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Angiogenic signaling aberrantly induced by tumor hypoxia

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1. ABSTRACT

Tumor growth, invasion and metastasis are largely dependent on the development of tumor vasculature. A great number of pro- and antiangiogenic molecules, have been identified. Bone marrow-derived cells are mobilized and recruited to angiogenic sites, by a variety of growth factors and cytokines, to promote angiogenesis and the formation of new blood vessels. The hypoxic microenvironment that is inevitably generated in solid tumors is a major contributor to tumor angiogenesis. Tumor hypoxia aberrantly modulates the expression of many potent pro- and antiangiogenic molecules, primarily through the action of heterodimeric transcription factors

termed hypoxia-inducible factors, HIF-1 and HIF-2. The disruption of the balance between pro- and antiangiogenic activities eventually leads to a shift in balance to a more angiogenic state. These findings have provoked considerable interest in HIFs as attractive targets for cancer therapy. Consequently, the development of small molecule HIF inhibitors is currently moving ahead at a fast pace.

2. INTRODUCTION

In the early phase of tumor progression, a tumor lies in a dormant state in an avascular area where the rate of cell proliferation is in balance with that of cell apoptosis (1). Subsequently, a transition from a non-angiogenic state to an angiogenic state occurs. This phenomenon has been called the angiogenic switch (2). In addition to genetic changes, one of the crucial factors implicated in the triggering of the angiogenic switch is the tumor microenvironment in which tumor cells are placed and interact with cellular, humoral and extracellular components in this environment.

Tumor vascular development involves two complicated processes. The one process is local sprouting (angiogenesis), in which tumor- and stromal-cell–derived angiogenic factors promote nascent capillary formation by endothelial cells in pre-existing blood vessels. The other process is the formation of new blood vessels (vasculogenesis), in which endothelial progenitor cells (EPCs) are mobilized and recruited from the circulation to tumor angiogenic sites where they differentiate into endothelial cells. Several other bone marrow-derived cells (BMDCs), such as monocytes, which eventually differentiate into tumor-associated macrophages (TAMs), are also recruited to further accelerate active angiogenesis/vasculogenensis and promote invasion and metastasis (3).

Unlike normal microvessels, tumor microvessels are structurally and functionally abnormal because of the imbalance between pro- and antiangiogenic factors (4, 5). Tumor microvessels characteristically have irregular branching and uneven diameters. They lack a normal basement membrane and have increased permeability. The associated perivascular cells also have abnormal morphology. Tumor endothelial cells have an abnormal karyotype and respond to epidermal growth factor (EGF) sensitively, which is not the case in normal endothelial cells (6). Despite being disorganized, chaotic and poorly functioning, tumor microvessels ensure the delivery of oxygen, nutrients, and growth factors to tumor cells to some extent, and they facilitate the hematogenous spread of tumor cells. The microvessel density in primary tumors is significantly associated with metastasis and poor prognosis in several tumor types, including carcinomas of breast and prostate (7), and in murine tumor models (8, 9).

Despite active angiogenesis, tumor growth often exceeds the speed of formation of new blood vessels. Aberrant microvessels are often crushed by high interstitial pressure in tumor tissues. Rapidly proliferating tumor cells show a high rate of oxygen consumption, and oxygen concentration decreases in the distant lesion from local capillaries as tumor cells increase. Together, these factors cause an inadequate supply of oxygen (hypoxia), and most solid tumors possess both acute and chronic hypoxia areas. A hypoxic microenvironment affects tumor cell characteristics in many ways including glucose metabolism, angiogenic and invasive/metastatic potential, and resistance to chemotherapy and radiotherapy (10–13). A vast number of reports have shown that hypoxia-inducible factor-1 (HIF-1) is the key regulatory transcription factor in these hypoxia-induced processes. Hypoxia is also known to activate HIF-independent signaling pathways (14).

This review focuses on the recently reported regulatory mechanisms of HIF-1 activity, summarizes the hypoxia-inducible tumor angiogenesis-related molecules whose expression is regulated by HIF-dependent and -independent mechanisms. In addition, the prospect of HIF inhibitors as antiangiogenesis and anticancer drugs is discussed in brief.

3. HYPOXIA-RESPONSIVE TRANSCRIPTION FACTORS INVOLVED IN TUMOR ANGIOGENESIS

3.1. Hypoxia-inducible factor (HIF)

HIFs are composed of the HIF-alpha subunit and the HIF-beta subunit (also known as aryl hydrocarbon receptor nuclear translocator, ARNT). HIF-1alpha was initially identified by Semenza *et al* (10), and HIF-2alpha (also known as EPAS1) and HIF-3alpha (also known as IPAS) were subsequently identified (15, 16). Under hypoxic conditions, HIF-1alpha activity is primarily regulated at the levels of protein stability and transactivation while HIF-1beta is constitutively expressed. In addition to hypoxia, HIF-1alpha protein and mRNA levels are also controlled by many other factors such as dysregulation of growth factors and their cognate receptors, loss-of-function mutations in tumor suppressor genes, gain-of-function mutations in oncogenes (10), specific microRNA (miRNA) levels (17), HIF-1alpha gene dosage (18), mitochondrial DNA mutations (19), viral components (20), heat shock protein 90 (HSP90) and the receptor of activated protein C kinase (RACK1) (21). Since there are many excellent reviews concerning the molecular mechanisms underlying the regulation of HIF activity (10–13), I focus on the hypoxia-dependent regulation of HIF-1alpha activity here.

Under normoxic conditions, HIF-1alpha is hydroxylated at the proline residues Pro^{402} and Pro^{564} by specific Fe^{2+}-, oxoglutarate- and oxygen-dependent prolyl hydroxylases (PHD1, 2 and 3). The hydroxylated subunit is then recognized and ubiquitinated by an E3 ubiquitin ligase complex consisting of the tumor suppressor von Hippel-Lindau (pVHL), elongin B and elongin C, and is rapidly degraded through the ubiquitin-proteasome pathway. Under hypoxic conditions, PHD activity is reduced and the HIF-1alpha protein is stabilized, which allows its nuclear translocation and dimerization with HIF-1beta. In the nucleus, HIF-1 binds to hypoxia response elements (HREs), which contain the core binding sequence 5’-

(A/G)CGTG-3', in hundreds of hypoxia-inducible genes including those described below, and transactivates their transcription. HIF-1alpha is also hydroxylated at the asparagine residue Asn^{803} under normoxic conditions by an asparaginyl hydroxylase, factor inhibiting HIF-1 (FIH-1), thereby inhibiting the interaction between HIF-1 and its co-activator cyclic AMP response element binding protein (CREB)-binding protein (CBP)/p300. This leads to the repressed activity of HIF-1alpha transcriptional activity (22). Kato *et al* (23) have reported that histone deacetylase 7 (HDAC7) forms a complex with HIF-1alpha and CBP/p300 in hypoxia, thereby enhancing the transcriptional activity of HIF-1.

