Hypoxia Inducible Factor-1–Independent Pathways in Tumor Angiogenesis

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Abstract Among the factors that can stimulate angiogenesis, vascular endothelial growth factor has emerged as one of the most important, and inhibition of vascular endothelial growth factor has recently shown efficacy in the treatment of advanced colorectal cancer. Hypoxia develops within solid tumors and is one of the most potent stimuli of vascular endothelial growth factor expression. This effect is mediated primarily by hypoxia inducible factor-1 (HIF-1), often considered a master regulator of angiogenesis in hypoxia. Consequently, inhibition of HIF-1 has been proposed as a strategy to block tumor angiogenesis therapeutically. However, accumulating evidence indicates that HIF-independent pathways can also control angiogenesis. This review highlights some of the key signaling pathways independent of HIF-1 that can stimulate angiogenesis in hypoxia. Understanding the full spectrum of molecular pathways that control tumor angiogenesis is critical for the optimal design of targeted therapies.

Background

Angiogenesis is a hallmark feature of human malignancies. The induction of vascular endothelial growth factor (VEGF) is an essential component of tumor angiogenesis, and this is mediated by multiple interacting genetic and environmental signals (1). Oncogenic mutations that are critical for the tumorigenic process in general can stimulate VEGF in particular, and hypoxia dramatically enhances this up-regulation. Hypoxia inducible factor-1 (HIF-1) is a primary regulator of VEGF during hypoxic conditions.

HIF-1 is a heterodimeric basic helix-loop-helix transcription factor composed of two subunits, HIF-1α and HIF-1β (ARNT), and HIF-1α is the key regulatory component (2). In the presence of oxygen, HIF-1α is hydroxylated on conserved prolyl residues within the oxygen-dependent degradation domain by prolyl hydroxylases and binds to von Hippel-Lindau protein, which in turn targets it for degradation through the ubiquitin-proteasome pathway (3). However, in hypoxic conditions, prolyl hydroxylase is inactive, resulting in stabilization of HIF-1α. HIF-1 transcriptional activity is also enhanced in hypoxia by "factor-inhibiting HIF-1," an oxygen-sensitive enzyme that hydroxylates asparagine residues at the C-terminal transactivation domain of HIF-1α to displace p300/CBP coactivator proteins (4). The HIF-1 complex recognizes a consensus hypoxia response element in the promoter of a broad range of target genes, including VEGF, platelet-derived growth factor, and transforming growth factor-α, that mediate hypoxic responses including angiogenesis. Early xenograft studies of embryonic stem cells from *HIF-1α*$^{-/-}$ mice showed that VEGF levels and markers of vascularization were significantly reduced (5), indicating a key role for HIF-1 in angiogenesis.

Angiogenesis Is Preserved in HIF-1–Deficient Tumor Xenografts

To better delineate the role of HIF-1 in human tumors, *in vivo* xenograft studies using cells, in which HIF-1 was targeted genetically, have been done. Knockdown of HIF-1α through small interfering RNA in a DLD-1 colon cancer cell xenograft reduced tumor growth but surprisingly did not block tumor angiogenesis (6). The microvessel density of HIF-1–deficient xenografts was equivalent to control xenografts expressing HIF-1 (26.1 ± 6.3 per field versus 28.7 ± 8.6 per field, respectively). In addition, microvessel perfusion, as visualized by intravascular lectin, was not altered in HIF-1–deficient tumors. Surprisingly, the induction of VEGF was not abrogated in HIF-1–deficient xenograft tissue or in HIF-1–deficent cells *in vitro*. VEGF levels were reduced ~50%, indicating that substantial amounts of VEGF were still produced. Independent studies of HIF-1α$^{-/-}$ ES cells confirmed that angiogenesis, as measured by microvessel density, was preserved when HIF-1α was knocked-out. There was an ~50% decrease in VEGF mRNA levels, but no significant changes in VEGF protein levels were observed (7).

The persistent expression of VEGF can potentially explain the preservation of the angiogenic phenotype, but an additional angiogenic factor, interleukin 8 (IL-8), was found to be induced specifically in these HIF-1–deficient tumors (6). When neutralizing antibodies to IL-8 were given to mice bearing HIF-1–deficient tumors, there were reductions in microvessel density

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(from 27.5 ± 3.2 per field to 14.7 ± 3.5 per field) and diameter (from 22.4 to 5.9 μm), indicating the functional significance of IL-8 in the regulation of angiogenesis. A lectin perfusion study verified that vascular integrity was severely compromised in HIF-1 – deficient xenografts when IL-8 was simultaneously blocked, and vessels were markedly narrowed and fragmented. These findings suggested that HIF-1 was not the only factor regulating angiogenesis in hypoxia. Furthermore, the angiogenic response seemed to be highly adaptable, as targeting a single angiogenic factor resulted in the induction of an independent factor. Thus, combinations of antiangiogenic agents that target different factors may be necessary to offset such compensatory responses and maximize therapeutic outcomes.

