PERSPECTIVE

Building better vasculature

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Throughout embryogenesis, establishment of the vascular network is mediated by a complex and highly regulated interplay between positive and negative growth factors. In adults, this vascular network is relatively stable and formation of new microvasculature is generally associated with pathophysiological conditions such as tumorigenesis, ischemic insult, and chronic inflammatory diseases. Vascular endothelial growth factor (VEGF) is a critical positive regulator of both physiological and pathophysiological neovascularization. As such, VEGF has become an important therapeutic target in the fight against cancer, blindness resulting from age-related macular degeneration or diabetic retinopathy, and pulmonary hypertension. Conversely, induction of VEGF with the intent to promote angiogenesis may benefit patients suffering from myocardial ischemia, limb ischemia resulting from diabetes, and insufficient wound healing. While overexpression of VEGF alone has been shown to promote angiogenesis, the resulting capillaries are often leaky and accompanied by edema, inflammation, and spontaneous hemorrhagic ulcers. In contrast, the article authored by Elson and colleagues in this issue (Elson et al. 2001) demonstrates that induction of VEGF expression in basal keratinocytes by a constitutively active, gene-specific transcription factor results in increased dermal vascularization devoid of leakage or inflammation. These findings suggest important new therapeutic avenues for promoting angiogenesis and provide a context in which to assess the relative contributions of VEGF to the formation of new vasculature.

VEGF stimulates angiogenesis

New vasculature networks can arise via *de novo* assembly (vasculogenesis) or by branching from preexisting vessels (angiogenesis). VEGF is a potent inducer of both processes. VEGF forms active, disulfide-linked homodimers that are recognized by the VEGF receptors (VEGFR-1 and VEGFR-2) expressed in endothelial cells (de Vries et al. 1992; Terman et al. 1992). Five VEGF isoforms, VEGF₁₂₁, VEGF₁₄₅, VEGF₁₆₅, VEGF₁₈₉, and VEGF₂₀₁, are generated by alternative splicing from a single gene (Houck et al. 1991; Tischer et al. 1991). Although the roles of individual isoforms have not been fully determined, it is clear that VEGF elicits a strong

angiogenic response by affecting the survival, proliferation, and migration of vascular tissue (for reviews see Ferrara 1999; Neufeld et al. 1999; Daniel and Abrahamson 2000). For example, VEGF stimulates both the survival and expansion of endothelial cells for assembly into vascular structures (Alon et al. 1995; Gerber et al. 1998a,b). VEGF promotes the expression of specific proteases required for degradation of endothelial basement membrane (Unemori et al. 1992), initiating the early activation stage of angiogenesis (Daniel and Abrahamson 2000). Initially characterized as an inducer of vascular leakage (Senger et al. 1983), VEGF consequently promotes the leakage of plasma proteins, leading to the formation of an extracellular fibrin gel platform that supports angiogenic branching and tumor cell growth (Dvorak et al. 1987). Following these events, endothelial cells migrate to, and subsequently invade, the vascular stroma. VEGF administration has been shown to promote the recruitment of circulating endothelial progenitor cells into sites of vascular assembly (Asahara et al. 1999) and to activate proteases required for invasion (Pepper et al. 1991).

In fact, VEGF is such a potent angiogenic factor that lone overexpression of individual VEGF isoforms results in the formation of increased vasculature. When overexpressed in keratinocytes by the keratin-6 promoter, VEGF₁₂₀ stimulates hypervascularization in the skin of transgenic mice (Larcher et al. 1998). However, the resulting tortuous vessels have abnormally large diameters and were found to be leaky and associated with swelling due to severe edema (Larcher et al. 1998). Similarly, keratin-14 promoter-driven overexpression of VEGF₁₆₄ was shown to increase the density of these same winding, hyperpermeable capillary-like vessels that lead to inflammation of the surrounding skin tissue and the appearance of hemorrhagic ulcers (Detmar et al. 1998; Thurston et al. 1999). Interestingly, when both angiogenic factors VEGF₁₆₄ and angiopoietin-1 were simultaneously overexpressed by the keratin-14 promoter, the numerous resulting vessels were no longer leaky (Thurston et al. 1999). Together these studies show that VEGF must work in conjunction with other angiogenic factors to produce stable and healthy vasculature.

