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ANGIOGENESIS AND ITS ROLE IN THE BEHAVIOR OF ASTROCYTIC BRAIN TUMORS

Shirley I. Stiver

Beth Israel Deaconess Medical Center, Departments of Surgery and Pathology, Research North, Room 289, 330 Brookline Avenue, Boston, MA 02215

TABLE OF CONTENTS

1. ABSTRACT

Angiogenesis, the development of new vessels from a pre-existing vasculature, accompanies the growth and malignant transformation of astrocytic brain tumors. Neovascularization is essential for sustained tumor growth, and with increasing grade, astrocytic tumors undergo an "angiogenic switch" manifested by marked increases in vessel density and changes in vascular morphology. In the quiescent state, endogenous anti-angiogenic factors including endostatin, thrombospondin, and soluble vascular endothelial growth factor receptor-1 (sVEGFR-1) balance the actions of pro-angiogenic stimuli and restrain the angiogenic switch. Once activated, pro-angiogenic factors including most notably basic fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF-A), and platelet-derived growth factor (PDGF) incite robust astrocytoma neovascularization. Recent studies have also explored the expression patterns and functional importance of the angiopoietins, Tie2 and neuropilin receptors, and hepatocyte growth factor/scatter factor (HGF). Together these angiogenic factors have diverse actions on endothelium and perivascular supporting cells that engender tumor neovessels with a unique phenotype, distinct from normal vessels. Properties of the astrocytoma neovasculature contribute to tumor growth, malignant progression, invasion, hemorrhage, and edema formation. Thus, the mechanistic actions of angiogenic factors on cerebral microvessels and the nature of the resultant tumor neovasculature establish a framework for understanding many of the characteristic behaviors of astrocytoma tumors.

2. INTRODUCTION

Astrocytomas represent the most common primary adult neoplasm of the central nervous system. While traditionally presumed to derive from mature, differentiated astrocytes, their cell of origin remains elusive (1, 2). By comparison, gliomas represent a broader class that also encompasses oligodendrogliomas and tumors of ependymal origin (2). The WHO classification divides astrocytic brain tumors into 4 grades (2, 3). This review focuses on the more common Grade II-IV lesions and does not address the distinct group of grade I pilocytic astrocytomas. Grade II astrocytomas are the least malignant and are characterized by a proliferation of diffusely infiltrating, neoplastic astrocytes without obvious mitoses. Grade III tumors, known as anaplastic astrocytomas, exhibit mitoses and pleomorphism, but lack the necrosis and endothelial proliferation that characterize the most malignant of astrocytic tumors, the grade IV glioblastoma multiforme lesion. Glioblastomas may be primary, arising *de novo*, or they may develop secondarily by malignant progression from low-grade lesions (4-7). Angiogenesis,

the growth of new vessels from a preexisting vasculature, has been extensively studied in astrocytic tumors (for reviews see 8-13). This review explores the importance of angiogenesis, angiogenic factors, and the tumor neovasculature from the perspective of their contribution to the behavior patterns of astrocytic tumors.

3. ANGIOGENESIS AND TUMOR GROWTH

3.1. Co-option, neovascularization, and the angiogenic switch in astrocytomas

Because oxygen can diffuse only 0.1 to 0.2 mm from a blood vessel, tumor growth is highly reliant upon neovascularization (14-18). Astrocytic tumor growth in animal models is characterized by two vascular phases (19, 20). In the first phase, small collections of tumor cells co-opt or parasitize pre-existing blood vessels from the host, and within a week of inoculation, tumor cells become distributed preferentially around vessels. Co-option of the preexistent host vasculature occurs in the absence of angiogenesis. With exponential tumor cell proliferation, the co-opted vasculature quickly fails to meet the metabolic demands of the growing tumor mass, and co-opted vessels at the center of the tumor begin to regress. In the second phase of astrocytic vascularization, tumor cells in the vicinity of the degenerating vessels become hypoxic and upregulate expression of vascular endothelial growth factor (VEGF-A). VEGF-A is a potent stimulus for new vessel growth and induces a neoangiogenic response, predominantly at the tumor periphery, that rescues the tumor and facilitates further tumor growth (19-21). Studies using the arterial marker, ephrin B2, have further shown that the tumor vasculature acquires a normal arterial and venous circulatory tree (22).

The astrocytic tumor vasculature manifests distinctive patterns that vary with tumor grade. Low-grade astrocytomas (grade II) exhibit vessel densities slightly greater than normal brain and their vessel morphology is unremarkable. As the malignant phenotype advances to grade III, vessel density increases dramatically and with continued transition to a grade IV glioblastoma, marked changes in vessel morphology emerge (Figure 1). Thus the progression from a low-grade astrocytoma to a high-grade glioblastoma is accompanied by an “angiogenic switch” (23). The angiogenic switch is defined as the point during tumor growth at which dramatic upregulation of growth factors and receptors act to induce increased tumor vascularization. Based on the hypothesis that angiogenesis is regulated by a balance of pro- and anti-angiogenic factors in the tumor microenvironment, a multitude of environmental, genetic, and metabolic factors can trigger the angiogenic switch (14, 23, 24). Despite intense research effort, evidence that astrocytic neovascularization plays an independent, causal role in tumor growth and malignant transformation remains to be established.

