[Frontiers in Bioscience 8, d100-116, January 1, 2003]

GENETIC MODULATION OF HYPOXIA INDUCED GENE EXPRESSION AND ANGIOGENESIS: RELEVANCE TO BRAIN TUMORS

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1. ABSTRACT

Angiogenesis is required for the development and biologic progression of infiltrative astrocytomas and takes the form of "microvascular hyperplasia" in glioblastoma multiforme, the most malignant astrocytoma. This pathologic term refers to an abnormal vascular proliferation that is often associated with necrosis and likely originates in hypoxic zones. Both the physiologic response to hypoxia and genetic alterations contribute to this process. The presence of hypoxic regions within an expanding tumor mass leads to upregulation of pro-angiogenic factors, such as vascular endothelial growth factor (VEGF), through increased activity of the transcriptional complex HIF-1 (hypoxia-inducible factor-1). HIF-1 mediated gene expression may be directly or indirectly modulated by alterations in oncogenes/tumor suppressor genes that occur during astrocytoma development, including *PTEN, TP53, p16(CDKN2A), p14^{ARF}, EGFR,* and *PDGFR*. Genetic alterations are also believed to influence the HIF-independent expression of pro- and anti- angiogenic factors, such as basic fibroblast growth factor (bFGF) and thrombospondin-1 (TSP-1), respectively. Thus, genetic events that occur during the progression of infiltrating astrocytomas promote angiogenesis, both by modulating hypoxia induced gene expression and by regulating of pro- and anti- angiogenic factors.

2. INTRODUCTION

Infiltrative astrocytomas are common, clinically problematic brain tumors that range from low grade to highly malignant. Their widely invasive properties make them difficult to totally resect, and incompletely resected tumors eventually progress to a higher grade despite adjuvant therapy. With rare exception, patients will die due to progression of astrocytomas to their highest grade form, glioblastoma multiforme (GBM), as a result of tumoral mass effect, CNS herniation, and disruption of nervous system elements critical for life. Understanding the

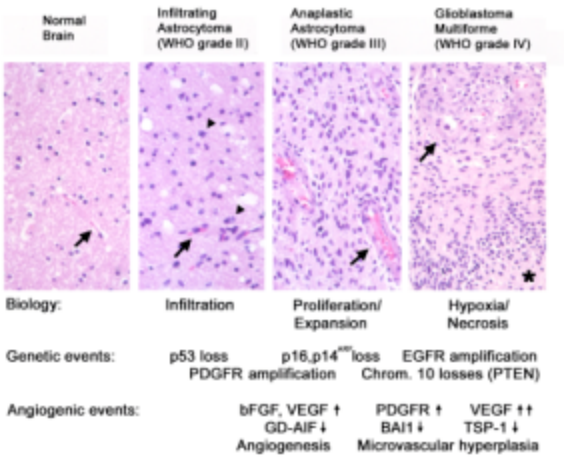

Figure 1. Histopathologic progression of infiltrating astrocytoma (WHO grade II, left) to glioblastoma multiforme (GBM; WHO grade IV, right). In grade II infiltrating astrocytomas, individual tumor cells percolate through CNS parenchyma (arrowheads). Vascular architecture and density are similar to that in normal brain white matter (arrows). Astrocytoma cells become more numerous, atypical, and show occasional mitotic figures in anaplastic astrocytoma (AA; WHO grade III). Vessels in AA are more numerous and often are dilated or have thickened walls (arrows). In glioblastoma multiforme, microvascular hyperplasia, including glomeruloid vascular proliferation (arrow), and necrosis with pseudopalisading tumor cells are seen (asterisk within necrotic center). Microvascular hyperplasia is often noted in zones surrounding pseudopalisading necrosis. Listed below the diagram are the concurrent biologic, genetic, and angiogenic events that occur during the progression of astrocytic neoplasms to glioblastoma multiforme. Not all astrocytomas progress though each of these events, and not all genetic events occurs within the same neoplasm.

biologic events of progression is essential for the development of effective interventions. This review focuses on our current knowledge of the biologic features of infiltrative astrocytomas, with special emphasis on glioma angiogenesis. The contributions of genetic alterations and the physiological hypoxia-response pathway in this process are discussed.

3. ANGIOGENESIS: CREATING NEW VESSELS

Continued growth and biologic progression of astrocytomas requires angiogenesis, the formation of new blood vessels from pre-existing ones, and/or the recruitment of endothelial precursor cells. It has been estimated that solid tumors can not grow larger than 1-2 mm without the formation of new blood vessels (1). It is unclear if these estimates apply to infiltrative astrocytomas, since neoplastic cells can migrate toward existing vascular supplies within the brain (2,3). Nonetheless, the similarity of angiogenic events that occur during the progression to GBM argues that new vessels are required (4-6). The consistency of angiogenesis is more remarkable when considered in the context of morphologic and molecular genetic diversity among astrocytomas (7).

While consistent, the formation of new blood vessels within glial neoplasms is not cursory. In fact, the biological steps of angiogenesis are highly complex and tightly regulated (8-11). Although angiogenesis is a normal occurrence during development, in the adult it is largely restricted to menstrual cycle events and pathologic states such as wound healing. Under developmental and reparative conditions, a delicate balance exists between pro- and anti-angiogenic factors (table 1). The angiogenic balance is tipped in favor of new vessel growth in neoplasms.

Morphological and biochemical investigations of new blood vessel growth in response to the pro-angiogenic cytokine vascular endothelial growth factor (VEGF) have demonstrated a well-defined pattern of events (12-14). Initially, mature vessels show increased permeability, leading to extravasation of plasma, plasma proteins, and deposition of pro-angiogenic matrix proteins. Basement membranes and extracellular matrices are then modified by a limited proteolytic digestion in order to accommodate the budding of new vessels. Dilated, pericyte-poor vessels ("mother vessels") emerge and give rise to daughter vessels through complex series of endothelial rearrangements. In response to the mitogenic effects of VEGF and other pro-angiogenic cytokines, endothelial cells undergo cell division, and begin to migrate along a chemotactic gradient leading to the extracellular matrix previously modified for their entrance. Once established in their new matrix, endothelial cells assemble themselves to form a central lumen, elaborate a new basement membrane, and eventually recruit pericytes and smooth muscle cells to surround the mature vessels. Thus, the expression of pro-angiogenic factors results in numerous tightly controlled signaling cascades and structural changes that evolve to a stable, new vasculature.

4. THE VASCULAR PHENOTYPE IN ATROCYTOMAS

During astrocytoma progression to GBM, the accelerated rate and unique morphologic configurations of angiogenesis suggest that tight regulation is lost or altered to favor neoplastic growth (15). Low grade, infiltrating astrocytomas (WHO grade II) have a vessel density that is only slightly greater than non-neoplastic brain and vessels are normal in structure (figure 1) (4). Tumor cells are diffusely scattered and show little tendency to aggregate around blood vessels. As astrocytomas become more cellular and atypical (AA; WHO grade III), the density of vessels increases noticeably, but their morphology is only slightly altered (5). Neoplastic cells in AA often form hypercellular clusters that are most evident around blood vessels. In the transition from AA to GBM (WHO Grade IV), the most dramatic vascular changes occur, both in quantity and quality, leading to one of the most highly vascularized of all neoplasms (6).