The modification of HIF-1alpha protein by the small ubiquitin-related modifier (SUMO) (SUMOylation) has been demonstrated. However, whether this process increases or decreases HIF-1alpha stability remains controversial. Bae *et al* (24) reported that HIF-1alpha is SUMOylated at Lys^{391} and Lys^{477}, enhancing HIF-1alpha stability. Carbia-Nagashima *et al* (25) showed that RSUME, a SUMOylation enhancer containing a RWD domain, is induced by hypoxia and enhances the SUMOylation of HIF-1alpha, thereby promoting its stabilization and transcriptional activity during hypoxia. In contrast, Chen *et al* (26) reported that hypoxia-induced SUMOylation of HIF-1alpha at Lys^{391} and Lys^{477} promotes its ubiquitination and degradation in a proline hydroxylation-independent manner, and that SENP1, a nuclear sentrin/SUMO-specific protease 1, de-conjugates SUMOylated HIF-1alpha, and stabilizes HIF-1alpha during hypoxia. Thus, the role of SUMOylation is ill-defined and awaits further studies.

The activity of sphingosine kinase 1 (SphK1), an oncogenic lipid kinase that converts sphingosine to sphingosine 1-phosphate, is stimulated by hypoxia via a reactive oxygen species (ROS)-dependent mechanism and stabilizes HIF-1alpha (27). The SphK1-dependent stabilization of HIF-1 levels relies on the phosphorylation of Akt and glycogen synthase kinase-3beta (GSK-3beta). In pVHL-deficient renal cancer cells, HIF-1 level was not influenced by SphK1, suggesting that SphK1 drives HIF-1 accumulation through a pVHL-dependent mechanism (27), though it is not clear how SphK1/Akt/GSK-3beta signaling pathway leads to the prevention of pVHL-dependent degradation of HIF-1alpha.

HIF-2alpha protein stability is also regulated by the hydroxylation at Pro^{405} and Pro^{531} by PHDs and by pVHL-mediated ubiquitination. Unlike HIF-1alpha, which is expressed in all cell types and tissues, HIF-2alpha expression is restricted to certain cell types such as endothelial cells and bone marrow macrophages, indicating that it has a different role in cellular physiology (11, 12). Supporting this notion, HIF-1 and HIF-2 have distinct transcriptional targets, even though HIF-2 also binds to the HREs of hypoxia-inducible genes (28). Importantly, a recent study has demonstrated that HIF-2alpha, but not HIF-1alpha, promotes the autonomous proliferation of genetically and pathologically diverse cancer cells through the activation of receptor tyrosine kinases including EGF receptor (EGFR) and insulin like growth factor 1 receptor (IGF1R), as well as by downstream ERK/Akt signaling (29).

In contrast to HIF-1alpha and HIF-2alpha, much less is known about HIF-3alpha. At present, HIF-3alpha is thought to act as a competitive inhibitor that blocks dimerization of HIF-1alpha and HIF-2alpha with HIF-1beta (11, 12).

3.2. Nuclear factor-kappaB (NF-kappaB)

NF-kappaB can mediate the hypoxic response via a HIF-independent pathway. One of the mechanisms underlying NF-kappaB activation by hypoxia is the change in redox state in hypoxic cells caused by the generation of ROS in mitochondria (30). ROS activate NF-kappaB transcriptional activity primarily through the classical IkappaB-kinase (IKK)-dependent pathway, though the molecular mechanisms leading to IKK activation are cell-type specific (31). NF-kappaB activity is also increased by the activation of IKKbeta, a subunit of IKK, through the inhibition of PHD1. Cummins *et al.* (32) reported that IKKbeta, a subunit of IKK, is negatively regulated by hydroxylation at Pro^{191} by PHD1. Hypoxia activates IKK by decreasing PHD1 activity, leading to phosphorylation-dependent degradation of IkappaBalpha, an inhibitor of NF-kappaB (32). Cross-talk exists between the HIF-1alpha and NF-kappaB signaling pathways; that is, on the one hand NF-kappaB acts as a critical transcriptional activator of HIF-1alpha, and on the other hand basal NF-kappaB activity is required for HIF-1alpha protein accumulation under hypoxia (33). Supporting the existence of cross-talk, there is a canonical NF-kappaB binding site in the HIF-1alpha promoter (34). As discussed later, NF-kappaB plays a critical role in the expression of several molecules important for tumor angiogenesis.

3.3. Other hypoxia-responsive transcription factors

Besides HIF and NF-kappaB, hypoxia activates other transcription factors such as CREB, activating protein-1 (AP-1), specificity protein (SP)1, SP3 and early growth response protein (EGR)-1. These transcription factors also take part in regulating the expression of angiogenesis-related molecules. These have been extensively reviewed elsewhere (14).

4. REGULATION OF TUMOR ANGIOGENESIS-RELATED MOLECULES BY HYPOXIA

Under hypoxic conditions, some tumor angiogenesis-related molecules are directly or indirectly regulated by HIF-1 or HIF-2, or by both HIF-1 and HIF-2. These molecules, together with other molecules that are induced in a HIF-independent manner, are summarized in Table 1.

