Hypoxia in the tumorigenesis of gliomas and as a potential target for therapeutic measures

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✓ In this article, the author provides a brief description of the role of hypoxia in the tumorigenesis of gliomas and suggests potential ways of exploiting this role to design treatment modalities. Tumor hypoxia predicts the likelihood of metastases, tumor recurrence, resistance to chemotherapy and radiation therapy, invasive potential, and decreased patient survival for many human malignancies. Various methods of measurement of tumor hypoxia are discussed, including direct measurement and imaging methods.

The role of hypoxia-responsive molecules, especially hypoxia-inducible factor-1 (HIF-1), in glioma tumorigenesis is explored. Treatment modalities regulated by hypoxia are proposed and some potential strategies reviewed. The progression of a low-grade astrocytoma to a glioblastoma multiforme may be mediated by hypoxia-induced phenotypic changes and subsequent clonal selection of cells that overexpress hypoxia-responsive molecules, such as HIF-1. In this model, intratumoral hypoxia causes genetic changes that produce a microenvironment that selects for cells of a more aggressive phenotype.

KEY WORDS • hypoxia • tumorigenesis • glioblastoma multiforme • vascularization

GLIOMASTOMA multiforme is the most common and most malignant brain tumor in humans, with a survival rate of only 0 to 5% at 5 years after presentation despite therapy. Although LGAs usually have a much better prognosis, most eventually progress to become higher-grade tumors. It is not understood what triggers this transformation, but features such as intratumoral necrosis and vascular endothelial proliferation distinguish high-grade from low-grade tumors.^{75,105}

In this paper, I propose the hypothesis that as an LGA outgrows its blood supply, small areas of hypoxia develop. The cells best able to respond to this hypoxic stress are clonally selected, and the tumor progresses to become a GBM. These cells have the ability to promote angiogenesis, a higher proliferation potential, and a decreased response to therapeutic measures. I hypothesize that this effect is mediated by HRMs such as HIF-1 α . A better understanding of this process and, eventually, the development of treatments that target HIF-1 α or similar HRMs in human gliomas could result in a better prognosis for patients with this devastating disease.

Abbreviations used in this paper: GBM = glioblastoma multiforme; HIF-1 = hypoxia-inducible factor-1; HRE = hypoxia response element; HRM = hypoxia-responsive molecule; LGA = low-grade astrocytoma; shRNA = short hairpin RNA; siRNA = small interfering RNA; VEGF = vascular endothelial growth factor; VHL = von Hippel-Lindau.

Hypoxia and Tumorigenesis

Glioblastoma multiforme is one of the most highly vascularized human tumors, but this microcirculation is functionally very inefficient compared with that of normal brain tissue.^{14,50,60,64,150} Intratumoral necrosis, the hallmark of GBM, is found in both small and large tumors, suggesting that necrosis may not simply be due to inadequate vascular supply but instead may be a result of intrinsic molecular or genetic changes within the tumor.¹¹⁷ Direct and indirect measurements of tumor hypoxia in human GBMs have not cleared up this controversy.^{51,100,113,151}

Evidence suggests that hypoxia plays a role in the malignant transformation of cells and subsequent tumor growth (Fig. 1). In a number of different human cancers, tissue hypoxia predicts the likelihood of metastases,¹⁴⁴ tumor recurrence,⁵⁹ resistance to chemotherapy and radiation therapy,^{41,118,130,159} invasion,^{31,48} and decreased patient survival.^{58,156} In fact, the degree of necrosis within a GBM correlates inversely with patient outcome and survival.^{52,76,80} Hypoxic stress has been linked to several phenotypic changes that are fundamental to malignant progression.^{129,131} This can be mediated through genomic instability, loss of apoptotic potential, alterations of gene expression, and induction of angiogenesis.^{47,67,117,126,141,145} All of these processes are exhibited in GBMs.^{42,53,72,88} I hypothesize that hypoxic stress causes a clonal expansion of cells with increased angiogenic potential and decreased apoptotic potential and that the result is a more aggressive tumor phenotype, in this case GBM (Fig. 2).^{43,110}

FIG. 1. Diagram showing known cellular responses to hypoxia that promote tumorigenesis.

