The Role of Angiopoietins During Angiogenesis in Gliomas

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The formation of new blood vessels plays an important role in human disease development and progression. For instance, it is well established that the growth of most cancers critically depends on the supply of nutrition and oxygen by newly recruited blood vessels. Similarly, malignant gliomas, the most common primary brain tumors occurring in humans are highly dependent on angiogenesis. In recent years, there has been tremendous effort to uncover the molecular mechanisms that drive blood vessel growth in adult tissues, especially during cancer progression. Vascular endothelial growth factor (VEGF) and other morphogens, such as angiopoietins and ephrins have been shown to be critically involved in the formation of new blood vessels during both developmental and pathological angiogenesis as evidenced by genetic studies in mice. In this review, we focus on angiopoietins, a family of growth factor ligands binding to Tie tyrosine kinase receptors with emphasis on their functional consequences during the growth and progression of experimental tumors and malignant human gliomas.

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INTRODUCTION

Malignant gliomas are neuroectodermal tumors that commonly arise in the white matter of cerebral hemispheres. In humans, gliomas contribute to 30% to 45% of all intracranial tumors (25a). Characteristic features that accompany disease progression are rapid growth and invasiveness. In addition, gliomas are angiogenesis-dependent, and thus, highly vascularized. They express growth factors and receptors that are typically induced during angiogenesis, most importantly VEGF and angiopoietins (11, 31, 41, 65). During the progression of low-grade to high-grade gliomas, increased vascularization is characteristic. Despite high microvessel densities, massive areas of necrosis are common. As a consequence of elevated oxygen demands, VEGF is highly upregulated in the tumor periphery of gliomas to counterbalance tissue hypoxia. Consequently, the formation of new blood vessels is initiated. In addition, angiopoietin-2 (Ang-2) has been shown to support the actions of VEGF during vessel sprouting as indicated by the overlapping expression patterns in a number of tumor models (22, 53). Thus, the tightly regulated interplay of both growth factors seems to be critical for the progression of experimental tumors but is also evidenced in human intracranial tumors as will be discussed in this review (56, 61, 63).

IMPORTANCE OF THE ANGIOPOIETIN/TIE SYSTEM DURING DEVELOPMENTAL ANGIOGENESIS

Angiogenesis, the formation of new blood vessels from the pre-existing vasculature, requires the coordinated interaction between endothelial and mesenchymal cells, and involves the complex signaling between multiple angiogenic growth factors. Most importantly, VEGF possesses a pivotal role during the formation of the primary capillary plexus (vasculogenesis) as indicated by gene dosage and the early lethality of VEGF-deficient mice (3, 9), (for review, see 4, 42). The extension of the primitive vascular network involves the proliferation and migration of endothelial cells. During the development of the immature vasculature, angiopoietin-1 (Ang-1) and angiopoietin-2 (Ang-2) are critically involved in angiogenesis, and influence processes such as remodeling, maturation and maintenance of the vascular plexus (Figure 1; 32, 55). Both proteins are ligands for the receptor tyrosine kinase with immunoglobulin and epidermal growth factor homology domains 2 (Tie2; 5, 6, 32, 44) with preferential expression in endothelial cells. There are 2 highly related members of the Tie receptor tyrosine kinase family, Tie1 and Tie2. They display unique extracellular domains (epidermal growth factor repeats, immunoglobulin-like domains, fibronectin-type III repeats) (Figure 1), and a separated tyrosine kinase domain in the cytoplasmic region (7, 40, 43, 46). Ang-1 and Ang-2 are the predominant ligands of Tie2 and are able to interfere with the receptor activity. In concert with VEGF, they control physiological and pathological angiogenesis (22). In addition, Ang-1 has recently been shown to be an activating ligand for Tie1 on lymphatic endothelial cells (35, 57). Engagement of Tie2 by Ang-1 ligand is responsible for receptor phosphorylation and the induction of survival signals in endothelial cells (25, 39). Ang-1 also provided evidence for an active role in vessel sprouting, as Ang-1 overexpression in transgenic mice increased vessel density and branching (55). Ang-1-mediated endothelial cell sprouting and migration has also been demonstrated using in vitro models (2, 19, 26). Consistent with these findings, interactions between endothelial cells and pericytes/smooth muscle cells are stabilized only in the presence of Ang-1 and decreased association of endothelial cells with support cells is evident in Ang-1 mutant mice (54). In the adult vasculature Ang-1 binding to Tie2 is constitutive and essential to maintain endothelium in the quiescent state (62). By contrast, opposing functions have been described for Ang-2. Binding of Tie2 by Ang-2 antagonizes receptor phosphorylation thereby disrupting contacts between endothelial cells and perivascular support cells (32). This process is fundamental for the initiation of vessel sprouting or regression.

