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Vasculogenesis, Angiogenesis, and Arteriogenesis: Mechanisms of Blood Vessel Formation and Remodeling

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Abstract In this review, the concept of oxygen homeostasis will be presented as an organizing principle for discussion of the phylogeny, ontogeny, physiology, and pathology of blood vessel formation and remodeling, with a focus on molecular mechanisms and potential therapeutic applications. J. Cell. Biochem. 102: 840–847, 2007.
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OXYGEN HOMEOSTASIS

All metazoan species are dependent upon a continuous supply of O_2 in order to survive. The roundworm *Caenorhabditis elegans* consists of less than one thousand cells, which receive O_2 by simple diffusion from the air. In the fruit fly *Drosophila melanogaster*, the presence of multiple cell layers necessitates the existence of a specialized system of tracheal tubes to conduct air to all cells of the organism [Gorr et al., 2006]. In vertebrates, the much larger body size requires an even more complex strategy of O_2 delivery involving a respiratory system, which provides a large pulmonary alveolar surface area for gas exchange, and a circulatory system, consisting of blood, heart, and blood vessels to deliver O_2 to all cells of the body. Although the circulatory system also functions to deliver nutrients and remove toxic metabolic wastes from the tissues, its principal function is to maintain oxygen homeostasis by precisely modulating O_2 delivery to meet the demands imposed by cells within every tissue. Thus, the requirement to maintain oxygen homeostasis represented an important driving force for the evolution of organisms with increasing biological complexity.

In mammals, the requirement for a functioning circulatory system occurs early in development when diffusion from blood vessels in the surrounding uterine tissues becomes insufficient to supply adequate O_2 to all cells of the growing embryo. In mice, failure to establish a functioning circulatory system by embryonic day 10 results in lethality. In humans, all of the major disease causes of mortality involve changes in tissue vascularization and O_2 delivery. Thus, oxygen homeostasis is an organizing principle for understanding evolution, development, and disease pathophysiology. This review will examine the molecular mechanisms underlying angiogenesis and related processes of vessel formation and remodeling from this physiological perspective.

PROCESSES OF VESSEL FORMATION AND REMODELING

Three major processes by which blood vessels are formed and remodeled are referred to as vasculogenesis, angiogenesis, and arteriogenesis [Carmeliet, 2004]. Vasculogenesis denotes de novo blood vessel formation during embryogenesis, in which angiogenic progenitor cells migrate to sites of vascularization, differentiate

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into endothelial cells, and coalesce to form the initial vascular plexus [Risau and Flamme, 1995; Carmeliet, 2003]. The budding of new capillary branches from existing blood vessels is termed angiogenesis. Arteriogenesis refers to the remodeling of an existing artery to increase its luminal diameter in response to increased blood flow [Heil et al., 2006].

Capillaries are tubes formed by endothelial cells, which are supported by vascular pericytes. Arteries and veins are tubes that consist of multiple layers: the intima, which is composed of endothelial cells, pericytes, and a basement membrane; the media, which is composed principally of smooth muscle cells and their extracellular matrix; and, in the largest vessels, the adventitia, which is composed principally of fibroblasts and their extracellular matrix. The formation of the initial vascular plexus within each tissue, as well as the later formation of the major blood vessels that conduct blood to and from the heart, appear to occur in a hard-wired, stereotypical fashion that is independent of O_2 concentration. In contrast, the pattern of capillaries that develops within each tissue of an individual is unique and is driven by local O_2 demand.

VASCULAR RESPONSES TO HYPOXIA/ISCHEMIA

In adults with systemic hypertension, the left ventricle must pump against increased resistance. In order to do so, the heart hypertrophies and the increase in cardiac muscle mass is associated with an obligate increase in O_2 consumption. The imbalance between O_2 supply and demand leads to hypoxia, which is a physiological stimulus that induces cells to produce angiogenic cytokines such as vascular endothelial growth factor (VEGF). These secreted proteins bind to cognate receptors (VEGFRs) on endothelial cells and activate signal transduction pathways that stimulate the cells to undergo sprouting angiogenesis (Fig. 1). Thus, in order to satisfy this increased demand for O_2, angiogenesis occurs to provide new capillary branches. VEGF is produced early in the angiogenic cascade and is responsible for initial activation of endothelial cells [Carmeliet, 2003; Ferrara et al., 2003].

