Role of Ang1 and Its Interaction with VEGF-A in Astrocytomas

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Abstract. Angiopoietins (Ang1 and Ang2) modulate the activity of the endothelial cell (EC)-specific receptor tyrosine kinase Tie2, which together with vascular endothelial growth factor (VEGF-A) and its EC-specific receptors, VEGFR1 and VEGFR2, regulate normal physiological vessel development. The functional role of angiopoietins in tumor angiogenesis, in particular astrocytoma angiogenesis, remains unclear. In this study, we focus on the specific contribution of Ang1 to the vascular growth of glioblastoma multiforme (GBM) and its interactive role with VEGF-A. Subcutaneous and intracranial GBM xenografts were generated using 3 established astrocytoma cell lines (U87, U373, and U343) that were transfected to stably over-express Ang1. GBM xenografts were also generated to express low levels of VEGF-A and high Ang1. We found that Ang1 increases the vascular growth of both subcutaneous and intracranial xenografts of GBM by approximately 3-fold. However, the increased vascular growth was only seen in xenografts with concurrent VEGF-A elevation, since decreasing VEGF-A expression resulted in a loss of the pro-angiogenic growth advantage seen with Ang1. Collectively, our data suggest that Ang1 regulates GBM vascularity in a VEGF-A dependent manner, synergizing the initial pro-angiogenic response that is triggered by VEGF-A and promoting the vascular growth of GBM.

Key Words: Astrocytoma; Ang1; Ang2; Glioblastoma multiforme (GBM); Tie2; Tumor angiogenesis; vascular endothelial growth factor (VEGF-A).

INTRODUCTION

Growth and malignant progression of human cancer has been firmly linked to tumor angiogenesis (1–4), leading to an increasing interest in targeting modulators of angiogenesis for cancer therapy. Of the various pro- and anti-angiogenic factors that contribute to tumor angiogenesis, vascular endothelial growth factor (VEGF-A) and angiopoietins have an angiogenic-specific role in modulating both physiological and pathological angiogenesis, due to expression of their receptors almost exclusively on endothelial cells (ECs) (1, 2). VEGF-A was the first EC-specific cytokine to be identified, which through activating its EC-specific receptor tyrosine kinases VEGFR1 and VEGFR2, promotes EC proliferation, migration, and primitive tubule formation (5, 6). VEGF-A is a known potent regulator of tumor angiogenesis, edema, and metastasis and considered to be the principle regulator of the florid angiogenic process seen in malignant human astrocytomas (7, 8). Various therapeutic strategies have targeted VEGF-A, with the hope of decreasing tumor angiogenesis and thereby tumor growth; however, none have yet conclusively demonstrated significant clinical benefit in astrocytomas. This, in part, is due to the complex interactions that exist between VEGF-A and other angiogenic factors in modulating tumor angiogenesis. A more comprehensive understanding of the combinatorial impact of relevant angiogenic pathways in tumor angiogenesis, we believe, will result in designing more efficacious anti-angiogenic therapies.

Angiopoietins, together with their cognate receptor Tie2, are the second class of EC-specific angiogenic factors that act in close coordinated manner with VEGF-A to produce normal physiological vessels (7–10). Unlike VEGF-A, angiopoietins are not mitogenic for ECs; however, they are involved in maturation of the vasculature by regulating the interactions between ECs and pericytes and smooth muscle cells (SMCs) of the extracellular matrix (10, 11). Ang1, through activation of Tie2, signals stabilization of the primitive vessels (10, 11), while Ang2, the naturally occurring antagonist to Tie2, inhibits the action of Ang1 and leads to vessel destabilization by disrupting the connections between ECs and supportive cells of the extracellular matrix (10, 11). Ang2 sensitizes ECs to VEGF-A, which in turn mediates EC proliferation, sprouting, and neo-angiogenesis (10, 11). Angiopoietins have been shown to play a complex and highly context-specific role, with recent physiological experiments proving the angiogenic capacity of Ang1 to be closely dependent on VEGF expression (12–17). Similar to physiological angiogenesis, the role of angiopoietins in tumor angiogenesis is also proving to be tumor type-dependent and somewhat contradictory, as a number of studies in various tumor models demonstrate contradictory responses to Ang1 and Ang2 (18–27). Results to
date suggest that angiopoietins act as secondary modifying angiogenic factors, and have a potentially dual angiogenic role based on the tumor type, stage, microenvironment, and epigenetic factors. Specifically, the role of Ang1 has been investigated in mammary, lung, colon, gastric, hepatic, and squamous cell cancers (19, 28–30), with no reports to date on the functional contribution of Ang1 and its interactions with VEGF-A in astrocytoma angiogenesis.