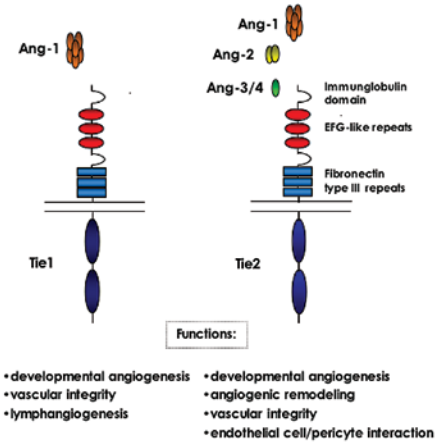

Figure 1. *Tie Receptor tyrosine kinases participating in angiogenic remodeling.* Angiopoietin ligand binding to the closely related tyrosine kinases with Ig and EGF homology domains Tie1 and Tie2 are displayed. While Ang-1 is an activating ligand of Tie2, Ang-2 antagonizes the actions of Ang-1. In the adult vasculature, receptor phosphorylation is induced by Ang-1 in endothelial cells. Ang-1 can also exert activating functions on the previous orphan receptor Tie1 in lymphatic endothelial cells. Angiopoietin-mediated functions are indicated. Actions of Ang-3 and -4 are less well understood.

The importance of the angiopoietin/Tie2 system has been demonstrated in genetic experiments as evidenced by the early lethality of Ang-1 or Tie2 mutant mice (6, 44, 54). Embryos lacking Tie2 receptor tyrosine kinase or Ang-1 ligand display aberrant vascular development and die around embryonic day E11 as a consequence of insufficient remodeling of the primary capillary plexus. Analysis of the vasculature of mice deficient for Tie2 or Ang-1 has indicated abnormal interactions between endothelial cells and peri-endothelial support cells (6, 44, 54). Contrary to these findings, mice with targeted expression of Ang-1 in the skin exhibit larger and more numerous branched vessels that are resistant to vascular leakage induced by permeability factors, such as VEGF (55). Based on these findings it has been proposed that the angiopoietin/Tie2 system plays an important role in the interaction between endothelial and mural cells. Angiogenic remodeling of the mature vasculature requires a progressive disengagement of endothelial cells from the surrounding support cells and this destabilization can result in vessels sprouting or vessel regression. The distinct expression pattern of Ang-2 at sites of active vascular remodeling (32) and in highly vascularized tumors (22, 53) has implicated Ang-2 in blockade of the Ang-1 stabilizing function to facilitate angiogenesis. In addition, transgenic overexpression in embryonic endothelial cells resulted in a similar phenotype as the deletion of the Tie2 gene, supporting the view that Ang-2 is an antagonistic ligand (32). However, genetic ablation of Ang-2 in mice resulted in a less severe phenotype which is compatible with life, as such, providing further evidence that Ang-2 is not redundant with Ang-1 (12). Furthermore, Ang-2 is selectively upregulated in tumor vessels before the onset of VEGF in adjacent tumor cells and can synergize with VEGF to enhance neovascularization. This indicated that Ang-2 might be an agonist in particular environments, such as in postnatal remodeling or pathological angiogenesis (12, 22).