Table 1. Angiogenic and anti-angiogenic factors in neoplastic progression

Endogenous Promoters of Angiogenesis
• Vascular endothelial growth factors A, B, C, D (VEGF-A, -B, -C, -D)
• Angiopoietins
• Angiogenin
• Basic fibroblast growth factor (bFGF)
• Ephrins
• Hepatocyte growth factor (HGF; scatter factor)
• Interleukin-8
• Platelet derived growth factor (PDGF)
• Transforming growth factor-beta (TGF- beta)
• Tumor-necrosis factor-alpha (TNF- alpha)
Endogenous Inhibitors of Angiogenesis
• Angiostatin
• Brain angiogenesis inhibitor-1 (BAI1)
• Endostatin
• Glioma derived angiogenesis inhibitory factor (GD-AIF)
• Interferons
• Platelet factor-4 cleavage products
• Prolactin fragment (16 kD)
• Thrombospondin-1
• Vascular endothelial growth inhibitor (VEGI)
• Vasostatin

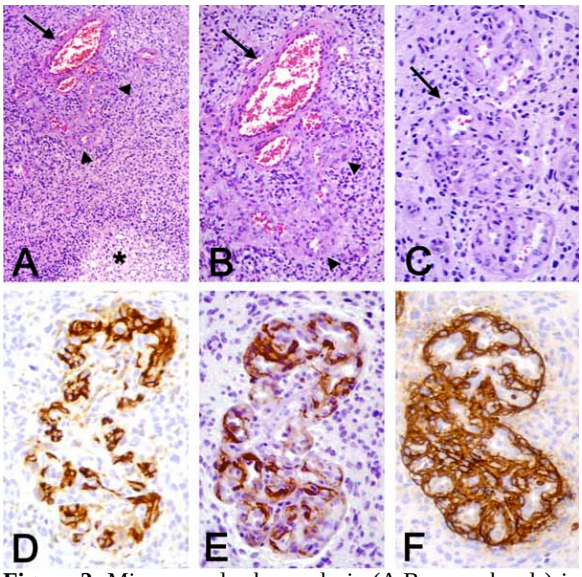

Figure 2. Microvascular hyperplasia (A,B; arrowheads) is seen arising from a parent blood vessel (A,B; arrow) in a GBM in a region surrounding pseudopalisading necrosis (A, asterisk). Microvascular proliferation frequently takes the form of glomeruloid bodies (C, arrow), which are discrete collections of proliferating endothelial cells and pericytes that form an aggregate of new vessels that resemble a renal glomerulus. Immunohistochemical stains for CD31 (D) and smooth muscle actin (E) highlight the distribution of endothelial cells and pericytes, respectively, in glomeruloid proliferations. Stains for collagen type IV demonstrate the large amount of basal lamina investing endothelial cells. (F).

The emergence of a highly proliferative form of angiogenesis, referred to by neuropathologists as "microvascular hyperplasia", is a pathologic hallmark of GBM (figure 2). It consists predominantly of rapidly dividing endothelial cells that form tufted micro-aggregates at the leading edge of sprouting vessels (15-16). Immunohistochemical and ultrastructural studies have shown that collections of endothelial cells in newly formed vessels are incompletely surrounded by layers of pericytes and smooth muscle cells and invested by a basal lamina. Some have mistakenly considered microvascular hyperplasia to refer to an increased number of blood vessels. While increased numbers of vessels are indeed present in high grade astrocytomas, for the purposes of grading astrocytic neoplasms, microvascular hyperplasia takes on specific meaning and is a terminology that refers to the type of angiogenesis defined by budding proliferations of endothelial and perivascular cells.

When microvascular hyperplasia in GBMs is in the form of compact collections of small vascular lumina, all contained within a single aggregate and resembling a renal glomerulus, the structures are referred to as "glomeruloid" bodies (figure 2) (15). Tumoral angiogenesis differs in quality and quantity depending on the organ system and is thought to be dependent upon differential expression of organ-specific stromal cytokines (17-20). The CNS "stroma" consists of a complex network of oligodendrocytes, astrocytes, and numerous intertwining neuronal processes. The distinctive stroma and its associated cytokines in the CNS may partially explain the frequent formation of glomeruloid bodies in gliomas compared to other neoplasms (21-23). Presently it isn't clear if these bodies represent a rapidly emerging form of functional blood vessels or a dysfunctional, perhaps abortive, type of proliferation.

Microvascular hyperplasia, often showing glomeruloid bodies, is tightly linked spatially and temporally with the presence of pseudopalisading necrosis. The latter is a specific type of necrosis found in some GBMs in which neoplastic astrocytes are tightly packed around necrotic centers (3,6). Grading schemes for astrocytic neoplasms once required the finding of pseudopalisading necrosis, but not microvascular proliferation, within a high grade astrocytoma for the diagnosis of GBM. Recent studies have shown that GBMs with microvascular hyperplasia but not pseudopalisading necrosis have the same prognosis as tumors containing both features, suggesting that microvascular hyperplasia signals an aggressive growth phase. Survival of patients with GBMs is less than one year, whereas patients with AAs, tumors lacking vascular proliferation and pseudopalisading necrosis, usually survive more than twice as long.

Precise explanations for the pseudopalisading configuration of astrocytoma cells around foci of necrosis are not at hand. It is possible that cells populating the pseudopalisade simply represent a remnant of a hypercellular zone whose center has become necrotic. Tumor necrosis could result from increased apoptosis (programmed cell death) or increased growth beyond the

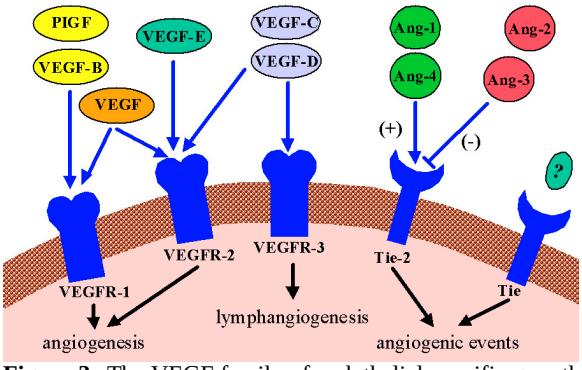

Figure 3. The VEGF family of endothelial-specific growth factors includes VEGF (VEGF-A) placental growth factor (PlGF), VEGF-B, VEGF-C, VEGF-D, and VEGF-E. These bind differentially (indicated by arrows) to receptors VEFGR-1, -2, and -3, promoting either angiogenesis (VEGFR-1 and 2) or lymphangiogenesis (VEGFR-3). The angiopoietins Ang-1, -2, -3, and –4) interact with the Tie-2 receptor. Whereas Ang-1 and Ang-4 are agonists, with binding causing angiogenic events, Ang-2 and Ang-3 are antagonists, and cause inhibitory effects at this receptor, leading to vascular regression. The role of Tie-1 is less well defined.

capacity of the emerging blood supply (infarction). However, tumor cells within pseudopalisades are not significantly more proliferative than those in adjacent regions (24). It is also not clear which viable cells in the pseudopalisades are prone to apoptosis and which are resistant. Another possibility is that the pseudopalisade appearance results from tumor cells having migratory capacities that allow their centifugal movement from the emerging necrotic center towards a new vascular supply (i.e “tumor cells fleeing hypoxic regions”).

The temporal and spatial relationship between necrosis and microvascular hyperplasia in GBMs is better understood, and the evidence indicates that the angiogenic response is due to hypoxic conditions associated with necrotic zones (25). Neoplastic astrocytoma cells seen palisading around necrotic foci in GBM express high levels of hypoxia-inducible regulators of angiogenesis, such as VEGF. In the partially necrotic regions of a GBM, concentrations of secreted VEGF protein are 200-300-fold higher than concentrations in serum (26).

VEGF (VEGF-A), a glycoprotein with mitogenic effects on endothelial cells, belongs to a larger family of secreted proteins with endothelial-specific stimulatory properties, including placental growth factor (PlGF), VEGF-B, VEGF-C, VEGF-D, and VEGF-E. Family members share substantial sequence homology but receptor binding properties, tissue specificity, and biological properties differ (12,18, 27). Adding to the complexity, each of these factors appears to have a variable number of splice forms. Alternative splicing of the *VEGF* gene yields isoforms of 121, 145, 165, and 189, and 206 amino acids, and at least in some experimental systems, these isoforms have differential activity in tumor neo-vascularization (28,29). Most studies of hypoxia inducible gene expression of angiogenic factors have focussed on VEGF, since its secretion appears to be most critical to the formation of new blood vessels in neoplasms. Other members of the VEGF family, their receptors (VEGFR-1, -2, and -3), angiopoietins (Ang-1, -2, -3, and –4) and Tie receptors (Tie and Tie-2) play central roles in angiogenesis and lymphangiogenesis, but the hypoxia responsiveness of these other angiogenic factor is only beginning to be defined (figure 3) (refer to review by Zadeh and Guha in this issue).