Although VEGF is necessary for vessel formation it may not be sufficient. In addition to promoting angiogenesis, VEGF promotes increases vascular permeability [Dvorak, 2006]. Transgenic expression of VEGF in mouse skin results in increased numbers of blood vessels that manifest excessive permeability, whereas expression of both VEGF and angiopoietin 1 (ANGPT1) results in increased vessels without excessive permeability [Thurston et al., 1999], an effect that is probably due to the ability of ANGPT1 to recruit pericytes to the endothelium [Hanahan, 1997]. In contrast, transgenic coexpression of ANGPT1 in the heart blocks the angiogenic effect of VEGF [Visconti et al., 2002]. Placental growth factor (PLGF) plays a critical role in ischemia-induced angiogenesis and has synergistic effects with VEGF in some tissues [Carmeliet et al., 2001; Luttun et al., 2002]. Synergistic effects of combined treatment with platelet-derived growth factor (PDGF)-BB and fibroblast growth factor-2 have also been reported [Cao et al., 2003]. Two important conclusions can be drawn from these data: (i) Increased expression of a single angiogenic factor may not be sufficient for functional vascularization. (ii) The effects of angiogenic factors may be tissue-specific.

In adults with atherosclerosis, narrowing of large conduit vessels in the heart (coronary artery) or limb (femoral artery) leads to reduced perfusion of tissue downstream of the stenosis. In response to the hypoxia that results from inadequate perfusion (ischemia), cells produce VEGF and other angiogenic cytokines, which stimulate local endothelial cells to proliferate and to undergo sprouting angiogenesis as described above. However, because the primary cause of the ischemia is stenosis of a large conduit vessel, the formation of additional capillaries cannot correct the problem. Thus, in addition to angiogenesis, pre-existing collateral vessels are remodeled by an increase in luminal diameter to accept increase blood flow and thereby provide a means to bypass the stenotic vessel.

In addition to their ability to activate vascular endothelial cells within the ischemic tissue, certain angiogenic cytokines, such as VEGF, PLGF, and stromal-derived growth factor 1 (SDF-1), stimulate the mobilization of a heterogeneous population of angiogenic cells from the bone marrow and other tissues (Fig. 1), and their recruitment to sites of angiogenesis and arteriogenesis [Ceradini et al., 2004; Grunewald et al., 2006; Jin et al., 2006]. Although this process of angiogenic cell recruitment is

