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NEOANGIOGENESIS IN HUMAN ASTROCYTOMAS: EXPRESSION AND FUNCTIONAL ROLE OF ANGIOPOIETINS AND THEIR COGNATE RECEPTORS

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1. ABSTRACT

Since the introduction of the concept of an "angiogenic switch" driving tumor growth and malignant progression by Judah Folkman in 1971, there have been numerous scientific reports confirming the central concept that tumor growth is angiogenesis dependent. Various angiogenic genes and gene products, from both neoplastic and normal tissues, have been isolated, purified, and cloned that contribute to the 'angiogenic switch'. Of these various molecules, two have been identified that act specifically on endothelial cells. First is Vascular Endothelial Growth Factor (VEGF), the cognate receptors of which are almost specifically expressed on endothelial cells. VEGF plays a crucial role in the development of the embryonic vasculature by providing differentiation and mitogenic signals to endothelial cells and their mesodermal precursors. Second are the Angiopoietins and their cognate receptor, Tie2. Angiopoietins are primarily involved in maturation of both embryonal and adult vasculature, with Angiopoietin 1 & 2 being naturally occurring agonists and antagonists of Tie2 respectively, indicating a very precise level of regulation in-vivo. In this review we summarize what is known of the biological role of Angiopoietins and Tie2, their interaction with VEGF in normal and tumor related angiogenesis, with emphasis on their functional consequence in the progression and growth of malignant human astrocytomas.

2. INTRODUCTION

2.1. Tumor angiogenesis

Since the introduction of the concept of an 'angiogenic switch' driving tumor growth and metastasis by Judah Folkman (1), multiple therapies in various tumor types have targeted angiogenic pathways to inhibit solid tumor growth and metastasis. The 'angiogenic switch' is the result of complex interactions between multiple pro- and anti-angiogenic factors, secreted by both host and tumor cells, which governs the ultimate status of tumor angiogenesis and thereby tumor growth (1, 2). Although it is thought that tumor angiogenesis recapitulates embryological angiogenesis in many ways, it is not as well organized or matured since tumor vessels are structurally and functionally abnormal. The relative contribution and interplay amongst the angiogenic regulators of tumor angiogenesis is not precisely known and requires further characterization. Various pro- and anti-angiogenic factors have been identified as important modulators of tumor angiogenesis, which include pleotropic factors such as PDGF, TGFβ, bFGF and integrins plus endogenous anti-angiogenic factors such as angiostatins and endostatin (3). To date, three groups of angiogenic factors have been identified that act more specifically on the endothelial cells (EC) as their receptor tyrosine kinases (RTKs) are found almost exclusively on EC (3). The first identified and most extensively studied group is Vascular Endothelial Growth Factor (VEGF) and it's EC-specific RTKs; VEGFR1 and VEGFR2. The second group are the Angiopoietins together with their cognate RTK Tie2/Tek, which is the focus of this review. The third and more recently identified group is the Ephrins with their Ephrin RTKs (4).

2.2. Astrocytoma angiogenesis

Astrocytomas are the most common primary adult brain tumors accounting for 4-5% of all cancer related deaths annually in North America (5). The World Health Organization (WHO) grades astrocytomas histopathologically

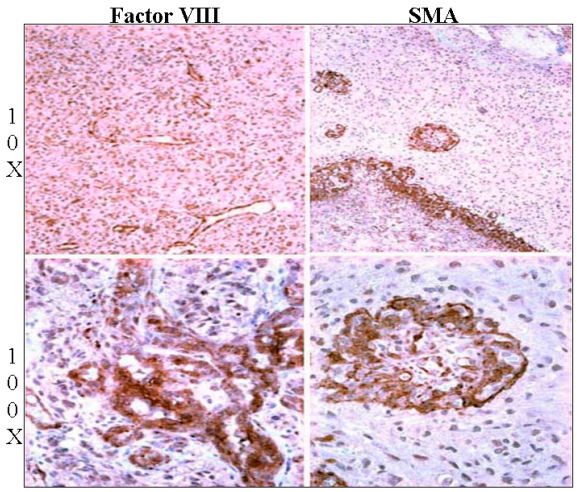

Figure 1. GBM vascular structure. GBM tumors are characteristically heterogeneous tumors, with endothelial proliferation as a pathological hallmark of these tumors. A) Factor VIII stain for detection of endothelial cells (EC) demonstrates hyperproliferation of EC and B) Smooth Muscle Actin (SMA) stain detecting the smooth muscle cells and pericytes surrounding the vessels, demonstrating abnormal proliferation in GBMs. [upper panel 10X, lower panel 100X magnification].

into four grades of increasing malignancy. Collectively Grade I and II astrocytomas can be considered as low grade astrocytomas, while Grade III (anaplastic astrocytomas or AA) and Grade IV (glioblastoma-multiforme or GBM) astrocytomas are highly malignant with increased cellularity, marked nuclear pleomorphism, increased mitotic activity and increased tumor vascularity (6). Unfortunately, GBMs are the most common primary adult brain tumors, with only a 9-12 month median survival from the time of diagnosis (7, 8).