VEGF expression is induced by hypoxia

Owing to the critical and varied consequences of VEGF expression, a multitude of signaling pathways regulate VEGF production. In addition to growth factors and cytokines (for reviews, see Ferrara 1999; Neufeld et al. 1999), VEGF expression is potentially up-regulated in re-

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sponse to a low-oxygen, or hypoxic, environment. Initially, VEGF mRNA levels accumulate following exposure to hypoxic conditions. Transcriptional activation of many genes by hypoxia is dependent upon the hypoxia-inducible transcription factor, HIF-1 (Semenza 1999). VEGF has been shown to be a bona fide HIF-1 target gene (Carmeliet et al. 1998; Iyer et al. 1998; Ryan et al. 1998), as the VEGF gene contains a hypoxia response element (HRE) within its promoter that is responsive to hypoxia-induced, HIF-1-mediated transcriptional activation (Forsythe et al. 1996). In addition to inducing VEGF gene transcription, hypoxic conditions promote VEGF expression through two additional posttranscriptional mechanisms. First, hypoxia promotes formation of a VEGF mRNA–protein complex within the 3′ untranslated region that enhances the stability of VEGF mRNA (Stein et al. 1995; Levy et al. 1996a,b, 1998; Damert et al. 1997; Claffey et al. 1998). Finally, the 5′ untranslated region of the VEGF transcript contains an internal ribosome entry site that may enhance VEGF translation during hypoxic stress (Akiri et al. 1998; Stein et al. 1998).

The gene-specific transcription factor HIF-1 is a central component of the hypoxia response pathway

The ability to sense and respond to changes in oxygen availability is critical for many developmental, physi-

ological, and pathological processes including angiogenesis, control of blood pressure, cerebral and myocardial ischemia, pulmonary hypertension, preeclampsia, and tumorigenesis (Semenza 2000a). In mammalian cells, exposure to a low-oxygen environment triggers an evolutionarily conserved hypoxia response pathway based on the regulated expression of HIF-1 (for review, see Semenza 1999). As shown in Figure 1, HIF-1 is an obligatory heterodimeric protein composed of two members of the basic-helix-loop-helix (bHLH)-containing PER–ARNT–SIM (PAS) domain family, HIF-1 α and the aryl hydrocarbon receptor nuclear translocator (ARNT) (Wang et al. 1995). Whereas ARNT expression is not regulated by hypoxia, HIF-1 α is constitutively expressed under normoxic conditions but is rapidly ubiquitinated, targeting the protein for degradation by the proteasome (Salceda and Caro 1997; Huang et al. 1998; Kallio et al. 1999). This regulated instability of HIF-1 α is mediated through its oxygen degradation domain (ODD) (Huang et al. 1998). The ODD contains a conserved proline residue that is hydroxylated under normoxic conditions (Ivan et al. 2001; Jaakkola et al. 2001). When hydroxylated, this proline residue is recognized by the product of the von Hippel-Lindau (pVHL) tumor suppressor gene, a component of a multisubunit ubiquitin-protein ligase (Maxwell et al. 1999; Cockman et al. 2000; Kamura et al. 2000; Ohh et al. 2000; Tanimoto et al. 2000) that polyubiqui-

Figure 1. A model for the regulation of the HIF transcription factor. HIF-1 α (or its homologs HIF-2 α and HIF-3 α) is constitutively expressed. Under normoxic conditions, a proline residue within the oxygen degradation domain (ODD) is hydroxylated by a HIF-prolyl hydroxylase. The modified HIF-1 α polypeptide is recognized by a complex containing pVHL, which targets HIF-1 α for degradation by the proteasome via a polyubiquitin tag. Exposure to a hypoxic environment blocks prolyl hydroxylation, allowing HIF-1 α to accumulate and translocate to the nucleus, where it dimerizes with its partner ARNT. Hypoxia also relieves suppression of the HIF-1 transactivation domains (CTAD and NTAD), promoting transcription of downstream HIF-1 target genes.

tinates the HIF-1 α polypeptide. Following a shift to a low-oxygen environment, prolyl hydroxylation is blocked and HIF-1 α is stabilized and subsequently translocated into the nucleus (Kallio et al. 1998).