The Ras Oncogene and HIF-1 – Independent Regulation of VEGF

The specific molecular pathways that underlie the HIF-1 – independent regulation of VEGF have begun to be elucidated, and the RAS oncogene seems to play a pivotal role (Fig. 1). This was first shown in *H-RAS* transformed embryonic fibroblasts from *HIF-1α*$^{-/-}$ mice (8). Surprisingly, angiogenesis in these "tumors" was well preserved, implying that oncogenic *H-RAS* can compensate for the loss of HIF to maintain angiogenesis *in vivo*. In the absence of HIF-1α, the hypoxic induction of hypoxia-responsive genes, such as glucose tranporter-1 and phosphoglycerate kinase was abolished, but the induction of VEGF was still observed. Mouse hepatoma cells deficient in ARNT, the binding partner of HIF-1α, display a persistent hypoxic induction of VEGF mRNA, again indicating that pathways independent of HIF-1 may regulate VEGF in epithelially derived cancer cells (9). Subsequent studies have shown the specific role of the *K-RAS* isoform in human cancer cells using small interfering RNA against HIF-1α and site-directed mutagenesis of HIF-1 binding sites in the human VEGF promoter (10).

Several mechanisms for RAS-mediated regulation of VEGF in hypoxia have been proposed. Oncogenic *K-RAS* in combination