The main pathological hallmark of progression from a lower grade astrocytoma to GBM is florid, abnormal and heterogeneous tumor angiogenesis (9-12). Cerebral angiograms of GBMs illustrate an extensive increase in cerebral blood flow and marked upregulation of tumor vascularity (13). Within GBMs the blood flow is heterogeneous, with areas of increased flow and decreased flow in the central and presumably hypoxic-necrotic regions respectively (13, 14). GBM associated vessels are structurally and functionally abnormal. Pathological correlation demonstrates tortuous vessels that have increased EC proliferation compared to normal brain, with increased vessel wall thickness due to multiple layering of smooth muscle cells (SMC) and pericytes (PC) in the central and more hypoxic regions (6, 12-14) (Figure 1). There is a high degree of arterio-venous shunting present, which leads to intra-tumoral hemorrhage and an absent blood-brain-barrier (BBB) due to lack of tight junctions (5, 12-14). These structural and functional aberrations are postulated to be due to an imbalance of angiogenic regulators secreted by the GBM cells and/or cellular elements of the vasculature. Therefore, GBMs provide an ideal solid tumor model for studying and increasing our understanding of the molecular mechanisms of tumor angiogenesis, in hopes of improving our anti-angiogenic treatments for the highly malignant GBMs and perhaps other human cancers.

The principal theories that have culminated from studying the role of various angiogenic factors in GBMs can be summarized as follows: GBMs overexpress VEGF and PDGF-B. Tumor associated EC also express PDGF-B and VEGF-R1 and VEGF-R2, while SMC and PC express PDGF-Rβ. It is therefore thought that EC proliferation is VEGF driven and SMC/PC proliferation is PDGF-B driven (15-18). VEGF as a potent angiogenic factor is thought to be the primary signal for astrocytoma angiogenesis (19-21). There is increased expression of VEGF in the more malignant and angiogenic forms of astrocytomas, Anaplastic Astrocytoma (AA) and Glioblastoma Multiforme (GBM), coupled with over-expression of their cognate receptors in the tumor endothelium (20, 21). In GBMs, hypoxia is thought to be the main physiological stimulant of VEGF, with VEGF typically highly expressed in the pseudopalisading hypoxic zones around areas of necrosis (discussed in section 3.2) (21, 22). Blocking the VEGF receptor pathway inhibits the growth of a number of murine tumors and human tumor xenografts (23-26). Various strategies aimed at blocking VEGF or its receptors in astrocytomas and other nervous system tumors have shown promise at a pre-clinical level (23-26), however, the clinical trials have not proven an unequivocal benefit, suggesting additional relevant angiogenic modulators have to be targeted for more effective anti-angiogenic treatment of astrocytomas. The second most promising candidate is the Angiopoietin-Tie2/Tek pathway.

3. ANGIOPOIETINS AND TIE RECEPTORS

3.1. Tie2/Tek receptor

Tie2/TEK (tunica interna endothelial cell kinase) and Angiopoietins are EC specific angiogenic cytokines that are essential for the maturation of physiological vessels during both embryonal and adult angiogenesis (4). The role of this pathway however in tumor biology remains unknown and their biological consequence in astrocytomas has been the focus of our investigation and is summarized in this review. Tie1 & Tie2/Tek are a family of EC receptor tyrosine kinases, which are structurally divergent from VEGFRs (27-30), (Figure 2). The extracellular domains of Tie receptors consist of three different structural motifs. Two Ig-like loops separated by three tandem epidermal growth factor-like cysteine repeats followed by three fibronectin type III-like motif (27-30). The intracellular domains are similar to VEGFRs with two tyrosine kinase domains split by a short stretch of amino acids (27-31).

Tie2/Tek is critical for normal embryonic vessel development and is expressed at E:7.5 (Table 1) (29, 31). Knockout of Tie2/Tek or ablation with a dominant negative mutant causes embryonic lethality (E:9.5-10.5) due to a plethora of defects in the vasculature (29, 32, 33). The vessels are characterized by a reduction in EC number and lack of recruitment of periendothelial cells (29, 32, 33).