Hypoxia is one of the most potent stimulators of VEGF expression and leads to dramatic increases in VEGF mRNA, which is due both to increased gene transcription and mRNA stabilization. Enhanced gene transcription is mediated by a hypoxia responsive element (HRE) within the *VEGF* promoter that allows binding of transcription factors of the HIF (hypoxia-inducible factor) family (30-33). Inhibition of this pathway has been demonstrated to prevent tumor growth (34). Molecular mechanisms of VEGF mRNA stabilization are not understood as well, but involve specific elements at the 3’ end (35). Extracellular VEGF binds to its high affinity tyrosine kinase receptors, VEGFR-1 and VEGFR-2 (Flt-1 and Flk-1/KDR, respectively) which are upregulated in endothelial cells of high grade gliomas, but are not expressed on endothelial cells of normal brain (30,33). Binding of VEGF to its receptors, in turn, initiates a cascade of signaling events that, in combination with other pro-angiogenic forces, results in new vessel formation in surrounding hypoxic regions. Thus, hypoxia is thought to be a critical physiologic stimulus for new blood vessel formation during the progression of astrocytomas and exerts many of its effects through altered gene expression.

5. HYPOXIA-INDUCED GENE EXPRESSION

Gene expression changes within hypoxic regions of GBMs is but one example of the adaptive response to low oxygen levels. In all human cell types, oxygen must be maintained within a narrow concentration range for normal function and hypoxia induces a host of biologic changes necessary for adaptation and for restoring normal oxygen levels. As with VEGF upregulation in GBMs, many of the most important changes in gene expression during hypoxia are mediated by the HIF transcription factors.

5.1. HIF Targets

Many target genes transactivated by HIF-1 in normal and tumoral tissues (including gliomas) have been identified and most are known for their role in adapting to hypoxia (32,36,37). The effects of HIF-1 on gene expression are mediated through hypoxia response elements (HRE) within the promoter of target genes. The HRE consensus core has the DNA sequence 5’-RCGTG-3’, although adjacent flanking sequence is necessary for it to enhance transcriptional activity under hypoxic conditions. Interestingly, this consensus overlaps with *Myc* E-box consensus core sequence. Erythropoietin (EPO), transferrin and its receptor, growth factors, glucose transporters, and a large number of glycolytic enzymes are all upregulated by HIF-1 mediated transcription of their respective genes

Table 2. Target Genes Regulated by Hypoxia Inducible Factors (HIF)

Upregulated Genes
• Vascular endothelial growth factor (VEGF)
• VEGF receptor-1 (VEGFR-1; Flt-1)
• Endothelin-1
• Adrenomedullin
• Nitric oxide synthase-2 (NOS-2)
• Heme oxygenase-1
• Erythropoietin (EPO)
• Ceruloplasmin
• Transferrin
• Transferrin Receptor
• Insulin-like growth factor binding protein-1, -2, -3
• Insulin-like growth factor II
• Transforming growth factor-beta3
• Cyclooxygenase (COX)-1
• p21
• NIP3
• Aldolase A,C
• Phosphoglycerate kinase (PGK)-1
• Phosphofructokinase (PFK)
• Glucose transporters 1, 3
• Hexokinase 1, 2
• Glyceraldehyde-3-phosphate dehydrogenase (G-3-PD)
• Enolase 1
• Pyruvate kinase M
• Lactate dehydrogenase (LDH) A
• Adenylate kinase 3
• Prolyl-4-hydroxylase-alpha1
Downregulated Genes
• Alpha-fetoprotein

(table 2). HIF-1 transcription elicits a strong pro-angiogenic effect. Perhaps most significant to astrocytoma progression, *VEGF* transcription is rapidly and dramatically increased under hypoxic conditions. Although the majority of HIF-1 target genes are induced by hypoxia, some genes appear to be repressed. For example, hypoxia reduces the expression of the *alpha-fetoprotein* gene in hepatoma cells through mechanisms dependent on HIF-1 (38).

5.2. HIF Structure/Function

HIF-1 is a DNA binding heterodimer consisting of HIF-1alpha and HIF-1beta (aryl hydrocarbon nuclear translocator; ARNT)(36,39,40). Both subunits belong to the PER-ARNT-SIM (PAS) family of basic helix-loop-helix (bHLH) transcription factors. The bHLH domains of alpha and beta subunits mediate dimerization and DNA binding (figure 4). HIF-1alpha can also dimerize with ARNT2 or ARNT3, which share homology with HIF-1beta but are not as widely expressed. To date, HIF-1alpha, HIF-2alpha, and HIF-3alpha subunits have been identified as key oxygen sensitive regulators of HIF-mediated transcriptional activation, but HIF-1alpha has been more extensively studied. HIF alpha subunits 2 and 3 contain similar functional domains as HIF-1alpha and dimerize with HIF-1beta under hypoxic conditions. Knock out mice lacking either HIF-1alpha or HIF-2alpha are embryonic lethal, suggesting that each form is critical (41,42). However, the variety of HIF related proteins suggests at least a mild degree of biologic redundancy, presumably as a safeguard, within the critical adaptive response to hypoxia.

Specific regions of the HIF-1 protein structure have been shown to direct nuclear translocation, dimerization, DNA binding, and transactivation. Translocation of HIF-1alpha to the nucleus depends on the nuclear localization signal (NLS) within the C-terminal end and nuclear import can be inhibited by either deletion or amino acid substitution within the NLS. HIF-1alpha exists in phosphorylated and dephosphorylated forms, with the phosphorylated preferentially localizing to the nucleus (43). The study of truncated mutants of HIF-1alpha suggests that the bHLH domain and N-terminal half of the PAS domain are required for the form of dimerization that allows DNA binding and transactivation (36,40). In addition to DNA binding, HIF-1 mediated transactivation requires the function of specific transactivational domains (TADs) located in the C-terminus. The minimal TADs consist of amino acids 531–575 (TAD-N) and 786–826 (TAD-C) (44,45). Amino acids 576–785, located between TAD-N and TAD-C, are thought to represent a transactivation inhibitory domain (TID) that represses TAD function under normoxia.

TADs interact with co-activators required for transcriptional activation. Upon DNA binding of HIF-1 under the direction of the HRE, co-activators mediate the recruitment of transcription factors associated with RNA polymerase II. Co-activators also possess histone acetyltransferase activity necessary for the chromatin remodeling required for transcription. Creb binding protein (CBP)/p300 is one co-activator that is recruited by nuclear, activated HIF-1 (46).

5.3. Regulation of HIF by Oxygen

The beta subunit of HIF-1 shows constitutive nuclear expression that is not substantially altered by oxygen levels (34,39). Protein levels of the alpha subunit, on the other hand, are minimal under normoxic conditions, but rapidly increase under hypoxia. Return to normoxia following hypoxia leads to a rapid decline in HIF-1alpha protein, with a half-life on the order of 5 minutes. In vitro studies have demonstrated that hypoxia induces dramatic increases in HIF-1alpha protein levels without comparable changes in mRNA levels, suggesting that protein degradation plays a major role in its regulation under hypoxic conditions. However, some in vivo studies have shown that HIF-1alpha mRNA expression also increases in response to hypoxia or ischemia (47). HIF-1alpha expression and transcriptional activity are most likely regulated at multiple levels as a function of oxygen concentration.

Under normoxic conditions, HIF-1alpha protein levels are maintained at low levels by the continuous degradation by the ubiquitin dependent proteosome pathway (48,49). Inhibitors of the proteosomal pathway cause dramatic increases in HIF-1alpha but not HIF-1beta. The tumor suppressor protein von Hippel Lindau (VHL) is