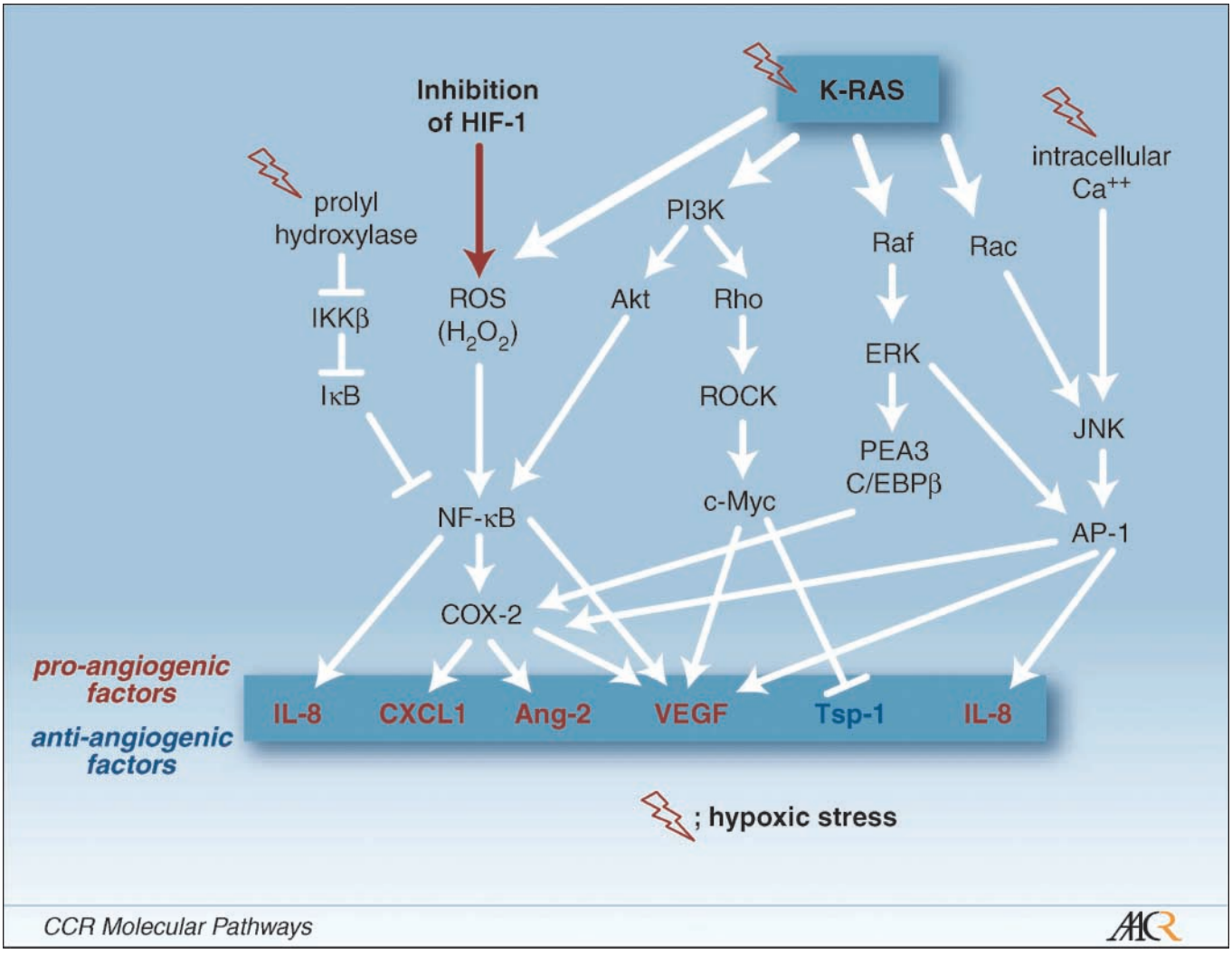

Fig. 1. Signal transduction pathways that can stimulate angiogenesis independently of HIF-1. Oncogenic Ras plays a central role in these HIF-1 – independent pathways; multiple effector pathways, including PI3K, Raf, and Rac, can be induced by Ras, which in turn stimulate transcription factors through protein kinases to up-regulate proangiogenic factors and down-regulate antiangiogenic factors. It should be noted that many of these factors can be synergistically regulated by hypoxia and Ras. In addition, NF-kB is induced by hypoxia specifically through accumulation of hydrogen peroxide when HIF-1 is blocked, and this compensatory pathway plays an important role to maintain angiogenesis in the absence of HIF-1 by up-regulating IL-8. Ang-2, angiopoietin-2; CXCL-1, CXC1 chemokine ligand-1; ERK, extracellular signal-regulated kinase; H_2O_2, hydrogen peroxide; IkB, inhibitor of NF-kB; IKKb, IkB kinase; JNK, c-Jun kinase; ROCK, Rho kinase; Tsp-1, thrombospondin-1.

with hypoxia can synergistically up-regulate VEGF (10). Hypoxia can activate multiple RAS effector pathways, including extracellular signal-regulated kinase, c-Jun-NH_2 kinase, p38, Akt, and Rho. Among these pathways, extracellular signal-regulated kinase and Akt, but not c-Jun-NH_2 kinase and p38, were activated by hypoxia in colon cancer cells (11). Akt is a major down-stream target of phosphoinositide-3 kinase (PI3K), and inhibition of PI3K either by a dominant negative construct or specific inhibitors, such as LY-294002, strongly down-regulated the hypoxic induction of VEGF. Despite the crucial role of Akt in the regulation of VEGF under normoxic conditions (12, 13), inhibition of Akt did not attenuate the hypoxic induction. Instead, an alternative target of PI3K, the Rho/Rho kinase pathway, mediated the hypoxic induction of VEGF (11). The combination of hypoxia and oncogenic *K-RAS* synergistically increased levels of GTP-bound Rho via PI3K. Induction of Rho by hypoxia has also recently been shown in endothelial cells (14), indicating a universal role of Rho signaling in hypoxic stress. The synergistic up-regulation of the VEGF promoter by hypoxia and *K-RAS* was attenuated by the inhibition of either PI3K or Rho kinase, and this was observed in HIF-1 deficient colon cancer cells.

VEGF promoter reporter assays and electrophoretic mobility shift assays identified a *cis*-regulatory element that was responsive to signaling by PI3K/Rho/Rho kinase. This pathway converged on the c-Myc oncogene (11). Hypoxia induced phosphorylation of c-Myc through PI3K/Rho/Rho kinase, and this was required for the induction of VEGF in the absence of HIF-1. A role for c-Myc in angiogenesis has been previously illustrated by the widespread vascular abnormalities seen in Myc-deficient mouse embryos (15) and by a pancreatic β-cell carcinogenesis model in which the angiogenic switch is regulated by Myc through induction of IL-1β (16). Hypoxia and c-Myc are linked to the regulation of VEGF through the activation of a novel *cis*-regulatory element in the VEGF gene promoter (11, 17). As an aside, signaling through this PI3K/RHO/Rho kinase/MYC pathway can simultaneously down-regulate thrombospondin-1, an endogenous antiangiogenic factor (18).

