Molecular Pathways

Molecular Pathways: Hypoxia Response in Immune Cells 
Fighting or Promoting Cancer

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Abstract

Both malignant and stromal components in tumors are influenced by the physiologic conditions of the microenvironment. Hypoxia is a prominent feature of solid tumors as a result of defective vascularization and intense metabolic activity. The gene-expression control mechanisms that adapt tissues to hypoxia are exploited by tumors to promote angiogenesis and vasculogenesis. The functions of infiltrating immune cells (macrophages and lymphocytes) and other stromal components are also influenced by a limited O2 supply. Hypoxia-inducible factors (HIF) are the main molecular transcriptional mediators in the hypoxia response. The degradation and activity of HIF-1α and HIF-2α are tightly controlled by the fine-tuned action of oxygen-sensing prolyl and asparaginyl hydroxylase enzymes. Recent evidence indicates that hypoxia can modulate the differentiation and function of T lymphocytes and myeloid cells, skewing their cytokine-production profiles and modifying the expression of costimulatory receptors. This conceivably includes tumor-infiltrating lymphocytes. Hypoxia not only directly affects tumor-infiltrating leukocytes but also exerts effects on tumor cells and vascular cells that indirectly cause selective chemokine-mediated recruitment of suppressive and proangiogenic T-cell subsets. This review focuses on changes induced by hypoxia in immune cells infiltrating solid malignancies. Such changes may either promote or fight cancer, and thus are important for immunotherapy. Clin Cancer Res; 18(5); 1207–13. ©2011 AACR.

Background

O2 supplied by hemoglobin from the blood stream is intracellularly present in normal tissues at concentrations of ~10% and even less in metabolically active tissues (1). Under these normoxic conditions, there is sufficient O2 to ensure the correct function of ATP synthesis in mitochondria. In tissue culture experiments, immune cells are placed under normoxic conditions, ignoring the fact that immune cells must work under hypoxia (0.1–3% O2) when they perform their functions in infected tissues, tissues undergoing autoimmunity, or solid tumors (2). Even in secondary lymphoid organs, such as the spleen, the O2 concentration is <5% (3).

Hypoxia-inducible factor 1α (HIF-1α) is the main regulator of the adaptation to a shortage of O2 (4). It was originally discovered in cells regulating erythropoietin production (5). As a way to preserve cell metabolism, most genes under the control of HIF-1α and HIF-2α are related to glucose transport and metabolism, diverting aerobic to anaerobic ATP production. In addition, the HIF-1α response induces soluble enhancers of vascularization such as VEGF (6) and adrenomedullin (7).

Figure 1 shows that O2 concentration-sensitive hydroxylase enzymes govern the molecular machinery that regulates the function of HIF-1α (8). Under normoxic conditions, prolyl-4-hydroxylase isoforms (PHD1, PHD2, and PHD3) act on prolyl residues of HIF-1α (Pro-402 and Pro-564), thereby defining 2 oxygen-dependent degradation domains in a reaction that is catalyzed by iron (Fe2+) and 2-oxoglutarate [2-OG (ref. 9)]. Once HIF-1α is prolyl-hydroxylated, a protein complex that contains Von Hippel-Lindau tumor-suppressor protein (pVHL), which acts as an E3 ubiquitin ligase, can build up ubiquitin chains linked to HIF-1α (Fig. 1). Lys-48 polyubiquitination in HIF factors leads to rapid proteolytic degradation by the proteasome (9). If the O2 is below a certain threshold (~3%, but the exact figure is probably dependent on cell type and species), the PHDs become nonfunctional (Fig. 1). The HIFs are then not efficiently degraded and translocate to the nucleus to dimerize with HIF-β. HIF-1α and HIF-2α form dimers with HIF-β and find short nucleotide sequences (referred to as hypoxia-responsive elements) on which these proteins act as transcription-promoting factors (10). The crucial function of HIF transcription factors is illustrated by the lethal phenotypes of HIF knockout mice, which die as embryos from vascularization failures (11). Experimentation to
clarify the role of the different molecular elements in the pathway has required tissue lineage–restricted conditional knockout mice of HIF and the 3 PHDs (12).

Hydroxylation on asparaginyl residues (e.g., Asn-804) located on the C-terminal transactivation domain of HIF-1α by factor-inhibiting HIF (FIH) constitutes another level of regulation (13). FIH activity is also dependent on O2, Fe^{2+}, and 2-oxoglutarate. When oxygen is available, hydroxylation on Asn-804 blocks the interaction of HIF-1α with the nuclear transcriptional coactivator protein p300 (Fig. 1; ref. 14). The...
HIF-1α has important functions mainly in erythropoiesis and vascularization (15, 16), as well as in macrophages (17), because expression of HIF-2α mRNA is cell-type–dependent (18). Because the focus of this review is immune cells involved in inflammation.