Astrocytomas are the most common primary brain tumors in adults, with the most malignant grade being glioblastoma multiforme (GBM) (31, 32). GBMs are one of the most highly vascularized tumors in adults, with florid angiogenesis and microvascular proliferation being a pathological hallmark of increasing malignancy (31, 32). GBM vessels are both structurally and functionally aberrant, characterized by EC hyper-proliferation, which can often be multi-layered and lack the full repertoire of supportive pericytes and SMCs present in a mature vessel (31). Studies by several groups have demonstrated elevated levels of VEGF-A and VEGFRs contributing to the angiogenic growth of GBMs (7, 8); however, the functional role of angiopoietin-mediated activation of Tie2 in astrocytomas remains to be established. We have previously demonstrated that expression of Ang1 and VEGF-A by tumor cells increases with increasing malignancy grade of astrocytomas, with GBMs expressing the highest levels of both Ang1 and VEGF-A (33). In addition, we have shown that inhibition of Tie2 activation by a Tie2 dominant-negative mutant (ExTek) significantly decreases GBM xenograft growth through disruption of tumor vascularity (34). Collectively, these findings support a crucial contribution of angiopoietin-mediated Tie2 activation towards the vascular development and growth of GBMs. The contribution of the individual ligands, Ang1 and Ang2, remains unknown and in this study we have focused on the role of Ang1 in astrocytoma angiogenesis. We found that Ang1 regulates the pathological vascularization of GBMs and increases tumor growth in a VEGF-A-dependent manner. The VEGF-A-dependent impact of Ang1 in GBM angiogenesis and growth has significant therapeutic implications and provides important clues to improve our anti-angiogenic treatment strategy for these highly malignant tumors.

MATERIALS AND METHODS

Cells and Reagents

Established human U-87 MG GBM cells were obtained from American Type Culture Collection (ATCC, Rockville, MD) and U-373 MG and U-343 MG GBM cell lines were a gift from B. Westermark (Uppsala, Sweden). These GBM lines, maintained in Dulbecco’s minimal essential medium (DMEM; Cellgro, Herndon, VA) supplemented with 10% FBS and penicillin-streptomycin antibiotics, were chosen as they provide variability in their degree of baseline angiopoietin, Tie2, and VEGF-A expression (Fig. 1A) (33), in addition to their tumorigenicity potential and other genetic aberrations. The 3T3-Tie2 cells were generated by stable transfection of Tie2 receptor in NIH-3T3 cells and maintained in DMEM with 250 µg/ml of G418. All above cells were grown in a 37°C, 5% CO2 incubator.