Another important transcription factor that can mediate hypoxic responses is nuclear factor-κB (NF-κB). The mechanism of NF-κB activation by hypoxia is not straightforward. Changes in the redox potential in hypoxic cells due to the generation of reactive oxygen species (ROS) in mitochondria can result in NF-κB activation (19). In addition, activation of NF-κB during hypoxic conditions can be mediated by decreased prolyl hydroxylation and subsequent degradation of IκB kinase-β (20). Finally, NF-κB can also be activated by oncogenic RAS, primarily through PI3K-Akt (21).

Previous studies have clearly shown that NF-κB can regulate VEGF transcription (22–24). Curiously, analyses of the VEGF promoter have not identified consensus and functional κB sites (25, 26), and NF-κB may regulate VEGF indirectly through other transcription factors. An essential role of activator protein-1 in NF-κB–dependent regulation of VEGF has been described (26), and there are at least two activator protein-1 sites involved in the hypoxic induction of VEGF transcription that do not depend upon HIF-1 (27). However, there is a complex link between NF-κB and HIF-1, and other studies have shown NF-κB–dependent induction of HIF-1. For example, NF-κB can induce HIF-1 when cells are stimulated by IL-1β (28), lipopolysaccharide (28), or ROS (29). Thus, NF-κB seems to induce VEGF through both HIF-1–dependent and HIF-1–independent mechanisms. As discussed later, a more critical role for NF-κB may be its induction of the angiogenic factor IL-8, and this process is HIF-1 independent (6).

Activated RAS can also control VEGF protein activity. RAS can stimulate the expression of several proteases, including matrix metalloproteases (matrix metalloproteinase-2 and matrix metalloproteinase-9) and urokinase-type plasminogen activator. As a consequence, the cellular release and activation of VEGF protein is enhanced, thereby increasing its extracellular levels (30). The hypoxic regulation of matrix metalloproteinase, urokinase-type plasminogen activator, and VEGF expression may also be independently mediated by the nonreceptor tyrosine kinases Syk and Lck (31).

HIF-1–Independent Activation of Angiogenic Factors other than VEGF

Although VEGF is one of the primary angiogenic factors induced in tumors, there are additional factors that play important roles, many of which do not depend upon the activity of HIF-1. For example, oncogenic *H-RAS* can induce IL-8, a potent angiogenic factor, through PI3K/Akt/NF-κB and Raf/extracellular signal-regulated kinase/activator protein-1 (32). In human ovarian cancer cells, hypoxia induces IL-8 through activation of NF-κB that is controlled by RAS-effector pathways including PI3K/Akt and p38 (33).

In colon cancer cells deficient in HIF-1, a strong induction of IL-8 was observed. This induction of IL-8 was mediated by the enhanced production of ROS under hypoxia and subsequent activation of NF-κB. Hypoxic conditions can lead to the increased production of ROS, and scavenging of H_2O_2 is often achieved by the increased production of pyruvate that occurs when cells shift from oxidative to glycolytic metabolism (34). Importantly, this shift depends upon HIF-1α (35, 36). The induction of NF-κB was blocked by ROS inhibitors. Exogenous administration of H_2O_2 stimulated the induction of IL-8, which was blocked by the NF-κB inhibitor BAY 11-7082. Furthermore, the *K-RAS* oncogene, which is commonly mutated in colon cancer, plays a critical role in this pathway. Knockdown of oncogenic *K-RAS* strongly attenuated the hypoxic induction of NF-κB reporter activity and IL-8 mRNA in HIF-1–deficient cells. In Caco2 cells that carry a wild-type *K-RAS* gene, expression of mutant *K-RAS* enhanced the induction of NF-κB by ROS. As an aside, NF-κB can also contribute to the induction of placental growth factor in RAS-transformed embryonic fibroblasts in concert with metal responsive transcription factor-1 in hypoxia (37).