3.2. Mechanisms of Astrocytic Tumor Neovascularization

Vascularization of solid tumors has long been accepted to occur through the sprouting of new capillaries from a preexisting host vasculature in a process termed tumor angiogenesis (14). Sprouting is a complex process that involves proteolysis of the extracellular matrix to create a path for subsequent passage of proliferating and migrating endothelial cells. Metalloprotease-2 (MMP-2, gelatinase A) and metalloprotease-9 (MMP-9, gelatinase B) are expressed in normal brain as well as in glioblastoma tumors (25, 26). Vascular endothelial growth factor (VEGF-A), a major angiogenic cytokine expressed in astrocytic tumors, has been shown to upregulate expression of matrix metalloproteases and plasminogen activators (27-29). Sprouting endothelial cells that have proliferated and migrated from host vessels then undergo morphogenic changes in which a new capillary lumen and new basement membrane are formed (30). While sprouting accounts for the increased vessel density in Grade II and III astrocytic tumors, mechanisms involving complex remodeling of the vasculature engender the more striking morphological changes observed in grade IV glioblastoma tumors (Stiver - unpublished data).

Recent studies suggest that circulating endothelial precursor cells (EPCs) may also contribute to the tumor vasculature in a *de novo*, vasculogenic mechanism (31-33). Following intravenous injection of EPCs into mice harboring C6 glioma implants, approximately 5% of the precursor cells were observed to incorporate into the endothelium of the tumor vasculature (32). E-and P-selectin played important roles in the homing of EPCs to the tumor endothelium (32). Following adhesion, EPCs were reported to extravasate into the tumor interstitium and to subsequently form endothelial clusters and neoangiogenic sprouts (32). In a similarly designed study, the contribution of EPCs to the tumor vasculature was less profound. Using Rosa 26-labeled bone marrow transplants and GL261 glioma cell tumors, only 0. 6% of tumor vessels co-expressed the bone marrow marker lacZ and the endothelial marker CD-31 (33). In this study the majority of lacZ positive cells within the tumor vessels and stroma were identified as microglia/macrophages. EPCs are a current topic of intense research investigation and future studies will further delineate their contributions to the tumor neovasculature.

3.3. Characteristics of astrocytic tumor vessels

Two patterns of astrocytic tumor angiogenesis have been described (12, 34). Infiltrative low-grade and anaplastic astrocytomas manifest a “classic” pattern of normal-appearing, delicate capillary sprouts, which are uniformly distributed at increased density compared to normal brain (35). By contrast, there is significant heterogeneity in the microvasculature of glioblastoma multiforme tumors. In addition to the classic microvascular sprouting pattern, glioblastoma tumors also exhibit vascular clusters, garlands, and bizarre glomeruloid proliferative structures (Figure 1) (34, 35). Glomeruloid structures are capillary tufts that resemble renal glomeruli and are comprised of small vascular channels lined by a mixture of hyperplastic endothelial cells and pericytes (36-39). Mitotic figures, positive bromodeoxyuridine (BrdU) incorporation, and ^{3}H-thymidine labeling demonstrate a high proliferative index within glomeruloid bodies (40, 41). Collectively, vascular clusters, garlands, and glomeruloid structures are

