Molecular Pathways

Hypoxia and Hypoxia-Inducible Factors: Master Regulators of Metastasis

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Abstract

Hypoxia is a common condition found in a wide range of solid tumors and is often associated with poor prognosis. Hypoxia increases tumor glycolysis, angiogenesis, and other survival responses, as well as invasion and metastasis by activating relevant gene expressions through hypoxia-inducible factors (HIF). HIF-1α and HIF-2α undergo oxygen-dependent regulation, and their overexpression is frequently associated with metastasis and poor clinical outcomes. Recent studies show that each step of the metastasis process, from the initial epithelial-mesenchymal transition to the ultimate organotropic colonization, can potentially be regulated by hypoxia, suggesting a master regulator role of hypoxia and HIFs in metastasis. Furthermore, modulation of cancer stem cell self-renewal by HIFs may also contribute to the hypoxia-regulated metastasis program. The hypoxia-induced metastatic phenotype may be one of the reasons for the modest efficacy of antiangiogenic therapies and may well explain the recent provocative findings that antiangiogenic therapy increased metastasis in preclinical models. Multiple approaches to targeting hypoxia and HIFs, including HIF inhibitors, hypoxia-activated bioreductive prodrugs, and gene therapies may become effective treatments to prevent or reduce metastasis.

Background

The occurrence of metastasis in solid tumors signals the breakdown of normal tissue homeostasis and dramatic rearrangement of tumor-stromal interactions. It has been increasingly recognized that hypoxia, which commonly refers to a condition in tissues where the oxygen pressure is less than 5 to 10 mmHg, is a powerful driving force for such transitions in tumor progression (1). The best-characterized hypoxia response pathway is mediated by hypoxia-inducible factor-1 (HIF-1; ref. 2). HIF-1 is a heterodimer with the HIF-1β subunit constitutively expressed and the HIF-1α subunit regulated in an oxygen-dependent manner (Fig. 1A). Under normoxia, prolyl hydroxylases (PHD) modify Pro-402 and Pro-564 of HIF-1α in a reaction that uses O2 as a substrate. Hydroxylated HIF-1α interacts with von Hippel-Lindau (VHL), which is part of an E3 ubiquitin ligase complex targeting HIF-1α for 26S proteasomal degradation. Under hypoxia, HIF-1α is stabilized because of the lack of O2 and dimerizes with HIF-1β to bind to the hypoxia response element (HRE; 5′-G/ACGTG-3′). By interacting with the coactivator CBP/p300, HIF-1 activates transcription of target genes that fall into four major categories: glucose transporters and glycolysis, angiogenesis, survival and proliferation, and invasion and metastasis (Fig. 1A; ref. 2). Gene-expression profiling analysis in distinct cell types revealed tissue- and cell-specific variations in hypoxia response, although a consensus hypoxia response signature was shown to be a general poor-prognosis indicator for diverse cancer types (3–5). Binding of HIF-1 to CBP/p300 is also regulated in an oxygen-dependent manner, because O2 is the substrate for HIF-1α hydroxylation at Asn-803 by factor inhibiting HIF-1α (FIH-1), and this modification prevents binding to CBP/p300 (1). Two HIF-1α homologs, HIF-2α and HIF-3α, have been identified (1, 2). Similar to HIF-1α, HIF-2α is also regulated by oxygen-dependent hydroxylation. HIF-1α and HIF-2α are structurally similar in DNA binding and dimerization domains but differ in their transactivation domains. Consistently, they share overlapping target genes, whereas each also regulates a set of unique targets. HIF-3α lacks the transactivation domain and may function as an inhibitor of HIF-1α and HIF-2α.