Direct Measurement of Tumor Hypoxia

Intracellular hypoxia can be measured by biochemical markers of tissue hypoxia. The nitroimidazole derivatives EF5⁸⁷ and pimonidazole^{7,121} have been used to label hypoxic cells in animal and human tumors. At low O₂ concentrations, these markers undergo bioreduction by nitroreductases and bind to cellular macromolecules, which can be measured using immunohistochemical methods.^{121,155} As a demonstration of this technique, we grew U251 GBM cells subcutaneously in athymic mice and after 60 days injected the mice with pimonidazole hydrochloride (Hypoxyprobe-1; Natural Pharmacia International, Inc., Belmont, MA). This drug is bioreductive—it becomes charged and trapped within the cell under hypoxic conditions.^{111,158} The tumors were removed and fixed in formalin. The tissue was placed on glass slides, and cells with pimonidazole (hypoxic cells) were visualized using immunohistochemical methods with an antipimonidazole monoclonal antibody.^{7,112} On similar sections, the tumor vasculature was visualized using antibodies directed against a vascular marker (CD31 monoclonal antibody; Santa Cruz Biotechnology, Santa Cruz, CA). The U251 cells showed significant areas of necrosis with surrounding areas of hypoxia that correlate with pimonidazole staining (Fig. 3).

Polarographic O₂ microelectrodes have also been used to determine intratumoral hypoxia in various tumors but have produced mixed results in the limited number of brain tumors studied.^{9,29,55,69,71,113,155} These studies of polarographic O₂ microelectrodes lack spatial correlation with preoperative imaging or intracellular hypoxia measurement. The lack of direct correlation between measurements of polarographic O₂ microelectrodes, biochemical data, and imaging studies might account for the discrepancy of the prior work. With current stereotactic techniques and the ability to register anatomical location by

using the preoperative imaging studies described later, this may be overcome in the future.

Imaging Measurement of Tumor Vascularity, Hypoxia, and Perfusion

Peritumoral edema is a condition of increased vascular permeability associated with GBM that can be measured using MR imaging. This imaging technology has also been used to map changes in blood volume fraction and vascular functionality associated with angiogenesis.^{1,2,19,25,}

FIG. 2. Diagram showing the mechanism by which hypoxia promotes genetic instability and the clonal selection of a more aggressive phenotype.

FIG. 3. Photomicrographs showing the immunohistochemical findings in U251 glioma cells grown subcutaneously in athymic mice. a and d: Tissue sections with pimonidazole staining (brown areas, arrows) in presumed hypoxic regions surrounding areas of necrosis (arrowheads). b and e: Tissue sections obtained in negative controls (animals not injected with pimonidazole before tumor removal). c and f: Tissue sections stained with CD31 antibody demonstrating intratumoral vasculature. High-power view demonstrating vessel diameter and intraluminal area (c). Lower-power view demonstrating vessel number and vascular area in relationship to total tumor area (f). Original magnifications $\times 10$ (a, c, and f); $\times 40$ (b, d, and e).

^{32,46,49,77,143} Dynamic contrast-enhanced MR imaging can provide measurements of tumor perfusion and vascularity by using kinetic modeling of tumor contrast uptake over time.^{10,38,94,101,142,149} It has the potential to predict intratumoral blood flow and vascular permeability preoperatively. After data collection, the relative cerebral blood-volume maps are derived on a voxel-by-voxel basis from the dynamic imaging sets. One disadvantage is that the significant amount of postprocessing necessary for data acquisition is time consuming and requires technical expertise.^{10,32,104,143}

Magnetic resonance spectroscopy is an emerging technology that allows for in vivo chemical analysis of tissue during MR imaging.⁹⁶ With this technology, the distributions of various metabolite concentrations including *N*-acetyl aspartate, *N*-phosphocholine (choline), and lactate can be determined. Increased lactate, choline, or creatinine and decreased *N*-acetyl aspartate reflect the neuronal tissue loss and increased membrane synthesis that is known to occur in GBM.^{6,44,162} At present, whether increased lactate peaks are due predominantly to hypoxia or to increased tumor metabolism is not known.³⁴