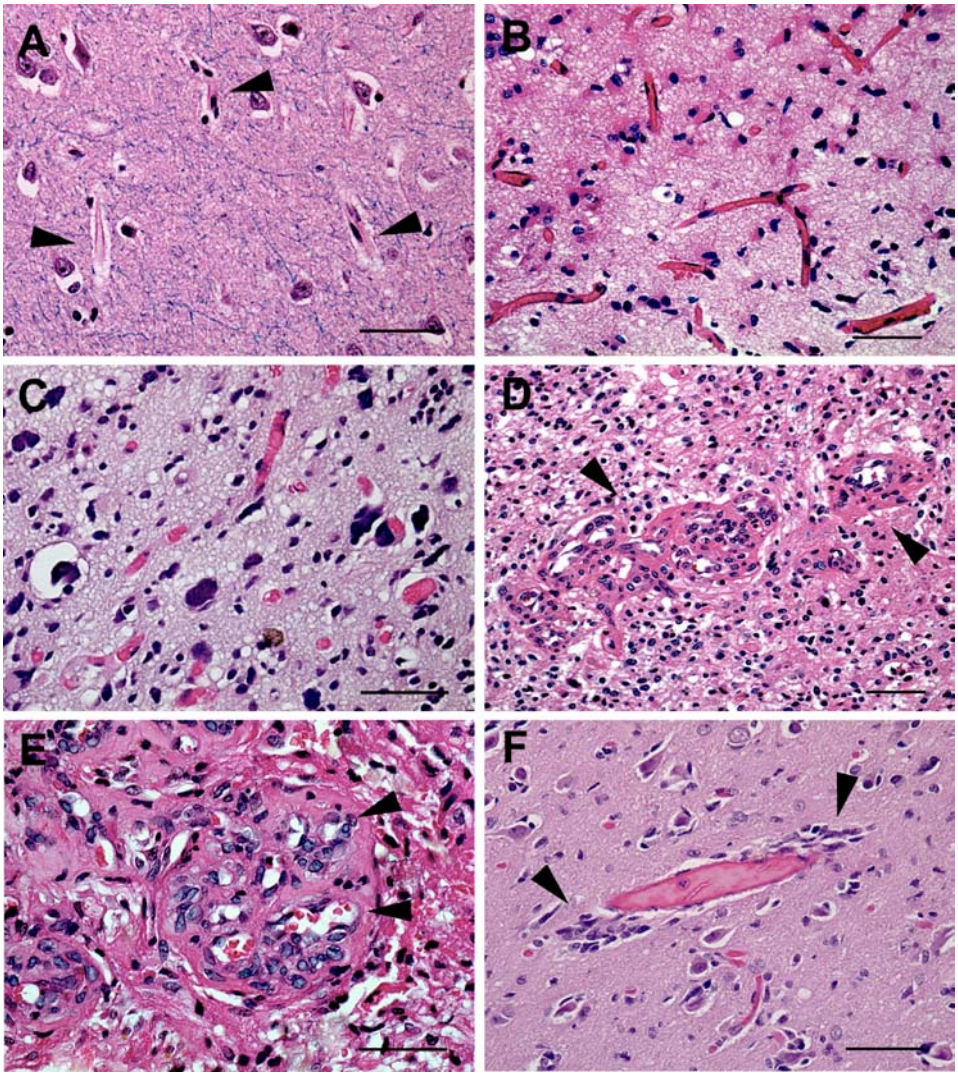

Figure 1. Angiogenic patterns in human glioblastoma multiforme tumors. A) Small delicate vessels (arrows) characterize the vasculature of normal human cortex. B) Prominent vessels in brain parenchyma juxtaposed to a glioblastoma tumor give the appearance of sprouting angiogenesis at the invading tumor front. C) An angiogenic sprouting pattern is further evidenced as an increased density of small vessels within a field of pleomorphic malignant cells. D) Endothelial proliferation, manifested as a garland of vessels with hyperplastic endothelial cells (arrows), is a diagnostic criterion for grade IV glioblastoma tumors. E) Glomeruloid structures are a bizarre form of endothelial proliferation that comprise capillary tufts (arrows) dispersed amidst a proliferation of endothelial cells and pericytes. F) Invasive tumor cells (arrows) of glioblastoma tumors show a predilection for perivascular cuffing. Hematoxylin and eosin stained paraffin sections. Scale bars: 50 μm.

referred to as vascular endothelial proliferation and represent a diagnostic criterion for grade IV glioblastoma multiforme tumors (3).

Tumor vessels are structurally and functionally distinct from normal vessels (42). Co-culture of U87 astrocytoma cells with human umbilical vein endothelial cells (Huvecs) has been shown to induce proliferative, migratory, and morphological changes in endothelial cells that recapitulate many facets of the tumor endothelial phenotype and simulate the angiogenic switch (43). In glioblastoma tumor vessels, endothelial cells exhibit altered levels of thrombomodulin and anti-thrombin III (44, 45), induction of $\alpha_v\beta_3$ integrin (46), and a different profile of adhesion molecules (47, 48). The neoangiogenic vessels of glioblastoma tumors are fragile and prone to spontaneous hemorrhage (49). Pericytes, supporting cells of normal capillaries, are characteristically abnormal with reduced coverage in tumor vascular beds, and the resultant lack of vessel maturation may be an etiologic factor in astrocytic tumor hemorrhage (50-53). Furthermore, extracellular components of the vessel matrix are altered and notably, the glycoprotein tenascin, rarely seen in the normal brain vasculature, is found around malignant astrocytoma tumor

vessels (54). Tenascin expression increases with increasing grade and correlates inversely with patient survival (54, 55).

The astrocytic tumor vasculature is not only morphologically aberrant but also functionally abnormal. The blood brain barrier is incompetent and leads to significant morbidity from tumor edema (for reviews see 56, 57). Loss of cerebral autoregulation contributes to hyperperfusion, as evidenced in positron emission tomography (PET) and magnetic resonance imaging (MRI) studies, and may play a role in tumor hemorrhage, edema formation, and local brain ischemia (58-60). Moreover, consideration should also be given to the functional outcome of the tumor neovascularity in terms of blood flow, oxygenation, and the degree to which it meets the metabolic demands of the tumor (61, 62). Indeed the paradoxical association of glomeruloid structures and microvascular proliferation juxtaposed to areas of necrosis may suggest that this profuse vascular response does not contribute to nutritive sustenance of the tumor (34).

4. ANGIOGENIC GROWTH FACTORS AND ASTROCYTIC TUMOR GROWTH

Numerous factors elaborated directly or indirectly by tumor cells play a role in tumor angiogenesis. Basic fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF-A), and platelet-derived growth factor (PDGF) are noteworthy for their significant role in astrocytoma vascularization. In addition, further studies have recently explored the contributions of angiopoietin 1 and angiopoietin 2 (Ang1, Ang2), neuropilin, hepatocyte growth factor/scatter factor (HGF), and a small group of endogenous anti-angiogenic factors.

4.1. Basic Fibroblast Growth Factor (FGF)

In *in vitro* angiogenesis assays, basic FGF is an essential and highly potent inducer of capillary endothelial tube formation (63, 64). Immunostaining of basic FGF expression in human astrocytomas has been reported to correlate with histological tumor grade and vessel density as well as with angiographic assessments of vascularity (65-67). However, basic FGF can be bound and stored in the extracellular matrix, and studies employing methodologies to extract basic FGF from astrocytoma tumor tissue, have reported no difference in expression levels between low- and high-grade lesions (64). In view of this finding, it has been suggested that basic FGF alone is insufficient to induce astrocytoma angiogenesis, but rather that it acts synergistically with VEGF-A and hepatocyte growth factor/scatter factor (HGF) to evoke robust neovascularization (64, 68). In addition, changes in FGF receptor expression have been suggested to play a role in malignant progression (69-71). However, in overall comparison to vascular endothelial growth factor (VEGF-A), basic FGF is a much less powerful regulator of astrocytoma angiogenesis.

4.2. Vascular Endothelial Growth Factor (VEGF-A)

VEGF-A is the chief member of the VEGF family of growth factors, which also comprises VEGF-B, C, D, E and placental growth factor (PlGF). VEGF-A mRNA is upregulated in low-grade astrocytomas, and in glioblastoma tumors it is over-expressed 50-fold compared to normal brain (72, 73). Alternative splicing of the VEGF-A gene yields isoforms of 121, 145, 165, 189, and 206 amino acids (74). The VEGF-A^{121} isoform is freely diffusible, whereas VEGF-A^{189} remains bound to the cells that secrete it (75). The 165 isoform predominates in astrocytic tumors and is partly diffusible and partly bound to the cell surface and extracellular matrix (75, 76). In addition, most glioblastoma tumors strongly express VEGF-D, an X-linked angiogenic factor regulated by the nuclear oncogene, c-*fos* (77, 78). PlGF mRNA has also been identified at low levels in astrocytic brain tumors (79). While it has been suggested that PlGF may act to potentiate VEGF-A, the role of PlGF in astrocytoma growth and angiogenesis has not been explored (79).