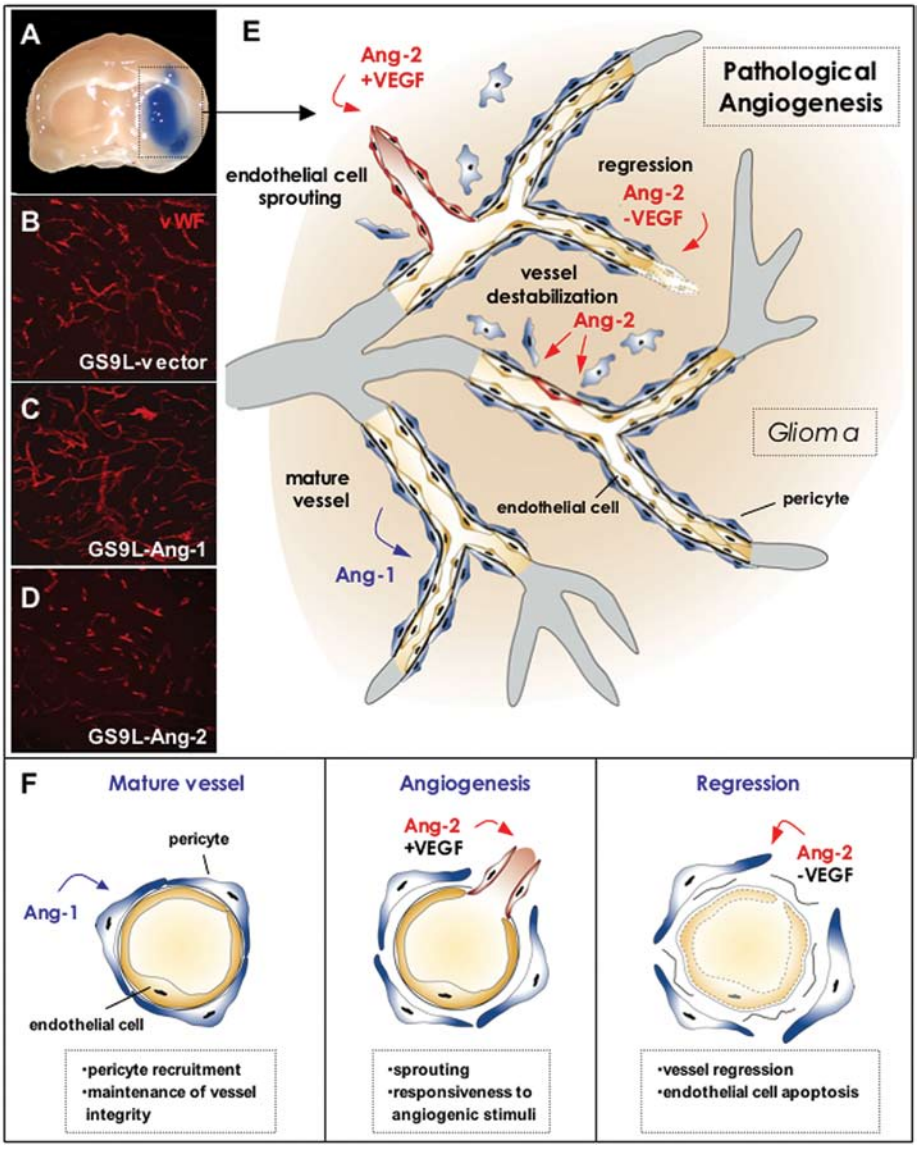

Figure 2. *The angiopoietin/Tie system regulates the formation of new blood vessels during pathological angiogenesis in tumors.* GS9L glioma cells induced to express Ang-1 or Ang-2 were implanted in syngeneic rat brains (**A**). Depending on the presence of Ang-1 or Ang-2 different effects were exerted on the tumor vasculature as determined by immunofluorescence with markers directed against von Willebrand factor (vWF) in endothelial cells (**B, C, D**). In GS9L glioma, overexpression of Ang-1 leads to enlarged (**C**), more functional vessels which often result in better glioma perfusion. Vessels derived from Ang-2 tumors are smaller, indicative for vessel sprouting (**D**). A schematic drawing of angiopoietin/Tie mediated functions in tumors is illustrated in (**E**). In concert with VEGF, which is highly induced in glioma, Ang-2 destabilizes the vasculature and leads to vessel sprouting or regression. Ang-1 contributes to the stabilization of pre-existing vessels (in the adult vasculature) and contributes to the maturation of new blood vessels in tumors, such as glioma. A higher magnification of (**E**) indicating functions of each angiopoietin on individual vessels is depicted in (**F**, modified after [15]).