Positron emission tomography provides the ability to measure the concentrations of positron-emitting radioisotopes within the tissues of living patients. Positron emission tomography of 2-nitroimidazole compounds, such as [¹⁸F]fluoromisonidazole, has been used to identify hypoxic tumors (including gliomas) in humans.^{9,114–116} The most studied of these compounds is [¹⁸F]fluoromisonidazole,

^{122,148} although its slow clearance from normoxic tissues and the difficulty in obtaining it has led to a search for newer radiotracers. Another compound, copper diacetyl-bis[N4-methylthiosemicarbazone] was recently discovered to have higher uptake in hypoxic tissues than the nitroimidazoles do, and it can yield high-quality images as soon as 20 minutes after injection.^{84,85,97} It is also flexible in that it can be labeled with any of several positron-emitting copper radioisotopes. In addition to these advantages, it already has been used in a few human clinical trials.^{13,33,39}

Molecules associated with tumor vascularity, such as VEGF, have been correlated with peritumoral edema and an increase in brain tumor malignancy.⁴⁵ No direct imaging techniques are available to measure the presence of VEGF or similar molecules. At present, it appears that correlating imaging characteristics with tumor histology and biology, although indirect, is the best method available for examining these relationships. Among molecules that are thought to regulate angiogenesis are those under the control of hypoxic regulation.

Hypoxia-Inducible Molecules

The transcription factor HIF-1 is the best-characterized HRM. It is composed of two subunits, HIF-1 β and HIF-1 α , both of which are constitutively expressed and not significantly altered by hypoxia. Although HIF-1 β protein is found in normoxic cells, almost no HIF-1 α protein can be

FIG. 4. Schematic representation of hypoxic control of HIF-1 α regulation and downstream HIF-1 α activity. The HIF-1 is composed of two heterodimeric subunits, HIF-1 α and HIF-1 β , which are constitutively expressed. Under normoxic conditions, HIF-1 α undergoes proteasomal degradation by a mechanism that involves hydroxylation of proline residues on HIF-1 α by prolyl-4 hydroxylases and subsequent ubiquitination by the VHL protein E3 ubiquitin ligase system. Under hypoxic conditions, HIF-1 α is stabilized and heterodimerizes with HIF-1 β to regulate transcription of downstream genes. EPO = erythropoietin.

detected under these conditions⁶⁶ because of regulation by two independent mechanisms. The first involves hydroxylation of proline residues, which leads to proteasomal degradation mediated by the VHL protein.^{26,28,62,146} The second involves hydroxylation of an asparagine, which inhibits interaction between HIF-1 and the nuclear coactivator CBP/p300.^{20,57,89} Under conditions of hypoxia (1–2% O₂), these regulatory mechanisms are inhibited and HIF-1 activates HREs, which induce transcription of a number of genes that help the cell “cope” with low O₂ conditions (Fig. 4).^{18,61,63,82,132,157} Hundreds of genes are responsive to either the HIF system or hypoxia.^{81,109,161} In addition to VEGF, other well-known HRMs include erythropoietin, glucose transporter–1, carbonic anhydrase IX, enolase, lactate dehydrogenase, tyrosine hydroxylase, aldolase A, phosphoglycerate kinase, transferrin and its receptor, and certain growth factors.^{20,73,81,133}

Tumor Growth and HRMs

Constitutive expression of HIF-1 α has been detected in the cancer cells found in the hereditary syndrome VHL disease,^{79,98,119,165} and restoration of wild-type VHL protein returns HIF-1 α regulation.^{78,91} Given the importance of the VHL protein in HIF-1 regulation, it is not surprising that VHL disease is characterized by highly vascularized tumors. Most GBMs do not have somatic mutations of the