HIF-1α can also be regulated through oxygen-independent mechanisms in a cell type–specific manner (1, 2). It can be activated by mutations of PTEN, VHL, succinate dehydrogenase (SDH), or fumarate hydratase (FH). In addition, HIF-1α can be activated by hyperactivity of ERBB2, SRC, endothelin-1 (ET-1), the RAS/MAPK pathway, and the phosphoinositide 3-kinase (PI3K)-Akt-mTOR pathway. Moreover, HIF-1α can be stabilized by reactive oxygen species (ROS) through blocking the PHD activities. HIF-1α is overexpressed in many cancer types and is associated with poor prognosis in cancers of breast, brain, oropharynx, cervix, ovary, and uterus (2). Interestingly,
HIF-1α is expressed at a higher fraction (69%) in metastases compared with primary tumors (29%) for breast cancer (6). HIF-2α overexpression is associated with poor patient outcome in renal cell carcinoma, non–small cell lung cancer (NSCLC), and neuroblastoma (7).

**Hypoxia Regulation of Metastasis**

Metastasis consists of a series of rate-limiting steps (Fig. 1B; ref. 8). The initiation of metastasis involves the acquisition of a motile phenotype by tumor cells in a process called epithelial-mesenchymal transition (EMT). Tumor cells then disrupt the integrity of the basement membrane and interact directly with the underlying extracellular matrix to eventually penetrate the blood vessel walls (intravasation) and become circulating tumor cells (CTCs). Successful dissemination depends on the survival of CTCs in blood circulation and subsequent extravasation at the secondary organs. Most disseminated tumor cells (DTCs) remain solitary or as dormant micrometastases until they adapt to the microenvironment of the secondary organs. Tumor-initiating capabilities [or cancer stem cell (CSC) properties] and productive tumor-stromal interactions underlying metastasis organotropism are believed to have fundamental importance in the successful outgrowth of clinically significant metastasis (8). Recent studies implicate regulatory functions of hypoxia in each of these steps (Fig. 1B).

**Epithelial-mesenchymal transition**

E-cadherin is a major component of the adherens junctions that maintain epithelial integrity and polarity. Loss of E-cadherin is a hallmark and functional requirement for EMT. Hypoxia induces EMT by HIF-dependent upregulation of transcription repressors of E-cadherin, such as SNAIL (9), TWIST1 (10), TCF3, ZEB1, and ZEB2 (11).

**Invasion, extracellular matrix modulation, and cell motility**

Basement membrane disruption was shown to be promoted by HIF-1α–dependent upregulation of cathepsin D (CTSD), urokinase-type plasminogen-activator receptor (uPAR), and matrix metalloproteinase-2 (MMP2) through a proteolytic cascade (12). Hypoxia promotes the formation of a fibronectin-rich matrix that can be recognized by integrins expressed in the tumor cell surface (12). Hypoxia also upregulates expression of lysyl oxidase (LOX), an extracellular enzyme that covalently modifies collagens to increase focal adhesion kinase activity, cell migration, and metastasis (13). Another mechanism of increasing cell motility is through the activation of MET by HIF-1 (14). By overexpressing MET, tumor cells respond to hepatocyte growth factor, the ligand for MET that is produced at an elevated level by fibroblasts under hypoxia (15), with increased cell migration through tissue parenchyma. Autocrine motility factor (AMF) is another tumor-secreted cytokine that is induced by HIF-1 and VEGF under hypoxia to enhance proliferation, migration, and angiogenesis through autocrine or paracrine mechanisms (16).

**Intravasation, circulation, and extravasation**

Besides promoting angiogenesis and lymphangiogenesis, a high level of VEGF induced by hypoxia is also associated with increased microvascular permeability and interstitial fluid pressure, both of which contribute to an increased chance of intravasation (17). Hypoxia may also promote intravasation by increasing the production of metalloproteinases. For example, MMP1 and MMP2, synergistic mediators of vascular permeability and intravasation (18), were shown to be HIF-1 targets (19). Moreover, HIF-1 increases expression of microRNA miR-372/373, which targets the MMP inhibitory protein RECK to promote intravasation (20).