VEGF-A is a key mediator of neovascularization in astrocytoma tumors (80). Multiple studies have shown a positive correlation between VEGF-A mRNA levels and microvessel density in astrocytomas (64, 73, 76, 81-83). VEGF-A mRNA is produced by the tumor cells, but the largest amount of VEGF-A protein is found on the vasculature (73, 81, 82), presumably in association with its two endothelial specific receptor tyrosine kinases VEGFR-1 (Flt-1; fms-like tyrosine kinase) and VEGFR-2 (KDR/Flk-1; kinase insert domain containing receptor or KDR in humans and fetal liver kinase-1 or Flk-1 in rodents) (84, 85). VEGFR-1 and VEGFR-2 are upregulated in astrocytic tumor vessels (73, 81, 86, 87), but in both low- and high-grade astrocytomas, only VEGFR-2 levels have been found to correlate with microvessel density (88).

VEGF-A mRNA is strongly expressed in the palisading tumor cells lining necrotic zones, evidence that hypoxia drives VEGF-A expression in glioblastoma tumors (72, 73, 81). Tumor growth, that exceeds the metabolic supply afforded by the tumor's vasculature, likely engenders these zones of hypoxia. Hypoxic transcriptional regulation of VEGF-A is mediated by hypoxia-inducible factor (HIF-1α), which binds to HIF-response elements in the VEGF-A promotor (89). Under hypoxic conditions, HIF-1α does not undergo oxidative degradation, leading to upregulation of VEGF-A transcription as well as increased VEGF-A mRNA stabilization (72, 89-92). However, high levels of VEGF-A expression can also be found at the tumor periphery, remote from zones of central necrosis and hypoxia (93). Furthermore, hypoxia is not a characteristic feature of low-grade astrocytomas, which also upregulate VEGF-A (94). Together, these findings suggest that additional factors and mechanisms may contribute to the induction of VEGF-A expression in astrocytic tumors.

Numerous oncogenes and tumor suppressor genes, including *Src,* (95, 96), *Ras* (97), p53 (98), and von Hippel Lindau (99) have been shown to play a role in the regulation of VEGF-A in astrocytoma tumors. In addition, the mechanisms by which the PTEN tumor suppressor gene controls angiogenesis and astrocytoma growth are of particular relevance. Mutations in the PTEN tumor suppressor gene on the long arm of chromosome 10 are

found in approximately 20-40% of glioblastoma tumors, more frequently in primary as compared to secondary lesions (4, 100-105). High levels of PTEN in glioblastoma tumors have been shown to correlate with a better patient prognosis (106). The tumor suppressor functions of PTEN are closely linked to the PI3Kinase/Akt signaling pathway, which plays a critical role in regulating cell growth, survival, and migration, as well as angiogenesis (107-109). PTEN encodes a lipid phosphatase (phosphatidylinositol-3,4,5trisphosphate phosphatase) that acts to negatively regulate PI3Kinase and thereby inhibits Pi3Kinase activation of VEGF-A and the oncoprotein AKT (107, 108, 110-112). Astrocytoma cell cultures treated with PTEN have been shown to exhibit a marked decrease in VEGF-A protein expression at the transcriptional level (110). Further studies have demonstrated PTEN's significant role in inhibiting angiogenesis, even in the face of additional pro-angiogenic factors such as p53 mutations and epidermal growth factor receptor (EGFR) overexpression (113). This suggests that loss of PTEN function is a potent contributor to the angiogenic switch. Additionally, PTEN mutations lead to increased activation of AKT, which has been shown to induce malignant transformation of anaplastic astrocytomas to glioblastomas (114-118).

4.3. Angiopoietins and Tie Receptors

The angiopoietins are a small family of proteins that exert their effects in the later phases of vessel development, acting to remodel and sculpt the mature vasculature. Ang1 appears to mediate vessel maintenance by stabilization of contacts between endothelial cells and perivascular pericytes and smooth muscle cells (119-121). In angiogenesis models, Ang1 has also been shown to inhibit vascular permeability (122, 123). By contrast, Ang2 reportedly destabilizes vessels and acts to sensitize endothelial cells to angiogenic stimulation (20, 124). Both Ang1 and Ang2 bind to the Tie2 receptor; but whereas Ang1 induces receptor phosphorylation, Ang2 acts to block Ang1 activation of Tie2 (124). Ang1 and Ang2, as well as Tie2 receptor, are expressed in malignant astrocytomas (125-130). Ang1 is expressed primarily by tumor cells (125, 126, 130), while Ang2 is localized to small tumor vessels (125, 127, 130). Levels of Ang1, Ang2, and Tie2 expression have been shown to correlate with tumor grade (128, 130).

The functional importance of the angiopoietins in tumor biology is an ongoing area of active interest. Murine implantation of U87 astrocytoma cells engineered to overexpress Ang1 has been shown to engender a modest increase in both microvascular density and tumor growth (128). The tumor vessels themselves were structurally normal. In contrast, tumor growth was inhibited following inoculation with U373 cells overexpressing Ang1, and differential levels of Ang1 expression may have been responsible for the variable results between the two cell lines (128).