4.1. Proangiogenic factors and mediators

4.1.1. Vascular endothelial growth factor (VEGF)

VEGF, also referred to as VEGF-A, is the most well-known and critical tumor angiogenic factor, and has a strong mitogenic effect on vascular endothelial cells (35, 36). VEGF not only stimulates growth of endothelial cells

Table 1. Tumor angiogenesis-related molecules regulated by hypoxia

Angiogenesis-related molecules	Hypoxia-responsive transcription factor	Recruitment of BMDCs to tumor site	References
Proangiogenic factors and mediators			
Vascular endothelial growth factor (VEGF)	HIF-1, HIF-2, NF-kappaB	Monocyte, VEGFR1$^+$ hemangiocyte, EPC	35, 37-39
Angiopoietin-2 (Ang-2)	HIF-1	TEM	46, 52, 164, 166
Platelet-derived growth factor-B (PDGF-B)	HIF-1 (?)		55, 56
Placental growth factor (PlGF)	NF-kappaB	EPC	59, 172
Basic fibroblast growth factor (bFGF)	?		61
Stem cell factor (SCF)	HIF-1		62
Osteopontin (OPN)	?		68
Adrenomedullin (AM)	HIF-1		72, 73
Endothelin (ET)	HIF-1		77
Semaphorin 4D (Sema4D)	HIF-1		83
Cyclooxygenase-2 (COX-2)	HIF-1, NF-kappaB		87, 88
Membrane type 1-matrix metalloproteinase (MT1-MMP)	HIF-2		92
Plasminogen activator inhibitor-1 (PAI-1)	HIF-1		95
Nitric oxide synthase (NOS)	HIF-1		100
Endosialin	HIF-2 (Ets1)		104
Adenosine A2A receptor	HIF-2		108
Oxygen-regulated protein-150 (Orp150)	?		109
Chemokines			
Stromal-derived growth factor 1 (SDF-1)	HIF-1	Monocyte, VEGFR1$^+$ hemangiocyte	114, 116, 170
Interleukin-8 (IL-8)	NF-kappaB (HIF-1, AP-1)	Neutrophil	120, 121
Chemokine (C-C motif) ligand 11 (CCL11)	?	Eosinophil	126, 130
Endothelial-monocyte-activating polypeptide II (EMAP-II)	?	Monocyte	134
Antiangiogenic factors and mediators			
$VEGF_{xxx}b$	HIF-1, HIF-2, RNA splicing factors		136, 141, 142
Delta-like ligand 4 (Dll4)	HIF-1		143
Vasohibin-1	?		150
Thrombospondin-1 (TSP-1)	?		153, 156
Regulator of G protein signaling 5 (RGS5)	HIF-1		157

BMDC: bone marrow-derived cells, TEM; Tie2-expressing monocyte, EPC: endothelial progenitor cell

but also promotes their survival and motility. There are at least five known VEGF isoforms in humans, $VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{189}$, and $VEGF_{206}$, which are generated by alternative splicing of exons 6 and 7 of the VEGF gene. The different isoforms have different heparin binding affinities and perform distinct roles in tumor angiogenesis (37). VEGF binds to the receptors, VEGFR1 (Flt-1) and VEGFR2 (Flk-1), which are expressed on endothelial cells. Upon binding of VEGF to VEGFR, several signaling pathways, including the Akt/PI3K/MAPK pathway, are initiated and result in endothelial cell proliferation, migration and invasion. Furthermore, tumor-associated VEGF recruits large numbers of primary monocytes from the circulation to the tumor tissue, and these monocytes eventually differentiate into TAMs (35, 37). VEGF also enhances the mobilization and supports the survival of circulating EPCs (38). The VEGF promoter contains a HRE, and both HIF-1 and HIF-2 activate the transcription of the gene under hypoxic conditions (39). VEGF expression is also regulated by the miRNAs, miR-15b, miR-16, miR-20a, and miR-20b, which bind to the VEGF 3'-UTR and repress its expression. Under hypoxic conditions, the expression of these miRNAs is markedly down-regulated; this might be mediated by the accumulation of p53, or the stabilization of HIF-1alpha, causing the up-regulation of VEGF (40). HIF-1 also up-regulates the VEGFR1 gene whose promoter contains a HRE, but not the VEGFR2 gene (41). Thus, the HIF-1/VEGF/VEGFR1 axis plays a key role in promoting tumor angiogenesis.

VEGF also binds to the VEGFR coreceptor neuropilin-1 (NRP-1). Upon binding of VEGF, NRP-1 interacts with VEGFR2 to mediate vascular development. Blocking NRP-1 function with a monoclonal antibody against the VEGF-binding domains of NRP-1 reduces angiogenesis and vascular remodeling (42).

Lee *et al* (43) reported that progressive endothelial degeneration occurs in mice bearing a deletion of the VEGF gene specifically in endothelial cells. In these mice, no detectable changes in the whole body levels of VEGF mRNA or protein were detected, indicating that the autocrine-acting VEGF–VEGFR-2 signaling pathway mediates endothelial cell survival and vascular homeostasis.

4.1.2. Angiopoietin-2 (Ang-2)

Angiopoietins, Ang-1 and Ang-2 are ligands of the endothelial cell-specific receptor tyrosine kinase 2, Tie2. Ang-1, which is widely expressed in normal tissues in adult humans, promotes angiogenesis, recruits pericytes, and stabilizes vessels (44). Ang-2 is a natural antagonist of Tie2 and disrupts *in vivo* angiogenesis (45). Hypoxia up-regulates the expression of both VEGF and Ang-2 (46), and VEGF itself stimulates Ang-2 expression (47). Therefore, the concomitant up-regulation of Ang-2 and VEGF has been observed in many tumors including human colorectal cancer cells (46-48). VEGF and Ang-2 play a pivotal role in tumor angiogenesis. In the absence of significant level of VEGF, Ang-2 leads to endothelial apoptosis and vessel regression (49). In the presence of VEGF, however, Ang-2 shows proangiogenic activity and promotes endothelial cell

migration and invasion (47, 49). Thus, VEGF and Ang-2 act complementarily and coordinately during tumor angiogenesis. Ang-2 inhibits VEGF expression at both the mRNA and protein levels in Tie2-expressing glioma cells by decreasing HIF-1alpha expression and HIF-DNA-binding activity (50). Further investigations are required to clarify whether this is also the case in endothelial cells. Recently, Simon *et al* (51) demonstrated that hypoxia up-regulates Ang-2 in human microvascular endothelial cells through HIF-1 binding to a HRE located in the first intron of the Ang-2 gene (52). Ang-2 gene expression is also up-regulated by the transcription factor ETS-1, the expression of which is regulated by HIF-1 (53).

4.1.3. Platelet-derived growth factor B (PDGF-B)

PDGF-B and its receptor PDGFR are overexpressed in tumors including gliomas, and these proteins influence tumor angiogenesis by mediating VEGF activity. When human glioblastoma cells are implanted into nude mouse brains, exogenous overexpression of PDGF-B enhances intracranial glioma formation by stimulating VEGF expression in neovessels and by attracting vessel-associated pericytes. As a result of pericyte recruitment, these neovessels induced by VEGF in the tumor vicinity migrate into the central region of the tumor (54). Hypoxia up-regulates PDGF-B expression in glioma, bladder carcinoma and hepatocellular carcinoma cells (55, 56). Short interference RNA (siRNA) targeting HIF-1alpha mRNA inhibits hypoxia-induced PDGF-B expression at the transcriptional level in glioma cells (55). However, one report indicates that a putative HRE in the PDGF-B promoter is not sufficient for hypoxic induction (56). Therefore, the exact molecular mechanisms underlying hypoxia-regulated PDGF-B expression remain to be determined.