Upon entering the nucleus, HIF-1 α dimerizes with ARNT and binds to HREs within the promoters of its downstream target genes. HIF-1 α contains two transcriptional activation domains bridged by an inhibitory domain (Jiang et al. 1997; Pugh et al. 1997). Exposure to a hypoxic environment relieves suppression of the activation domains, leading to functional association with co-activators such as CBP, p300, SRC-1, and TIF-2 (Kallio et al. 1998; Ema et al. 1999; Carrero et al. 2000) and increased transcription of target genes (for a list of target genes, see Semenza 1999). Two HIF-1 α homologs, designated HIF-2 α /endothelial PAS domain protein (EPAS) (Tian et al. 1997; Wiesener et al. 1998; O'Rourke et al. 1999) and HIF-3 α (Gu et al. 1998; Srinivas et al. 1999), appear to be regulated in a similar manner.

A great deal of work by a number of laboratories has focused on elucidating the molecular mechanism by which cells sense changes in O₂ levels and relate those changes to the regulation of the HIF transcription factors. Early evidence suggested that changes in oxygen levels might be detected by a heme-based sensor. The actual nature and identity of the oxygen sensor has remained elusive, however. Several cellular components have been proposed to sense changes in oxygen levels or serve as signaling intermediates which mediate hypoxia response pathway, including the electron transport chain, NADPH oxidoreductases, reactive oxygen species, phosphorylation cascades, NO and CO, and redox-regulated pathways (for review, see Semenza 1999). Although the relative contribution of these various components to the regulation of HIF-1 stability/activity remains unclear, it is likely that HIF activity is regulated by the integration of many signals. It was recently reported that hydroxylation of a proline residue within the ODD mediates interaction with pVHL under normoxic conditions (Ivan et al. 2001; Jaakkola et al. 2001). Prolyl 4-hydroxylases are known to modify collagen in the endoplasmic reticulum (Kivirikko and Pihlajaniemi 1998); however, these enzymes are unable to modify the ODD (Jaakkola et al. 2001). Like these well characterized enzymes, the HIF prolyl 4-hydroxylase requires Fe²⁺, ascorbate, 2-oxoglutarate, and O₂ (Ivan et al. 2001; Jaakkola et al. 2001), leaving open the possibility that the HIF-prolyl hydroxylase can serve as a direct oxygen sensor.

Overexpression of HIF-1 promotes hypervascularization with fewer defects

Elson and colleagues surmised that as a potent regulator of VEGF transcription, constitutive expression of HIF-1 might induce angiogenesis. However, moderate overexpression of HIF-1 under normoxic conditions would be expected to have little or no effect on activation of downstream target genes, as the HIF-1 α polypeptide is rapidly degraded. Indeed, placement of the entire HIF-1 α coding region behind the keratin-14 promoter resulted in no ob-