VHL tumor suppressor gene,⁷⁰ but alterations of components of the VHL-mediated HIF-1 α degradation pathways are plausible. In addition to VHL, other tumor suppressor genes, oncogenes, and growth factors have been reported to affect HIF-1 function.^{27,36,65,92,120,147,164,166}

Tumors derived from cells with HIF-1 α knockout show decreased cell growth in vivo and in vitro.^{25,90,127,128} Also, HIF-1 α is expressed even in normoxic conditions in human cancers like prostate,¹⁶⁵ squamous cell,⁸ lung,¹⁵³ breast,^{17,83} bladder,⁶⁸ and pancreatic cancers.⁵ Overexpression of HIF-1 α is associated with more aggressive tumor grade, tumor invasion, resistance to radiation therapy, metastatic potential, and a worse prognosis.^{3,12,16,17,103,109,123,140} The expression of other HRMs and their association with cancer is less studied, although carbonic anhydrase IX and glucose transporter–1 expression reportedly correlates with poor response to adjuvant treatments.^{30,108,139}

In higher-grade brain tumors, HRMs seem to be overexpressed (unpublished data).^{138,152,163} Our data from the study of 175 gliomas suggest that high-grade gliomas are more likely to be immunohistochemically positive for HIF-1 α , VEGF, glucose transporter–1, and carbonic anhydrase IX than are low-grade tumors ($p = 0.0001$ by chi-square testing; (RL Jensen, et al., in press). The expression of HIF-1 α does not seem to be confined to perinecrotic areas (Fig. 5). Increased expression of HRMs in high-grade tumors was confirmed by Western blot analysis performed at the time of surgery on protein extracts from

FIG. 5. Photomicrographs showing HIF-1 α and VEGF immunohistochemical findings. A: Tissue from a GBM prepared with immunohistochemical stain for HIF-1 (anti-HIF-1 α monoclonal antibody; Novus Biologicals, Littleton, CO), with 3', 3'-diaminobenzidine tetrahydrochloride as the developing substrate and toluidine blue as the counterstain. Sections demonstrate necrotic areas (*arrowhead*) with pseudopalisading cells (*arrow*) surrounding the necrotic areas. The HIF-1 α nuclear staining is strong throughout, not confined to perinecrotic regions. B: Tissue from a GBM showing strong nuclear staining for HIF-1 α throughout. C: Tissue from an LGA negative for HIF-1 α . D: Tissue from a GBM (negative control; nonimmune serum substituted for antibody) for HIF-1 α showing no nuclear staining. E: Tissue from a GBM positive for VEGF. Section showing necrotic area (*arrow*) and strong VEGF expression. F: Tissue from an LGA negative for VEGF in a specimen prepared using same technique as in other examples in this figure. Original magnifications $\times 10$ (A, C, E, and F); $\times 40$ (B and D).

human brain tumors (Fig. 6). Benign tumors such as meningiomas, vestibular schwannomas, and endolymphatic sac tumors were included with the various grades of glioma tumors for comparison. A correlation has been suggested between brain tumor grade, vascularity, and HIF-1 α expression, based on a small series of brain tumors.¹⁶³

Glioma cell lines in culture express HRMs in response to hypoxic conditions (RL Jensen, et al., in press).^{40,74,81,166} Our data suggest that HIF-1 α is expressed even under normoxic conditions and that the hypoxic response is exaggerated in a number of GBM cell lines (RL Jensen, et al., in press), especially compared with more benign intracranial tumors (Fig. 7).