ANGIOPOIETINS AND TUMOR-ASSOCIATED ANGIOGENESIS

The essential role of angiogenesis for the expansion of solid tumors is demonstrated by the observation that avascular tumors are not able to grow beyond a certain size unless they acquire new blood vessels for the supply of nutrients and oxygen (10, 16, 63). Cooption of existing vessels from the neighboring tissue thereby displays one possible mechanism to promote tumor growth (22). In addition, tumor cells provide endothelium-specific growth factors such as vascular endothelial growth factor (VEGF) and angiopoietins for the recruitment of new blood vessels.

During development, Tie2 expression is present on almost all endothelial cells (6, 44). In addition, Tie2 expression is increased during physiological and pathological angiogenesis in the adult. However, endothelial cells of the vasculature normally remain quiescent during adult life. Numerous studies have demonstrated altered expression patterns for angiopoietin ligands and their Tie receptors in a variety of tumors. This clearly indicated important roles for angiopoietin/Tie signaling beyond development in experimental models of tumor growth (56). Tumor vessels are known to have abnormal phenotypes that include changes in the architecture and assembly of the vessel wall (34, 61). These vessel abnormalities are likely the cause for increased vascular permeability within the tumor. In perspective of potential targeted interventions of angiogenesis in tumors it is required to decipher the mechanisms that promote or inhibit the vessel growth. Regarding the current knowledge of angiopoietin biology during pathological and tumor angiogenesis, results are controversial and include pro- and antiangiogenic functions for both, Ang-1 and Ang-2. In more detail, overexpression of Ang-1 in experimental tumors induces maturation by the recruitment of pericytes and smooth muscle cells to recently formed vessels (for review, see 56). Consequently reduced tumor growth or tumor stasis has been reported by a number of research laboratories in experimental tumors, such as colon, lung, mammary and squamous cell carcinoma (1, 18, 20, 50, 51, 59, 64). However, findings derived from some tumor types, including our own results, indicate proangiogenic functions when overexpressing Ang-1 (30, 48). These

