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The role of hypoxia and acidosis in promoting metastasis and resistance to chemotherapy

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1. ABSTRACT

By a multiplicity of mechanisms, hypoxia and acidosis create a nurturing environment for tumor progression and the evolution of metastatic, drug-resistant cells. Acidosis drives mutagenesis and promotes the subversion of checkpoints and apoptotic mechanisms. Hypoxic tissues secrete cytokines that undermine normal anti-tumor surveillance by macrophages, turning them into accomplices and facilitators of invasion and angiogenesis. Invasiveness is also abetted by acidosis, the result of shifting to an anaerobic glycolytic metabolism. These factors explain the generally poor prognosis indicated by tumors expressing hypoxia-inducible factor-1 (HIF-1). However, these insights into the physiology of hypoxic tumors have inspired the development of new chemotherapeutic approaches directed at these tissues, including bioreductive drugs and gene therapies, some of which are in clinical trials. The ability to target the hypoxic compartment should allow longer progression-free survival and overall survival of patients bearing solid tumor malignancies.

2. INTRODUCTION

Unbridled tumor growth creates a stressful microenvironment, both for the neoplastic cell and for adjacent tissues, including fibroblasts, endothelia, and macrophages. As the tumor volume expands beyond the ability of neighboring vasculature to support it, oxygen is depleted, and hypoxia causes a metabolic shift that leads to extracellular acidification. Hypoxia and acidosis in turn lead to genomic instability, which potentiates further tumor progression. (1-5)

In this review, we describe the mechanisms by which hypoxia and acidosis affect the course of tumor development. To do so, we must first consider how normal tissues respond to these stresses and the players involved. Then we will consider how these responses differ in the growing tumor. In particular, the contribution of hypoxia-stressed macrophages to tumor progression will be described in depth. The hypoxia-inducible factor (HIF) and its client genes are central to these processes and will also be explored in some depth. Finally, possible drug therapies

Table 1. Agents that induce macrophage transition

M1 to M2	M2 to M1	Reference
IL-41	Interferon gamma	23, 24
IL-10 1,2	HCMV	23, 24
IL-13	Microbial products	24
TGF-beta-1 1,2		23, 24
Steroids		24
Prostaglandin e2 (PGE2) 1,2		23, 24

[1]Secreted by tumor cells along with MDG, IL-6 23, 96, [2]Suppress expression of MHC Class II by Macrophages 23

will be presented for the treatment of hypoxic tumors. Properties of hypoxic tumors that are being exploited to facilitate drug delivery will also be examined.

3. HYPOXIA IN NORMAL AND TUMOR TISSUES

In order to understand the impact of hypoxia on the tumor cell, we must first consider the normal cellular response to hypoxic conditions. Hypoxia in normal tissues can be termed as chronic or acute. Chronic hypoxia occurs when tissues are deprived of oxygen for long periods of time whereas acute hypoxia is used to define times of sudden oxygen deprivation. Chronic hypoxia plays a role in diseases such as diabetes where reduced blood supply to the extremities leads to necrosis of tissues. Stroke and heart attack, where there is a short disruption of oxygen and then re-oxygenation, are examples of acute hypoxia. (6)

Hypoxia activates the transcription factor HIF-1α (described in sections 7 and 8) leading to the activation of p53 and expression of cyclin-dependent kinase inhibitors (CKI) (7). It has been shown that HIF-1α directly associates with, activates and stabilizes p53 (8, 9). When HIF-1 alpha is activated, it prevents cells from progressing through the G1 phase of the cell cycle into S phase by increasing expression of CKIs $p21^{cip1}$ and $p27^{kip1}$ (7). p21 and p27 suppress cyclin/Cdk2 activity, causing dephosphorylation of the retinoblastoma protein, pRb, blocking the transcription factor E2F and cell cycle progression. At this check point, if the cells are damaged, DNA repair ensues. Upon repair, pRb becomes phosphorylated, releasing E2F, allowing transcription and progress of undamaged cells with undamaged DNA to S phase. It has also been suggested that hypoxia-induced cell cycle arrested may be accompanied by decreased expression of specific cyclin dependent kinase (CDK) complexes as well as dephosphorylation of pRb (7, 10).

Bennin *et al.* showed that cyclin G2 is induced by hypoxia as a result of HIF-1 activation. Cyclin G2 binds to protein phosphatase 2A resulting in negative cell cycle regulation (7, 11, 12). When cells are ready to exit the G1 phase and enter S phase, cyclin E binds to Cdk2, phosphorylating p21(7). This results in the degradation of cyclin E and promotes expression of cyclin A, which can then bind to Cdk2, releasing cells into S phase. HIF-1 regulates cyclin E expression, but not cyclin A. Goda *et al.* have shown that HIF-1 alpha-deficient MEFs cultured under hypoxic conditions demonstrate enhanced and continuous accumulation of cyclin E but not cyclin A. In addition, Cdk2 activity is elevated, and cells enter S phase regardless of the hypoxic stress (7). In contrast, p53 played a negligible role in hypoxia-induced arrest. These findings suggest that HIF-1 is the primary oxygen-sensitive gate-keeper at the G1/S boundary (7).