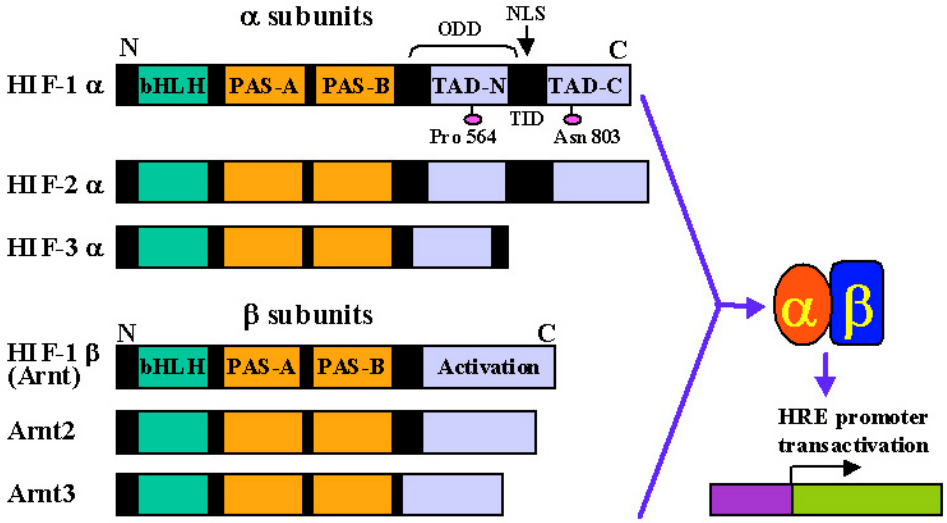

Figure 4. The HIF family of transcription factors are DNA binding heterodimers consisting of alpha and beta subunits. Both subunits contain PER-ARNT-SIM (PAS) and basic helix-loop-helix (bHLH) domains, which are responsible for heterodimer formation and DNA binding. HIF-1alpha, HIF-2alpha, and HIF-3alpha are the alpha subunits, which can bind with HIF-1beta (Arnt), Arnt2 or Arnt3. Binding of heterodimers to HRE containing promoters initiates transcription of hypoxia inducible target genes. In addition to PAS and bHLH domains, alpha subunits contain transactivation domains (TAD-N and TAD-C), a transactivation inhibitory domains (TID), an oxygen dependent degradation (ODD) domain (also known as the PSTD domain), and a nuclear localization signal (NLS). Also shown on HIF-1alpha are the locations of Pro 564 and Asn 803, which are sites of hydroxylation critical to the oxygen dependent regulation of the ubiquitination and co-activator (CBP/p300) interaction, respectively.

a component of a complex with ubiquitin E3 ligase activity, which is responsible for steady-state ubiquitination of HIF-1alpha under normoxic conditions (50). In addition to VHL, this complex contains elongin B, elongin C, Cul2, Rbx1, and E2 (figure 5). The alpha subdomain of VHL binds directly to elongin C, which facilitates the binding of VHL to elongin B, while the beta subdomain of VHL binds directly to HIF-1alpha (51). Ubiquitination of HIF-1alpha by VHL occurs within the oxygen-dependent degradation (ODD, amino acids 426-608) domain of HIF-1alpha (figure 4) and requires the hydroxylation of a key proline (amino acid 564) within the ODD (52-54). This modification occurs by the action of a family of Fe(2+)-dependent prolyl hydroxylases, using oxygen as a substrate. Since proline hydroxylation is oxygen dependent, this post-translational modification may be critical to the upregulation of HIF-1alpha protein and transactivation in response to low oxygen. The recent identification of specific prolyl hydroxylases should lead to their genetic and functional characterization in disease states such as neoplasia (54). Loss of prolyl hydroxylase activity could lead to enhanced HIF mediated transcriptional activity of target genes under normoxia.

A second ubiquitin ligase, MDM2, is believed to regulate HIF-1alpha protein levels as well. MDM2 forms a protein complex with p53 and is responsible in large part for the ubiquitin mediated degradation of p53. It is believed that p53 recruits MDM2 to HIF-1alpha and enhances the latter's ubiquitination and degradation, even under hypoxic conditions (34,40).

Recent studies suggest that dimerization of alpha and beta subunits may be regulated by another hypoxia induced protein with structural similarity to HIF-1 alpha and HIF-1beta. Referred to as inhibitory PAS domain protein (IPAS), this protein contains the bHLH and PAS DNA binding domains, but not the transactivational domains of other HIF related proteins (55). IPAS inhibits HIF mediated transactivation, presumably by binding to HIF-1alpha (but not HIF-1beta) through its complementary bHLH/PAS dimerization domain, thus acting as a dominant negative inhibitor. While further studies are necessary, IPAS may modulate transcriptional activity of HIF under hypoxia (figure 5). It will be of particular interest to examine IPAS expression and gene integrity in brain tumors.

Oxygen also regulates HIF target gene transcription though co-activators. CBP/p300 interacts with the C-terminal transactivation domain of alpha subunits and is dependent upon the hydroxylation status of a conserved asparagine within the TAD-C (Asn^{803} in HIF-1alpha; Asn^{851} in HIF-2alpha) (56). Hydroxylation at this site requires both oxygen and Fe(2+) and inhibits the transactivational capacity of TAD-C. Mutation of this asparagine to alanine leads to constitutive activity of TAD-C and a loss of oxygen sensitivity. Recent in vitro studies have shown that FIH (Factor Inhibiting HIF) is an Fe(2+) and 2-oxoglutarate (2-OG) dependent oxygenase capable of hydroxylating HIF-1alpha at Asn^{803} (57,58; see below). Thus, hydroxylation of asparagine within the TAD-C by FIH, and perhaps other hydroxylases, represents another

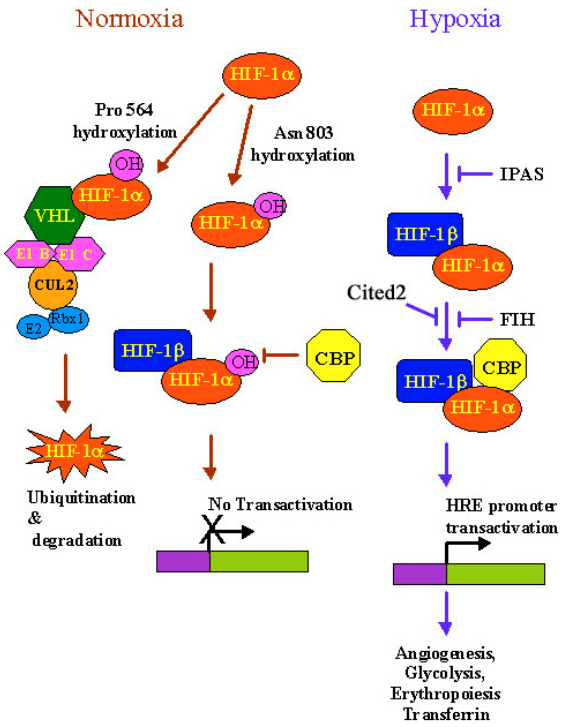

Figure 5. HIF-1alpha is differentially regulated under hypoxia and normoxia. Under normoxia, two key oxygen dependent hydroxylation events regulate HIF. Proline 564 within the ODD domain of HIF-1alpha is hydroxylated by a family of prolyl hydroxylases, leading to increased VHL mediated ubiquitination and proteosomal degradation (left). Hydroxylation of asparagine 803 of HIF-1alpha inhibits the interaction of HIF with the co-activator CBP/p300, thereby blocking HIF mediated transactivation (middle). Under hypoxic conditions, the oxygen dependent hydroxylations of proline 564 and asparagine 803 do not occur (right). However, IPAS (inhibitory PAS domain protein) is capable of dimerizing with HIF-1alpha and inhibiting HIF mediated transactivation, presumably by preventing dimerization of HIF-1alpha and HIF-1beta. FIH has been shown to have asparagine hydroxylase activity, but has also been shown to bind with HIF-1alpha and inhibit HIF transcription under hypoxia by mechanisms that may include enhanced VHL mediated degradation of HIF-1alpha and the recruitment of histone deacetylases (HDACs) to HIF (right). Cited2 is a nuclear regulatory protein that binds to CBP/p300, competitively inhibiting its interaction with HIF-1alpha

oxygen dependent mechanism that regulates HIF-1 mediated transcription, in this case by inhibiting the TAD-C dependent recruitment of the co-activator CBP/p300. It will be interesting to examine whether mutations might occur in regulatory hydroxylases such as FIH during the development of tumors or whether HIF might acquire mutations at these hydroxylation sites that render it resistant to downregulation.

CBP/p300 also interacts with the transcriptional modulator Cited2, a nuclear protein that binds to the CH1 domain of CBP/p300 in a location similar to that of HIF-1alpha (59). It is believed that Cited2 competes with HIF-1alpha for CBP/p300 binding and interferes with hypoxia induced transcription that depends on the interaction of CBP/p300 and HIF. Interestingly, hypoxia causes increased expression of Cited2, which may indicate that it could regulate HIF transactivation of target genes under both normoxic and hypoxia conditions. Cited2 knockout mice are embryonic lethal, having severe cardiac abnormalities and neural tube defects similar to those seen in mice transgenic for VEGF (60). Tissues from Cited2 -/- embryos show upregulation of HIF target genes and cultured fibroblasts demonstrate enhanced hypoxic induced gene expression of VEGF. Thus, Cited2 appears to be an essential negative regulator HIF mediated transcription by competitively inhibiting HIF-1alpha interactions with CBP/p300.