One unanswered question is whether HRM overexpression is simply a result of hypoxic stimulation. Colocalization of HIF-1 α expression and pimonidazole staining of U87 glioma cell lines have been demonstrated in vitro but not in tumor xenograft experiments.^{154,155} The expression of carbonic anhydrase IX and glucose transporter-1 is inversely distributed relative to vascular perfusion and correlates with polarographic O₂ microelectrodes and pi-

monidazole staining^{4,86,99,108,160} and with HIF-1 α expression, but there is debate over which "hypoxia marker" is the best.^{4,21} There are also concerns that HRM expression represents a real-time marker of hypoxia (that is, quick changes of cellular levels of HIF-1 α depending on the cellular O₂), whereas biochemical markers of hypoxia bind to hypoxic tissues as a function of drug availability and tissue oxygenation and more likely represent cumulative or "historic" cellular hypoxia.¹⁵⁵

Hypoxia Response Element–Controlled Cancer Treatments

A fundamental problem for cancer therapy is the lack of a tumor-selective delivery system. One approach to overcome this, at least in part, is to develop tumor-specific gene expression systems.¹³⁶ An HRE is a short segment of nucleotides within a gene's promoter region that is recognized by transcription factors of the HIF family. The HREs from mouse phosphoglycerate kinase and human *VEGF* or erythropoietin genes have been used to control

the expression of marker and therapeutic genes in vitro and in vivo.^{11,123,124,135} Multimerized HREs coupled to minimal viral promoters have increased the efficiency of these systems.^{15,136} This approach has been used to increase apoptosis, produce an enzyme necessary for prodrug activation, and rescue postischemic neurons.^{15,23,124,136,137}

Post and Van Meir¹⁰⁶ have developed novel HIF-activated systems for cancer therapy, including a bidirectional hypoxia/HIF-responsive expression vector to target gene expression to hypoxic cells. They showed that these vectors have moderate to high inducibility at 1% O₂ but maintain tight regulation under normoxic conditions. With this system, they have produced a conditionally replicative oncolytic virus that can specifically lyse hypoxic tumor cells.¹⁰⁷

In our laboratory, we have used RNA interference technology to target expression of HIF-1 (RL Jensen, unpublished data). We are working to adapt this technology to be under the control of hypoxic stimulation. The RNA interference is triggered by double-stranded RNA in a cell and results in the rapid destruction of messenger RNA that contains an identical or nearly identical sequence.^{37,54} Several reports describe successful gene inhibition in mammalian cells that has been achieved with this technique.^{22,24,35,93} The ability to induce RNA interference from stably integrated plasmids or virus-producing shRNAs has been demonstrated in vitro and in vivo.^{56,95,102,125} Using this approach, *p53* gene silencing was accomplished in breast and lung carcinoma cell lines by using adenovirus-delivered siRNA.¹³⁴

We have designed siRNAs for HIF-1 α by searching the coding sequence of HIF-1 α for two adenines followed by 19 nucleotides that had a GC content of less than 45% and did not contain more than three thymines or adenines in a row. These sequences were tested for possible homology to other human and mouse genes with the Basic Local Alignment Search Tool (<http://www.ncbi.nlm.nih.gov/BLAST>). Four potential siRNA sequences were selected, and siRNA was prepared using a Silencer siRNA Construction Kit (Ambion, Inc., Austin, TX). Two DNA primers that contain the sense and antisense siRNA sequence and a short sequence complementary to the T7 Klenow DNA polymerase promoter were annealed and filled in with separate reactions. The T7 polymerase is used for in vitro transcription of the sense and antisense templates, which are then annealed and treated with RNase to digest the GGG overhangs left by T7 polymerase. The product is a 19-nucleotide double-stranded RNA with a UU overhang on each end. A negative control was designed by randomizing the sequence of one of the siRNAs and checking for nonhomology to any human or mouse gene by using the Basic Local Alignment Search Tool.

These constructs were tested for inhibition of HIF-1 activity and VEGF secretion (data not shown). To study stable HIF-1 α suppression, the pSilencer 2.1 U6-hygro plasmid (Ambion, Inc.) was used to express an shRNA that was controlled via the U6 promoter. Two DNA oligonucleotides containing the sense and antisense siRNA sequences separated by a short loop sequence and having *Bam*HI- and *Hind*III-compatible overhanging ends were annealed and ligated into the linearized pSilencer plasmid. Two siRNA sequences that were found to best suppress HIF-1 α transiently were used, along with the negative control. U-251 cells were transfected with

FIG. 6. Western blot analysis in which HIF-1 α monoclonal antibody was used to measure HIF-1 α in various human brain tumors. High-grade tumors such as GBM show higher levels of HIF-1 α than LGAs or benign tumors such as vestibular schwannoma, meningioma, or endolymphatic sac tumors. Endolymphatic sac tumors can occur in patients either sporadically or as part of VHL syndrome. The patient with a known *VHL* gene mutation (*VHL*–) has increased HIF-1 α compared with the patient without *VHL* loss (*VHL*+).