servable affect on dermal vascularization despite substantial HIF-1 α mRNA accumulation (Elson et al. 2001). Alternatively, inhibition of the HIF-1 α degradation pathway might be expected to result in HIF-1 α accumulation under normoxic conditions and subsequent transcription of HIF target genes. Transgenic mice engineered to constitutively express from the keratin-14 promoter a HIF-1 α polypeptide lacking the 200 amino acids that comprise the ODD (K14-HIF-1 α Δ ODD) featured increased levels of both VEGF and Glut-1 mRNAs as well as a 66% increase in dermal capillaries (Elson et al. 2001). Unlike the microvasculature resulting from VEGF overexpression, these perfused dermal capillaries were structurally indistinguishable from normal capillaries in both diameter and morphology. This contrasts sharply with the tortuous vasculature induced by VEGF overexpression alone (Thurston et al. 1999). Because VEGF causes blood vessel permeability, up-regulation of individual VEGF isoforms results in a leaky microvasculature, particularly when exposed to inflammatory stimuli. Despite increased levels of VEGF transcripts detected in the K14-HIF-1 α Δ ODD mice, no increase was observed in vessel leakage or in the number of leakage sites relative to nontransgenic mice, even after treatment with an inflammatory agent (Elson et al. 2001). Consequently, histopathological analysis revealed no associated edema or inflammation with the dermal vasculature (Elson et al. 2001). Unlike the K14-VEGF transgenic mice, K14-HIF-1 α Δ ODD mice did not develop hemorrhagic ulcers or skin tumors as they aged (Elson et al. 2001).

The underlying basis for the differences in vasculature quality arising from overexpression of either VEGF or HIF-1 α Δ ODD has not been determined. Individual VEGF isoforms likely play distinct roles throughout angiogenesis which cannot be fully recapitulated by a single VEGF isoform. RT-PCR analysis of the K14-HIF-1 α Δ ODD mice demonstrated an increase in the levels of all VEGF splice variants and a relative expression pattern similar to that of nontransgenic mice (Elson et al. 2001). In addition to VEGF, the HIF-1 α Δ ODD transcription factor activates the expression of other genes including *Glut-1* (Elson et al. 2001). Angiopoietin-1 and -2 are also known to be up-regulated in response to hypoxia. When co-overexpressed with VEGF in the skin, these factors promote hypervascularization without the accompanying leakage (Thurston et al. 1999). However, the resulting vasculature is morphologically distinct from normal vessels in both vessel diameter and the number of leakage sites (Thurston et al. 1999). RT-PCR analysis of the K14-HIF-1 α Δ ODD showed no increase in angiopoietin-1 and -2 message levels (Elson et al. 2001). Nevertheless, the HIF-1 transcription factor does induce transcription of other factors that influence angiogenesis, including the VEGF receptor Flt-1, nitric oxide synthase-2, endothelin-1, adrenomedullin, heme oxygenase 1, plasminogen activator inhibitor-1, and the α_{1B} -adrenergic receptor (summarized in Semenza 2000a). One or more of these factors could operate in conjunction with VEGF to produce normal, nonleaky vasculature. Whatever the mechanism, constitutive overexpression of stable HIF-1

in keratinocytes appears to be more effective in promoting hypervascularization in the skin than VEGF alone. Although HIF-induced angiogenesis in pathophysiological settings such as tumorigenesis or wound repair is often associated with vessel leakage and inflammation, these conditions are also accompanied by differences in the physical and biochemical microenvironment that may impinge upon HIF-induced angiogenesis. Therefore, growth of physiologically normal vasculature may be of therapeutic benefit when locally induced via manipulation of the HIF-dependent hypoxia-response pathway.