Because duration from intravasation to extravasation can be as short as several hours, transient hypoxia response in the primary tumor may persist long enough to affect later steps of intravascular movement and extravasation (17). Indeed, transient "priming" of tumor cells with hypoxia in vitro before intravenous inoculation into mice increased colonization (17). Possible mediators of this priming effect include hypoxia responsive factors that regulate resistance to anoikis, such as TrkB (a suppressor of anoikis; refs. 21, 22) and integrin α5 (a sensitizer of anoikis; ref. 23), which plays a role in regulating the survival of CTCs. Extravasation may be promoted by hypoxia response factors VEGF, MMP1, and MMP2 in a similar way to intravasation. ANGPTL4, a key molecule for extravasation in lung, is upregulated by both TGF-β (24) and hypoxia (25), suggesting a possible synergistic priming effect of the two pathways.

**Homing and the premetastatic niche**

Chemokine receptor CXCR4 plays a key role in metastatic homing of tumor cells to organs expressing high levels of its ligand SDF1 (26). Hypoxia may increase metastatic homing by inducing CXCR4 expression in renal cell carcinoma (27), ovarian cancer (28), breast cancer (25), and NSCLC (29). Hypoxia in the primary tumor may influence the metastatic seeding at distant organs even before tumor cell dissemination. The function of hypoxia in promoting seeding is achieved through the regulation of the premetastatic niche that was reported to form in distant organs by hematopoietic bone marrow–derived cells under the influence of soluble factors released from primary tumors (30). Interestingly, a critical mobilizing factor is LOX, a known hypoxia target, which recruits CD11b+ myeloid cells to form the niche to facilitate the colonization of metastatic tumor cells (31).

**Proliferation at the secondary organs**

Some organs contain relatively hypoxic areas, including the hematopoietic stem cell niche region in the bone marrow and the pericentral region of the liver. Metastases in these organs may experience hypoxia immediately after extravasation. Otherwise, metastasis colonies that continue to grow will eventually become hypoxic when the growing tumor outstrips its blood supply. Indeed, we recently showed the existence of hypoxia in early bone and lung metastases of breast cancer in a xenograph model using a dual-color bio-
Angiogenesis: ANGPT1/2, FGF3, PGF, TGF-β1/3, VEGFA, VEGFR1

Glucose metabolism: ALDA, GLUT1/3, GAPDH, HK1/2, LDHA, PFKL, PGK1, PKM

Survival/proliferation: ADM, Cyclin G2, EPO, IGF2, IGF-BP1/2/3, NOS2, NOTCH1, TGF-α

Metastasis: See below

Hypoxia-independent regulators of HIF-1α

Proteasomal degradation

Cofactor recruitment

HIF-1α

Hypoxia-dependent regulators of HIF-1α

Target genes

Hypoxia

Normoxia

(1) Primary tumor
Angiogenesis: VEGFA
EMT: SNAIL, TCF3, TWIST1, ZEB1/2
CSC: HIF-2α, DLK1

(2) Motility and invasion
Motility: LOX, MET, AMF
Invasion: CTSD, uPAR, MMPs, Fibronectin

(3) Intravasation
VEGFA, miR372/373, MMPs

(4) Circulation
TRKB, ITGA5

(5) Metastatic seeding
Homing: CXCR4
Extravasation: VEGFA, MMPs, ANGTL4
Pre-metastasis niche: LOX

(6) Metastatic outgrowth in secondary organs
Angiogenesis: VEGFA
Tumor-stromal interactions: CTGF, OPN, IL6, IL8, MCP1, ANGPTL4, DUSP1

Non-tumor epithelial cell
Epithelial tumor cell
Mesenchymal tumor cell
Cancer stem cell
Stromal cell
Extracellular matrix