4. MACROPHAGES AND TAMS IN NORMOXIC AND HYPOXIC ENVIRONMENTS

The lymphatic system plays an important role in the immune response by trafficking immune cells and interstitial fluid in and out of tissues (15). Interstitial fluid consists of dendritic cells, macrophages, lymphocytes and mast cells, all of which are necessary for the body to initiate an effective immune response to foreign material, including dead and mutated cells that populate tumors. Tumors grow in confined spaces. As the tumors grow larger, they put mechanical stress on the structures within, including blood vessels and lymphatic vessels causing interstitial fluid to "leak" out of the tumor (15). The loss of interstitial fluid within the tumor means that an effective immune response cannot be implemented because macrophages, mast cells, and dendritic cells are lost into the surrounding tissues (6, 16).

4.1. Anti-tumor and pro-tumor functions

Macrophages are extremely important in maintaining the normal tissues and are the body's first line of defense against invading microbes (17, 18). Macrophages have the unique ability to aid in normal cell turnover, repair injured sites and aid in tissue remodeling (18, 19). Tumor associated macrophages (TAMs) are abundantly found in all stages of tumor progression (20). TAMs like normal macrophages are phagocytes. Since hypoxia itself causes cell death, it has been suggested that TAMs are attracted to these areas (21). Once TAMs reach the hypoxic microenvironment, hypoxia inhibits their migration, keeping the macrophages from doing their intended job of clearing out all the dead and damaged cells (21).

Macrophages can be grouped into two categories: M1 and M2. M1 macrophages are efficient immune effector cells that kill microorganisms as well as tumor cells and efficiently present surface antigens, resulting in the secretion of high levels of T-cell stimulatory cytokines (22). They are typically high in IL-12 but low in IL-4, 10 and 13, while M2 macrophages are low in IL-12 but high in IL-4, 10 and 13. In normal tissues under normoxic conditions, macrophages identify tumor cells as foreign and initiate their removal by expressing antigens that stimulate the anti-tumor functions of T-cells and NK cells. On the other hand, macrophages from tumor tissues under hypoxic conditions or exposed to tumor derived cytokines such as IL-4, IL-10, TGF-β and prostaglandin E2 (PGE_2) (Table 1) convert from M1 to M2 macrophages (22, 23). M2 macrophages are poor antigen presenters, promote angiogenesis, are scavengers and, unlike M1's, produce factors that inhibit T cell proliferation and activity (22). Thus, rather than attacking tumor cells, these macrophages promote their survival.

TAMs exposed to hypoxia acquire a pro-angiogenic phenotype by expressing vascular endothelial