HIF-1α does more than control gene transcription directly, and a number of alternative functions and mechanisms of this protein are being revealed. These include (i) protein-to-protein association with other transcription factors, such as RAR-related orphan receptor-γt [RORγt (19)] and activator protein-1 [AP-1 (Fig. 1; ref. 20)]; (ii) induction of microRNAs (miRNA), such as miR-210 (21); and (iii) induction of the expression of transcription factors that in turn control other genes and/or epigenetic mechanisms, such as histone acetylation involving p300/CBP (22).

HIF-1α can also decrease the levels of gene transcription by different mechanisms (4). Surprisingly, HIF-1α can also induce the degradation of proteins by targeting them to polyubiquitination, as was recently observed with the FOXP-3 transcription factor with CD4⁺ T lymphocytes (Fig. 1; refs. 19). The PHDs that hydroxylate HIF have also been discovered to prolyl hydroxylate members of the canonical NF-κB pathway (23) that are critically involved in inflammation.

**Innate immune system and inflammation under hypoxic conditions**

Tumor stroma contains vascular cells, myeloid cells [e.g., tumor-associated macrophages (TAM)], tumor-infiltrating lymphocytes (TIL), and activated fibroblasts associated with the tumor. Vascular cells, including endothelium and pericytes, undergo functional changes due to the hypoxic microenvironment and have been extensively studied in tumors (24). Moreover, components of tumor stroma have been shown to outperform malignant cells in the production of VEGF, and thus play a key role in angiogenesis and aberrant vascularization of tumors (Fig. 2; refs. 25, 26).

Myeloid cells are highly represented in intratumoral environments, and macrophages are frequently the most abundant of these cells that usually invade the intratumoral hypoxic environment. Indeed, hypoxic tumor areas induce tumor-homing of bone marrow–derived CD45⁺ myeloid cells (27). These effects have been reported to be partly dependent on the production by hypoxic tumor cells of stromal cell–derived factor 1α (SDF1α), which binds to CXCR4. These cells are also sensitive to interleukin (IL)-8 (CXCL8)–guided chemotaxis (28), and IL-8 is abundantly produced in the tumor at least partly in response to hypoxia.

TAMs normally contribute to tumor progression (Fig. 2; ref. 29). Although the cytotoxicity of macrophages in the early immune response contributes to the death of tumor cells, the presence of macrophages has also been correlated with a poor prognosis for patients. Macrophages that have been recruited to inflammatory sites undergo distinct forms of activation depending on the cytokine signals encountered in the surrounding milieu. For example, cytokines or bacterial compounds that usually accumulate during bacterial infection, such as lipopolysaccharide and helper type-1 (Th1) cytokines (e.g., IFN-γ), induce classic macrophage polarization [M1 (30)]. This M1 polarization is chiefly characterized by enhanced intracellular bacterial killing, production of nitric oxide (NO), and tissue destruction, but also with resistance to tumor progression. By contrast, helper type-2 (Th2) cytokines such as IL-4, as well as other signals, induce alternative macrophage polarization (M2) oriented mainly toward immunoregulation, tissue remodeling, and tumor promotion (30–32).

Recent evidence suggests that HIF-1α and HIF-2α may play contrasting roles in macrophage M1 and M2 polarization. In this regard, HIF-1α is activated in response stimuli that induce M1 polarization but is not altered under conditions in which macrophages undergo M2 polarization in response to IL-4 (33, 34). Such HIF-1α upregulation seems to be dependent on a specific increase in HIF-1α mRNA levels, which involve the NF-κB transcription factor (17, 35, 36). In addition, HIF-1α induces markers of M1 polarization such as inducible NO synthase. Moreover, loss of HIF-1α induces M2 markers such as macrophage scavenger receptor 1 (37, 38).

In contrast, a recent study indicated that HIF-2α is induced specifically during M2 polarization, and showed that HIF-2α is a critical regulator of arginase 1 gene expression, a molecular marker of M2 polarization that reduces the intracellular l-arginine pool required to produce NO (33, 39). This could suggest opposite roles of the HIF1 and HIF2 systems in NO generation and potentially in M1/M2 polarization. However, controversy surrounds the role of HIF-2α in M2 polarization and arginase expression, probably reflecting the different conditions of the in vitro models employed (40).