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CCR Molecular Pathways
Hypoxia response molecules that promote survival, invasion, and angiogenesis in the primary tumor may function similarly in the secondary site. In addition, hypoxia may upregulate proteins that mediate interactions with unique stromal cells in the secondary organ. Compatibility of tumor cells with the microenvironment, first proposed as the “seed and soil” hypothesis by Paget (32), plays a predominant role in determining the organ distribution patterns of metastasis (33). Metastatic organotropism was investigated for breast cancer in a series of studies by identifying organ-specific metastasis gene signatures (34). It was shown that tumor cells use distinct sets of genes when colonizing different organs. However, how hypoxia influences the organ-specific metastasis was unknown. We recently found that hypoxia affects lung- and bone-metastasis gene signatures in different ways: whereas hypoxia enhances the expression of a large percentage of genes involved in lung metastasis, it activates a more limited number of bone metastasis genes, such as CXCR4 and DUSP1 (25). Several known hypoxia responsive genes, including connective tissue growth factor (CTGF; ref. 35), osteopontin (OPN; ref. 36), interleukin-6 and -8 (IL-6 and -8; refs. 37, 38), may also contribute to the bone metastasis–promoting function of hypoxia. CTGF may promote bone metastasis through its function in osteostelialosis and bone resorption (39, 40). OPN may promote osteolytic metastasis by interacting with α/β3-expressed osteoclasts (41). IL-6 and IL-8 have pleiotropic functions in promoting metastasis including angiogenesis, migration, tumor self-seeding, and osteolysis during bone metastasis (42–44). Interestingly, a 43-gene hypoxia response signature was identified and showed prognostic power for lung metastasis, but not for bone metastasis, when applied to transcription profiling data of primary breast tumors, suggesting the selection of the lung metastatic phenotype by hypoxia in the primary site. Despite the different mechanisms of action in organ-specific metastasis, blocking HIF-1α significantly inhibited metastases to both bone and lung in animal models, establishing HIF-1α as a promising therapeutic target for treating metastasis at multiple sites (25, 45).

Cancer stem cells

By definition, metastasis should originate from CSCs with tumor-initiating capabilities that allow DTCs to constitute growing, heterogeneous secondary tumors (46). Therefore, mechanisms enhancing the CSC phenotype should also increase the efficiency of metastasis. In neuroblastoma, whereas HIF-1α was upregulated in both CSCs and non-CSCs under hypoxia, HIF-2α upregulation was restricted to CSCs (48). Targeting of either HIF-1α or HIF-2α in CSCs suppressed self-renewal and tumorigenicity (48). Despite the emerging links between hypoxia and CSC regulation, direct demonstration of the enhancement of metastasis by hypoxia in a CSC-dependent manner has not been reported. Cancer types with higher incidence of distant metastasis should be used for such studies.

In addition to the impact of hypoxia in different steps of metastasis discussed above, hypoxia may also promote metastasis through other mechanisms. For example, hypoxia response in stromal cells may be an integral part of the metastasis program. For instance, the expression of monocyte chemoattractant protein-1 (MCP-1), a prometastatic factor in multiple organs (49), is induced by hypoxia in fibroblasts (50) and astrocytes (51). MCP-1 produced by the first-tier stromal cells such as fibroblasts may help recruit the second-tier stromal cells, such as macrophages or osteoclast precursors, to promote metastasis. A poorly appreciated, yet potentially significant, cellular mechanism that promotes metastasis is cell fusion between tumor and host cells or between two tumor cells (52, 53). Hypoxia enhances fusion between hematopoietic progenitor cells and cardiomyocytes (54) and may promote metastasis by increasing frequency of cell fusion in cancer.

Clinical-Translational Advances

As summarized above, hypoxia can activate many steps of metastasis. This connection may partially explain the recent provocative observations that antiangiogenic drugs...
increased invasiveness and metastasis of tumor cells in preclinical models (55, 56), because these drugs induced hypoxia by blocking neovascularization. Therefore, targeting hypoxia through different approaches (Table 1), at appropriate time windows, may significantly reduce tumor-intrinsic and treatment-induced metastasis.