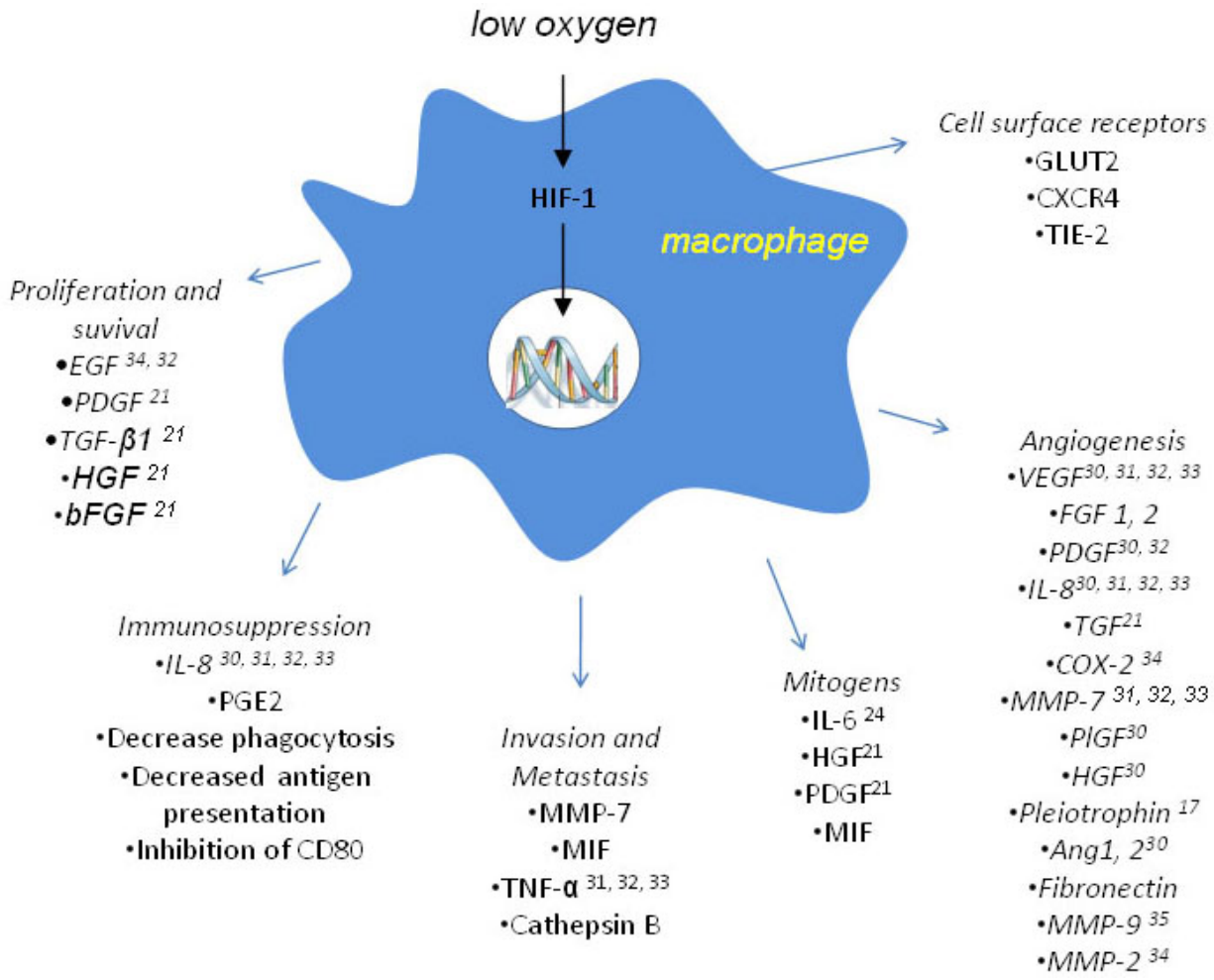

Figure 1. Changes in macrophage phenotype induced by hypoxia. When a macrophage enters a hypoxic tumor, HIF transcription factors activate a multiplicity of adaptive responses, some of which may benefit the tumor. Factors are secreted that promote growth and survival, invasion, and angiogenesis. Adapted from Lewis (17).

growth factor (VEGF) and become pro-invasive through increased expression of proteinases such as matrix metalloproteinase-7 (MMP-7) (20, 25-28). They also express other pro-tumor cytokines and cell surface receptors (21). Hypoxia associated TAMs have also been shown to up-regulate HIF-1 and -2 both *in vitro* and *in vivo* (28, 29). Figure 1 lists some of the phenotype changes seen in macrophages exposed to hypoxia.

4.2. TAMs and Prognosis

A high level of TAMs is generally associated with poor prognosis (32), with the exception of stomach, colorectal cancer and melanoma (36-38). Hypoxia induces Tie-2 expression by macrophages and modulates its response to Ang-2 (4, 39). Ang-2 is up-regulated by hypoxic tumor cells. Exposure of Tie-2 positive macrophages to both hypoxia and Ang-2 has a noticeable inhibitory effect on the release of IL-12, a potent anti-angiogenic cytokine whose expression is down-regulated in macrophages that have migrated into hypoxic tumors (17, 39). Instead of an anti-angiogenic response (17), these TAMs promote rapid angiogenesis and the re-oxygenation of the hypoxic cells (40). The combined action of Ang-2 and hypoxia also inhibits the release of TNF-alpha (40). In high concentrations, TNF-alpha is known to promote apoptosis in both tumor and endothelial cells (39, 40). Table 2 supplies a list of some cancers, their association with TAMs, and the correlation of TAMs with prognosis.

5. SOURCES OF GENOMIC INSTABILITY

Re-oxygenation of hypoxic tissues results in its own set of problems. The activation of HIF-1alpha should induce apoptosis through activation of p53. This program is foiled when p53 is mutant (62).

5.1. Reactive Oxygen Species

One source of mutation and genomic instability is the creation of reactive oxygen species (ROS) that cause DNA damage. In acute hypoxia, the sudden disruption of oxygen and then reoxygenation results in the production of free radicals that lead to "reperfusion injury" or

Table 2. Occurrence of HIF-1alpha and TAM in human cancers and correlation with prognosis

HIF expression detected in tumors	Cancers where increased TAM populations correlate with good prognosis	Cancers where increased TAM populations correlate with poor prognosis	Cancers where number of TAMs has no correlation with prognosis	References
Brain	Stomach	Breast [5,6]	Colon carcinoma	38, 41-45
Bladder	Colorectal	Prostate [5]	High-grade astrocytomas	36, 41, 46, 47
Breast1	Melanoma	Endometrial [5]	Lung carcinoma	19, 25, 28, 37, 38, 41, 49-51
Colon		Bladder [5,6]	Cervical carcinoma	41, 52, 53
Ovarian[1]		Kidney [5]		25, 28, 41, 48, 49, 51, 54
Pancreatic		Esophageal		41, 55
Clear cell renal carcinoma [3,4]		Squamous cell carcinoma [5]		41, 48, 51, 56, 57
Prostate		Malignant uveal melanoma [5]		41, 58
Oligodendroglioma1		Follicular lymphoma		25, 28, 48, 49, 51
Oropharyngeal1				25, 28, 48, 49, 51
Esophageal1				25, 28, 48, 49, 51
Non-small cell lung cancer 2				60, 61
Hemangioblastoma 4				41, 56

1 Indicates overexpression of HIF-1alpha and correlation with high aggressiveness, poor prognosis and treatment failure, 2 HIF-1alpha expression correlates with apoptosis and patient survival, still controversial, 3HIF-2alpha overexpression, 4 HIF-1alpha / HIF-2alpha expression independent of hypoxia, 5 correlates with increased tumor angiogenesis, 6correlates with increased involvement of local lymph nodes.