Lessons for therapeutic stimulation of angiogenesis

Because the oxygen supply in primary tumors is limited by diffusion, these cells adapt to the hypoxic environment by promoting the formation of new vasculature. Examination of human gliomas has demonstrated a correlation between HIF-1 expression, tumor vascularization, and tumor grade. Furthermore, loss of HIF-1 can result in tumor latency and decreased vascular density (for review, see Semenza 2000b). Consequently, much attention has been focused on the identification of inhibitors of the hypoxia-response pathway, particularly those that target VEGF expression/activity (for review, see Ferrara and Alitalo 1999; Ferrara 2000). However, patients suffering from a number of pathophysiological disorders resulting from inadequate vascularization may benefit from therapeutic strategies aimed at localized induction of angiogenesis. Preliminary studies have begun to examine the ability of exogenous VEGF to promote angiogenesis in subjects suffering from myocardial or limb ischemia (summarized in Ferrara and Alitalo 1999; Ferrara 2000). Typically, these protocols either administer recombinant VEGF or introduce plasmid DNA encoding VEGF via somatic gene therapy (Baumgartner and Isner 2001). Whereas animal models have shown promise for these therapeutic avenues, early clinical studies have been less encouraging (Ferrara 2000). As the work summarized here suggests, even successful induction of angiogenesis by a single VEGF isoform may still give rise to poor microvasculature accompanied by edema, inflammation, vascular leakage or spontaneous hemorrhagic ulcers. Administration of VEGF in combination with other angiogenic factors such as angiopoietin-1 may alleviate some of the vascular defects (Thurston et al. 1999, 2000). In addition to the practical drawbacks of administering multiple factors, much work remains in order to resolve the relative contributions and context-dependence of these factors. Of equal concern is that prolonged exposure to high levels of VEGF in the skin (Larcher et al. 1998) or myocardium (Lee et al. 2000) has been shown to accelerate tumor development. VEGF administration was also recently found to be associated with the development of atherosclerosis, possibly by promoting vessel formation or inflammation within atherosclerotic plaques (Celletti et al. 2001). The clinical significance of these observations remains unclear.

The findings presented by Elson et al. (2001) suggest an alternative strategy. Induction of a key upstream transcriptional regulator of VEGF and other proangiogenic

factors may result in a more physiological and coordinated induction of angiogenesis. Transfection of naked DNA encoding a chimeric transcription factor composed of the HIF-1 α DNA-binding domain fused to the VP16 transactivation domain in a rabbit hindlimb ischemia model led to enhanced vascular perfusion (Vincent et al. 2000). Although it may also be feasible to attempt to overexpress the constitutively stable HIF-1 α Δ ODD protein via somatic gene therapy, a much more appealing approach will be to develop small molecule inhibitors of the HIF-1 α degradation pathway. The recent discovery that hydroxylation of a proline residue within the ODD is required for VHL-mediated ubiquitination of HIF-1 α revealed an attractive target for such small molecules.

Of particular interest is the finding that a macrophage-derived polypeptide, PR39, induces enhanced formation of functional myocardial vasculature in mice (Li et al. 2000). Under normoxic conditions, PR39 administration resulted in accumulated HIF-1 α protein levels similar to those observed following exposure to hypoxia. HIF-1 α protein accumulation was due to inhibition of ubiquitin-proteasome degradation of HIF-1 α and was accompanied by a dose-dependent increase in VEGF mRNA levels (Li et al. 2000). Because PR39 is a proline-rich polypeptide (19 of its 39 residues are proline; Agerberth et al. 1991), it is possible that this peptide blocks the activity of the HIF prolyl hydroxylase. Proline-rich peptides are inhibitors of other prolyl-4-hydroxylases (for review, see Kivirikko and Pihlajaniemi 1998). However, the HIF-1 α protein that accumulates following PR39 treatment is polyubiquitinated (Li et al. 2000), indicating that the block in the degradation pathway may be downstream of the pVHL-HIF-1 α interaction. Unlike the accumulated HIF-1 α induced by proteasome inhibitors (Kallio et al. 1999), the ubiquitinated HIF-1 α resulting from PR39 treatment was translocated to the nucleus and was capable of activating transcription of downstream target genes.

Even if PR39 does not affect prolyl hydroxylase activity, inhibitors of known prolyl-4-hydroxylases, including mimics of 2-oxoglutarate, have already been shown to block prolyl hydroxylation of HIF-1 α in vitro and to stabilize the protein under normoxic conditions in vivo (Jaakkola et al. 2001). Identification of the actual HIF-prolyl hydroxylase should allow for the identification of inhibitors demonstrating greater specificity for this enzyme. Although prolonged induction of HIF-1 could also predispose patients to tumor formation, localized and transient application of inhibitors of the HIF-1 degradation pathway might produce results similar to those observed by Elson and colleagues—induction of a stable and healthy microvasculature.

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