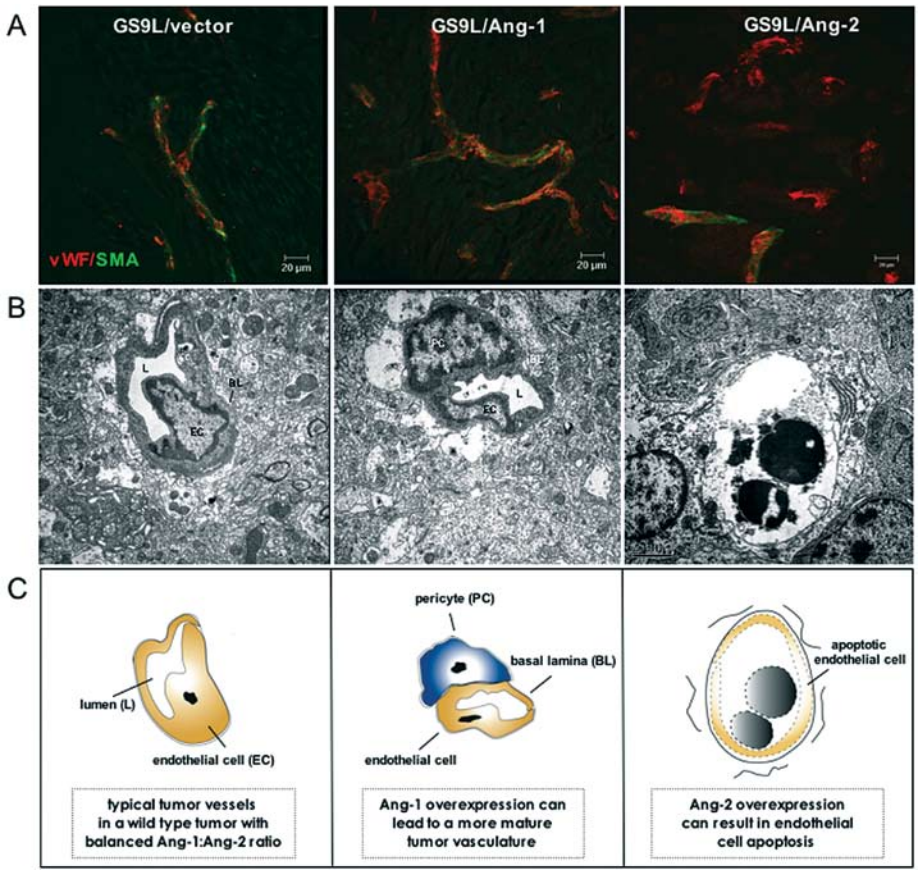

Figure 3. *Angiopoietin/Tie-induced phenotypes in the vasculature of gliomas.* Pericyte coverage (as indicated by smooth muscle actin [α-SMA] labeling) in glioma transfectants differs significantly depending on the availability of Ang-1 or Ang-2 (**A**). The presence of Ang-1 is reflected by increased pericyte covering (**A**). Vessels in Ang-2 gliomas are less mature and largely defective for α-SMA positive staining (**A**). At the ultrastructural level differences in the maturity/stability of tumor vessels dependent on the presence of Ang-1 or Ang-2 are evident (**B**). High order architecture in Ang-1 GS9L gliomas as compared to defective endothelial cell lining in Ang-2 GS9L tumors are clearly demonstrated (**B**). A model representing the regulation of morphological changes in the glioma vasculature during disease progression is deduced from our histological and ultrastructural analyses. Results are reflective of the present knowledge in angiopoietin/Tie biology in gliomas (**C**). Scale bars: 20 µm (**A**), 2 µm (**B**).

controversial findings may be related to differences in the presence of growth factors within the tumor types investigated. Although effector functions of Ang-1 on the net outcome of tumor growth are not completely resolved, the overall picture that can be drawn from the current literature involves an improved vessel architecture in the presence of Ang-1 (Figure 2). This is mainly exerted by a higher degree of pericyte coverage (Figure 3). Ang-2 in contrast, is necessary to initiate vessel sprouting and is associated with pericyte loss of the host tumor vasculature (1, 8, 23, 30, 58, 64, 69). This is achieved through the destabilizing actions on the previously quiescent vasculature (Figure 2, 3). At present, findings that have been reported for Ang-2 actions during tumor progression are not well recognized and controversial. However, what can be concluded from the current literature with regard to Ang-2 functions in tumors is a shift in the balance of Ang-1 and Ang-2 in favor of Ang-2. Consequently, instability of the host vasculature and aberrant, non-functional vessels were often observed (56, 68). In addition, angiogenic tumors with higher vessel densities and low grades of vessel maturation were prominent (Figure 3). Furthermore, Lewis lung carcinoma, mammary carcinoma, gastric and brain tumors overexpressing Ang-2 display increased frequencies of metastatic dissemination and are highly invasive (8, 23, 64). In summary, recent evidence from the literature implies that vessel destabilizing defects of Ang-2 might be caused by the disengagement of pericytes from the tumor vessels, and the defective cellular linings caused by openings between endothelial cells might be the reason for an increased permeability of tumor vessels (17).

Ang-2 is strongly regulated at the transcriptional level (21) and highly induced in endothelial cells in areas of active angiogenesis (22, 53) such as in tumors, making it an attractive target for therapeutic intervention. Moreover, Ang-2 has been associated with poor prognosis and lymph node metastasis in human tumors, pointing towards a need for therapeutic intervention (8, 23, 36, 47). Pharmacological inhibition of angiopoietin functions by sequestration with soluble Tie2 (28, 29, 49) or by the usage of dominant-negative Tie2 mutants has previously been shown to have a negative impact on tumor growth and progression. More recently, systemic administration of Ang-2-selective inhibitors (Fc-fusion proteins and antibodies) enabled the pharmacological inactivation of endogenous Ang-2 (38), and consequently suppressed tumor growth. Whether targeted intervention of Ang-2 will be applicable in human tumors as well remains to be elucidated in the future.