"reoxygenation injury" (63) . Mutations in pro-apoptotic genes like p53, BAX, and BAK, as well as overexpression of anti-apoptotic factors such as BCL2, make it possible for the damaged cells to survive (64-67) .

5.2 Polyploidy

Tumors that overexpress BCL2 exhibit changes in ploidy (64). Polyploidy occurs when mitotic checkpoints fail and chromosomal DNA is endoreplicated (64), i.e., the genome is duplicated without cell division. Polyploidy correlates with an increase in nuclear size and is considered a prerequisite for genomic instability (68, 69). Nelson *et al* have shown that hypoxia promotes polyploidy both *in vitro* and *in vivo* and that these cells have defects in their intrinsic apoptotic pathways, a hallmark of cancer (64, 70).

5.3. Underexpressed or nonfunctional repair enzymes

Since DNA repair enzymes do not function properly and are under-expressed in hypoxic tumors and cell cycle check points are also defective, what ensues is gene amplification of the damaged DNA (7). The more rounds of replication the damaged DNA completes, the more genomic instability arises within the cell population (2, 4, 5, 13, 71). In a Darwinian process, the increase in mutations leads to cells that are better suited to the atypical hypoxic microenvironment (71-75). Furthermore, those that eventually make up the tumor are overly simplified and equipped with only enough genetic information to survive and grow (72, 75-77). This dedifferentiation allows cells to thrive in microenvironments of low oxygen and low nutrients where normal cells could never survive (72, 75, 76).

5.4. Acidosis

The intracellular pH of hypoxic tumors is known to be neutral or slightly alkaline. In contrast, the extracellular pH is acidic. There are two sources of hydrogen ions that contribute to extracellular acidosis—lactic acid and carbonic acid (6, 15, 78) . The build up of lactic acid results from prolonged anaerobic glycolysis, and carbonic acid, from the conversion of carbon dioxide and water via carbonic anhydrase. Carbonic anhydrase and enzymes used in anaerobic glycolysis are all regulated by HIF-1 (6). Acidosis also creates a mutagenic microenvironment. Low pH induces topoisomerase II-dependent DNA strand breaks and is carcinogenic in mice (79).

6. ANGIOGENESIS, METASTASIS AND INVASION

Angiogenesis is under tight control in normal tissues. There is a strict balance between pro- and anti-angiogenic factors, often favoring anti-angiogenic factors. In tumor cells, however, the balance is offset toward pro-angiogenic factors. Tumors that outgrow their initial vasculature become dormant and must continually recruit more vasculature to resume expansion. Hypoxia induces the upregulation of angiogenic factors such as VEGF (vascular endothelial growth factor), ANG2 (angiopoeitin-2), PDGF (platelet derived growth factor), PLGF (placenta growth factor), TGF (transforming growth factors), IL-8 and HGF (hepatocyte growth factor) (51).

Cells that occupy nutrient-poor hypoxic tumors migrate to richer environments (80). This is facilitated by the lower extracellular pH of hypoxic tumors, which promotes the degradation of the extracellular matrix by proteases and increases cell death and genomic instability in surrounding tissues (77, 81). The cells that can survive in the acidic environment help the tumor metastasize and invade the surrounding tissues. Once the basement membrane is breached, cancer cells are able to invade the surrounding tissue (82).

7. HYPOXIA INDUCIBLE FACTOR (HIF)

The hypoxia inducible transcription factor-1 (HIF-1) is considered to be a crucial mediator of cell response to reduced oxygen levels (30, 83). HIF-1 is known to control the expression of many genes that have important

Table 3. HIF-1 Target Genes

Cell proliferation	TGF-alpha	WAF-1	Cyclin G2	Cyclin D	TGF-beta	IGF2				
Cell survival	TGF-alpha	VEGF	NOS2	IGF2	EPO	ADM				
Apoptosis	NIP3	NIX	P53	BAX	BAK					
Motility	ANF/GPI	C-MET	LRP1	TGF-alpha						
Cell adhesion	MIC2									
Erythropoiesis	EPO									
Angiogenesis	VEFG	ENG	LEP	LRP1	TGF-beta3					
ECM Metabolism	CATHD	Collagen type V	FN1	MMP2	PAI1	Prolyl-4-hydroxylases (PDH)				
pH regulation	Carbonic anhydrase 9									
Glucose metabolism	HK1, 2	ALD A, C	TPI	AMP/GPI	ENO1	GLUT1, 3	GAPDH	LDHA	PFKBF3	PFKL