Table 1. Summary of targeted transgene and knockout mice for Ang1, Ang2 and Tie2

Gene	Expression	Lethality	Phenotype
Knockout			
• **Tie2/Tek**	E7.5	E9.5-12	intravascular hemorrhage, loss of heart trabeculations, enlarged perivascular cavity, loss of branching and recruitment of perivascular cells (29, 31)
• **Ang1**	E9	E12.5	immature endocardium, Loss of branching, maturation of vessels, rounding of EC and separation from the underlying matrix (43)
• **Ang2**	P0	P14	peri/post natal lethality with variable picture
Transgenic			
• **Tie2/Tek**			Hyperproliferation of dermal vessels, altered dermal angiogenesis (34)
• **Ang1**			Larger, more numerous branched vessels of the skin, resistant to plasma leakage (53,60)
• **Ang2**			Poor development of endocardium and myocardium, loss of vessel branching, similar phenotype to Ang1 and Tie2/Tek knockout (41)

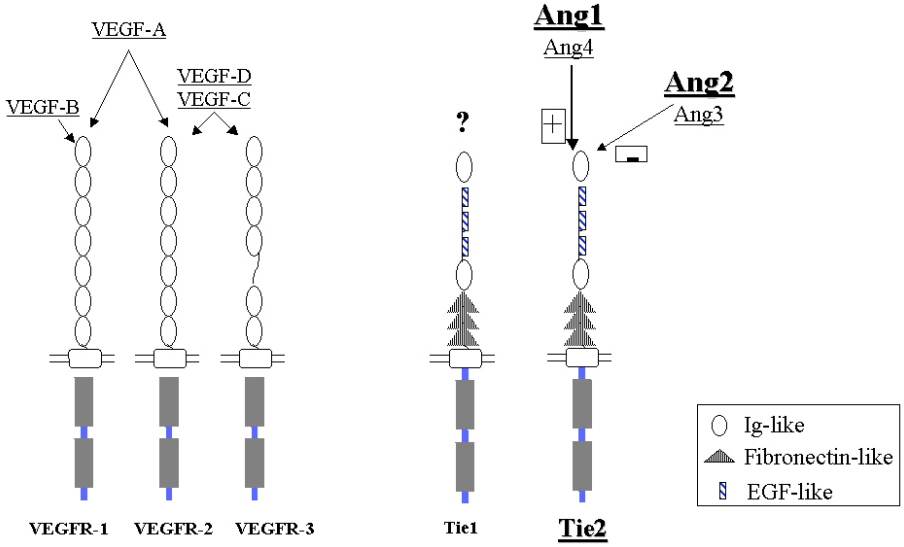

Figure 2. Diagram of endothelial-cell-specific receptor tyrosine kinases Tie and VEGFR. Schematic representation of the VEGF and Tie receptors that are primarily expressed on the endothelial cells. VEGFR contain seven Ig-like domains in their extracellular portion, which is divergent from the structure of Tie1 and Tie2/Tek receptors (two Ig-like loops separated by three tandem epidermal growth factor-like cysteine repeats followed by three fibronectin type III-like motif). The intracellular domains of Tie2 are similar to VEGFRs with two tyrosine kinase domains split by a short stretch of amino acids. Tie2 has the identified ligands of Ang1-4, while Tie1 remains an orphan receptor.

There is a decrease in sprouting and remodeling of the primitive vascular network leading to restricted growth of head and heart organs, together with a loss of heart trabeculations (29, 32, 33). A recent binary transgenic approach, based on the tetracycline repressor, to allow temporal expression of Tie2/Tek during mouse development, demonstrates the importance of Tie2/Tek signaling for maintaining a stable embryonal vasculature (34). Additionally, basal low levels of Tie2/Tek expression and activation are present in the quiescent adult vasculature, including the normal brain providing a constitutive stabilizing force (35, 36). During normal physiological processes such as wound healing and pathological angiogenic states such as tumor vascularization, Tie2/Tek expression and activation are increased (35, 37-39), indicating a requisite role for this pathway in neo-angiogenesis.

3.2. Angiopoietins

Angiopoietins are the principal ligands of Tie2/Tek, while Tie1 remains an orphan receptor (4) (Figure 1). Four distinct members of the Angiopoietin family have been characterized to date (Ang1-4). Ang1 and Ang2 are ~75kD in size and highly secreted proteins, with 60% homology in their amino acid sequence (40, 41). The more recently described Ang3 and Ang4 may represent widely diverged counterparts of the same gene locus in mice and humans (4, 42). Angiopoietins act either as agonists to Tie2/Tek (Ang1/Ang4) or antagonists (Ang2/Ang3) (4, 42). Evidence for Ang1 being a biological ligand for Tie2/Tek was initially supported by the similarities in Ang1 and Tie2/Tek knockout mice (43), (Table 1). Similarly, evidence for Ang2 being a natural antagonist for Ang1 mediated activation of Tie2/Tek came from transgenic mice over-expressing Ang2 which had a similar lethal phenotype to Ang1 and Tie2/Tek knockout mice due to a disrupted vascular development (41). The presence of a natural antagonist suggests a precise level of Tie2/Tek receptor regulation in vivo. It should be noted this antagonist function of Ang2 is cell specific to EC, since it activates Tie2/Tek ectopically expressed in other cell types (41).