In spite of the intense research on angiopoietin functions during physiological angiogenesis (32, 55) and tumor angiogenesis (22, 53) the biological actions of angiopoietins during tumor progression have not been fully ascertained. Clearly, molecular mechanisms for a more precise understanding of angiopoietin/Tie-mediated effector functions that may lead to increased vessel integrity or drive vascular remodeling/regression are largely missing. In more detail, it is well established that tumor vessels display highly permeable vessels, but only few studies focused on the cellular basis of tumor vessel permeability (17, 33, 34). For instance, it is largely unknown how Ang-1 prevents vessel leakiness although Ang-1 seems to interfere with cell-cell interactions and junctional proteins (13, 45). We recently investigated the cellular consequences of angiopoietin expression on tumor vessel morphology in 2 mouse mammary tumors which naturally display distinct Ang/Tie2 expression profiles (our own observations). Furthermore, we generated mammary tumor cell lines that express Ang-1 and Ang-2. Analyses of angiopoietin-overexpressing mammary xenografts in comparison to 2 different control mammary tumors, which display either high or low Ang-2 levels, at the ultrastructural level strongly supported the hypothesis that Ang-1/Tie2 signaling is essential for vessel stabilization and endothelial cell/pericyte interaction and suggested that Ang-2 is mainly responsible for the immature and disrupted vasculature. Furthermore, our findings further supported the hypothesis that Ang-2 can trigger important signals that are decisive for a switch of vascular phenotypes within tumors. Beyond this, current results also imply that disruption of cell-cell contacts between endothelial cells might be inversely regulated by Ang-1 and Ang-2. For instance, it has recently been shown that VEGF-mediated disruption of cell-cell interactions is attributed to the dissociation of β-catenin from VE-cadherin (60). Interestingly, this effect can be opposed by Ang-1. Future studies will help to unravel participating cellular elements in tumors more precisely.

ANGIOPOIETINS DURING GLIOMA PROGRESSION

During progression from a low-grade glioma to a high-grade malignant glioblastoma there is a remarkable change within the vascular phenotype. Malignant glioma vessels are characterized by high endothelial cell proliferation and chaotic association with perivascular cells (69). As vascular changes progress, vessels acquire an abnormal deposition of extracellular matrix (hyperplasia and vascular sclerosis). The expression pattern of angiopoietins correlates with the malignancy grade and changes in the vascular phenotype. The expression of Ang-1 and Ang-2 in human gliomas has been studied by in situ hybridization (53, 67). Ang-1 mRNA but not Ang-2 mRNA was detected in glioma cells. While Ang-2 is not expressed by normal human brain vessels, it is dramatically induced in neovascular endothelial cells of malignant gliomas. Hyperplastic but not sclerosed vessels displayed high levels of Ang-2, although even normal appearing glioblastoma vessels expressed high levels of Ang-2 (22). The fact that Ang-2 is expressed in the tumor vasculature but not in the normal brain vasculature identifies Ang-2 as an early tumor angiogenesis marker (22, 53, 67). Moreover, Ang-2 protein is not only restricted to vascular cells but can be present in glioma cells (27). Particularly high Ang-2 expression is displayed in the tumor periphery in sprouting vessels. Ang-2 expression is also increased adjacent to necrotic areas, suggesting that hypoxia might stimulate Ang-2 expression. Alternatively, Ang-2 expression around necrosis may be due to the induction by VEGF, rather than a directly mediated induction of Ang-2 by hypoxia (37). Thus, Ang-2 expression was prominent in areas of vessel regression as well as in regions with robust angiogeneic activity (27, 68).

A recent study suggested that Ang-2 induces glioma cell invasion (23). Intracranial xenografts of glioma cells engineered to express Ang-2 were highly invasive into adjacent normal brain. Overexpression of Ang-2 increased the levels of metalloproteinases (MMP) (23). Since glioma cells did not express detectable levels of Tie-2, the mechanisms by which Ang-2 overexpression induces invasivess remains to be established. Ang-2 might modulate integrins and other molecules involved in cell-cell adhesion, cell migration and in the activation of MMP-2 (23). Alternatively, elevated levels of vessel regression induced by the overexpression of Ang-2 might lead to increased vessel cooption, rendering the tumor cells to a more invasive phenotype. In analyses of human glioma biopsies, Guo et al (14) showed that tumors preferentially expressing Ang-2, MMP-2, membrane type 1-MMP (MT1-MMP) and laminin 5 γ 2 in the invasive edge, are associated with a more invasive phenotype. Increase levels of these proteins correlated with the invasiveness of malignant human gliomas.

