopoietin1 at cell-cell contacts also contribute to the homeostasis of quiescent endothelial cells (A), while Tie2-Angiopoietin1 signaling in the context of cell-matrix interactions leads to migration of endothelial cells (B). The association status of VEGF with the matrix dictates differential responses by VEGFR2. Activation of VEGFR2 by matrix-bound VEGF leads to its association with beta1-integrins and activation of p38. Soluble VEGF binding leads to VEGFR2 association with Neuropilins or beta3-integrins, leading to activation of Akt (B).