Abbreviations: TGF, transforming growth factor; IGF, insulin-like growth factor; VEGF, vascular endothelial growth factor; EPO, erythropoietin; ADM, adrenomedullin; ENG, endoglin; LEP,leptin; LRP1, LDL-receptor related protein; CATHD, cathepsin D; FN1, fibronectin 1; MMP2, matrix metalloproteinase 2; HK, hexokinase; ALD, aldolase; TPI, trisephosphate isomerase, ENO, enolase; GLUT, glucose transporter; GAPDH, glyceraldehyde-3-p-dehydrogenase; LDHA, lactate dehydrogenase A; PFKBF3, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphate-3; PFK, phosphofructokinase. Not an exhaustive

roles in erythropoiesis, glycolysis, cell survival, hindering apoptosis and promoting angiogenesis. Studies have shown that the loss of HIF-1 activity results in a dramatic decrease in tumor growth and vascularization (84). In addition, HIF-1 regulates enzymes of glucose metabolism (GLUT-1, GLUT-3, hexokinase I and II). Overexpression of any of these genes leads to increased glycolysis in tumors (74, 75, 76, 85). Table 3 lists target genes relevant to these processes.

7.1. HIF-1alpha

As a heterodimer, HIF-1 consists of an alpha and a beta subunit (86). The beta subunit of HIF is known as aryl hydrocarbon nuclear translocator (ARNT). Both subunits are members of the basic-helix-loop-helix (bHLH) PER, ARNT and SIM (PAS) superfamily of transcription factors found in eukaryotes (2, 84, 86-88). DNA binding is mediated by the bHLH domains, while the two PAS domains are required for heterodimer formation (86, 89). HIF-1α itself contains three domains—TAD-N, TAD-C and PAS (84). TAD-N is the N-terminal transactivation domain and contains an oxygen-dependent degradation subdomain (ODD). TAD-C is the C-terminal transactivation domain and requires p300/CBP for its activation.

7.2. HIF-2α

HIF-2 alpha, also termed EPAS/HRE (endothelial PAS) is primarily a cytoplasmic, constitutively expressed protein mainly found in vascular endothelial cells. It is not degraded in the presence of oxygen and is upregulated in cancers (86, 90-92). Endogenous HIF-2 alpha does not stimulate the transcription of the genes that HIF-1 alpha targets and is thought to be primarily responsible for hypoglycemia-induced gene expression (86, 93).

7.3. HIF-3α

Of the three types of heterodimers that can form with ARNT, HIF-3 alpha is by far the least characterized family member. It shows similarity within the bHLH and PAS domains to HIF-1 alpha and HIF-2 alpha but lacks the C-terminal transactivation structures. It is expressed in adult thymus, brain, heart and kidney (84). Surprisingly, even though HIF-3 alpha has been found to be upregulated and dimerize with ARNT (HIF-1 beta) in response to hypoxia, it has been shown *in vitro* that HIF-3 alpha is an inhibitor of HIF gene expression, possibly due to the inhibitory PAS domain (IPAS) that is a splice-variant of HIF-3 alpha (7). IPAS has both bHLH and PAS domains but lacks a transactivation domain (84). This inhibitor binds to the alpha subunit of HIF-1, resulting in a heterodimer that does not associate with HRE and as such is considered a negative regulator of HIF-1 alpha (84).

8. REGULATION OF HIF-1

HIF-1 is regulated post-transcriptionally by ubiquitination through its interaction with pVHL (von Hippel-Lindau protein, tumor suppressor and member of the E3 ubiquitination complex) that targets it for degradation by the 26s proteasome (94, 94) (Figure 2).

8.1. PHD

pVHL binds to two proline residues located in the ODD of the alpha subunit of HIF-1 (7, 84). This binding occurs in the presence of oxygen because the enzyme responsible, prolyl-4-hydroxlyase (PHD), is a deoxygenase that requires oxygen, iron and 2-oxoglutarate as substrates (84). These enzymes also mediate the detection of intracellular oxygen levels (84). Generally, PHDs are inactive in hypoxia because of the low levels of oxygen. Four PHDs have been identified with very different functions (84). For example, PHD-2 is upregulated in hypoxia but under normoxic conditions, it sets the "normal" HIF-1α levels (84). PHD-2 is also a critical oxygen sensor (84). Its upregulation in hypoxia provides a HIF-1 dependent auto-regulatory mechanism that is driven by oxygen levels. PHDs are inactivated by competitive