Ang1 is expressed by non-EC and acts in a paracrine fashion to activate Tie2/Tek, while Ang2 is expressed predominantly by ECs (40, 41). Ang1 is not a mitogen for EC in contrast to VEGF, and its role is principally chemoattraction of mesenchymal cells leading to vessel maturation (4). Ang1 plays a role in stabilizing the contacts between EC, PC and SMC of the ECM (31, 43). Ang2, however, by antagonizing Ang1 destabilizes vessels and is expressed primarily at sites of vascular remodeling (41, 44, 45). Experimental evidence suggests that Ang2 loosens capillary structures and sensitizes EC to angiogenic stimuli, such as VEGF. In the absence of EC mitogens such as VEGF, these active vessels regress and involute (44, 45).

3.3. Biological role of Angiopoietins in angiogenesis

The biological role of Angiopoietins and Tie2/Tek however is far more complex than the above postulated paradigm. Recent studies are revealing the various aspects of vessel formation in which Angiopoietins are involved with. Ang1 has an anti-apoptotic effect on EC by activating the PI3Kinase/Akt survival pathway through Tie2/Tek signaling (46-49). Other aspects of vessel formation that Ang1 has been implicated in include EC migration and cell adhesion. Ang1 has been found to facilitate interactions between fibronectin and integrin receptors (50). Also, Ang1 has the potential to directly interact with $\beta 1$ and $\alpha v \beta 5$ integrin receptors, however, at higher doses than is required for its modulation of Tie2/Tek (51). Regulation of EC tight-junctions has also been linked to Angiopoietins function, since Ang1 knock-out embryos have leaky vessels (Table 1) (43). A possible mechanism to explain this may involve Ang1 regulation of Platelet-EC-Adhesion-Molecule-1 (PECAM-1) and vascular endothelial cadherins, both of which are required to maintain EC tight-junctions and vessel integrity (52). Thurston et. al. have shown that the profound and well established permeability effect induced by VEGF (20) can be blocked by Ang1 (53). Similarly, Ang-1 renders vessels resistant to plasma leakage when the leakage is not due to excess VEGF but instead secondary to an inflammatory agent (53). These findings were seen without an angiogenic response from Ang1, indicating a dual role for this protein (53). Likewise, the biological function of Ang2 also remains ambiguous. Despite the original belief that Ang2 acts as an antagonist to Tie2/Tek is has been shown that at high non-physiological doses Ang2, similar to Ang1, activates the PI3Kinase/Akt pathway by phosphorylation of Tie2/Tek (54). Ang2 can also induce tubule formation of endothelial cells in-vitro, which is in contrast to its role as an Ang1 antagonist (55). Additionally, Ang2 deletion in knockout mice, show a phenotype that implicates Ang2 in regulation of lymphangiogenesis (56) and demonstrates phenotypes that are suggestive of a role for this ligand distinct from the Tie2/Tek receptor (56). The bioloy of Angiopoietins, in particular Ang2, is heterogeneous and remains an area requiring further investigation.

Additional factors that can add to the complexity of the Angiopoietin-Tie2 pathway and suggest that further fine-tuning of this pathway can exist in-vivo, are the identified isoforms of Angiopoietins. Four isoforms of Ang1 have been identified by one group (1.3kb,1.5kb,0.9kb and 0.7kb), with two of the isoforms shown to potentially act as dominant negatives to the full-length Ang1 (57). The expression pattern for Angiopoietins is variable in few tumor cell lines examined (discussed in section 3.1) and suggest an as of yet undetermined role for these different isoforms in pathophysiological scenarios (57). Similarly, an alternative splicing of Ang2 gene results in an Ang2 isoform whose expression pattern and biological function is not yet precisely known (58).

Another principle area of interest is the interaction of Angiopoietins with other angiogenic factors. Few studies have focused on understanding how Angiopoietins co-ordinate and orchestrate other relevant molecules. A recent publication focuses on the collaborative functions that exist between Ang1, Ang2 and VEGF (59). Transgenic mice over-expressing Ang1 in cardiac cells demonstrate no increase in angiogenesis, which is in contradiction to the findings of Ang1 over-expression in the skin (60). Double transgenic mice over-expressing VEGF and Ang1 showed a restricted angiogenesis and inhibition of the potent angiogenic response seen to VEGF over-expression alone (59). Both findings reiterate a very organ specific response to Ang1 and how depending on the tissue type in which Ang1 is expressed it can have a positive or negative regulatory role in angiogenesis. This rationale will be revisited in sections 2.4 and 3.3, where the role of Angiopoietins in tumor angiogenesis is discussed and described as being context dependent. Ang2 on the other hand enhanced the mitogenic signal of VEGF by approximately 50% in the heart of double transgenic mice over-expressing VEGF and Ang2 (59). This finding is consistent with the paradigm that Ang2 works in orchestration with VEGF to promote neo-angiogenesis (44).