In related studies, overexpression of Ang2 in U87 and U373 astrocytoma lines had no affect on tumor growth but promoted a dilated and abnormal vascular architecture (128, 129). Orthotopic implants of U87 cells overexpressing Ang2 have also been observed to promote angiogenesis at the invading tumor front (131). Concordant with this, evidence suggests that Ang2 plays an initiating role in the angiogenic switch and astrocytoma neovascularization. Ang2 is not expressed in low-grade astrocytomas, but is expressed at high levels in anaplastic astrocytomas and glioblastomas (126, 127, 130). Furthermore, Ang2 demarcates apoptotic endothelial cells in co-opted vessels undergoing regression**,** precedes the formation of necrotic foci, and heralds tumor cell associated expression of VEGF-A, all of which suggest that Ang2 plays a pivotal role in the angiogenic switch (20, 130, 132). In the presence of VEGF-A, Ang2 may further facilitate neoangiogenesis by loosening periendothelial contacts and making the vessels more susceptible to the angiogenic effects of VEGF-A (19, 20). The importance of the angiopoietin/Tie 2 signaling cascade as an anti-angiogenesis target has been validated in recent studies demonstrating that blockade of Tie2 receptor activity in glioblastoma xenografts effectively inhibits tumor vascularity and growth (129).

4.4. Neuropilin

Neuropilin is a transmembrane co-receptor for VEGF-A^{165} activation of VEGFR-2 that has been shown in other tumor types to promote angiogenesis as well as tumor cell migration and survival (133-136). Acting in association with plexins, neuropilin also has an important role in semaphorin signaling (reviewed in 137). Neuropilins 1 and 2 have been demonstrated by RT-PCR and immunoblot analysis in a number of human astrocytoma lines, with expression levels exceeding those in human breast and colon cancer (138, 139). In further studies, expression of neuropilins 1 and 2 co-localized with F-actin to cytoskeletal adhesion sites in U138 malignant astrocytoma cells (138); however, the biological importance of neuropilins in astrocytoma tumors remains largely unexplored.

4.5. Platelet-Derived Growth Factor (PDGF)

Weakly angiogenic, platelet-derived growth family members A and B form homo- and heterodimers that signal transduce through PDGF-Rα and -Rβ receptors (140). PDGF-B plays an important role in angiogenesis, enabling vessel maturation through its action as a strong mitogen and chemoattractant factor for the recruitment of pericytes and smooth muscle cells (141). PDGF is thought to be an early marker of tumorigenesis (142), and PDGF/PDGF-R expression in low-grade astrocytomas is associated with loss of p53 tumor suppressor (143, 144). PDGF-A and PDGF-B are expressed in low-grade, anaplastic and glioblastoma tumors with expression levels increasing with tumor grade (142, 145, 146). In malignant astrocytomas, the A isoform predominates over the B isoform and is expressed at levels up to 100-fold that of normal brain (146, 147). PDGF-A and PDGF-Rα expression localize to the tumor cells themselves suggesting a role for PDGF-A in autocrine stimulation of astrocytoma tumor growth (142). In contrast, PDGF-B and PDGF-Rβ are expressed in tumor endothelial cells and show a predilection for glomeruloid vascular structures (142, 148, 149).

PDGF-B has been shown to upregulate VEGF-A expression in both astrocytoma cells and endothelial cells *in vitro* (150, 151). Moreover, inoculation of mice with U87 astrocytoma cells overexpressing PDGF-B has been found to upregulate VEGF-A expression in the resultant tumors, leading to increased tumor angiogenesis and larger tumors (50). Of further interest, these PDGF-B overexpressing cells stimulated increased recruitment of pericytes to the developing tumor neovasculature (50). PDGF-B is present in low-grade astrocytomas, and the observation that it can upregulate VEGF-A expression has led to the hypothesis that PDGF-B plays an important role in regulating the angiogenic switch (50, 152).

4.6. Hepatocyte Growth Factor/Scatter Factor (HGF)

Hepatocyte growth factor (HGF) (originally termed scatter factor) functions as a potent angiogenic factor that signals through MET, a tyrosine kinase receptor encoded by the proto-oncogene c-*met* (153). In early studies, malignant astrocytoma cyst fluid was found to contain higher concentrations of HGF as compared to fluid from non-tumorous cysts (154). Further studies have gone on to show that HGF exhibits strong chemotactic action on numerous astrocytoma cell lines (68, 154). HGF is also mitogenic for astrocytoma cells, but this action is less robust that its effect on motility and invasion (68). Experimental tumors grown from astrocytoma cell lines transfected with HGF were observed to be larger and to exhibit higher vessel densities (68, 155). In human specimens, expression levels of HGF and Met receptor have been shown to correlate with tumor grade (64, 68, 156, 157). Compared to low-grade tumors, anaplastic astrocytomas and glioblastomas together expressed 7-fold higher levels of HGF and 11-fold higher concentrations of VEGF-A (64, 68). By regression analysis both HGF and VEGF-A functioned as independent predictors of tumor microvessel density (68).

4.7. Endogenous Anti-angiogenic Factors

The angiogenic switch from a quiescent vasculature to robust tumor neovascularization results from perturbation of the balance between pro-angiogenic and anti-angiogenic factors (23, 24, 158). Endogenous anti-angiogenic proteins playing important roles in the inhibition of astrocytoma angiogenesis and tumor growth include endostatin, thrombospondin (TSP), and soluble VEGFR-1 (sVEGFR-1).

Endostatin is a fragment of collagen XVIII, a constituent of vascular endothelial basement membranes. Endostatin is expressed in astrocytomas, and levels have been shown to correlate with the degree of malignancy (159, 160). Moreover, antibodies to endostatin have been detected in the blood of a glioblastoma tumor patient (161). In several animal models, endostatin treatment substantially reduced tumor burden through reductions in tumor neovessel density as well as by inhibition of tumor migration and invasion (162-164).