Fugene transfection reagent (Roche, Nutley, NJ), and transfectants were selected by hygromycin exposure. Stable transfectants were isolated using dilutional cloning and individual clones were tested for VEGF activity to find cell lines with various levels of HIF-1 inhibition (Fig. 8a). In a similar fashion, the same shRNA constructs were transfected into U251 glioma cell lines that were stably transfected with a luciferase reporter plasmid under the control of an HRE to test HIF-1 activity inhibition directly (Fig. 8b). All cell lines demonstrate various degrees of decreased VEGF secretion and HIF-1 expression under normoxic and hypoxic conditions (Student t-test, $p < 0.05$). However, clones such as RT21 appear to have more HIF-1 inhibition, making them especially interesting for further testing, including in vivo growth measurements, which will be published shortly. We plan to place this expression vector under the control of an HRE to allow for selective targeting of hypoxic cells with specific molecular targets.

FIG. 7. Bar graphs showing VEGF secretion in U251 GBM cell line and primary meningioma cultures measured under conditions of normoxia or hypoxia. Media taken from culture dishes growing each cell line were examined using enzyme-linked immunosorbent assay for VEGF protein and normalized to total protein in the media. Baseline VEGF production is much higher in the U251 glioma cell line (b) than in each of the five meningioma cell cultures (M1–5) (a) under normoxic and hypoxic conditions. Note the 100-fold difference in the axis between each plot. All cell lines demonstrate increased VEGF secretion after 24 hours of hypoxia (Student t-test, $p < 0.05$).

FIG. 8. Bar graph showing transfected U-251 cell lines. a: The U-251 cells were transfected with anti-HIF-1 siRNA-expressing plasmids. Transfectants were selected by hygromycin exposure. Stable transfectants were isolated with dilutional cloning, and individual clones were tested for VEGF activity to find cell lines with various levels of HIF-1 inhibition. b: In a similar fashion, the same shRNA constructs were transfected into a U251 glioma cell line stably transfected with a luciferase reporter plasmid under the control of an HRE. This allows for the direct testing of HIF-1 activity inhibition. All cell lines demonstrate various degrees of decreased VEGF secretion and HIF-1 expression under normoxic and hypoxic conditions (Student t-test, $p < 0.05$). However, clones such as RT21 appear to have more HIF-1 inhibition, making them especially interesting for further testing, including in vivo growth measurements.

FIG. 9. Diagram illustrating the hypothesis presented in this paper. The transition from a low-grade glioma to GBM involves dysregulation of HIF-1 α triggered by initial tumor hypoxia or the clonal selection of cells with increased HIF-1 α activity activated by nonhypoxic mechanisms. This occurs directly through HIF-1 α activity or indirectly through VEGF-mediated angiogenesis. This results in subsequent angiogenesis, endovascular proliferation, vascular permeability, peritumoral edema, and tumor invasion.

Conclusions

We hypothesize that the transformation of LGA to GBM is produced by hypoxia-induced phenotypic changes and subsequent clonal selection of cells that overexpress HRMs, such as HIF-1 α . In this model, intratumoral hypoxia causes genetic changes (oncogene activation or tumor suppressor gene inactivation) and produces a microenvironment that selects for cells of a more aggressive phenotype (Fig. 9). As reviewed earlier, there is convincing evidence that HRMs such as HIF-1 α , VEGF, carbonic anhydrase IX, or glucose transporter-1, may play a role in the tumorigenesis and angiogenesis of a number of malignancies, but their role is unclear in the case of malignant gliomas.

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