Thus far, our understanding of the Tie2/Tek-Angiopoietin pathway indicates that these cytokines have a multifaceted role in promoting and maintaining normal vessels. The regulatory effects of Angiopoietins and Tie2/Tek can vary according to the organ and context in which they are expressed. Therefore, we postulate that this pathway potentially contributes in a similar context dependant manner to the formation of the abnormal vasculature seen in GBMs.

3.4. Functional consequence of Angiopoietins and Tie2/Tek in tumor biology

Several studies have been published in the last year that examined the functional role of Angiopoietins and Tie2/Tek activation, in different tumor systems (61-66), however, none include astrocytomas. Ang1 has been shown to play a growth-promoting role in breast cancer models, where it is postulated that Ang1 increases stability of vessels resulting in increased tumor growth (63). In contrast, other studies have reported that Ang1 up-regulation has a growth inhibitory role in colon, lung and mammary cancer cell tumor xenografts (61, 66). The inhibition is postulated to be secondary to enhanced vascular stability and prevention of neo-angiogenesis by Ang1. Ang2 up-regulation increases growth of colon cancer and orthotopic stomach cancer xenografts, together with

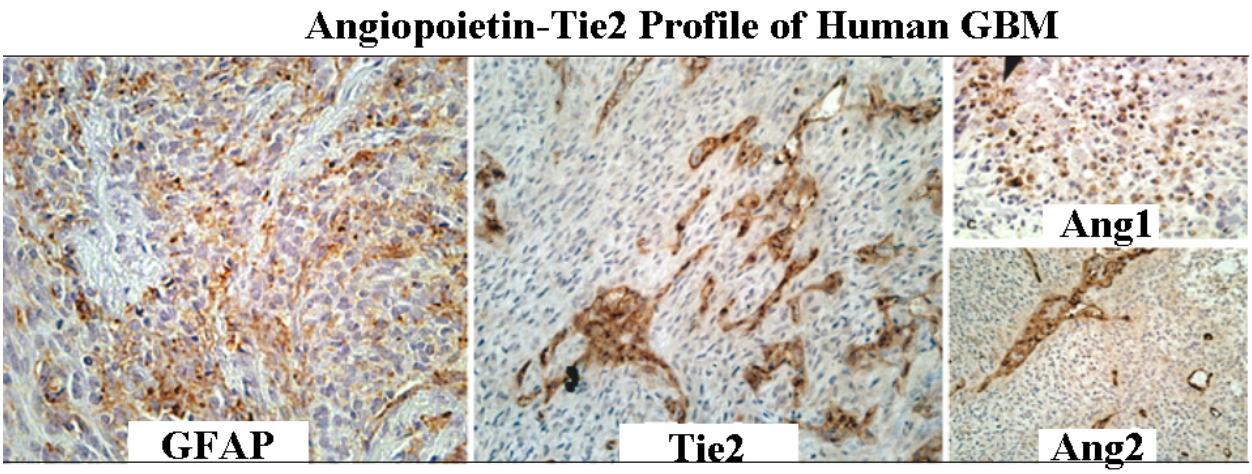

Figure 3. Expression profile of Angiopoietins and Tie2/Tek in human GBM specimen. Immunohistochemical analysis: A) GFAP stain, confirming the astrocytic origin of the GBM tumor used B) Tie2/Tek stain, demonstrates restricted expression by vessel endothelial cells which is markedly increased in GBM compared to low grade astrocytoma and normal brain, C) Ang1 stain, shows marked increase expression by astrocytoma cells and D) Ang2 expressed specifically by vessel endothelial cells has also an increased expression level in GBMs.

marked intra-tumoral hemorrhages (62). In contrast, Ang2 up-regulation in Lewis lung and mammary xenografts cause vessel regression and restriction of tumor growth (61). The role of Tie2/Tek in tumor angiogenesis and growth has been tested in mammary cancer cell xenografts (64, 65) and similarly show a variable response. Tie2/Tek inhibition using a dominant-negative receptor decreased tumor growth generated from one mammary cell line xenograft, without much effect in xenografts of other mammary cell lines (67). These variable roles of Angiopoietins and Tie2/Tek in these experimental tumor models emphasize the important regulatory influence of the tumor microenvironment on angiogenic factors that tumors elaborate and the tissue specific role of Angiopoietins and Tie2. In addition the temporal and spatial balance of angiogenic proteins are significant determining factors in pathological tumor angiogenesis. Hence, investigating the role of Tie2/Tek-Angiopoietins in astrocytomas is crucial to our understanding of the functional contribution of these cytokines to astrocytoma angiogenesis.