### Agents targeting HIF-1

Several ways target the function or the expression of HIF-1. Interaction of the transactivation domain of HIF-1α with CBP/p300 was blocked with either a dominant-negative polypeptide or the small compound chetomin, which were shown to reduce xenograft tumor growth (57). Anthracyclines (doxorubicin and daunorubicin) inhibited HIF-1 binding to DNA and reduced prostate cancer growth in a mouse model (58). HIF-1α transactivation domain was also inhibited by the proteasome inhibitor bortezomib (59), which is used to treat relapsed multiple myeloma and mantle cell lymphoma. It will be important to investigate how much of the efficacy of bortezomib is due to HIF-1 inhibition.

HIF-1 expression has been perturbed in many ways. Antisense oligonucleotide (60) or siRNA (61) targeting HIF-1α showed antitumor activity when applied to preclinical models. Agents that inhibit HIF-1α protein translation and showed antitumor effects include the topoisomerase I inhibitor topotecan, cardiac glycoside digoxin, and PX-478 (62). Disruption of microtubule polymerization by 2-methoxyestradiol reduced HIF-1α protein level and xenograft tumor growth (63). HIF-1α interacts with the chaperone Hsp90. and the Hsp90 inhibitor 17-AAG induces HIF-1α degradation in a VHL-independent manner (64). Because HIF-1α can be induced by hypoxia-independent signaling pathways, therapeutic agents targeting these pathways, such as mTOR, ERBB2, and MAP/ERK kinase (MEK), also decreased HIF-1α level, and this may partially account for their antitumor activities (2).
Hypoxia-activated bioreductive prodrugs

Hypoxia-activated bioreductive prodrugs display cytotoxicity in the absence of O_2 when the intermediate reaction product, instead of being converted back by O_2, further reacts to become toxic end product. Tirapazamine is a prototype of benzotriazole di-N-oxides and showed remarkable antitumor activity in animal models (65). However, results from phase III clinical trials were mixed (66). Tirapazamine is currently under evaluation in more phase III trials. Other bioreductive prodrugs under early phase clinical trials include CB 1954, SN 23862, PR-104 (nitroaromatics), mitomycin C, RH-1 (quinones), and AQ4N (tertiary amine N-oxides; ref. 67). Limitations of bioreductive prodrugs include requirement for reductase activity in the hypoxic areas, toxicity, and low efficacy due to rapid consumption (67).

Hypoxia-targeted gene therapy

HRE can be used to drive expression of proteins with intrinsic cytotoxicity to kill hypoxic tumor cells, for example, the apoptosis-inducing factor BAX (68). Similarly, HRE can drive expression of a protein that converts a prodrug into a cytotoxin. This approach, called gene-directed enzyme prodrug therapy, has been shown feasible by expressing HRE-driven flavoprotein cytochrome c P450 reductase (69), HSV thymidine kinase (70), and cytosine deaminase (71).

Conclusions

Mounting evidence suggests hypoxia as an important driving force and master regulator for the multistep process of metastasis. Targeting hypoxia may become an effective approach in preventing or reducing metastasis. In particular, combining anti-angiogenic drugs with HIF-α inhibitors may minimize the prometastatic effects elicited by antiangiogenesis-induced hypoxia and improve the efficacy of current antiangiogenic therapies. Nevertheless, many of the mechanistic and therapeutic investigations of the prometastatic role of hypoxia were based on preclinical animal models. Therefore, caution must be applied when extrapolating conclusions drawn from such studies to clinical settings. As hypoxia is likely to have complex and even opposing roles during different stages of tumor development, further studies should focus on clinically relevant tumor models to determine the most appropriate treatment window for targeting hypoxia. As hypoxia is a notoriously heterogeneous and constantly evolving phenotype of a solid tumor, better molecular, pathologic, and, in particular, noninvasive imaging markers are also needed to improve patient selection and treatment-response monitoring for clinical uses of hypoxia inhibitors.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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