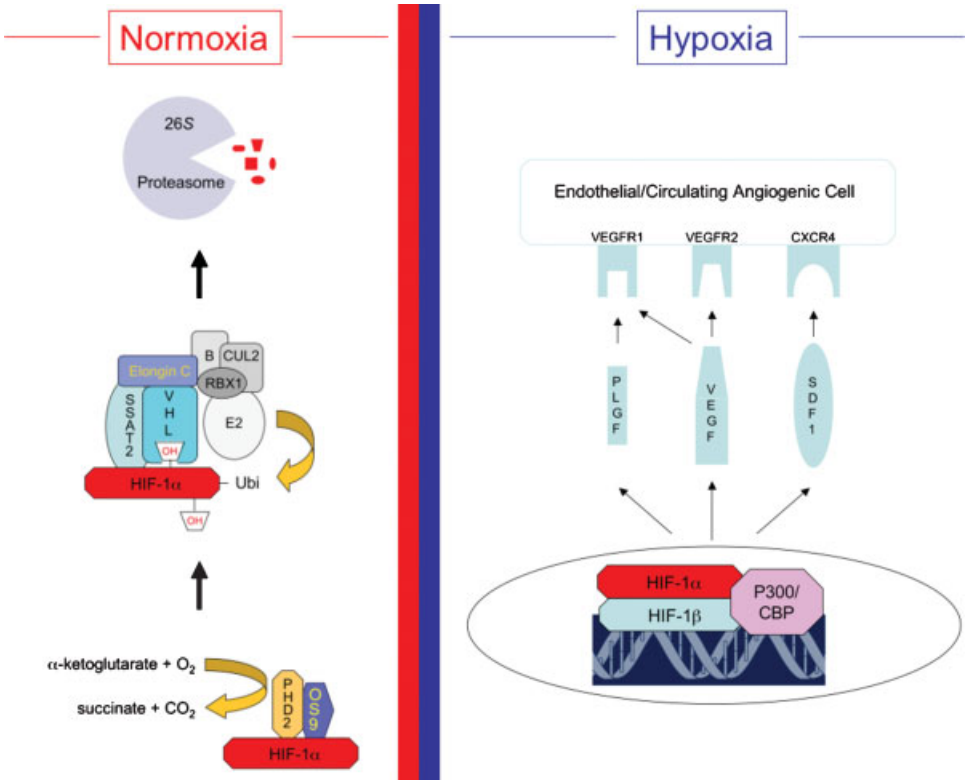

Fig. 1. Molecular mechanisms of oxygen homeostasis. **Left**, under normoxic conditions, HIF-1α is synthesized, but is rapidly subjected to prolyl hydroxylation by the PHD2-OS9 complex and asparaginyl hydroxylation by FIH-1 (not shown). Prolyl hydroxylated HIF-1α is bound by VHL, which together with SSAT2 recruits Elongin C, which in turn recruits a ubiquitin ligase complex containing Elongin B (B), ring box protein 1 (RBX1), cullin 2 (CUL2), and an E2 ubiquitin conjugating enzyme. Ubiquitination of HIF-1α targets the protein for degradation by the 26*S* proteasome. **Right**, under hypoxic conditions, HIF-1α accumulates, dimerizes with HIF-1β, recruits the coactivators CBP/p300, and activates the transcription of genes encoding angiogenic cytokines including PLGF, VEGF, and SDF-1. The protein products of these genes are secreted and bind to their cognate receptors (VEGFR1, VEGFR1/VEGFR2, and CXCR4, respectively), which are located on the plasma membrane of vascular endothelial cells and circulating angiogenic cells. The diagram is simplified: cells may express one, two, or three of the receptors shown, as well as receptors for other cytokines, which are not shown.

sometimes referred to as vasculogenesis, it appears to be quite different from the process that occurs during the formation of the initial embryonic vascular plexus. Among the important distinctions are the following: (i) Formation of the initial vascular plexus in the embryo is not driven by oxygen gradients. (ii) Many of the cells recruited to sites of postnatal angiogenesis or arteriogenesis are inflammatory cells that are not directly incorporated into the new capillaries or remodeling arteries, respectively.

Among the cell types that may participate in these responses are endothelial progenitor cells [Asahara et al., 1997], which by definition incorporate into the endothelium of new or remodeling vessels [Yoder et al., 2007], as well as myeloid, mesenchymal, and hematopoietic progenitor cells, which promote vascular growth and remodeling through production of additional angiogenic cytokines. We designate this heterogeneous population of cells that is mobilized in response to ischemia as circulating angiogenic cells. The most important prerequisite for a cell to be directly recruited to ischemic tissue in response to the production of an angiogenic cytokine is that the recruited cell must express the cognate receptor on its plasma membrane (Table I).

TABLE I. Angiogenic Growth Factors/ Cytokines and Their Cognate Receptors

Receptor	Ligand(s)
C-KIT	SCF
CXCR4	SDF-1
TIE2	ANGPT1, ANGPT2
VEGFR1	PLGF, VEGF
VEGFR2	VEGF

Clinical trials of individual angiogenic factors, such as VEGF, for treatment of ischemic cardiovascular disease have failed to demonstrate efficacy [Henry et al., 2003]. This result is consistent with the studies in mice cited above that indicated a requirement for multiple angiogenic factors [Blau and Banfi, 2001; Bruick and McKnight, 2001]. Attempts to stimulate tissue vascularization and repair following myocardial infarction by administration of autologous bone marrow cells as a source of angiogenic cells have not met with great success [Rosenzweig, 2006]. It appears that the angiogenic response to ischemia is impaired at multiple levels in patients, resulting in decreased production of angiogenic cytokines in response to hypoxia/ischemia, decreased numbers of angiogenic cells bearing receptors for these cytokines, and decreased ability of these cells to home to ischemic tissue and promote vascular remodeling and repair. The multifactorial causes of these impaired responses include aging, hyperlipidemia, and diabetes [Dimmeler and Zeiher, 2004] as well as differences in genetic background [Rohan et al., 2000; Resar et al., 2005; Shaked et al., 2005]. Multiplex therapies may be required to overcome these multifactorial impairments [Gallagher et al., 2007].