4. ROLE OF ANGIOPOIETINS IN ASTROCYTOMAS

4.1. Expression profile of Angiopoietins in astrocytomas

Expression profile of Angiopoietins across increasing grades of malignancy of astrocytomas has been studied by a few groups including our own (36, 68-72), (Figure 3). All studies have found an increase in expression of Ang1, Ang2 and Tie2/Tek with increasing malignancy grade of astrocytomas. These results are in keeping with our own findings that show Angiopoietin and Tie2/Tek expression increases with increasing grade of malignancy, with GBMs showing the highest level of Ang1 and Ang2 expression (36). Additionally, we have shown a robust increase in Tie2/Tek expression, restricted to the EC of GBMs compared to low-grade astrocytomas and normal brain. Associated with the robust Tie2/Tek expression is an increase in phosphorylation of the receptor (36). This data suggests that an increase in activation of the Tie2/Tek receptor contributes significantly to the florid angiogenic response mounted by GBMs and targeting this pathway may potentially inhibit the angiogenic growth of the tumors. We have found that human malignant astrocytoma cell lines (U87, U373, U343) show a variable amount of Ang1 mRNA and protein expression. U87 cells expressed the highest levels of Ang1 mRNA followed by U373, with U343 cells having no detectable levels as confirmed by Northern blot and PCR analysis (36). Levels of Ang1 mRNA and protein secretion paralleled those of VEGF, and the in-vivo growth capacity of these cell lines as tumor xenografts (36). Furthermore, we have found the Angiopoietin-Tie2/Tek expression profile to parallel that of VEGF quite closely, in operative tumor specimens (36). This indicates an involvement and interaction of these two EC specific pathways are occurring in astrocytomas to develop the characteristic hyper-proliferative vessels seen in GBMs.

The cellular source of Ang1 and Ang2 has been debated as suggested by recent publications (68, 72). Using techniques of immunohistochemistry (IHC) and in-situ-hybridization (ISH) most reports have revealed Ang1 to be expressed by both neuronal and astrocyte cells in normal brain and by tumor cells in GBMs. However, one recent report shows Ang1 to be expressed by both astrocytoma cells and tumor associated EC (68). The Ang1 expressed by astrocytoma cells is shown to be biologically active in-vitro, promoting EC spreading and interaction with the ECM (68). Consistently, all reports have found Ang1 in high concentrations in areas of high vascular density and present throughout all stages of GBM progression, which is in keeping with its physiological role of maintaining vascular stability.

Ang2 expression is reported by most authors to be absent or scant in normal brain and low-grade astrocytomas, however, expressed robustly by EC in GBMs. Using double labeling techniques (IHC together with ISH) Stratmann et al established Ang2 mRNA to be expressed unequivocally by EC as opposed to SMC/PC (69). Contradictory to findings by us and others, where no Ang2 expression is seen in astrocytoma cells both in-vitro and in-vivo, confirmed by RT-PCR, Northern, western and IHC analysis, a more recent paper reports Ang2 to be expressed by human astrocytoma cells (72). They have found astrocytoma cell lines (U105, U251, U373) to also express both Ang2 mRNA and protein (72). This latter finding by Koga et al suggests that astrocytoma cells up-regulate Ang2 in order to regulate angiogenesis and tumor progression directly (72). Differences in these results can be postulated to stem from transcriptional and translational regulation that can vary according to tumor stage and tumor cell subtypes studied, and underscoring the inherently heterogeneous nature of GBM tumors. Additionally, whether this discrepancy in findings can be explained by Angiopoietin isoforms has not thus far been investigated. The initial publications demonstrating the presence of four Ang1 and two Ang2 isoforms (57, 58), have not been followed up by additional functional studies on their biological role in physiological or pathological process and is an area that warrants further attention.