In summary, the signaling mechanisms that transduce low cellular oxygen into increased HIF-1 alpha protein and transcriptional activity continue to be defined (34,40). Relevant mechanisms include: 1) the oxygen-dependent hydroxylation of proline within the ODD of HIF-1alpha, which enhances the VHL mediated ubiquitination and degradation of HIF-1 alpha; and 2) the oxygen dependent hydroxylation of asparagine within the TAD-C, which inhibits the recruitment of CBP/p300 and resulting transcriptional activity. Other theories of oxygen-sensing by HIF-1 speculate that a deoxygenated hemoprotein promotes hypoxic induced signal transduction, whereas the oxygenated state inhibits it. Another possibility is that reactive oxygen species, such as superoxide, hydrogen peroxide, and hydroxyl radical, are the signals. Nitric oxide (NO) and carbon monoxide (CO), which both inhibit hypoxia-inducible gene expression by interfering with HIF-1alpha DNA-binding, could serve as rapid signals, independent of HIF-1alpha protein levels. Other transduction candidates include the mitochondrial electron transport chain complex I, the global cellular redox status, and protein phosphorylation.

5.4. Oncogenic Modulation of HIF-1

Hypoxic regions occur in many neoplasms and thus HIF-1 mediated gene expression in tumors might be explained by hypoxia alone (61). However, it has become clear that a variety of tumor suppressor genes and oncogenes can modulate HIF-1 activity, thereby leading to increased target gene expression and angiogenesis. For example, oncogenic forms of *Ras*, which occur in over 40% of all human solid tumors (but are rare in primary brain tumors), have been shown to lead to increased levels of VEGF and tumoral angiogenesis, especially under hypoxic conditions (62,63). One study showed a 15-fold induction of *VEGF* promoter transactivation in *Ha-Ras* transformed cells (62). This effect was abolished by both mutant inhibitory forms of *Ras* and by mutations in the *VEGF* promoter region. Mazure, et al then showed that hypoxia-induced expression of VEGF in *Ha-ras* transformed fibroblasts could be blocked by the phosphatidylinositol 3-kinase (PI(3)K) inhibitor, wortmannin, as well as by dominant negative mutants of the PI(3)K subunit p85 (64). Thus, oncogenic *Ras* appears to activate HIF-1 by upregulating the PI(3)K signaling pathway—a mechanism now known to be central to HIF activity (see below).

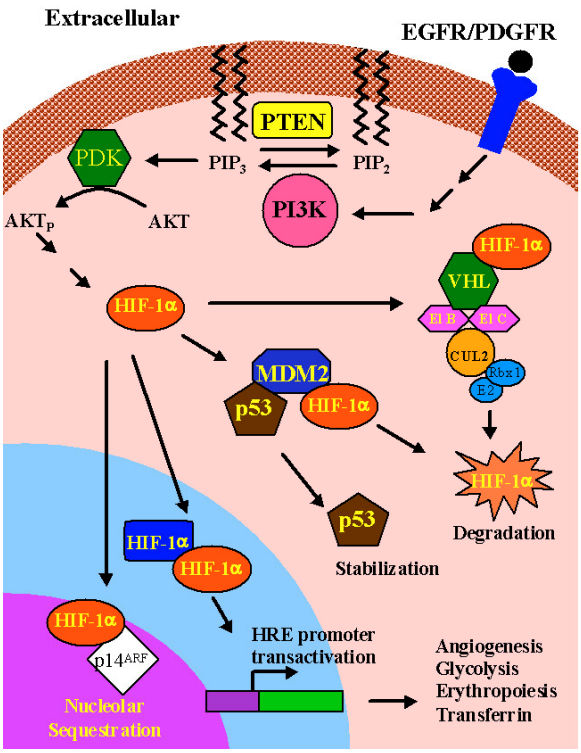

Figure 6. Schematic representation of the regulation of HIF mediated transcription by intracellular signaling pathways that are disrupted in brain tumors. *PTEN*, *TP53*, *p14^{ARF}*, *EGFR*, *and PDGFR* genes are altered during the progression of astrocytomas to glioblastoma multiforme in manners that may enhance HIF induced transcription of target genes, including *VEGF*. Increased activity of EGFR and PDGFR stabilize HIF-1alpha through upregulation of the PI(3)K/Akt pathway. Loss of PTEN activity leads to elevated PIP3(3,4,5), which in turn activates Akt and stabilizes HIF-1alpha. Loss of p53 leads to decreased p53-MDM2 mediated degradation of HIF-1alpha and increased HIF mediated transcription. HIF activity is normally inhibited by nucleolar sequestration of HIF-1alpha by p14^{ARF} and therefore loss of p14^{ARF} would be expected to enhance HIF mediated transcription. Functional *VHL* is lost from tumor types associated with von Hippel-Lindau disease, including cerebellar hemangioblastomas. Loss of VHL mediated ubiquitination and degradation of HIF-1alpha leads to constitutively active HIF target gene transcription under normoxic conditions.

Expression of the *v-Src* oncogene can also cause HIF-1alpha protein stabilization and enhanced target gene expression (*VEGF* and *enolase 1*) (65). Increased target gene expression in v-Src expressing cells was seen under both hypoxic and normoxic conditions and required an intact HIF-1 DNA binding site. Elevated HIF-1alpha protein levels appeared to be due to increased HIF-1alpha mRNA as well as decreased protein degradation and correlated with tumor growth in vivo. The finding that v-Src enhances HIF-1 target gene expression is intriguing since c-Src expression does not appear to be required for HIF-1 mediated transcriptional activation (66,67).

5.5. Regulation of HIF-1 by Phosphatidylinositol 3-kinase (PI(3)K) and Akt

While precise oxygen transducing signals for HIF-1 continue to be defined, an intracellular signaling pathway central to HIF-1 activation has been uncovered. Phosphatidylinositol 3-kinase (PI(3)K) phosphorylates inositol lipids, which generates the cellular signaling molecules phosphatidylinositol 3,4-bisphospate [PIP2 (3,4)] and phosphatidylinositol 3,4,5-trisphosphate [PIP3(3,4,5)]. The of the product of the tumor suppressor gene *PTEN* has lipid phosphatase activity that opposes the activity of PI(3)K, causing dephosphorylation of PIP2(3,4) and PIP3(3,4,5). One of the main downstream targets of PI(3)K activity is the serine-threonine kinase Akt (protein kinase B), which is recruited to the cell membrane and activated by phosphorylation by another serine-threonine kinase, phosphatidyl inositol dependent kinase 1 (PDK1) (figure 6). In studies of human carcinoma cells, hypoxia induced HIF-1alpha protein stabilization could be specifically reduced by the PI(3)K inhibitors LY294002 and wortmannin or by the overexpression of PTEN (68-70). In addition, activation of PI(3)K signaling by growth factors or by the loss of PTEN function caused increased protein levels of HIF-1alpha, but not HIF-1ß. Results from many experimental systems have shown that PI(3)K activity does not effect the level of HIF-1alpha mRNA, suggesting that it exerts most of its effects by stabilizing the protein. More recent studies demonstrated that increased HIF-1alpha protein levels in response to PI(3)K activity may be at least partially due to enhanced protein synthesis (71,72). Constitutively active forms of PI(3)K and Akt are both able to increase HIF-1 dependent transactivation of the endogenous *VEGF* promoter and HRE reporter constructs. PTEN expression is able to block the effects of constitutively active PI(3)K but not of Akt, suggesting that Akt is a downstream signal of PI(3)K sufficient for inducing HIF-1 mediated transactivation (68,70). The exact mechanism whereby Akt stabilizes HIF-1alpha protein is not known; stabilization is not due to direct phosphorylation of HIF-1alpha by Akt. Increased HIF-1 mediated VEGF expression by insulin and HER2 (neu) signaling depends on both the activation of the PI(3)K/Akt pathway and the downstream kinase FRAP/TOR (FKBP-rapamycin-associated-protein/target of rapamycin) (71,72). Thus, activation of the PI(3)K/Akt pathway increases HIF-1 alpha protein levels under hypoxic conditions by mechanisms that may include, but are not restricted to, activation of FRAP/TOR, and leads to HIF-1 dependent transactivation of target genes.