4.1.4. Placental growth factor (PlGF)

PlGF is a member of the VEGF family, and it primarily binds to the receptor VEGFR1, on endothelial cells. PlGF is expressed in a variety of tumors including breast (57), and non-small cell lung cancers (58). PlGF expression is induced by hypoxia, but the mechanism of induction is poorly defined. A complex of metal response element-binding transcription factor 1 (MTF-1) and NF-kappaB is involved in PlGF induction in hypoxic cells (59). PlGF promotes the release of endothelin-1 (ET-1) from human pulmonary microvascular endothelial cells (HPMVECs) and increases the expression of the endothelin-B receptor (ET-BR) in monocytes via phosphatidylinositol (PI)-3 kinase, ROS and HIF-1 (60). PlGF-mediated ET-1 release from HPMVECs and expression of the ET-1 cognate receptor ET-BR in monocytes create a PlGF–ET-1–ET-BR loop, leading to increased expression of monocyte chemotactic protein (MCP-1) and interleukin (IL)-8 via the activation of HIF-1alpha, in a hypoxia-independent manner (60).

4.1.5. Basic fibroblast growth factor (bFGF)

Calvani *et al* (61) reported that human umbilical vein endothelial cells (HUVECs), cultured on Matrigel in the absence of exogenous growth factors, form tube-like structures when incubated under hypoxic conditions. This hypoxic induction of tube-like formation requires HIF-1-dependent induction of bFGF, though a consensus HRE sequence has not so far been identified in the regulatory region of the bFGF gene.

4.1.6. Stem cell factor (SCF)

SCF and its receptor tyrosine kinase c-Kit (also known as CD117) are overexpressed in some human malignancies including breast cancer and glioma (62, 63). Binding of SCF to c-Kit results in autocrine activation of c-Kit intrinsic tyrosine kinase activity and leads to tumor cell growth. SCF acts as an angiogenic factor that directly promotes the survival, migration and capillary tube formation of HUVECs (64). SCF has also been implicated in the mobilization of EPCs required for neovascularization (65). Activation of c-Kit leads to a predominantly HIF-1alpha–mediated enhancement of VEGF expression (66), which would predict further enhancement of angiogenesis. HIF-1 up-regulates the expression of SCF through binding of HIF-1alpha to a HRE located proximal to the transcription start site of the SCF gene in human breast cancer (62).

4.1.7. Osteopontin (OPN)

OPN is a glyco-phosphoprotein that is expressed and secreted into the extracellular matrix by numerous human cancers (67). It shows multifaceted functions, one of which is to act as an angiogenic factor by promoting neovascularization through alphavbeta3 integrin-mediated endothelial cell migration, the prevention of endothelial cell apoptosis, and vascular lumen formation (67). OPN is induced by a variety of stimuli including hypoxia in a HIF-independent manner (68).

4.1.8. Adrenomedullin (AM)

AM is a peptide belonging to the calcitonin gene-related peptide superfamily (69). It promotes endothelial cell migration, invasion and differentiation into tube-like structures on Matrigel, and it inhibits apoptosis by binding to the calcitonin receptor-like receptor/receptor activity-modifying protein-2 (CRLR/RAMP2) and -3 (CRLR/RAMP3) complexes (70). AM not only enhances the differentiation of EPCs into endothelial cells but also facilitates the formation of mature vessels (71). AM expression is up-regulated by hypoxia in several cancers including renal cell cancer (72), through binding of HIF-1 to the HREs present in the AM promoter (73). Hypoxia also up-regulates the transcription of the CRLR gene in microvascular endothelial cells via HIF binding (74). Thus, simultaneous transcriptional up-regulation of AM and CRLR in endothelial cells could lead to a potent survival loop and therefore might play a significant role in angiogenesis.

4.1.9. Endothelin (ET)

ETs comprise a family of three vasoactive peptides, ET-1, ET-2 and ET-3, which show a variety of physiological and pathological roles by binding to the G-protein–linked transmembrane receptors, ET-RA and ET-RB (75). One or more endothelin isoforms are produced by

many tumor types. These peptides activate the expression of genes whose products are involved in angiogenesis, such as VEGF, resulting in the modulation of endothelial cell proliferation, migration, invasion and tube formation. They also protect tumour cells, macrophages and endothelial cells from apoptosis induced by hypoxia and serum starvation (76). Hypoxia induces ET-1 expression via HIF-1 binding to a HRE located in the antisense strand of the ET-1 promoter. ET-2 is also induced by hypoxia in a HIF-1-dependent manner in breast cancers (77). ET-1 stabilizes HIF-1alpha in ovarian carcinoma cells, thereby potentiating hypoxia-induced VEGF production (78). Several cell types in the tumor microenvironment such as fibroblasts and TAMs may also contribute to the expression of endothelins (76).

4.1.10. Semaphorin 4D (Sema4D)

Sema4D is a member of the semaphorin family and is a potent proangiogenic molecule that acts by binding to its receptor plexin B1, which is expressed on endothelial cells, thereby activating c-Met (79, 80). Sema4D is expressed in many different aggressive cancers, including head and neck squamous cell carcinoma (HNSCC), as well as in TAMs, and it promotes growth and vascularity of some tumor xenografts *in vivo* (79, 81, 82). A recent study showed that Sema4D is strongly induced in hypoxia in a HIF-1-dependent manner, and that there is a correlation between HIF-1 activity and Sema4D expression in HNSCC (83).