4.2. Hypoxic regulation of Angiopoietins

Although hypoxia is a major regulator of VEGF mRNA, Ang1 mRNA is not upregulated by hypoxia. The pattern of Ang1 expression is in contrast to that of VEGF, since Ang1 is not seen around necrotic areas, suggesting that in-vivo regulation of Ang1 is not hypoxia dependant. In fact Ang1 is known to be down-regulated by hypoxia (73) and in-vitro we have shown a decrease in Ang1 expression after 8hrs of hypoxia, while during this time we have observed maximal VEGF expression (36). On the other hand Ang2 signal increases around necrotic zones of GBM (72). However, since VEGF is known to induce Ang2 expression (74) whether glioma Ang2 is induced directly by hypoxia or by VEGF, is not known. A sequential and staged expression of Ang2, which in part results from tumor hypoxia, is implied. Astrocytomas are postulated to initiate their growth by co-opting existing host vessels, subsequent to which the host mounts a defensive response and chokes off this vascular supply (45). This in turn elicits a hypoxic response, which induces increased expression of VEGF and Ang2 that together are necessary for neo-angiogenesis. Therefore, Ang2 is thought to play an important role in the initiation of neo-angiogenesis (70, 71). However, elevated levels of Ang2 persist throughout GBM late stages, indicating a continuous role for Ang2. Whether Ang2 is upregulated by EC or astrocytes depending on the stage and zone of astrocytomas, and whether the overall level of VEGF and Ang1 influence Ang2 expression are questions that need to be addressed. We have therefore undertaken experiments to address the question of the functional role that Angiopoietins and Tie2/Tek play in astrocytoma angiogenesis.

4.3. Functional role of Angiopoietins and Tie2/Tek in astrocytomas angiogenesis

We have studied the functional role of Angiopoietins in astrocytomas by over-expressing Ang1 and Ang2 in human GBM cell lines and studying their growth pattern and vascularity as subcutaneous Nod-Scid xenografts (75). Ang1 over-expression in these astrocytoma models demonstrated a variable effect of Ang1 on tumor angiogenesis and growth, depending on the astrocytoma cell line studied. We propose that the balance of angiogenic factors present in the tumor microenvironment governs the regulatory role that Ang1 plays. In the context of U87:Ang1 xenografts; moderate VEGF levels that are secreted by U87 cells promotes EC proliferation and new vessel formation. In these tumors the over-expression of Ang1 leads to increased EC survival and vascular stability of newly formed tumor vessels, thereby enhancing tumor growth. However, in the context of U373:Ang1 xenografts, where low levels of VEGF is present, there is comparatively less proliferative signal to EC. In this situation over-expression of Ang1 translates to an increased stabilizing force on the existing tumor vasculature, preventing neo-angiogenesis and limiting tumor growth. Similar seemingly contradictory responses to Angiopoietin-Tie2 modulation in xenografts generated from different human tumor cell lines have been found and described in section 2.4. Our results along with the aforementioned studies emphasize a context dependant regulatory role of Ang1 in tumor angiogenesis and perhaps a variable role depending on tumor subtype. As discussed above in section 3.2, VEGF and Ang2 together are necessary for neo-angiogenesis, survival and progression of astrocytomas (70, 71). However, in our experimental models of astrocytomas, we have shown that Ang2, as an independent angiogenic factor does not alter the overall growth of astrocytomas if not accompanied by an equal elevation of Ang1 and VEGF (70).

We investigated the biological function of Tie2/Tek in GBMs by injections of purified ExTek protein locally into human GBM explants from operative samples grown as xenograft models that maintain a high expression profile for Angiopoietins and Tie2/Tek (Figure 4). ExTek contains the extracellular portion of receptor Tie2/Tek and is a soluble protein that is purified using a baculorvirus expression vector, (Figure 4) (64). ExTek acts as a dominant negative mutant and competes with the parental ligands for Tie2/Tek. We have illustrated that ExTek can in fact decrease Tie2/Tek phosphorylation both in-vitro and in-vivo (75). This led to a 30% reduction in GBM growth, a ten-fold lowering of the proliferation rate, decreased EC proliferation and microvascular density (MVD) over a ten-week growth course of the GBMs (Figure 4). These findings suggest that modulation of Tie2/Tek activation by Ang1 and Ang2 contributes to GBM vascularization and overall growth. In keeping with these results, we have found that ExTek treatment in intracranial models of GBMs increases the overall survival by approximately 35%. These results are promising for eventual translation to clinical use of Tie2 inhibitors as a treatment option for GBMs.