Thrombospondin (TSP) is a tumor suppressor gene found on chromosome 10 that has important anti-angiogenic properties. PTEN and the transcription factor p53 regulate TSP expression and may thereby influence angiogenesis in astrocytomas (165). Normal brain, low-grade astrocytomas, and anaplastic astrocytomas generally retain chromosome 10 and stain strongly for TSP (166). Loss of chromosome 10 is largely restricted to glioblastoma lesions (167), and in a small series, 12 of 13 glioblastomas were shown to lack TSP expression (166). One case followed serially demonstrated conversion to negative TSP immunostaining coincident with malignant progression (166). In these studies, normal chromosome 10 was re-introduced back into several glioblastoma cell lines. Tumor growth from chromosome 10-reverted cells was inhibited and culture media failed to stimulate endothelial cell migration or rat corneal neovascularization (166). TSP neutralizing antibodies restored the potent angiogenic properties of the parent cells. Together these studies implicate TSP as an important inhibitor of the angiogenic switch in astrocytomas.

sVEGFR-1, a soluble, secreted form of VEGFR-1 (Flt-1) produced by alternative splicing, has recently been found to function as an endogenous anti-angiogenic protein in astrocytic tumors (168). sVEGFR-1 has a high binding affinity for VEGF-A and can act in a dominant negative fashion to inhibit VEGFR-2 signaling (169, 170). Together these data indicate that sVEGFR-1 functions as a negative regulator of VEGF-A bioavailability. In animal models, adenoviral expression of sVEGFR-1 has indeed been demonstrated to inhibit angiogenesis and tumor growth (171, 172). Interestingly, VEGF-A has been shown to upregulate sVEGFR-1 expression (168). Levels of sVEGFR-1 were also found to correlate with astrocytic tumor grade and patient survival (168). Specifically, sVEGFR-1 was found in 40 of 46 glioblastomas (87%) and 6 of 14 (43%) anaplastic astrocytomas. sVEGFR-1 expression was 12-fold higher in glioblastomas as compared to anaplastic astrocytomas ($p < 0.001$). However, at the same time the sVEGFR-1 to VEGF-A ratio decreased due to substantially greater increases in VEGF-A concentrations. None-the-less, in a small cohort of newly diagnosed glioblastoma patients, levels of sVEGFR-1 expression correlated with patient survival (168).

5. ANGIOGENESIS IN ASTROCYTIC TUMOR PROGRESSION AND MALIGNANT TRANSFORMATION

Anti-angiogenic strategies for treatment of astrocytic tumors are predicated on the theory that angiogenesis plays a causal role in tumor growth and malignant progression. Evidence that tumor growth is angiogenesis-dependent is comparatively well established. In astrocytoma models in rodents, neutralizing antibodies against VEGF-A (173, 174) as well as VEGFR-2 (175) have been shown to reduce tumor vascularity and inhibit tumor growth. In more recent studies, treatment of U87 astrocytoma implants with the PI3kinase inhibitor, LY294002, caused a substantial reduction in tumor burden through inhibition of tumor angiogenesis (165). Importantly, VEGF-A has been shown to have negligible effects on proliferation and signal transduction in astrocytoma cells, despite the presence of low levels of

both VEGFR-1 and VEGFR-2 (176). However, similar control studies are needed for all factors contributing to astrocytoma angiogenesis in order to separate out the importance of angiogenesis to tumor growth. In addition, studies should control for the fact that angiogenic factors may also act to promote extracellular matrix degradation and tumor invasion thereby facilitating tumor growth, independent of angiogenesis (27, 29). Thus, rigorous definition of angiogenesis as an independent determinant of tumor growth is a challenging experimental endeavor.

Malignant progression from a low- to high-grade astrocytoma is accompanied by florid tumor angiogenesis. Few studies, however, have examined whether the angiogenic switch is a causal factor in malignant transformation. In early studies, C6 rat glioma cells transfected with sense and antisense murine VEGF-A^{164} yielded tumors that modeled high- and low-grade lesions respectively (177). However, this correlation does not prove causation, as findings of necrosis, hemorrhage, and edema denoting the high-grade lesions are not diagnostic of malignant transformation and may have been caused by increased tumor growth and the co-morbid effects of VEGF-A expression. In more recent studies, Sonoda *et al*. (178) demonstrated that tumors resulting from implantation of H-ras cells engineered to express VEGF-A^{121} and VEGF-A^{165} were more vascularized than anaplastic astrocytomas arising from control H-ras cells. The VEGF-A 121 and 165 overexpressing tumors did not, however, show evidence of malignant transformation to glioblastomas, evidencing that neither VEGF-A nor increased angiogenesis engendered tumor progression in this model.

In clinical studies, establishing causation between angiogenesis and tumor progression is even more complex and difficult. Many studies have shown a correlation between microvascular density and either tumor grade or patient survival (94, 179-182). However, interpretation of these data is complicated by the fact that more aggressive tumor cells, capable of stimulating more robust angiogenesis, may also elaborate additional factors that promote malignant transformation. Moreover, increased morbidity attributable to vessel permeability, hyperemia with associated ischemic steal, and capillary fragility leading to hemorrhage may confound the interpretation of survival data.