MOLECULAR MECHANISMS OF ISCHEMIA-INDUCED VASCULARIZATION

As described above, the circulatory system evolved as a mechanism for optimal O_2 delivery to all cells despite the greatly increased body size of vertebrates. The vascular responses to ischemia are primarily based on the sensing of reduced O_2 concentrations by cells within ischemic tissue, which leads to the increased expression of genes encoding angiogenic growth factors. At the center of this response pathway is hypoxia-inducible factor 1 (HIF-1), which is a heterodimeric transcription factor that is composed of a constitutively expressed HIF-1β subunit and an oxygen-regulated HIF-1α subunit [Wang and Semenza, 1995; Wang et al., 1995]. The HIF-1α subunit is continually synthesized and degraded within adequately perfused cells with normal oxygenation (Fig. 1). Under hypoxic conditions, the degradation of HIF-1α is inhibited, leading to its accumulation, dimerization with HIF-1β, DNA binding, recruitment of co-activators, and transcriptional activation of target genes.

HIF-1α is subjected to O_2-dependent ubiquitination that is initiated by the binding of the von Hippel-Lindau tumor suppressor protein (VHL) and its recruitment of an E3 ubiquitin-ligase complex [Salceda and Caro, 1997; Maxwell et al., 1999] that contains Elongin C, Elongin B, Cullin 2, and RBX1. The binding of VHL is dependent upon the hydroxylation of proline residue(s) 402 and/or 564 of HIF-1α [Ivan et al., 2001; Jaakkola et al., 2001; Yu et al., 2001]. The HIF-1α prolyl hydroxylases that perform this reaction are dioxygenases that utilize O_2 as a substrate [Schofield and Ratcliffe, 2005]. One oxygen atom is inserted into the HIF-1α prolyl residue to form a 4-hydroxyl group and the other oxygen atom is inserted into the co-substrate α-ketoglutarate, forming succinate and CO_2 as side products. Under hypoxic conditions, the hydroxylase activity is inhibited and the half-life of HIF-1α increases as a result of decreased hydroxylation, ubiquitination, and degradation. Although three HIF-1α prolyl hydroxylases have been identified, the activity of PHD2 determines the basal levels of HIF-1α under aerobic conditions. Changes in O_2 concentration are very rapidly transduced to changes in HIF-1α protein levels. The speed and precision of this O_2-dependent regulation appears to reflect the involvement of multiprotein complexes in hydroxylation and ubiquitination (Fig. 1). OS-9 binds to both HIF-1α and PHD2 and is required for efficient hydroxylation [Baek et al., 2005], whereas SSAT2 binds to HIF-1α, VHL, and Elongin C and is required for efficient ubiquitination [Baek et al., 2007].

In addition to prolyl hydroxylation, HIF-1α is also subjected to O_2-dependent hydroxylation of asparagine residue 803 in the transactivation domain by factor inhibiting HIF-1 (FIH-1), which is another dioxygenase that utilizes O_2 and α-ketoglutarate [Peet and Linke, 2006]. Hydroxylation of asparagine-803 prevents the interaction of HIF-1α with the co-activators p300 and CBP. Thus, both the half-life and transcriptional activity of HIF-1α are regulated by O_2-dependent hydroxylation events that provide a direct mechanism by which changes in O_2 concentration can be transduced to the nucleus as changes in the activity of HIF-1.

HIF-1α expression is required for proper vascularization of the mouse embryo [Iyer et al., 1998; Ryan et al., 1998]. HIF-1α is necessary and sufficient for the hypoxia-induced expression of

multiple angiogenic growth factors including VEGF, PLGF, ANGPT1, ANGPT2, and PDGFB [Kelly et al., 2003]. Administration of AdCA5, an adenovirus encoding an engineered form of HIF-1α that is constitutively active due to the presence of a deletion and point mutations that block O_2-dependent degradation of the protein, promotes the recovery of blood flow following limb ischemia by stimulating increased angiogenesis and arteriogenesis [Patel et al., 2005]. Administration of vector encoding a chimeric protein containing the dimerization and DNA binding domains of HIF-1α fused to the VP-16 transactivation domain has also been shown to promote recovery of blood flow following femoral artery ligation [Vincent et al., 2000] and is currently being evaluated as a therapy for critical limb ischemia in no-option patients [Rajagopalan et al., 2007].