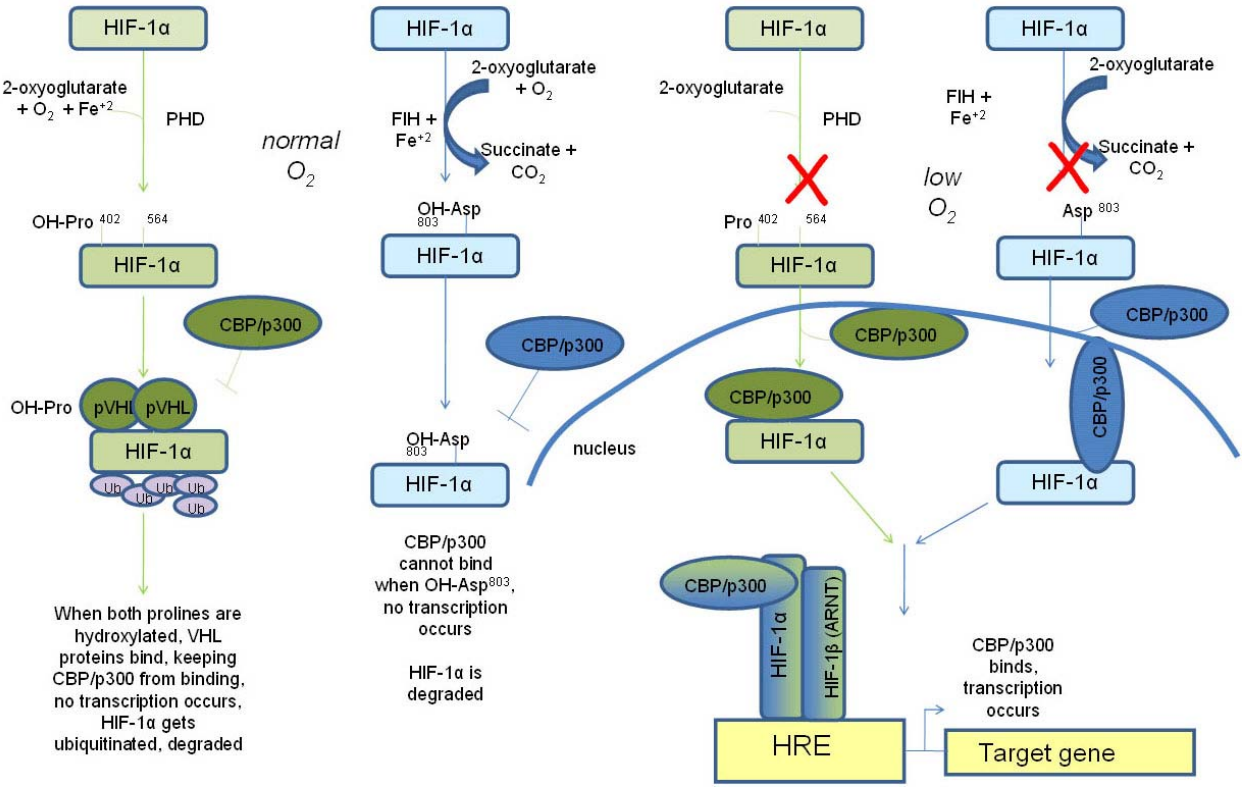

Figure 2. Tandem regulation of HIF-1 activity by oxygen sensors. Green, the PHD pathway. In normoxia, PHD hydroxylates two proline residues in the ODD domain of alpha subunit. Hydroxylation allows pVHL to bind, preventing p300/CBP binding, and resulting in ubiquination and degradation of the alpha subunit. In hypoxia, PHD is inactive, the proline residues remain unmodified, and pVHL cannot bind, allowing p300/CBP to bind alpha and enable it to complex with beta. This complex then binds to HRE (Hypoxia Response Element) and activates transcription of HIF-1 target genes. Blue, the FIH (asparaginyl-aspartyl hydroxylase) pathway is similar except that its target is Asp803.

substrate analogues (84). When PHDs are inactivated, the HIF prolines remain unmodified and do not bind to pVHL, causing HIF-1α levels to increase.

8.2 FIH

Under normoxic conditions, an asparagine (located at N^{803} in HIF-1 alpha and N^{851} in HIF-2 alpha) is hydroxylated by cofactors 2-oxoglutarate, oxygen and the enzyme FIH-1 (Fe^{+2} dependent HIF- alpha asparagine hydroxylase) (7, 95). This hydroxyl group prevents CBP/p300 complex from binding to TAD-C and activating gene expression (7, 95). Figure 2 shows how PDH (green) and FIH (blue) control gene expression in normoxia and hypoxia.

8.3. Other regulation

Other pathways regulate HIF-1 alpha levels independently of pVHL (96, 97). MDM2 ubiquitin ligase is recruited to HIF-1 alpha through its interaction with p53. Binding of MDM2-p53 to HIF-1 alpha causes HIF-1 alpha to become ubiquinated and degraded (97). As one would expect, a loss of p53 in tumor cells results in an increase in HIF-1 alpha levels.

Another factor regulating HIF-1 alpha stability is heat shock protein-90 (Hsp-90) (84). Hsp-90 interacts directly with HIF-1 alpha and is thought to impart a conformational change to the alpha subunit upon heterodimerization with the beta subunit (HIF-1 beta) (85, 84). Inhibitors of Hsp 90 promote the loss of HIF-1 alpha protein even in cell lines lacking pVHL (98). Mutations in prolines 402 and 564 do not stop Hsp-90 inhibitor-induced degradation of HIF-1 alpha (84).