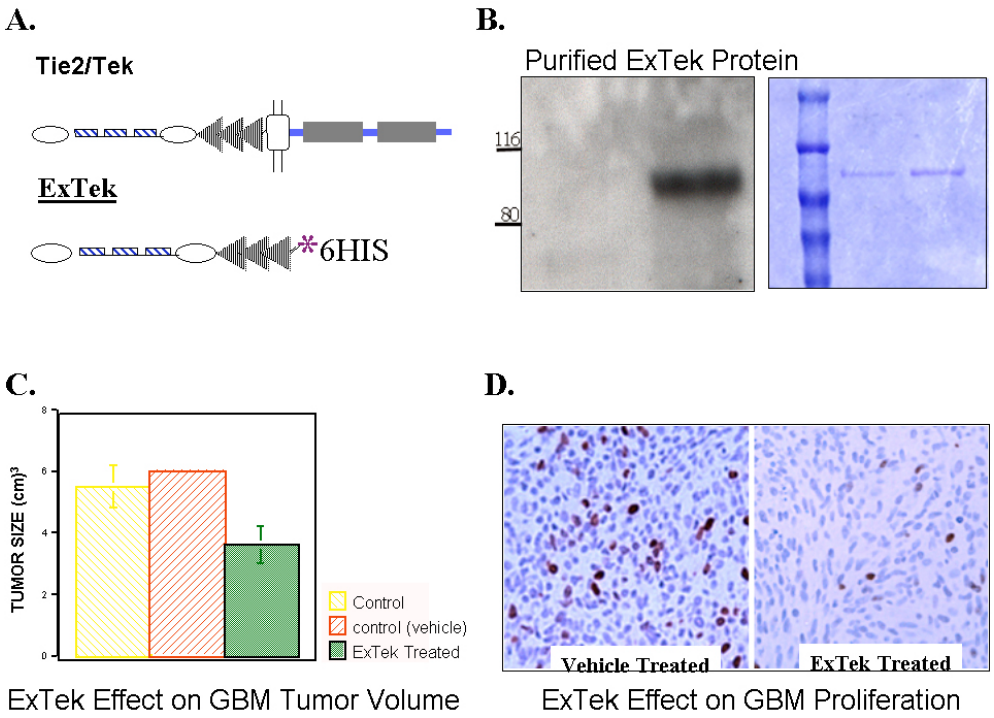

Figure 4. ExTek protein purification and biological effect on GBMs. ExTek protein contains the extracellular portion of Tie2/Tek receptor and acts as a dominant negative mutant to inhibit the receptor and A) outlines the structure of ExTek, containing a 6HIS tag which is used for purification of the protein, B) Western blot analysis of the purified ExTek using the specific antibody to ExTek epitope and Cooomasie stain of the purified ExTek shows the purified protein running at approximately 96kD, C) ExTek treated explants of human GBMs, reached a final tumor volume that was approximately 30% less than the control and vehicle treated GBMs over a ten week period, D) Associated with the decreased growth was a decrease in proliferation rate of the ExTek treated tumors as seen with the extent of BrDu staining.

5. CONCLUSION AND PRESPECTIVES

In conclusion, Angiopoietins are clearly involved in the angiogenic progression of GBMs. Similar to their biological role that has thus far been deciphered, their effect in tumor biology is also complex and conflicting. The interaction of Angiopoietins with other angiogenic factors, in specific VEGF, appears to strongly influence the regulatory role that they play in astrocytoma angiogenesis. Furthermore, the findings support a tumor context dependent role for these angiogenic proteins. We also believe that the tumor-stage and cell type from which Angiopoietins and Tie2/Tek are expressed governs the ultimate impact these proteins leave on the tumor vasculature. Whether a dose dependent response to Angiopoietins, in particular Ang2, exists and whether Ang2 acts as agonistic or antagonistic ligand to Tie2/Tek remains unknown. Whether interactions with other relevant angiogenic factors govern the eventual effect of Angiopoietins is also an area requiring more comprehensive investigation. The presence of cofactors and co-receptors in this signaling pathway can potentially explain some of the variability in functional consequences of this pathway reported by various groups. In astrocytomas it is imperative to enhance our understanding of the role that Ang1 plays in tumor edema and how this can translate to therapeutic strategies to decrease GBM associated edema, which is one of the main causes of patient morbidity. A unified picture for the role of Angiopoietin-Tie2 pathway can not be drawn due to the variability in data presented so far in both in-vitro and in-vivo studies. However, collectively the results indicate and emphasize a highly complex and precisely regulated pathway in-vivo that warrants continued investigation, since it holds promise to act as a novel therapeutic target for GBMs, in addition to enhance our understanding the molecular mechanisms of tumor angiogenesis.

As part of our future investigations we are examining the effect of ExTek in a spontaneous transgenic mouse model of malignant astrocytomas that we have developed in our laboratory (76). These transgenic astrocytomas have a strong angiogenic expression profile for VEGF, Angiopoietins and Tie receptors, which are similar to human malignant astrocytomas, including increased vascularization, necrosis and intra-tumoral hemorrhage. We are exploiting this fact to study the role of Tie2/Tek activation in the vascularization and overall growth of astrocytomas, using adenoviral mediated and stereotactic injected ExTek delivery. These experiments will shed insight into the influence of Tie2/Tek signal transduction in various stages of tumor development.

A second area of research involves micro-dissection of human GBM specimens with Laser Capture Micro-Dissection (LCM). LCM will allow isolation of pure cell populations of endothelial cells and astrocytoma cells. Coupled with real-time PCR the expression levels of Angiopoietins and Tie2/Tek can be compared amongst the various regions GBMs. These experiments provide novel information on the expression profile of angiogenic factors as they are influenced by the tumor micro-environment.

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