6. p53 PATHWAY

6.1. Regulation of Hypoxia Induced Gene Expression by p53

The p53 tumor suppressor pathway is altered in a substantial percentage of low grade infiltrating astrocytomas (60-70%) as well as the high grade tumors that arise from them (2,6). The most relevant mechanisms of p53 pathway disruption are: 1) *TP53* point mutation combined with the loss of the second allele; 2) *p14^{ARF}* losses; and to a lesser extent 3) *MDM2* amplification (73-77).

Early evidence that p53 might modulate HIF-1 target gene expression was the finding that wild type p53 could down-regulate VEGF expression (78,79). Direct evidence of the influence of p53 on hypoxia induced gene expression was demonstrated using human colonic cancer cells (HCT116) that have targeted homozygous deletion of *TP53* (80). These cells showed enhanced growth and angiogenesis when established as tumor xenografts in nude mice. Both HIF-1alpha levels and HIF-1 dependent VEGF expression in these cells were upregulated in response to hypoxia. Restoration of wild type *TP53* into these cells inhibited HIF-1 activity by promoting ubiquitination and proteosomal degradation of the HIF-1alpha subunit. This effect was then shown to be independent of p53 mediated transactivation but dependent on its MDM2 binding domain. Subsequently, it was shown that HIF-1alpha forms a physical complex with p53 under hypoxic and normoxic conditions in vivo and that this interaction destabilizes HIF-1alpha and reduces HIF mediated gene transcription (80,81).

In contrast to the destabilizing effect of p53 on HIF-1alpha, the physical interaction between these proteins augments the level of p53 and consequently increases overall p53 transcriptional activity in the cell (43,82). Mechanisms that are independent of HIF can also lead to accumulation of p53 under hypoxia (83,84). It is unclear whether p53 accumulation under hypoxia increases MDM2 mediated degradation of HIF-1alpha or whether increased HIF-1alpha levels under hypoxia stabilize p53 by displacing MDM2 from p53 (43,82,85).

Taken together, the data suggest that the destabilizing effect on HIF-1alpha by p53 is due to the formation of a ternary complex between p53, HIF-1alpha and MDM2, leading to ubiquitination of HIF-1alpha by MDM2 (figure 6). This mechanism is further regulated by phosphorylation. Dephosphorylated HIF-1alpha preferentially binds to p53, leads to stabilization of the latter, and has been shown to enhance p53 mediated apoptosis under hypoxia (43,85). Phosphorylated HIF-1alpha, on the other hand, enters the nucleus and initiates HIF mediated transcription (43).

Mutations of *TP53* that occur in human astrocytomas most often involve the DNA binding region and would be predicted to retain inhibitory properties on HIF-1. However, such mutations are also associated with a prolonged p53 protein half-life due to decreased MDM2 mediated degradation. This may indicate that such *TP53* mutations attenuate p53-MDM2 protein interaction, despite the fact that most *TP53* mutations are in the DNA binding domain and not the MDM2 binding domain. The interactions of specific p53 mutant proteins, MDM2, and their overall effects on HIF-1 activity in human astrocytomas remain to be defined.

The $p14^{ARF}$ tumor suppressor protein is known to interact with the MDM2-p53 complex and inhibit MDM2 mediated degradation of p53 (77). A portion of the inhibitory function of $p14^{ARF}$ on MDM2 is thought to be due to the ability of $p14^{ARF}$ to promote translocation of MDM2 from the nucleus to the nucleolus, where it cannot facilitate p53 degradation (86). Thus, one might expect $p14^{ARF}$ to increase HIF-1 activity due to a reduction of HIF-1alpha degradation through the p53-MDM2 pathway. Unexpectedly, $p14^{ARF}$ has been found to associate with HIF-1alpha and strongly inhibit HIF-1 related gene transcription, independent of its regulation of p53-MDM2 mediated degradation of HIF-1alpha (87). This might explain the ability of $p14^{ARF}$ to mediate vascular regression during mouse eye development (88). Decreased transcriptional activity of HIF-1 by $p14^{ARF}$ was shown to be due to nucleolar sequestration of HIF-1alpha facilitated by $p14^{ARF}$ (figure 6). The portion of HIF-1alpha responsible for binding to $p14^{ARF}$ was localized to a proline-serine-threonine protein stability domain (PSTD; also known as ODD) within amino acids 463-652, while nucleolar translocation of HIF-1alpha required its N-terminal 199 amino acids. Given the frequent loss of $p14^{ARF}$ tumor suppressor alleles in gliomas, it will be important to investigate whether this step constitutes a critical pro-angiogenic switch in tumor progression.

6.2. Regulation of Angiogenesis by p53

The *TP53* gene product also appears to regulate the formation of new vessels in manners independent of HIF-1, by affecting the expression of pro- and anti-angiogenic factors more directly. For example, one study using U87MG glioma cells showed that expression of basic fibroblast growth factor (bFGF), a potent stimulator of angiogenesis, was repressed by transfection with wild type *TP53*, but upregulated by transfection with mutant *TP53* (89). Modulation of *bFGF* gene expression by wild type and mutant forms of p53 depended on direct transcriptional regulation of the *bFGF* promoter. This study suggests that mutations of *TP53*, which occur early in astrocytoma progression, cause direct upregulation of bFGF. Indeed, both bFGF mRNA and protein are overexpressed in human GBM samples and could in part contribute to an angiogenic phenotype (90).

Other investigations have suggested that inactivation of p53 might lead to a loss of angiogenesis inhibitors that are normally expressed. One such example is thrombospondin-1 (TSP-1), a potent inhibitor of angiogenesis (91). Expression of wild type p53 was found to positively regulate TSP-1 levels in fibroblasts from patients with Li-Fraumeni syndrome (92) but not in *TP53*-null LNZ308 glioblastoma cells (93). Another example is glioma derived angiogenesis inhibitory factor (GD-AIF), an anti-angiogenic factor found in conditioned medium of LNZ308 cells upon restoration of wild type p53 expression (94). Preliminary characterization of GD-AIF suggests that it is labile to pronase digestion and has a molecular weight greater than 100 kD. These data imply that GD-AIF is a large protein or protein complex, the synthesis and secretion of which is directly or indirectly regulated by p53.

Of the established inhibitors of angiogenesis that are likely candidates for GD-AIF, the gene product of *Brain Angiogenesis Inhibitor-1 (BAI1)* has properties that might be suggestive (95). *BAI1* was cloned in a screen for p53 regulated genes and its expression has been reported to

be induced by wild type p53 in T98G glioma cells. As its name implies, BAI1 mRNA and protein expression appear to be specific to brain. The predicted protein sequence of the *BAI1* gene product includes a 7 transmembrane spanning region and an extracellular domain with five TSP type-1 repeats. Recombinant proteins containing fragments of the latter have been shown to inhibit angiogenesis in a rat corneal model. Thus, the reported p53 modulated expression of BAI1, its specific expression in the brain, and its anti-angiogenic properties suggest that BAI1 could be critical to glial tumorigenesis. Initial studies suggest that BAI1 mRNA and protein expression are lost in the majority of human glioblastoma cell lines as well as GBM resection specimens (96). However, p53-mediated regulation of BAI-1 mRNA levels could not be confirmed (96). Whether loss of TSP-1, GD-AIF, or BAI1 expression through p53 pathway alteration is significant to glioma development remains to be established.

7. LOSSES ON CHROMOSOME 10

7.1. Regulation of Hypoxia Induced Gene Expression by PTEN

Some of the most frequent genetic alterations in GBMs (70-90%) are losses on chromosome 10, occurring either as complete chromosome loss or loss of the long or short arm (2,6). The *PTEN* gene, located at chromosome 10q23.3, was the first tumor suppressor gene identified on chromosome 10 and is mutated in 20-40% of GBMs (97,98). PTEN is a dual protein and lipid phosphatase. One of its main regulatory roles is the dephosphorylation of PIP3(3,4,5) to PIP2(4,5), thus opposing the action of PI(3)K, the enzyme responsible for PIP2 phosphorylation to PIP3 (99). PI(3)K and Akt activity are established modulators of hypoxia dependent stabilization of HIF-1alpha and expression of VEGF. It follows that PTEN should have a negative regulatory effect on hypoxia induced gene expression and that its loss during gliomagenesis should enhance VEGF expression and angiogenesis (figure 6). Transfection of wild type *PTEN* into a *PTEN* mutant glioblastoma line (U373MG) was found to block hypoxia induced Akt phosphorylation and kinase activity (100). Concomitantly, inhibition of hypoxia induced gene expression was demonstrated for *VEGF*, *cyclooxygenase (COX)-1*, *phosphoglycerate kinase (PGK)-1*, and *phosphofructokinase (PFK)*, all of which are known to be dependent on HIF-1 transactivation. An identical pattern of attenuated hypoxia induced gene expression was noted following exposure to the PI(3)K inhibitor wortmannin, consistent with PTEN opposing the activity of PI(3)K. HIF-1 dependent transactivation caused by constitutive activity of Akt could not be attenuated by wild type PTEN, suggesting that the actions of PTEN are upstream of Akt. The effects of PTEN on hypoxia induced gene expression were shown to be due to suppressed HIF-1alpha protein stabilization, which is dependent upon Akt (68-70,100).