Whereas transgene-directed expression of VEGF in the skin induces both hypervascularization and hyperpermeability [Thurston et al., 1999], expression of a constitutively active form of HIF-1α from the same transgene construct induces hypervascularization without hyperpermeability [Elson et al., 2001]. It is likely that this difference is due to the HIF-1-mediated expression of additional angiogenic factor(s) that suppress the effect of VEGF on vascular permeability. Administration VEGF is not sufficient to induce vascularization in the superficial capillary layer of the retina, whereas administration of AdCA5 induces robust vascularization, which is associated with increased expression of mRNAs encoding VEGF, PLGF, ANGPT1, ANGPT2, and PDGFB [Kelly et al., 2003]. These results are consistent with the conclusion from earlier studies that physiological angiogenesis occurs through the action of multiple angiogenic growth factors. Remarkably, these factors appear to be coordinately regulated by HIF-1.

In addition to its role in stimulating angiogenic growth factor expression in hypoxic tissues, HIF-1 plays important cell-autonomous roles in endothelial cells [Tang et al., 2004; Manalo et al., 2005; Calvani et al., 2006]. In bone marrow-derived mesenchymal stem cells (MSCs), HIF-1 is required for the expression of VEGFR1 and the chemotactic migration of MSCs towards a gradient of VEGF or PLGF [Okuyama et al., 2006]. HIF-1 has also been implicated in the expression of SDF-1 and the SDF-1-dependent recruitment of $CXCR4^+$ progenitor cells in a mouse skin flap model of ischemia [Ceradini et al., 2004].

ANGIOGENESIS IN CANCER

The growth of cancers is dependent upon angiogenesis [Folkman, 1995; Carmeliet and Jain, 2000]. Hypoxia-induced and HIF-1-mediated angiogenic growth factor production plays a major role in tumor vascularization [Acker and Plate, 2003; Pugh and Ratcliffe, 2003]. HIF-1 gain-of-function in human colon cancer cells and HIF-1 loss-of-function in human gastric cancer cells resulted in increased and decreased vascularization of tumor xenografts, respectively [Ravi et al., 2000; Stoeltzing et al., 2004]. The targeted knockout of HIF-1α expression selectively in endothelial cells also impaired the vascularization of tumor xenografts in mice [Tang et al., 2004]. Thus, HIF-1 activity is required in both tumor and stromal cells for maximal vascularization.

There is great interest in therapeutic targeting of the HIF-1 → VEGF → VEGFR signaling axis as a novel strategy for cancer therapy. Bevacizumab, a humanized monoclonal antibody against VEGF, is the first anti-angiogenic agent to be approved by the FDA for the treatment of cancer [Shojaei and Ferrara, 2007]. Low-molecular weight compounds, such as Sunitinib, which inhibit the tyrosine kinase activity of the VEGF, PDGF, and SCF receptors are also in clinical trials [Roskoski, 2007]. The cytotoxic effect of radiation therapy may be due in part to radiation-induced apoptosis of endothelial cells, which leads to reduced tumor cell perfusion and a secondary hypoxia-induced apoptosis of tumor cells [Garcia-Barros et al., 2003]. Recent studies of tumor xenografts in mice suggest that activation of HIF-1 following radiation may induce a protective angiogenic response that blocks endothelial cell death [Moeller et al., 2004]. Inhibition of HIF-1 [Moeller et al., 2004] or the downstream angiogenic response [Magnon et al., 2007] results in increased tumor cell killing.

CONCLUSION

Changes in tissue vascularization play critical roles in the pathophysiology of ischemic cardiovascular disease and cancer. Progress in understanding the molecular, biochemical, and physiological mechanisms that underlie

vascular responses to hypoxia and ischemia have led to novel therapeutic approaches for these diseases, which are the major causes of mortality in the developed world. However, because of the great complexity of these responses and the multifactorial changes imposed by disease states, our knowledge remains rudimentary. Further research is likely to increase our ability to discover new therapies and to identify, among patients with a particular diagnosis, the subgroup for which any particular therapy will do the greatest good.

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