4.1.11. Cyclooxygenase-2 (COX-2)

COX-2 is an enzyme involved in the synthesis of prostaglandins and thromboxanes, in particular thromboxane A2 and prostaglandin E2 (PGE2), which are regulators of biological processes such as inflammation, cell proliferation, and angiogenesis (84). In various tumors, the level of COX-2 correlates with microvessel density and VEGF level (84). Enforced expression of COX-2 in a colon cancer cell line resulted in an increase in VEGF production, and this was suppressed by NS-398, a specific COX-2 inhibitor (85). Also, the contribution of a COX-2/PGE2/HIF-1alpha/VEGF pathway to tumor angiogenesis is suggested by the results of the following experiments in a gastric cancer cell line (86). After exogenous PGE2 stimulation, HIF-1alpha increased concomitantly with VEGF. This effect was blocked by SC19220, a PGE2 receptor antagonist. In addition, pretreatment with NS-398 effectively suppressed HIF-1alpha protein accumulation. Stromal and endothelial COX-2 in tumors might also take part in tumor angiogenesis. In colorectal carcinoma cells, under hypoxic conditions, transcription of the COX-2 gene is up-regulated via HIF-1 binding to a HRE in the COX-2 promoter, and PGE2 enhances HIF-1 transcriptional activity and VEGF induction. (87). Hypoxia can also up-regulate COX-2 through the NF-kappaB pathway in vascular endothelial cells (88).

4.1.12. Matrix metalloproteinases (MMPs)

MMPs are a large family of zinc-binding endopeptidases. They are initially expressed as proenzymes by a variety of cells, including endothelial cells and tumor cells, and are then converted to active MMPs by proteolytic cleavage (89). For example, MMP-2 is secreted as a latent form, and then undergoes activation through interaction with membrane type (MT)1-MMP and the endogenous tissue inhibitor of MMPs (TIMP)-2. Numerous studies have shown that MMPs play a major role in the degradation of the extracellular matrix, which is essential for endothelial cells and tumor cells to migrate and invade through the matrix (89). In human endothelial cells, hypoxia followed by reoxygenation up-regulates MMP-2 and MT1-MMP mRNA expression, leading to enhanced secretion of active MMP-2 protein (90), thereby enabling the cells to migrate and invade. Hypoxia also increases the expression of MMP-2 and MMP-9 in oral squamous cell carcinoma(SCC) cells and in xenografts (91). MT1-MMP is reported to be a direct target of HIF-2 in pVHL renal cell carcinoma (92). It should also be noted that MMP-2 and MMP-9 play a role in converting plasminogen to angiostatin which is one of the potent antagonists of angiogenesis (93).

4.1.13. Plasminogen activator inhibitor-1 (PAI-1)

PAI-1 is a member of the serpin (serine protease inhibitor) family of protease inhibitors. It inhibits the activities of urokinase plasminogen activator (uPA) and tissue-type plasminogen activator (tPA), and thus limits plasmin generation from plasminogen. PAI-1 is synthesized by a large number of cancer cells and several normal cells including macrophages and endothelial cells (94). Hypoxia enhances PAI-1 expression through the action of HIF-1alpha (95). During angiogenesis, stimulated endothelial cells express both uPA and PAI-1 (94). Plasmin activates MMPs, degrades extracellular matrix, and releases growth factors and cytokines trapped within the extracellular matrix. Therefore, PAI-1 acts as an inhibitor of normal angiogenesis (94). Paradoxically, PAI-1 has been shown to enhance tumor angiogenesis and progression (94). Although the precise mechanism of this effect remains to be determined, Bajou *et al* (96) have recently provided evidence that PAI-1 protects endothelial cells from apoptosis mediated by Fas and its ligand FasL. They demonstrated that in PAI-1 deficient mice undergo increased plasmin generation, which stimulates the release of a soluble FasL fragment from the cell surface of endothelial cells. This soluble FasL fragment activates Fas expressed on endothelial cells and acts as a potent inducer of endothelial cell apoptosis (96).

4.1.14. Nitric oxide synthases (NOS)

The NOS family comprises neuronal ROS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS). NOS expression is observed in many types of tumors including breast, head and neck, prostate, and colon cancers, and its expression is positively correlated with angiogenesis, and tumor growth and aggressiveness (97, 98). NOS catalyzes the formation of nitric oxide (NO), which mediates a multiplicity of processes involved in angiogenesis such as endothelial cell survival, proliferation and migration (99). Hypoxia up-regulates NOS expression through the binding of HIF-1 to a HRE located in the NOS promoter (100).

4.1.15. Endosialin

Endosialin (also known as TEM1 or CD248) was identified as a specific cell surface tumor endothelial marker and is a C-type lectin-like cell surface receptor of unknown function (101). Endosialin is strongly expressed by stromal fibroblasts and pericytes during periods of active angiogenesis in tumors (101, 102) and by a population of $VEGFR2^{+}/CD31^{+}/CD45^{-}/VE\text{-}cadherin^{+}$ EPCs derived from human $CD133^{+}/CD34^{+}$ cells (103) Transcription of the endosialin gene is reported to be induced by hypoxia predominantly through a mechanism involving HIF-2 cooperating with the Ets-1 transcription factor (104). Although the function of endosialin still needs to be clarified, these reports indicate the involvement of endosialin in tumor angiogenesis.

4.1.16. Adenosine A2A receptor

Hypoxia is known to drive extracellular adenosine accumulation in the local tumor microenvironment (105), and adenosine stimulates both proliferation of HUVECs and expression of VEGF (106, 107). The biological effects of adenosine are mediated through the different G protein-coupled adenosine receptors, A1, A2A, A2B, and A3. A recent study has demonstrated that A2A receptor has angiogenic potential by stimulating endothelial cell proliferation, migration and tube formation (108). Moreover, the A2A receptor has been shown to be regulated by hypoxia via the binding of HIF-2, but not HIF-1, to a HRE in the gene promoter in human lung endothelial cells (108). Therefore, a hypoxia-mediated adenosine/A2A receptor axis may contribute to tumor angiogenesis.

4.1.17. Oxygen-regulated protein-150 (Orp150)

Orp150, also known as HYOU1, is an important inducible molecular chaperone of the endoplasmic reticulum in hypoxia (109). Orp150 stimulates tumor angiogenesis by facilitating post-translational transport and processing of VEGF through the endoplasmic reticulum (110). Because Orp150 is thought to be cytoprotective against hypoxia (111), it is possible that hypoxia-induced Orp150 suppresses apoptosis of TAMs, enabling them to continue producing VEGF.

4.2. Chemokines

4.2.1. Stromal-derived growth factor 1 (SDF-1)

SDF-1 (also known as CXCL12) belongs to the CXC subfamily of chemokines, and is expressed in many tumors including ovarian cancer, neuroblastoma, breast cancer, and prostate cancer (112, 113). The receptor for SDF-1, CXCR4, is constitutively expressed in numerous cell types including hematopoietic and endothelial cells. SDF-1 not only promotes angiogenesis by engaging with CXCR4 expressed on vascular cells, but it also supports mobilization and recruitment of proangiogenic $CXCR4^{+}VEGFR1^{+}$ hematopoietic cells to neo-angiogenic sites in tumors. HIF-1 up-regulates CXCR4 expression in various cell types including endothelial cells, cancer cells and TAMs (114). SDF-1 and VEGF act synergistically to mediate the expansion of vascular endothelial cells, and to protect them from serum starvation-induced apoptosis (115). A functional HRE in the promoter region of the SDF-1 gene has been demonstrated in endothelial cells (116).