Stable Cell Lines

Full-length human Ang1 cDNA (gift from K. Alitalo, Helsinki, Finland) was subcloned into the pSec vector (Invitrogen, Mississauga, ON, Canada) to allow generation of Myc-Histidine epitope tagged constructs. The Ang-Myc/HIS sequence was subcloned into the BamHI and EcoR1 sites of the pCAGG vector that contains a CMV promoter with a chicken β-actin enhancer element. Stable cell lines were generated by transfection of the vector “pCAGG-Ang-Myc/HIS-Zeoacin” into U87, U373, and U343 GBM lines using Lipofectamine 2000 (Gibco/BRL, Rockville, MD) as per the manufacturer’s instructions. Twenty stable clones, selected with 1 mg/ml of Zeocin (Inviron), were examined for Ang1 expression by Western blot analysis as described below (Fig. 1B). Two single clones with highest expression of Ang1 above baseline parental levels and 1 pooled clone of Ang1 were selected for each of the 3 GBM lines (U87:Ang1, U373:Ang1, and U343:Ang1). Corresponding control stable cell lines were generated using empty vector transfectants.

To evaluate interactions of Ang1 and VEGF-A, we decreased VEGF-A expression in U87 cells, which express high levels of endogenous Ang1 (Fig. 1A) (33). U87:Antisense-VEGF stable cell lines were obtained from Cheng et al (35). Briefly, a construct was generated containing the VEGF (189) cDNA subcloned in an antisense orientation under a CMV promoter. This construct was transfected into U87 cell lines to decrease all isoforms of VEGF, with stable clones selected using hygromycin selection antibiotic. The conditioned media (CM) of these cells demonstrated a 6-fold decrease in VEGF-A expression by ELISA and a significant decrease in inducing human microvascular endothelial cell migration. Furthermore, U87: Antisense-VEGF had a significant loss in their in vivo tumorigenicity potential compared to control U87 xenografts (35).

Northern Blot Analysis

In brief, total RNA was isolated from cultured cells using the RNeasy mini kit (Qiagen, Valencia, CA), with 10 to 30 µg of total RNA fractionated on 1% agarose-formaldehyde gels and transferred to Hybond nylon membranes (Amersham Life Science, Uppsala, Sweden). The human cDNA Ang1 probe was generously provided by Dr. Alitalo (494 bp Ang1 cDNA coordinates +5 to 499 of coding region), with the VEGF-A human cDNA probe as previously described (35), for use in Northern blot analysis (Fig. 1A). A human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe served as loading control. The cDNA probes were radiolabeled with α-32P dCTP using a random primer labeling kit (Pharmacia, Uppsala, Sweden). Hybridization was performed in ExpressHyb (Clontech, Palo Alto, CA), containing 2 × 106 cpm/ml probe as per the manufacturers instructions with the last wash in 0.1 × SSC, 0.1% SDS at 50°C for 40 min. The probed blots were exposed to Kodak Blue XB-1 films with intensifying screens at −70°C.
Fig. 1. Characterization of parental established human GBM cell lines (U87, U373, and U343) and stable clones overexpressing Ang1. A: Northern blot analysis demonstrating the baseline expression levels of Ang1 and VEGF-A mRNA (U87: High for both; U373: Moderate for both; U343: Minimal-nega- tive for both). B: Western blot analysis for Ang1 levels on the conditioned media (CM) of U87, U373, and U343 transfected stable clones, demonstrating over-expression of Ang1 compared to control cells. For each cell line, the 2 highest expressing clones (A1-1; A1-2) and 1 pooled clone (A1-p) were selected for in vivo studies. C: Tie2 phosphorylation assay using CM of Ang1 transfected stable clones. Tie2 phosphorylation was induced 5 min after stimulation with the CM of stable clones over-expressing Ang1 from all 3 GBM lines (representative lane for U87:Ang1, U373:Ang1, and U343:Ang1 clones are demonstrated). Controls included baseline phosphorylation of the receptor with media alone; CM from empty vector transfectants; CM from empty vector transfectants + orthovanadate. Specificity of Tie2 phosphorylation by Ang1 in the CM was demonstrated by inhibition of Tie2 phosphorylation to baseline levels by the soluble dominant-negative kinase dead Tie2 protein, ExTek (a representative lane with 500 ng of ExTek CM from U87:Ang1 cells is demonstrated).
with the most induction of Tie2 phosphorylation are shown (Fig. 1C). Controls included media only, media with orthovanadate, and CM from empty vector transfectant cells. Specificity of Ang1-mediated Tie2 phosphorylation was demonstrated by inhibiting the CM-induced Tie2 phosphorylation with the Tie2 dominant-negative mutant, ExTek (34). All experiments were repeated in triplicate.