7.2. Effects of FIH-1 (Factor Inhibiting HIF-1) on Hypoxia Induced Gene Expression

The gene encoding Factor Inhibiting HIF-1 (FIH-1) is located at chromosome 10q24 near the *PTEN* gene in the region that is often lost during the progression to glioblastoma multiforme (101). This gene was identified in a yeast two-hybrid screen for proteins that interact with HIF-1alpha. Subsequent analysis demonstrated that FIH-1 interacted most strongly with amino acids 531-826 of HIF-1alpha, which contains parts of the transactivation inhibitory domain (TID) and the TAD-C (figure 4). Expression of FIH-1 in human embryonic 293 cells inhibited hypoxia induced transcription mediated by HIF-1 in a dose dependent fashion. Importantly, it appeared that FIH-1 was capable of inhibiting transcriptional activity by HIF-1 in normoxic as well as hypoxic cells. More recently, FIH-1 has been shown to have Fe(2+) and 2-OG dependent oxygenase activity capable of hydroxylating HIF-1alpha within the TAD-C at Asn^{803}. Such hydroxylation inhibits HIF interactions with the co-activator CBP/p300 and attenuates HIF mediated transcription (56-58). Since asparagine hydroxylation requires oxygen, FIH-1 hydroxylase activity can potentially account for much of the diminished HIF transcription under normoxia, but other mechanisms may be relevant to its regulation of HIF under hypoxia. Different sites of FIH-1 protein are capable of binding to both VHL and HIF-1alpha, and therefore a portion of FIH-1 mediated inhibition of HIF-1alpha may be due to a ternary complex containing FIH-1, VHL, and HIF-1 alpha, resulting in enhanced HIF-1alpha ubiquitination and degradation. Alternatively, FIH-1 may act as a co-repressor by recruiting histone deacetylases (HDACs) to HIF-1alpha, a common mechanism for inhibiting the binding of transcription factors to DNA. FIH-1 has been shown to bind specifically with HDAC1-3, but less strongly than VHL. The cumulative data suggest that FIH-1 most likely regulates HIF under normoxia by Asn^{803} hydroxylation and could potentially modulate HIF function under hypoxia by mechanisms that include enhanced VHL mediated degradation of HIF-1alpha or increased recruitment of HDACs (figure 5). Loss of FIH-1 during the progression to high grade gliomas could potentially lead to derepressed transactivation by HIF-1, resulting in increased target gene expression including *VEGF*. The expression pattern of FIH in normal and tumoral tissue is currently unknown. Loss of FIH-1 expression or mutations in *FIH-1* could favor tumor growth. Given the location of *FIH-1* on chromosome 10q24, it will be highly relevant to examine its role in glioma development even if *PTEN* appears to be the main target of deletions in this region.

7.3. Regulation of Angiogenesis by PTEN

Losses on chromosome 10 that occur during the progression of astrocytomas may cause enhanced angiogenesis by mechanisms distinct from HIF-1 mediated transcription. Hsu et al demonstrated that reconstituting glioma cell lines with fragments of chromosome 10 by microcell-mediated transfer led to decreased tumor growth in nude mice and that clones with reconstituted chromosome 10 showed increased secretion of the angiogenesis inhibitor TSP-1 (102). Moreover, normal brain and low grade astrocytomas that retained chromosome 10 demonstrated immunoreactivity for TSP-1 protein, whereas GBMs with loss of chromosome 10 did not. These studies suggested that gene(s) on chromosome 10 might be inhibitory to tumor growth and regulate TSP-1

expression. The discovery of the *PTEN* gene on chromosome 10 raised the possibility its tumor suppressor activity might, in part, be due to activation of TSP-1. Indeed, expression of TSP-1 has been shown to decrease the tumorigenicity of human glioma cells (92). Wen, et al, recently addressed this issue by reconstituting *PTEN* cDNA into glioma cell lines lacking the gene, and found a significant decrease in tumorigenicity in nude mice (103). These tumors also demonstrated anti-angiogenic properties that were attributed to TSP-1 expression. The mechanism of TSP-1 induction by PTEN is unknown. Together these results suggest that loss of chromosome 10, and the *PTEN* gene in particular, may be causally related to the vascular hyperplasia associated with malignant transformation of astrocytomas. This may occur through the loss of TSP-1 mediated inhibition of angiogenesis as well as angiogenic stimulation due to Akt mediated HIF stabilization.

8. REGULATION OF HYPOXIA INDUCED GENE EXPRESSION BY EGFR AND PDGFR

Both epidermal growth factor receptor (EGFR) and platelet derived growth factor receptor (PDGFR) belong to the receptor tyrosine kinase (RTK) class of receptors (104). As such, they are transmembrane monomers that dimerize upon receptor binding, leading to autophosphorylation of specific tyrosine residues in their cytoplasmic domains. Autophosphorylation leads to recruitment and docking of proteins that contain SH2 (Src homology 2) and PTB (phosphotyrosine binding) sites. Some enzymes containing SH2 domains interact directly with RTKs (eg. phospholipase Cgamma), while other signaling cascades, such as the Ras/MAP kinase cascade, are initiated by SH2 containing adaptor proteins (e.g. Grb2). Most significant to HIF-1 activation, PI(3)K is activated by nearly all RTKs (figure 6). The regulatory subunit of PI(3)K, p85, contains two SH2 domains and one SH3 domain which interact directly with activated RTKs. The catalytic subunit, p110, is responsible for phosphorylating PIP(4) and PIP2(4,5) to PIP2(3,4) and PIP3(3,4,5), respectively. Following recruitment to the cell membrane, PIP4(3,4,5) activates the serine-threonine kinases PDK1 and Akt. Thus, upregulation or activation of either EGFR or PDGFR, both of which occur in a subset of malignant gliomas, leads to activation of Akt, raising the possibility that these pathways may stabilize HIF-1alpha.

8.1. EGFR

A large percentage (40-50%) of GBMs are characterized by amplification and/or overexpression of the *EGFR* gene (105,106). Either wild type or mutated forms can be amplified, and in either case, the levels of mRNA as well as protein expressed at the cell surface are markedly increased. The most common *EGFR* gene amplification in GBMs is a mutated form lacking exons 2-7 that results in a truncated cell surface protein with constitutive tyrosine kinase activity. Hypoxia may also indirectly upregulate EGFR protein expression. The early growth response factor (Egr-1) is a regulator of gene expression that is induced by hypoxia and its expression has been shown to enhance transcriptional activity at the *EGFR* promoter (107).

In human carcinoma cells, activation of EGFR by extracellular epidermal growth factor (EGF) causes increased HIF-1alpha protein levels, even under normoxic conditions (69). This effect is blocked by the PI(3)K inhibitors LY294002 and wortmannin, implicating the PI(3)K/Akt pathway as the mediator of EGFR's effect. Fibroblasts transfected with truncated, constitutively active forms of EGFR, such as those in GBMs, show enhanced hypoxia induced expression of VEGF compared to those transfected with the full length *EGFR* (108). Enhanced VEGF expression in these experiments was also inhibited by PI(3)K inhibitors, indicating that mutant forms of EGFR utilize the same signaling cascade to activate HIF-1 mediated gene expression.

Other investigations have suggested that EGFR induces VEGF expression via the PI(3)K pathway, but in a manner distinct from hypoxia (109). EGF was found to induce transcriptional activity at a *VEGF* promoter that lacked an HRE with similar effectiveness to that at a *VEGF* promoter with a retained HRE. EGF-induced transcription at the HRE-deleted promoter could be inhibited by PI(3)K inhibitors, suggesting that EGF acts though the PI(3)K pathway, but through mechanisms distinct from those utilized under hypoxia. Thus, hypoxia and EGFR activation may have synergistic effects on HIF-1 mediated gene expression and VEGF production.