4.2.2. Interleukin-8 (IL-8)

IL-8 (also known as CXCL8) is a member of the CXC subfamily of chemokines, which are associated with the promotion of neutrophil chemotaxis and degranulation. IL-8 binds to two specific G-protein-coupled receptors, CXCR1 and CXCR2 (117), and it has been shown to be a potent angiogenic factor (118, 119). Depending on the cell type, IL-8 production is highly induced by hypoxia and anoxia in a HIF-1-independent but NF-kappaB-dependent manner (120, 121). In human endothelial cells, hypoxia induces IL-8 mRNA expression through increased DNA-binding of HIF-1, NF-kappaB, and AP-1 to the IL-8 promoter (122). In this case, hypoxia-induced IL-8 expression requires activation of HIF-1. The expression of IL-8 at protein level is regulated by PHD2, in a HIF-independent manner, and IL-8 is involved in PHD2-mediated angiogenesis and BMDCs recruitment (123). Recent studies have shown that IL-8 up-regulates VEGF mRNA and protein levels in endothelial cells by acting on CXCR2 and activating NF-kappaB activity, resulting in the activation of VEGFR2 in an autocrine fashion (124). IL-8 also up-regulates the expression of VEGF in a human pancreatic cancer cell line (125).

4.2.3. Chemokine (C-C motif) ligand 11 (CCL11)

CCL11 (also known as eotaxin-1) exclusively binds to the CC chemokine receptor 3 (CCR3). CCL11 is a powerful eosinophil attractant and it also exerts a chemotactic effect on other leukocytes and human CCR^{+} microvascular endothelial cells, implicating in angiogenesis. (126, 127). A recent study has shown that human CCL11 signaling plays an important role in proliferation and invasion of human ovarian carcinoma cells that express both CCL11 and CCR3 (128). CCL11 is also expressed in stromal cells such as fibroblasts and leukocytes in colorectal cancers (129). Hypoxia might induce CCL11 expression in tumor-associated fibroblasts, because hypoxia leads to an increase in secretion of CCL11 in nasal polyp-derived fibroblasts (130). The role of CCL11 in tumor angiogenesis awaits further study.

4.2.4. Endothelial-monocyte-activating polypeptide II (EMAP II)

EMAP II is a proinflammatory cytokine and a chemoattractant for monocytes and granulocytes. Mature EMAP II is the proteolytic cleavage product of a precursor protein (proEMAP II), which is identical to the p43 subunit of the mammalian tRNA multisynthetase complex (131). ProEMAP II/p43 is secreted by several tumor cells including prostate adenocarcinoma cells (132) and induces migration of EPCs via the chemokine receptor CXCR3 (133), suggesting that it has a role in the regulation of angiogenesis. In contrast, mature EMAP II is secreted by late apoptotic cells, and it inhibits migration and angiogenic cord formation, and induces apoptosis of endothelial cell (131). In murine B16 melanoma cells, hypoxia does not cause an increase in EMAP II mRNA,

but it leads to the release of EMAP II protein (134). A recent study has demonstrated that EMAP II binds to the alpha5beta1 integrin receptor on the cell surface, and then is internalized into the cytoplasmic compartment where it interacts with its cytoplasmic partner PSMA7, a component of the proteasome degradation pathway. This interaction increases HIF-1alpha degradation under hypoxic conditions (135).

4.3. Antiangiogenic factors and mediators

4.3.1. $VEGF_{xxx}b$

Two families of proteins, $VEGF_{xxx}$ and $VEGF_{xxx}b$ (where xxx is the number of amino acids in the mature protein), are generated by alternative splicing of exon 8 of the VEGF mRNA (136). $VEGF_{xxx}b$ differs from $VEGF_{xxx}$ only in the carboxy-terminal six amino acids, but, in sharp contrast to $VEGF_{xxx}$, $VEGF_{xxx}b$ exhibits antiangiogenic activity. $VEGF_{165}b$ inhibits $VEGF_{165}$-mediated endothelial cell migration and proliferation *in vitro,* and it suppresses $VEGF_{165}$-induced angiogenesis and tumor growth *in vivo* (137-139). The exact mechanism of the inhibitory function of $VEGF_{xxx}b$ still remains to be clarified (136). Interestingly, Varey *et al* (140*)* have recently shown that over 90% of the VEGF in normal colonic tissue is $VEGF_{xxx}b$ and that the $VEGF_{xxx}b$ is downregulated in paired human metastatic colorectal carcinoma samples. Furthermore, normoxic colonic adenoma cells express predominantly $VEGF_{xxx}b$, whereas hypoxic adenoma cells and colonic carcinoma cells express predominantly $VEGF_{xxx}$ (139, 140). This hypoxia- and cancer-associated switch from anti- to pro-angiogenic VEGF isoform expression may be related to the state of phosphorylation and nuclear localization of RNA binding splice factors such as ASF/SF2, SRp40 and SRp55 (141, 142).

4.3.2. Delta-like ligand 4 (Dll4)

Dll4 is a ligand for Notch 1, 3 and 4 proteins, and is exclusively expressed in the developing endothelium of normal tissue and the endothelium of some tumors (143-145). The Dll4/Notch signaling pathway plays a key role in embryonic vascular development. In mice, heterozygous deletion of the Dll4 gene results in embryonic lethality due to major defects in arterial and vascular development (146). Patel *et al* (143) demonstrated that Dll4 is induced by VEGF and hypoxia, through the action of HIF-1, and that down-regulation of Dll4 expression by siRNA leads to the inhibition of endothelial cell proliferation, migration, and tube formation. Paradoxically, Williams *et al* (147) demonstrated that up-regulation of Dll4 in endothelial cells reduces proliferative and migratory responses to VEGF selectively by decreasing the expression of VEGFR2 and NRP1 in a gamma-secretase inhibitor–sensitive manner. This indicates that Dll4 is a selective inhibitor of VEGF activities. The neutralization of Dll4 with a selective antibody renders endothelial cells hyperproliferative in a VEGF-dependent manner, and accelerates non-productive angiogenesis, resulting in the retardation of angiogenesis and the inhibition of tumor growth (148). Thus, it seems likely that Dll4 acts as a regulator of VEGF-induced angiogenesis. Its overexpression or blockade may result in a striking uncoupling of tumor growth from vessel density, thereby retarding tumor growth (148, 149).