9. TREATMENT OF HYPOXIC TUMORS

Hypoxic tumors are known to be more chemoresistant and radioresistant than tumors exhibiting normal oxygen levels. Radiotherapy and many chemotherapeutic drugs rely on oxygen to generate cytotoxic ROS and are much less effective on hypoxic tumor tissue (99). The heterogeneity of blood flow within tumors is an important contributor to hypoxia and thus a major cause of resistance to radiation and some chemotherapeutics. Hypoxic tumors are known to be more aggressive, invasive and metastatic and thus correlate with poor prognosis (14, 80). Moreover, as mentioned previously, immunosurveillance is subverted in hypoxia (6). It is important to take into consideration these unique characteristics of hypoxic cells when devising therapies.

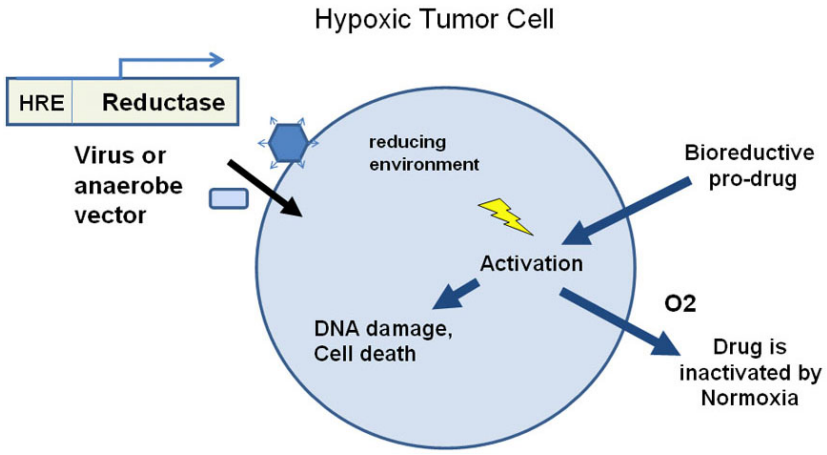

Figure 3. Targeted gene therapy in which genes delivered by adenoviruses or anaerobic bacteria potentiate the action of bioreductive drugs that exploit the hypoxic nature of the tumor. The drug is activated upon entering the cytosol but inactivated upon exit, thus minimizing damage to adjacent normal tissues.

Table 2 contains a brief list of cancers exhibiting HIF-1 expression and its correlation with prognosis and treatment.

9.1. Chemotherapy based on hypoxia and pH

Hypoxia can be seen as a physiological abnormality that is restricted to the tumor itself 100. Exploiting this unique characteristic of tumors is at the forefront of new treatment strategies. The so-called *bioreductive drugs* are preferentially cytotoxic to hypoxic cells. There are three classes of bioreductive drugs: quinines, nitro compounds, and benxotriazine di-N-oxides (101). One such bioreductive drug, mitomycin C, a quinine-alkylating agent, has been widely used in combination chemotherapy routines for breast cancer (101). In the reducing environment of hypoxic tissues, this drug becomes an alkylating agent that attacks the O6 and N7 positions of guanine, leading to intrastrand and interstrand cross-links in DNA (76). Triapazamine (TPZ), a benxotriazine di-N-oxide, is in phase II/III clinical trials in combination with cisplatin (101). According to Denny *et al.*, TPZ in combination with cisplatin is more effective on malignant melanoma and non-small cell lung cancer than cisplatin alone. The low extracellular pH of hypoxic tumors also affects their permeability to cytotoxins. The trans-membrane gradient hinders the uptake of weak base drugs like doxorubicin, reducing their efficacy in tumor tissues (6). The low extracellular pH can be exploited by using weak acid drugs like chlorambucil that can easily permeate the membrane in their uncharged state. Since the intracellular pH of hypoxic tumors is neutral or slight alkaline, the drug is then re-ionized and activated, resulting in an increased drug concentration within the tumor (102).

9.2 Hypoxia-regulated Gene Therapy

Bos *et al.* in 2001 found a crucial role for HIF-1 in breast cancer progression (48, 103). The study found that more aggressive forms of breast cancer correlated with the over-expression of HIF-1. All HIF-1-responsive genes have the hypoxia responsive element (HRE); this sequence can be used to confine expression of a therapeutic gene to hypoxic tissues (101). The first use of this concept was by Dachs *et al.* with HRE-regulated expression of the suicide gene cytosine deaminase (104). They showed an increased sensitization of hypoxic cells to the pro-drug 5-fluorocytosine (104, 105).

Gene therapy can be combined with bioreductive drugs, for example by using an HRE to drive expression of a reductase gene. This system would allow the specific activation of the bioreductive drug to produce DNA-damaging free radical species within the hypoxic cells. Once the bioreductive pro-drug is activated, it can diffuse into and kill other hypoxic regions of the tumor, but diffusion into healthy, normoxic tissue re-oxidizes it into its non-toxic pro-drug form (104). Figure 3 is a schematic of how an adenovirus or anaerobic bacteria can work together with targeted gene therapy for the activation of bioreductive drugs.

9.2.1. Viral vectors

Viral vectors can be used to specifically target surface receptors known to be upregulated by hypoxia, e.g., that of VEGF (104). To date, numerous viral vector systems have been proposed for cancer gene therapy including retroviral, adenoviral, adeno-associated virus, and herpesvirus (106).