A recent study in an adenocarcinoma model showed that hypoxic TAMs show signs of M2 polarization. Of interest, the majority of TAMs that resemble an M1 phenotype are located in normoxic areas, whereas M2 is found in hypoxic regions. This suggests that the proposed hypoxia-HIF2α-M2 polarization axis could dominate in the intratumoral hypoxic environment (32). In this regard, immunohistologic studies have found strong HIF-2α expression in TAMs (41). Moreover, genetic studies in myeloid HIF-2α–deficient mice showed altered migratory properties of HIF-2α–deficient macrophages that were associated with reduced tumor progression (40). It is also important to consider that the HIF activity of TAMs not only alters intratumoral endothelial cells but also regulates other cell types that act as immunosuppressors of T cells, such as myeloid-derived suppressor cells (Fig. 2; refs. 42, 43).

Of importance, metastatic niche formation was recently reported to depend on HIF-1α, which induces multiple members of the lysyl oxidase family that facilitates bone...
marrow–derived cell recruitment prior to metastatic colonization by malignant cells (Fig. 2; ref. 44).

The effects exerted by hypoxia on dendritic cells remain unknown (Fig. 2); however, hypoxia has been found to favor the immune and inflammatory response, thereby presumably enhancing their ability to prime and activate T cells (45). Moreover, dendritic cells cultured with hypoxic glioblastoma tumor lysates are more efficient at priming effector T cells compared with antigen uptake from normoxic tumor-cell lysates (46).

**TILs under hypoxia**

T lymphocytes in tumors come in different flavors (Fig. 2). Some are potentially endowed with tumoricidal properties that are restrained, whereas others can suppress the functions of effector T cells and natural killer cells. Such
immunosuppressor lymphocytes are called regulatory T cells [Treg (47)]. Among effector T lymphocytes, CD8 and CD4 can be found in the tumor stroma. TILs enter tumors from blood vessels. A variety of factors in the tumor repress their functional activity, although there is evidence for tumor antigen recognition by TILs. The balance between regulatory and effector T cells in the stroma correlates with the prognosis of patients and the response to immunotherapeutic interventions (48).

When T cells enter tumors, they reach progressively more hypoxic regions as they penetrate deeper into the tumor. Nonetheless, T cells infiltrating tumors tend to remain associated with vessels in subregions that ought to be less hypoxic. It is well known that areas of solid tumors can become completely anoxic and undergo necrosis. Very few T cells are found around these necrotic areas.

Unfortunately, little research has been carried out regarding the activation of T cells under hypoxia and the differences with activation under normoxic conditions. Some pioneer studies seem to suggest a less efficient activation by T-cell receptor (TCR)-derived signals and costimulation (49–51). Moreover, it has been reported that HIF-1α-deficient T cells respond with higher intensity to stimulation of the antigen receptor. In addition, a study of bacterial sepsis showed a functional advantage for T cells upon conditional deletion of the HIF-1α gene in T cells in the clearance of disseminated bacteria in mice (52). The CD4 and CD8 HIF-1α-deficient T cells of these mice also showed a more intense ability to proliferate and to produce IFN-γ (53). Therefore, it was concluded that HIF-1α is a repressor of T cells, and hypoxia should be an intrinsic immunosuppressive condition for T cells, including the ability to kill TCR-recognized targets (53, 54). In contrast, a recent study suggested that HIF-1α deficiency in T cells does not affect proliferation of Th1/Th-2 polarization (19). It was also shown that hypoxia leads to less efficient target cell killing by CTLs (54). Overall, the T-cell HIF1α-response to hypoxia can be considered a factor that contributes to immunosuppression in the tumor microenvironment. At present, there is a conspicuous lack of information about how hypoxia affects Treg functions in the tumor microenvironment or even in ex vivo culture studies.

The mechanisms involved in the modulation of effector T cells by hypoxia include the release to the extracellular milieu of adenosine, which acts on A2R inhibitory receptors (55), production of oxygen free radicals, and HIF-1α control of genes that downmodulate the immune response (Fig. 1).

Dying cells release ATP that is metabolized to adenosine by the coordinated extracellular enzymatic functions of CD73 and CD39, which are also upregulated by hypoxia (Fig. 1; ref. 56). Soluble adenosine in turn acts on surface receptors of T cells (A2Rs) to augment intracellular cAMP, a second messenger known to repress T-cell functions (Fig. 1; ref. 57).

Under hypoxic conditions, the respiratory chain in the mitochondria becomes less efficient, to the point of releasing reactive oxygen species that are produced both by tumor and stromal cells. There is evidence that reactive oxygen species can inhibit NF-κB nuclear translocation and thereby switch off cytokine production. In addition, this oxidative stress has been shown to promote the selective degradation of components of the TCR-CD3 complex, such as CD3ζ (58).