The modulation of Tie2 ligands in concert with other growth factors, such as VEGF, has been studied in glioblastoma multiforme (66). Zadeh and collegues (66) demonstrated that Ang-1 regulates angiogenesis in intracranial and subcutaneous glioblastoma multiforme xenografts in a VEGF-dependent manner. Ang-1 overexpression increased the microvascular density and generated more stable vessels only with concurrent VEGF elevation. Low levels of VEGF in these tumors resulted in loss of the proangiogenic activity of Ang-1. These results are in line with our own findings (30). In a syngeneic rat glioma model we demonstrated that in the presence of Ang-1, tumors induce a more functional, "normalized" vascular network, which led to better tumor perfusion and growth (24, 30).

CONCLUSION

Angiopoietins are essential to vessel development. In addition, it is now well established that they are important dur-

ing pathological angiogenesis. In glioma, angiopoietins are associated with tumor growth and disease progression. Currently, the exact role of the angiopoietins during tumor angiogenesis is still not entirely understood and actions of Ang-1 and Ang-2 are controversial. However, what is emerging from experimental tumors, as well as vascular development, is the predominant role for Ang-1 in stabilization and maturation of the tumor vasculature. Ang-1 is not significantly upregulated in the majority of tumors. In contrast, Ang-2 is highly induced in the tumor vasculature, even prior to the induction of VEGF. As such, a shift in the Ang-1:Ang-2 balance in favor of Ang-2 occurs. Therefore, it is evident that Ang-2 is the determining factor that triggers signals to initiate vessel sprouting. Consequently, what can be concluded from the current literature is that responses of endothelial cells to angiopoietins appear to be precisely regulated. Interestingly, no special attention has been attributed to the cellular consequences on vessel architecture and morphology so far. For instance, molecular mechanisms that lead to increased (in case of Ang-1) or reduced (with regard to Ang-2) endothelial cell integrity are not identified. Accordingly, the involvement of cell junction proteins has not been extensively studied. Further exploration of such components is particularly important in understanding glioma-associated leakage and subsequent peritumoral vasogenic edema, which are common in glioma due to defective vessel structures. Context-specific inducible expression of either angiopoietin—in tumor cells or for instance in endothelial cells in transgenic mice—would be advantageous to precisely identify angiopoietin functions on the vessel architecture at the molecular level. It also has to be taken into account that recent findings in gliomas and other tumors as well support the concept that vascular remodeling and angiogenic sprouting are at least in part the result of the differential display of growth factor and receptor systems. The heterogeneity of angiogenic growth factors—angiopoietins in concert with other factors such as VEGF—in tumors is a major challenge for cancer treatment. Further studies are needed to discern how angiopoietins cooperate with other molecules. Malignant gliomas are potential targets for antiangiogenesis therapies because of their poor prognosis despite of all available aggressive multimodality therapies. Experimental and clinical data in gliomas suggest that increased expression of angiopoietins is related to tumor progression. Therefore angiopoietin/Tie-2 pathway might be an attractive target for antiangiogesis therapy in malignant gliomas. Recently, Tie-2 activation has been implicated in glioma angiogenesis and growth. By using kinase-deficient Tie-2 current reports demonstrated that Tie-2 inhibition in gliomas disrupted tumor vascularity and decreased the growth of subcutaneous and intracranial tumors. These results provide evidence that targeting Tie-2 activation might be a reasonable therapeutic alternative together with other antiangiogenic strategies in order to inhibit angiogenesis-dependant glioma growth. In addition, results in preclinical pharmacology models using colon and squamous cell carcinoma indicated that systemic administration of Ang-2 inhibitors interferes with tumor growth. However, further studies and clinical trials are needed to demonstrate therapeutic efficacy in human disease.

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