Retroviruses are appealing because they have an inherent specificity for actively dividing cells. Murine Moloney leukaemia virus (MoMLV) was one of the earliest retroviral systems exploited for gene therapy (106). Replication-competent MoMLV was shown to fully transduce a glioma after a single intracranial injection and mobilize away from the injection site (107). No virus was detected in non-tumor tissues, indicating stringent tumor specificity (106, 107).

Adenoviral vectors (AV) have also been widely used to target solid tumors (106). AV can be produced at high titer and infect a wide range of cancer cells (108). Two

AV's have been approved for clinical use of head and neck cancers in China—one delivering wildtype p53 (109) and the other designed to replicate only in p53-deficient cells (106). However, a disadvantage of adenoviruses for targeting hypoxic tissues is that stressed cells downregulate functions required for viral replication, limiting their efficacy.

9.2.2. Anaerobic bacteria as vectors

Anaerobic bacteria have long been an appealing vector because of their natural preference for hypoxic tissues. Several strains have been used, either alone or in conjunction with chemotherapy or radiation (101). Of particular interest is the use of Clostridial spores. These gram-positive, rod-shaped bacteria are strict anaerobes that form spores under harsh conditions (106). Intravenously injected spores germinate only when they encounter an anaerobic environment (106). In 1947, one of the first experiments using Clostridia demonstrated that direct injection of *C. histolyticum* spores into a mouse sarcoma caused oncolysis and tumor regression (110). Later studies, using a different strain (*C. tetani*) demonstrated that when intravenously injected into healthy, non-tumor bearing mice, the mice remained healthy (106). When injected into tumor-bearing mice, all mice died within 48hrs after injection of *C. tetani* because the spores were allowed to germinate and systemically released the tetanus toxin (111). *C. sporogenes,* a non-pathogenic strain of Clostridia, was the first to be gene-modified (112).

Several Phase I clinical trials have been conducted using anaerobic bacteria as vectors for the treatment of solid tumors, but outcomes have not yet been posted. A series of Phase 1 trials was conducted by the National Cancer Institute to examine whether genetically modified *Salmonella typhimurium* (VNP20009) administered intravenously can inhibit the growth of advanced or metastatic cancers (e.g., see NCT00004988). Another, sponsored by Anza Therapeutics (NCT00585845, terminated), used an attenuated form of *Listeria monocytogenes* that has been genetically altered to release the antigen, mesothelin. Mesothelin is believed to be present primarily on tumor cells and as such, using bacteria to both colonize the hypoxic tumor and release more of the antigen could provoke an immune response. The last study (NCT00358397), sponsored by Sidney Kimmel Comprehensive Cancer Center, used a one-time intravenous infusion of *Clostridium novyi-NT* spores for the treatment of solid tumors that failed to respond to conventional therapy. This strain was designed at the Center to infect and kill hypoxic tumors but not to grow in normal tissue (113). A single IV injection of these bacteria resulted in dramatic tumor regression and prevented recurrence in 30% of the animals. However, the clinical trial is currently suspended. Details can be accessed at www.clinicaltrials.gov.

10. CONCLUSION

Here we have considered how tumor growth leads to hypoxia and acidosis and in turn creates a nurturing environment for tumor progression and the selection of metastatic, drug-resistant cells. We discussed how this mutagenic milieu promotes the subversion of checkpoints and apoptotic mechanisms, and how hypoxic tissues secrete cytokines that turn macrophages into facilitators of invasion and angiogenesis. Invasiveness is also abetted by acidosis, the result of shifting to an anaerobic glycolytic metabolism. Central to the process is the transcription program induced by activation of HIF-1, a program designed for escape from the primary tumor. Thus, hypoxia has come to be regarded as a necessary step in the education of the metastatic cell, and HIF-1 expression is regarded as a negative prognosticator. However, increased understanding of the physiology of hypoxic tumors has led to the development of new chemotherapeutic strategies directed at these tissues, including bioreductive drugs, gene therapies that target unique aspects of hypoxic tissues, and exploitation of bacterial anaerobes. The ability to target the hypoxic compartment should in principle allow us to subvert the maturation algorithm that produces the metastatically competent cell and keep the tumor manageable and local.

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Abbreviations: EGF: epidermal growth factor; PDGF: platelet derived growth factor; TGF-Beta: transforming growth factor beta; bFGF: basic fibroblast growth factor; IL-8: interleukin 8; PGE2: prostaglandin E2; MMP-7: matrix metalloproteinase-7; MIF: macrophage migration inhibitory factor; TNF-alpha: tumor necrosis factor alpha; IL-6: interleukin 6; HGF: hepatocyte growth factor; GLUT2: glucose transporter 2; CXCR4: CXC receptor type 4; TIE-2: TEK tyrposine kinase; VEGF: vascular endothelial growth factor; FGF: fibroblast growth factor; IL-8: interleukin 8; TGF: transforming growth factors; COX-2: cyclooxygenase 2; PlGF: placental growth factor; ANG 1, 2: angiopoietin 1, 2; MMP-9: matrix metalloproteinase-9; MMP-2: matrix metalloproteinase-2

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