One approach in clinical studies has been to look at a select group of low-grade lesions and to test whether microvessel density correlates with an indicator of malignant transformation, such as time to recurrence. In such studies, expression levels of VEGF-A and VEGFR-2 have been statistically associated with earlier times to recurrence ($p=0.0018$ and 0.024 respectively) (88). Furthermore, VEGF-A and microvascular density have been shown to be prognostic measures of survival in low-grade patients (180). Using a different approach, however, Kern *et al*. (183) found no relationship between the MIB-1 labeling index of endothelial cells in glioblastoma tumors and tumor progression, as determined by time to tumor recurrence. Similarly, Birner *et al*. (34) compared the two histological patterns of angiogenesis in glioblastoma multiforme tumors with patient prognosis. The presence of endothelial proliferation comprising garlands, clusters, and glomeruloid structures was associated with a poorer survival than that observed for tumors exhibiting a "classic" microvascular-sprouting pattern. MIB-1 labeling was similar in the two patterns, but tumor cell apoptosis was significantly higher in tumors with a classic sprouting pattern, accounting for their longer survival times. The authors hypothesized that tumors with endothelial hyperproliferation outpace their vascular supply such that growth and progression become independent of vascularization. This led them to further speculate that glioblastomas expressing a proliferative vascular pattern may not be responsive to anti-angiogenic therapy.

6. THE INVASIVE NATURE OF ASTROCYTOMAS FROM AN ANGIOGENIC PERSPECTIVE

Cure of astrocytic brain tumors is thwarted by their diffuse and widespread invasive nature. Astrocytic tumor cells disseminate along distinct anatomical pathways within the central nervous system, following components of the extracellular matrix that foster cell adhesion and migration (184, 185). Experimentally, C6 rat glioma cells injected into caudate-putamen demonstrate a high affinity for endothelial basement membranes and a predisposition to grow around blood vessels (186, 187). Furthermore, histopathological sections from patient specimens evidence that astrocytic tumor cells have a predilection to form perivascular cuffs (188). Despite this perivascular clustering, astrocytoma cells do not transgress the wall of cerebral blood vessels (189). This finding accounts for the characteristic lack of hematogenous spread of astrocytic brain tumors, and research to define the molecular elements responsible for the restrictive nature of cerebral microvessels is of great interest (190).

The interactions between astrocytoma cells and specific extracellular matrix components of the brain microenvironment are complex (191, 192). Brain parenchymal matrix is largely comprised of hyaluronic acid and various glycosaminoglycans; the more common extracellular components laminin, collagen, and fibronectin are restricted to the basement membranes of cerebral blood vessels (193). In *in vitro* models, laminin, fibronectin, and collagen type IV have been shown to foster astrocytoma cell migration and invasion (194-199). Interactions between integrin receptors on astrocytoma cells and these substrates are instrumental in promoting tumor cell dissemination (194, 196, 198). Furthermore, the matrix glycoprotein tenascin is expressed around vessels at the invasive front of malignant astrocytomas and may act through $\alpha 2\beta 1$-integrin receptors to promote tumor cell migration (200, 201). Recent evidence suggests that many of the extracellular components that act to foster tumor dissemination are, in fact, elaborated by the host brain tissue in response to the invading tumor cells (201, 202).

Discovery of new laminin isoforms has particular significance to astrocytoma cell invasion. Normal brain microvascular endothelial cells express small amounts of

the laminin-9 isoform ($\alpha4\beta2\gamma1$), while in human glioblastoma tumors, capillary basement membranes elaborate laminin-8, an isoform comprising $\alpha4$, $\beta1$, and $\gamma1$ chains (203). Interestingly, co-culture of astrocytoma cells with brain endothelial cells stimulated the endothelial cells to express the laminin-8 $\beta1$ chain (203). Furthermore, antisense oligos directed against the $\alpha4$ and $\beta1$ chains of laminin 8 significantly inhibited astrocytoma cell invasion in *in vitro* invasion assays (203). In related clinical studies, expression of laminin-8 has been found to be predictive of astrocytoma recurrence (204).

Angiogenic cytokines elaborated by astrocytic tumor cells also play a role in tumor cell migration and invasion. A detailed analysis of the distribution of VEGF-A in human astrocytoma biopsy specimens demonstrated high VEGF-A expression in tumor cells that had infiltrated the surrounding brain (93). In addition to upregulating metalloproteases that act to degrade the extracellular matrix, VEGF-A has also been shown to induce focal adhesion kinase (FAK), a mediator of early integrin functioning (205, 206). Human astrocytoma cells and tumor tissue exhibit increased levels of FAK expression (207); and glendamycin, a small molecule antagonist of FAK has been shown to inhibit astrocytoma cell migration (208).

Other angiogenic factors, including most notably Ang2 and HGF, have also been shown to promote astrocytoma cell invasion. Overexpression of Ang2 in U87 astrocytoma cell implants led to aggressive tumor cell invasion into adjacent brain parenchyma through activation of matrix metalloprotease-2 (131). Ang2 and MMP-2 were co-expressed at the invading tumor front. The functional significance of MMP-2 was evidenced by *in vitro* invasion assays showing that MMP-2 inhibitors blocked migration of U87 cells that overexpressed Ang2 (131). Boyden chamber migration studies have also demonstrated that HGF is chemotactic for many astrocytoma cell lines (154, 157, 209). Basic FGF, PDGF, and VEGF-A had markedly weaker efficacy in these assays, targeting HGF as a potent motility factor in astrocytoma invasion (209).

In early reports, Wesseling et al. (12) raised the possibility that anti-angiogenic therapy may not be efficacious in treating astrocytomas as their highly infiltrative nature allows them to derive much of their blood supply from the host brain. Indeed, subsequent studies have shown that, while inhibition of astrocytoma angiogenesis can impair tumor growth, invasion along the host vasculature can actually be enhanced by these treatments (174, 175, 210).