Cyclooxygenase-2 (COX-2) is another key mediator of angiogenesis, and it can be induced both by RAS and hypoxia. Although HIF-1 can play an important role in the induction of COX-2 in hypoxic conditions (38), hypoxia can also up-regulate COX-2 through HIF-1–independent pathways including NF-κB (39). In addition, RAS acts through Rac/c-Jun-NH_2 kinase to phosphorylate c-Jun (activator protein-1) and Raf/extracellular signal-regulated kinase to activate CCAAT/enhancer binding protein β (CAAT/enhancer binding protein β) and/or Ets transcription factor PEA3, all of which are key regulators of COX-2 expression (40, 41). RAS effectors also play a role in stabilizing COX-2 mRNA (42). COX-2 mediates most of its

proangiogenic effects through the induction of prostaglandin E2. Although prostaglandin E2 can signal through HIF-1 (43), it can also activate a variety of other pathways including mitogen-activated protein kinase and PI3K/Akt that can potentially induce VEGF (44, 45). Furthermore, prostaglandin E2 can induce other angiogenic molecules including CXCL1, a proangiogenic chemokine (46), as well as the vascular remodeling protein angiopoietin-2. This induction of angiopoietin-2 is stimulated by hypoxia and does not depend upon HIF-1 (47). Thus, there are multiple pathways that are both HIF-dependent and HIF-independent that regulate the angiogenesis mediated by COX-2 and prostaglandin E2.

Clinical-Translational Advances

Although disrupting the function of a transcription factor is a challenge, several potential approaches to inhibit HIF-1 for therapeutic purposes have been identified (48). Echinomycin and polyamides seem to block binding of HIF-1 to its DNA hypoxia response element (49, 50), and chetomin can block the interaction of HIF-1 with CBP/p300 (51). These reagents have shown promising antitumor effects in preclinical studies. Other compounds that have been tested *in vitro* including heat shock protein 90 inhibitor 17-allyl-aminogeldanamycin and endogenous metabolite of estrogen, 2ME2, seem to function by targeting signaling pathways that activate HIF (52, 53). Phase II trials of 17-allyl-aminogeldanamycin in a wide variety of solid and hematologic malignancies are under way, and phase I trials of the closely related compound 17-dimethylaminoethylamino-17-demethoxygeldanamycin have also been initiated. Some of these phase II trials will correlate the RAS mutation status with clinical outcome. Because these compounds have multiple functions in addition to the inhibition of HIF-1, it may be a challenge to assess the relative contribution of HIF-1 inhibition to the observed therapeutic effects. Nevertheless, it will be critical to determine which HIF-1–independent mechanisms may be responsible for adaptive angiogenic responses that emerge.

In addition to the preclinical data previously discussed, several independent observations support the concept that acquired resistance to single-agent antiangiogenic therapy can develop. In a mouse model of pancreatic islet tumorigenesis, inhibition of VEGF receptors 1 and 2 with monoclonal antibodies initially blocked angiogenesis and tumor growth. However, tumor regrowth accompanied by revascularization was observed at 4 weeks. This resistance to anti-VEGF receptor therapy was characterized by the compensatory up-regulation of VEGF-A, the fibroblast growth factors (FGF) FGF1, FGF2, FGF7, and FGF8, ephrin-A1, and angiopoietin-2. The functional significance of these FGFs was verified when the addition of a neutralizing FGF trap then resulted in regression of tumor growth and angiogenesis (54). Similarly, in a clinical trial of 10 rectal cancer patients treated with bevacizumab alone, significant decreases in blood flow and microvessel density were observed after a 2-week period. Not surprisingly, there was a strong induction of plasma VEGF levels after the introduction of neutralizing VEGF antibody. However, plasma levels of placental growth factor, another angiogenic factor that binds to VEGF receptor 1, increased nearly 3-fold after treatment (55). These observations underscore the importance of compensatory angiogenic responses. Ultimately, the most successful antiangiogenic approaches may require combinations of agents that simultaneously target these adaptive pathways. In particular, inhibition of HIF-1–dependent, as well as HIF-1–independent pathways, may prove to be a compelling strategy.

Concluding Remarks

Targeting tumor angiogenesis is an effective component of the treatment strategy for cancer patients. The regulation of angiogenesis is a complex interplay between tumor genotype and the environment. Hypoxia is a universal feature of solid tumors, and one nearly ubiquitous factor that seems to mediate the hypoxic regulation of angiogenesis is HIF-1. Although HIF-1 is clearly an important therapeutic target, there are multiple pathways other than HIF-1 that can respond to hypoxia. Furthermore, *in vivo* studies have introduced the concept that alternative angiogenic pathways can be induced when a single factor, such as HIF-1, is blocked. Collectively, these observations underscore the complexity and diversity of the tumor angiogenic response. Delineation of the full spectrum of angiogenic mechanisms that are HIF-dependent, as well as HIF-independent, is therefore a prerequisite to the design of optimal combinations of antiangiogenic agents.

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