4.3.3. Vasohibins

Vasohibins, vasohibin-1 and vasohibin-2 are expressed primarily in the endothelium, where they function as VEGF-inducible negative feedback regulators of angiogenesis (150-152). Overexpression of vasohibin-1 in Lewis lung carcinoma cells does not affect the growth of cancer cells *in vitro*, but it inhibits tumor angiogenesis and tumor growth when the cells are inoculated in mice (151). Hypoxia does not inhibit the expression of vasohibin-1 in endothelial cells, but it inhibits VEGF-stimulated vasohibin-1 expression at both the mRNA and protein levels (150).

4.3.4. Thrombospondin-1 (TSP-1)

TSP-1 belongs to a family of matricellular glycoproteins that mediate interactions between extracellular matrix molecules and integrin receptors such as alphavbeta3 integrin receptor (153). It is an endogenous angiostatic factor which inhibits VEGF-induced endothelial cell proliferation and migration, and it suppresses tumor angiogenesis and growth. The antiangiogenic effect of TSP-1 is linked to its ability to induce apoptosis of endothelial cells (153). The expression of TSPs is down-regulated in tumor cells and endothelial cells incubated under conditions of hypoxia, or hypoxia and glucose deprivation, thereby promoting the switch to an angiogenic phenotype (154, 155). The down-regulation of TSP-1 is HIF-1-independent and may be related to the degree of methylation of the promoter region (153, 156).

4.3.5. Regulator of G protein signaling 5 (RGS5)

RGS5 belongs to the R4 subfamily of RGS proteins and is enriched in cardiovascular tissues, especially in pericytes and endothelial cells. RGS5 induces apoptosis of endothelial cells by activating caspase-3 and increasing the Bax/Bcl-2 expression ratio, and it antagonizes the angiogenic effect of VEGF by increasing the activation of the p38 MAPK signaling pathway (157). Thus, RGS5 acts as a negative regulator of angiogenesis. Recently, RGS5 was reported to be induced by hypoxia via HIF-1 (157).

5. BONE MARROW-DERIVED CELLS MOBILIZED AND RECRUITED TO TUMORS

5.1. Myeloid lineage cells

Many types of $CD45^+$ BMDCs from the myeloid lineage such as monocytes, Tie2-expressing monocytes (TEMs) and $VEGFR1^+$ hemangioblasts, have been shown to be recruited to neoangiogenic sites and to actively engage in tumor angiogenesis and vaculogenesis. These cells also express CXCR4, the receptor for SDF-1. Other BMDCs from the myeloid lineage such as myeloid-derived suppressor cells, neutrophils, eosinophils and mast cells are also suggested to take part in these processes (158).

5.1.1. Tumor-associated macrophages (TAMs)

TAMs have been shown to accumulate in hypoxic and necrotic areas of a variety of human cancers including colorectal cancer (159). Large numbers as primary monocytes are recruited from the circulation into tumors by their response to gradients of the many chemoattractants secreted by both tumor and stromal cells. Such chemoattractants include hypoxia-inducible VEGF, ET-1, and SDF-1. Monocytes also respond to other chemoattractants such as MCP-1, colony-stimulating factor (CSF)-1, and tumor necrosis factor-alpha (TNF-alpha) (160). Once recruited into tumors, these monocytes differentiate into TAMs. In hypoxic areas, TAMs express many proangiogenic and angiogenesis-modulating factors such as VEGF, bFGF, Ang-2, IL-8, COX2, hepatocyte growth factor (HGF), VEGFR1, tissue factor (F3), MMP7 and MMP12 (161, 162), resulting in further acceleration of angiogenesis. Human carcinomas with high levels of TAM infiltration show higher rates of metastasis and a poorer patient prognosis (163).

5.1.2. Tie2-expressing monocytes (TEMs)

TEMs have recently been shown to play an important role in tumor angiogenesis (164, 165). They have been found in human cancer specimens, including kidney, colorectal, breast, gastric, pancreatic, and lung cancers, and in soft tissue sarcomas as well as in murine tumors (164, 166). Purified human TEMs markedly promote angiogenesis in xenotransplanted human tumors (164). Ang-2 has been reported to stimulate migration of TEMs, at least partially via activation of Tie2 (164, 166). Although TEMs mainly localize to well-vascularized areas (164-166), hypoxia makes them more responsive to Ang-2 by up-regulating Tie2 expression (167). Furthermore, hypoxia augments the inhibitory effect of Ang-2 on the expression of the antiangiogenic cytokine IL-12 in TEMs (166). The combination of Ang-2 and hypoxia inhibits the release of TNF-alpha by TEMs (166). As TNF-alpha shows a proapoptotic effect on endothelial cells (168), hypoxia has a profound effect on the survival of these cells.

5.1.3. VEGFR1$^+$ hemangiocytes

VEGFR1$^+$ hemangiocytes are recruited to angiogenic sites by the cytokine-mediated release of SDF-1 from platelets, and are involved in neovascularization in the ischemic hindlimb model (169). Du *et al* (170) demonstrated that HIF-1alpha, partly through up-regulation of SDF-1alpha, induces the recruitment of CD45$^+$ myeloid cells containing subpopulations that are VEGFR1$^+$, Tie2$^+$, CD11b$^+$, and F4/80$^+$, thereby promoting neovascularization in mouse glioblastoma. These results suggest that VEGFR1$^+$ cells are recruited into hypoxic areas of tumors where VEGF and SDF-1 are up-regulated.

5.2. Endothelial progenitor cells (EPCs)

EPCs are defined as precursor cells that can differentiate into mature endothelial cells when recruited to angiogenic sites, and are presently characterized by their expression of AC133 and endothelial cell surface molecules markers such as CD31 and VEGFR2 (171). Li *et al* (172) reported that VEGF and PlGF promote vasculogenesis by enhancing EPC recruitment and vessel formation at the site of tumor neovascularization, through the action of VEGFR1 expressed on the surface of the EPCs. There is currently a debate with regard to the degree of contribution of EPCs in tumor angiogenesis (173), and further studies are required to clarify this issue.