7. ANGIOGENESIS AND ANGIOGENIC FACTORS IN ASTROCYTIC TUMOR EDEMA AND HEMORRHAGE

Edema, the result of plasma extravasation through hyperpermeable vessels, forms around brain tumors at a rate of 14-78 ml per day (211). In the case of astrocytic tumors, permeability occurs through breakdown of the blood brain barrier of normal cerebral vessels together with leakage across abnormal angiogenic vessels of the tumor vasculature. VEGF-A is generally considered to be the key cytokine responsible for vascular permeability and edema formation in malignant astrocytic tumors. VEGF-A potently causes vascular permeability through the induction of caveolae/vesicular vacuolar organelles (VVOs), fenestrations, and transcellular gaps in endothelial cells (for reviews see 212, 213). In astrocytic tumors, VEGF-A mRNA levels have been shown to correlate with capillary permeability and vascular volume (82). However, attempts to use MRI imaging to compare the severity of tumor edema with levels of VEGF-A expression have not yielded uniformly consistent results (214, 215).

Derangements in several components of the blood brain barrier likely contribute to edema formation in malignant astrocytomas. Perivascular changes include the presence of astrocytic tumor cells that may not recapitulate the barrier function of astrocyte foot processes, and the less extensive pericyte coverage of tumor vessels may further potentiate tumor vessel hyperpermeability (52, 216). In glioblastoma multiforme tumors, electron microscopy studies have documented structural changes in the tumor endothelium including the presence of open inter-endothelial tight junctions, increased numbers of pinocytic vesicles/caveolae, and the induction of fenestrae and transendothelial gaps (217-221). Data demonstrating the open status of tight junctions should be interpreted cautiously, as many glioblastoma specimens derive from patients treated with mannitol, a hyperosmotic agent that reportedly opens inter-endothelial passages (222). Despite this, evidence from several studies does suggest that the integrity of endothelial tight junctions may be compromised and a source of vascular hyperpermeability in malignant astrocytomas. Transmembrane and associated proteins that bind adjacent endothelial cells maintain tight junctions. In astrocytoma tumors, the tight junction proteins occludin, claudins 1,3, and 5, as well as zonnula occludens-associated protein-1 (ZO-1) are downregulated (223-226). Expression levels of these tight junction proteins have been shown to correlate inversely with malignancy (223-226). Furthermore, VEGF-A-induced phosphorylation of occludin and ZO-1 has been shown to downregulate these tight junction proteins leading to an increase in endothelial permeability (227).

While the above results implicate abnormalities in tight inter-endothelial junctions, related studies point to the importance of caveolae and intra-endothelial vesicular transport in astrocytoma edema. VEGF-A has recently been shown to downregulate caveolin-1, a principle protein of caveolae and vesicular organelles (228). Caveolin-1 expression in endothelial cells isolated from glioblastoma tumors was decreased 75% compared to normal brain, with a 2-fold increase in phosphorylated caveolin-1 (228). In endothelial cell culture, VEGF-A has been shown to induce caveolae formation leading to increased permeability (229). Furthermore, VEGFR-2 has been found to localize to caveolae and to associate with caveolin-1 (230). Together these data identify caveolin-1 and VEGFR-2 as possible molecular targets for the treatment of vascular hyperpermeability and astrocytoma edema (228, 230).

Hemorrhage is a characteristic source of considerable morbidity in glioblastoma tumors. Tumor

vessels are fragile and their endothelium exhibits altered expression of pro-thrombotic and pro-coagulant proteins (44). Elaboration of high levels of the VEGF-A 165 isoform appears to be a causal factor in malignant tumor hemorrhage. In a mouse model, U87 astrocytoma cells expressing VEGF-A^{121} and VEGF-A^{165} induced rapid growth and breakdown of the vasculature leading to cerebral hemorrhage within 5 days (231). Cells expressing VEGF-A^{189} demonstrated a similar degree of vascularity, but the tumors did not undergo hemorrhagic conversion. Further studies demonstrated the importance of pericyte coverage in preventing tumor hemorrhage. Simultaneous overexpression of PDGF-B and VEGF-A^{165} in U87 astrocytoma tumors led to a 3.4 fold increase in pericyte coverage of the tumor vessels and completely prevented intracranial hemorrhage (50). These studies elegantly demonstrated that PDGF-B acts to recruit pericytes to the developing tumor neovasculature, facilitating their maturation and stabilization against hemorrhage (50).

8. CONCLUSIONS AND PERSPECTIVES

New research has advanced our understanding of specific genes that control angiogenic factors and promote the angiogenic switch in astrocytoma tumors. Mechanisms by which the more established angiogenic factors, vascular endothelial growth factor (VEGF-A), basic fibroblast growth factor (FGF), and platelet-derived growth factor (PDGF), regulate astrocytoma angiogenesis has been the focus of many investigations. New studies are also beginning to discover important roles for the angiopoietins, Tie and neuropilin receptors, as well as for a less well known but potent angiogenic factor, hepatocyte growth factor/scatter factor (HGF). Despite this intensive effort, an absolute link between angiogenesis and tumor growth and malignant progression remains to be rigorously proven and validated in clinical trial. In addition, preliminary findings that anti-angiogenic therapy may promote increased tumor invasion need to be carefully evaluated. Future studies focused on the molecular mechanisms underlying the structural and functional abnormalities of angiogenic vessels will enable research efforts to alleviate tumor edema and hemorrhage and to selectively target the tumor neovasculature in treatment strategies.

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Send correspondence to: Shirley I. Stiver M. D., Ph. D., Beth Israel Deaconess Medical Center, Departments of Surgery and Pathology, Research North, Room 289, 330 Brookline Avenue, Boston, MA 02215, Tel: 617-667-3637, Fax: 617-667-3591, E-mail: sstiver@bidmc.harvard.edu