Very recently it was discovered that HIF-1α favors the differentiation of Th17 cells via direct transcriptional induction of RAR-related RORγt and indirectly IL-17 (19). For these functions, HIF-1α partners with phosphorylated STAT-3, which enhances the transcription of HIF-1α and cooperates with HIF-1α at the RORγt promoter (Fig. 1). Moreover, CD4 T cells that are starting to differentiate toward FOXP3+ Treg cells experience another surprising effect of HIF-1α that involves degradation of the forkhead box P3 (FOXP3) protein (Fig. 1). Amazingly, HIF-1α binds protein-to-protein to FOXP3, and as a result recruits the VHL E3 ubiquitination machinery to mark FOXP3 for degradation (19).

We have observed that HIF-1α can modulate the expression of costimulatory receptors such as CD137 (A. Palazón et al.; unpublished data). Indeed, T-cell activation under hypoxia leads to a much more ready upregulation of surface CD137 upon antigen stimulation through a mechanism that is dependent on HIF-1α. This explains why TILs in tumors are brightly CD137+, including effectors and Tregs. Of importance, CD137 is a receptor that upon targeting by immunostimulatory monoclonal antibodies leads to a therapeutic enhancement of antitumor immunity (59). In the absence of any agonist ligand expressed by neighboring cells, CD137 is useless for the antigen-primed T cells. However, if immunotherapeutic agonist antibodies are received, a strengthened function of the TILs ensues and tumor eradication becomes a frequent outcome. Of note, recent findings indicate that CD137 expression on TILs inside human lymphomas has an important impact as a prognostic factor (60).

**Indirect mechanisms of hypoxia on T lymphocytes**

In addition to affecting T cells directly, hypoxia can modify other components of the tumor tissue that in turn dictate the behavior of T cells. Hypoxic tumor cells produce high levels of CC-chemokine ligand 28 (CCL28). This chemokine has been shown to selectively attract Tregs that express CXCR10, which also induces antigen presentation (60). This chemokine has been shown to selectively attract Tregs that express CXCR10, which also induces antigen presentation (60).

It has also been observed that tumor cells under the effect of hypoxia activate autophagy, and that autophagy protects malignant cells from being killed by cytotoxic T cells (63).

The overall picture is that TILs are restrained in tumors due to a partial deprivation of oxygen, which leads to HIF-1α stabilization and extracellular adenosine accumulation (55).
Clinical–Translational Advances

We must rethink tumor immunotherapy by taking local tumor hypoxia into consideration. Innate and adaptive immune cells are profoundly modified under hypoxia (Fig. 2). The response to hypoxia as mediated by HIF-1α in T cells and macrophages is entangled with key transcription factors that modify the intensity and quality of the immune/inflammatory response, including STAT-3, NF-κB, and RORγt (64).

Two immunotherapy strategies, adoptive T-cell transfer (65) and immunostimulatory monoclonal antibodies (66), currently stand out because of their potential benefit in the clinical arena. The impact of local tumor hypoxia on these therapies should be reexamined. In the most clinically effective form of adoptive T-cell therapy, TILs are taken from tumors, reinvigorated, and expanded to be given back to a host who has been preconditioned by chemotherapy and total-body irradiation (65). The TILs need to find their way back to the tumor and destroy it. The exact state of hypoxia in the tumor lesions, which modulates all of these phenomena (i.e., T-cell activation, memory differentiation, migration, and extravasation), needs to be experimentally addressed.

In the case of immunostimulatory monoclonal antibodies (66), we know virtually nothing about the function of costimulatory receptors of the TNF receptor and immunoglobulin superfamilies when the concentration of O2 drops. As in the case of CD137, even the expression of the targets for the immunotherapeutic antibodies can be modified by the hypoxia response.

If hypoxia is found to be a guilty accomplice of pernicious immunoregulatory networks in the tumor microenvironment, interventions with antioxidants, pharmacologic adenosine inhibitors, and drugs acting on the HIF response pathway, including proteasome inhibitors such as bortezomib, may be considered as interesting adjuvants for a variety of immunotherapies. In any case, the bottom line is that immunomodulatory mechanisms that are controlled directly or indirectly by the hypoxia response certainly do exist, and such mechanisms offer real opportunities for intervention.

Disclosure of Potential Conflicts of Interest

I. Melero has an ownership interest in Bristol-Myers Squibb and has served as a consultant to Bristol-Myers Squibb, Pfizer Inc., Merck-Kerexon, Digna Biotech, and Milestone Biotech. The other authors disclosed no potential conflicts of interest.

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