6. HIF INHIBITORS AS ANTIANGIOGENESIS AND ANTICANCER AGENTS

The fact that many angiogenic factors and mediators are regulated by hypoxia makes HIFs attractive targets for the development of anticancer drugs. Several small molecule inhibitors have recently been identified, including the cardiac glycoside digoxin, anthracycline anticancer drugs, and antibacterial acriflavine. Cardiac glycosides, anthracyclines and acriflavine markedly retard tumor vascularization and tumor growth by inhibiting HIF-1alpha synthesis, HIF-1 transcription factor activity and HIF-1 dimerization, respectively, and mobilization of CXCR4$^+$/Sca1$^+$, VEGFR2$^+$/CD34$^+$, and VEGFR2$^+$/c-Kit$^+$ BMDCs. Because these drugs are already applied in clinical settings, these results have important implications for the use of these drugs as antiangiogenesis agents in cancer patients (174-178). Recent studies have shown that antiangiogenic therapy targeting the VEGF pathway reduces primary tumor growth but increases local invasion and distant metastasis due to increased hypoxia in primary tumors (179, 180). Since HIF inhibitors can also inhibit hypoxia-induced invasion and metastasis, they might have an advantage over VEGF inhibitors in treating cancers. None of the HIF inhibitors identified so far is HIF-specific. Therefore, further careful studies are required to determine whether these HIF inhibitors have unfavorable effects *in vivo*.

7. SUMMARY AND PERSPECTIVE

Decades of research have identified many pro- and antiangiogenic mediators produced by a variety of cell types including tumor cells and stromal cells. More recent studies have demonstrated that BMDCs are mobilized and recruited to angiogenic sites to promote angiogenesis by a variety of growth factors and cytokines. The development of normal vasculature depends on the homeostatic balance between pro- and antiangiogenic mediators. However, in tumor angiogenesis, tumor hypoxia itself and genetic alterations that activate oncogenes and inactivate tumor suppressor genes aberrantly modulate the expression of many potent pro- and antiangiogenic molecules. HIFs and in some cases other transcription factors play a predominant role in promoting the net balance to more angiogenic state. Based on cumulative investigations, HIF inhibitors have emerged as promising antiangiogenesis and anticancer drugs (Figure 1). The realization that potent antiangiogenic therapy targeting the VEGF pathway can promote invasion and metastasis due to increased hypoxia in primary tumors may give

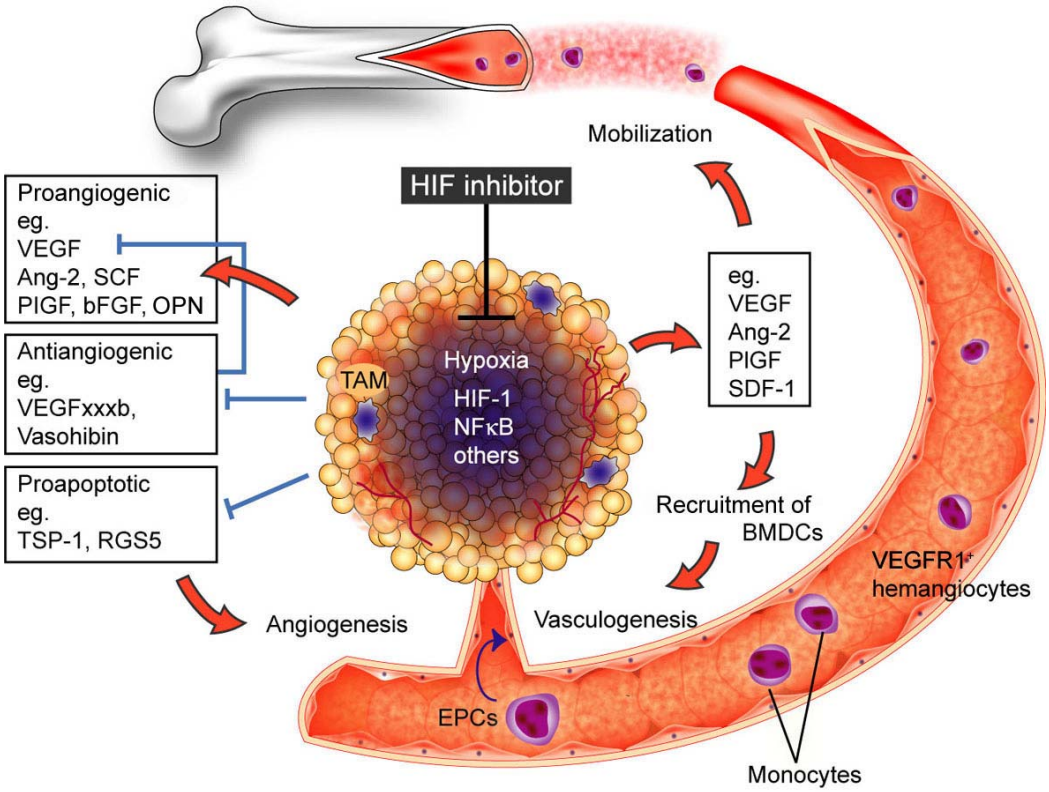

Figure 1. Schema of tumor angiogenesis induced by hypoxia. Tumor hypoxia up-regulates many proangiogenic molecules and chemokines such as VEGF, Ang-2, SCF, PlGF, bFGF, OPN and SDF-1 in cancer cells and stromal cells via the activation of HIFs and NF-kappaB. VEGF, Ang-2, PlGF and SDF-1 mobilize and recruit BMDCs and EPCs that actively engage in tumor angiogenesis and vaculogenesis. Monocytes and EPCs differentiate into TAMs and endothelial cells, respectively. On the other hand, tumor hypoxia down-regulates the expression of antiangiogenic factors and mediators such as $VEGF_{xxx}b$ and proapoptotic factors such as TSP-1 and RGS5 in endothelial cells. In this way, tumor hypoxia disrupts the balance between pro- and antiangiogenic activity, eventually promoting the shift in balance to a more angiogenic state. Drugs targeting HIFs (HIF inhibitors) can be utilized to suppress hypoxia-induced angiogenesis, thereby retarding tumor growth and metastasis.

HIF inhibitors the advantage as anticancer drugs, because HIF inhibitors may be able to inhibit hypoxia-induced malignant progression as well as the VEGF pathway. Therefore, development of HIF inhibitors with high specificity and less unfavorable effects is a burning issue. Concurrently, the effect of the combination of HIF inhibitors with conventional chemotherapy should be further defined.

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