8.2. PDGFR

The receptor for PDGF occurs in two forms, PDGFR-alpha and PDGFR-beta (110). While PDGFR-beta is typically expressed by blood vessels in glial neoplasms, PDGFR-alpha is often overexpressed in glial tumor cells. The level of PDGFR-alpha mRNA is elevated in all grades of astrocytomas, but is highest in GBMs (111,112). Overexpression of PDGFR-alpha in gliomas generally occurs in the setting of normal gene dosage; however, gene amplification has been documented in a small subset of GBMs (8-16%).

Activation of the PI(3)K/Akt pathway also occurs in cells exposed to extracellular PDGF and is mediated by PDGFR (113,114). Akt activation by these mechanisms depends upon specific tyrosine phosphorylation events of the PDGFR and can be blocked by inhibitors of PI(3)K. In vascular endothelial cells expressing PDGFR-beta, exposure to extracellular PDGF caused increased VEGF transcription and secretion, leading to autocrine growth stimulation of endothelial cells expressing VEGFR. The significance of the PDGFR and PI(3)K/Akt pathways in VEGF expression was demonstrated by showing that these effects could be blocked by PI(3)K inhibitors, mutated inactive forms of PDGFR, and dominant negative forms of PI(3)K (113).

Additional studies with fibrosarcoma cells have shown that PDGFR-beta is activated by extreme hypoxia in a ligand independent fashion (115). Under these conditions, PDGFR-beta interacted with the p85 regulatory subunit of PI(3)K within one hour and resulted in activation of Akt. Such activation was blocked by PI(3)K inhibitors, but not by the growth factor receptor poison suramin. These

experiments did not directly investigate whether HIF-1 mediated gene expression was affected by ligand independent activation of Akt through the PDGFR; however, the findings suggest that hypoxia induces adaptive gene expression through multiple interconnected mechanisms.

9. REGULATION OF ANGIOGENESIS BY p16(CDKN2A)

The retinoblastoma (Rb) tumor suppressor pathway is best known for its regulation of the cell cycle (2,6,116,117). Disruption of the Rb pathway during gliomagenesis can occur by loss of the *Rb* gene itself, homozygous deletion or promoter hypermethylation of the *p16(CDKN2A)* gene, or by amplification of either *CDK4* or *CDK6* genes. Inactivation of p16(CDKN2A) is the most frequent route of Rb pathway interruption, occurring in 60-70% of GBMs. Because *p16(CDKN2A)* is deleted or silenced in a high percentage of astrocytomas at a time of biologic progression and accelerated vascular proliferation, its role in angiogenesis has been investigated. In studies that used glioma cell lines lacking endogenous *p16(CDKN2A)* (U-251 MG and U-87 MG), adenoviral transfection of wild type *p16(CDKN2A)* was found to substantially reduce the level of VEGF mRNA and the amount of secreted VEGF protein (118). Decreased VEGF production occurred at periods when cell cycle arrest had not yet occurred and was therefore not thought to represent a secondary effect of growth inhibition. Secreted products from cells with restored p16(CDKN2A) were also found to greatly reduce the number of vessels that grew as a capillary network in an air sac fascia assay of angiogenesis. In this assay, restoration of p16(CDKN2A) to p16(CDKN2A) deficient cells was more effective at reducing angiogenesis than restoring p53 to p53 deficient glioma cells. The precise mechanism of VEGF down regulation and inhibition of angiogenesis by p16(CDKN2A) has not yet been determined.

10. VON HIPPEL LINDAU DISEASE

Von Hippel Lindau disease is an autosomal dominant disorder in which affected patients carry an increased risk for developing neoplasms involving multiple organ systems (119). Most typical are capillary hemangioblastomas of the central nervous system and retina; renal cell carcinoma; pheochromocytomas of the adrenal medulla; islet cell tumors of the pancreas; endolymphatic sac tumors of the inner ear; papillary cystadenomas of the broad ligament or epididymis; and benign cysts of the kidney and pancreas. The genetic predisposition to neoplasia is due to an inherited mutation of the tumor suppressor gene *VHL*, located on chromosome 3p25-26. Functional loss of the second allele is associated with the formation of neoplasms. *VHL* encodes a protein with 213 amino acids which functions as part of a ubiquitin ligase complex with elongin B and C, Cul2, E2, and Rbx1 (50,119) (figures 5, 6). The majority of *VHL* mutations that give rise to disease are located in the region encoding the alpha subdomain, which binds to elongin C, or the ß subdomain, which physically interacts with HIF-1 alpha. Loss of both *VHL* alleles and resulting loss of protein expression leads to decreased VHL mediated ubiquitination of HIF-1alpha and HIF-2alpha. Renal carcinoma cells lacking *VHL* constitutively express HIF-1alpha and HIF-2 alpha as well as HIF inducible genes under non-hypoxic conditions (120). Re-introduction of *VHL* into these cells restored the oxygen dependent expression of alpha subunits. The high vascularity of the neoplasms that arise in von Hippel Lindau syndrome, such as renal cell carcinomas and hemangioblastomas, reflect the pro-angiogenic genotype caused by unregulated HIF target gene expression, including VEGF. The finding of VHL mutations in the majority of VHL-associated tumors suggests that HIF activation following the loss of VHL cannot be fully compensated by the actions of IPAS, FIH-1, or Cited2 in these tissues. This may in part explain the tissue specificity of the disease.

10.1. Dysregulation of HIF-1 in Hemangioblastomas

Hemangioblastomas are low grade neoplasms that arise predominantly in the cerebellum and spinal cord of patients with von Hippel Lindau disease, but can also occur sporadically in patients without the syndrome (119,121,122). When occurring in VHL patients, hemangioblastomas generally occur at a younger age and are more often multiple. These tumors are composed of bland, highly lipidized stromal cells and display an extremely high density of small delicate blood vessels. Although the neoplasms have been named after their prominent vascular component, the neoplastic cells are, in fact, the fat-laden stromal cells. The latter lack functional VHL protein and therefore VHL mediated ubiquitination and degradation of HIF-1alpha and HIF-2 alpha does not occur properly under normoxia (121,122). Enhanced HIF transcription leads to excessive production of VEGF, which accounts for the high vascularity of hemangioblastomas, and to increased secretion of EPO, which can lead to polycythemia.

11. PERSPECTIVES

Understanding the angiogenic events that occur during the progression of astrocytomas will be critical for developing effective interventions. The past decade has uncovered a variety of genetic pathways that are disrupted in glial tumorigenesis. Current evidence strongly suggests that alterations in oncogenes and tumor suppressor genes directly and indirectly influence angiogenesis by modulation of the physiological response to hypoxia and by regulating the expression pro- and anti-angiogenic factors. A promising approach for therapy is the development of anti-angiogenesis strategies, broadly defined as genetic elements or chemotherapeutics that could suppress vascular proliferation in GBMs or other tumors. An obvious target for these interventions is the HIF mediated response to hypoxia, which appears central to angiogenesis and biologic progression of astrocytomas. Small molecular inhibitors of HIF-mediated transactivation are currently being identified in large screens using reporter genes under the control of HRE elements (123). A recent report indicates that the Hsp-90 specific inhibitor geldanamycin induced degradation of HIF-1alpha protein in prostate

cancer cells under both normoxia and hypoxia (124). A close analog of geldanamycin, 17-(allylamino)-17-demethoxygeldanamycin (17-AAG), is already in clinical trials. Other approaches use HIF-dependent gene therapies (125) or the delivery of anaerobic pathogens (126). Such approaches will need to carefully consider the potential side effects of blocking HIF targets that may be associated with the desired anti-tumor effects. For example, VEGF plays a critical role in both dendritic cell maturation and neuronal survival (127,128). Nonetheless, targeted disruption of HIF mediated angiogenesis is a rational and potentially promising approach for developing new therapies.

12. ACKNOWLEDGEMENTS

Supported in part by US Public Health Service National Institutes of Health (NIH) grants CA-86335, CA-87830, NS-41403 (EGVM), and NS-42934 (DJB).

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Key Words: Astrocytoma, Glioblastoma Multiforme, Hypoxia, HIF-1, Genetics, Angiogenesis, Review

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