In Vivo Xenograft Models

Subcutaneous Models: Subcutaneous xenografts were generated by growing the selected stable clones over-expressing Ang1 in the flanks of NOD-SCID mice. For each stable clone, 10 mice were injected with 10E7 cells suspended in 300 µl of PBS, with 5 mice injected with control empty vector transfectants. Tumor growth was measured bi-weekly, using calipers, by 2 observers in a blinded fashion. Tumor volume was calculated using the formula: (diameter² × length)/2. As per animal protocol, mice were killed by perfusion fixation following an in injection of a 100-mg/kg BrdU (Sigma-Aldrich, Canada Ltd., Oakville, ON, Canada). Tumors were dissected from mice, cut in cross sections, and kept in formaldehyde for paraffin blocks and immunohistochemical analysis. All in vivo xenograft studies were repeated in duplicate.

Intracranial Models: For orthotopic xenograft models, stable cell lines of U87:Ang1 and U373:Ang1 (10⁶) were stereotactically injected 3 mm deep into the frontal cortex of NOD-SCID mice. Mice were killed after BrdU injection by perfusion fixation when signs of failure to thrive developed as per institutional animal care protocols. U373 cells do not have a very strong growth potential in the intracranial compartment and grow inconsistently, whereas U343 cells do not grow in vivo at all in NOD-SCID mice.

Xenografts with Downregulation of VEGF-A and Upregulation of Ang1 Expression: Parental U87 cell lines have abundant VEGF-A expression and moderate levels of Ang1 (Fig. 1A) as we previously described (33). In order to generate U87 xenograft tumors that had decreased VEGF-A expression while maintaining high levels of Ang1, a 1:1 mixture of U87:Antisense-VEGF-A cells with U87:Ang1 over-expressing clones (“U87:AntisenseVEGF-A+Ang1”) were generated, as described for previous xenograft studies (28). Similarly, a control xenograft comprising a 1:1 mix of U87:Control cells with U87:Ang1 over-expressing cells (“U87:Control+Ang1”) was generated. An additional panel of control tumors was also grown simultaneously in NOD-SCID mice using U87:Antisense-VEGF-A, U87:Ang1, and U87:Control cell lines only. For each xenograft, 10E7 cells were injected subcutaneously in the right flank of 15 NOD-SCID mice as described above.

Immunohistochemistry

Standard immunohistochemical analysis was performed on the paraffin-embedded sections with antibodies to: Ki-67 (polyclonal rabbit #A0047, DAKO, 1:400) and Factor VIII (polyclonal rabbit #A0082, DAKO; 1:2,500). A goat anti-mouse antibody (Zymed; 1:200) was used as the secondary antibody and antigens were detected using the avidin-biotin complex method (Vector Laboratories, Burlingame, CA) and diaminobenzidine substrate. To evaluate tumor vascularity, consecutive 5-µm paraffin sections were stained with an anti-Factor VIII (DAKO; 1:200), followed by detection with an avidin-biotin complex method-3,3’-diaminobenzidine (VectorStain Elite; Vector Laboratories) system. Microvessel density (MVD) counts were derived by averaging the number of hollow lumen vessels stained by Factor VIII in 10 high-power fields (HPFs), in addition to counts made in 5 HPFs in the vascular “hot spots.” Areas containing abnormal vessel architecture were not included in the calculation of MVD (such as dilated, serpentine structures). All analysis was carried out using the MicroComputer Image Device (MCID, Imaging Research, Inc., St. Catharines, ON, Canada) linked to a color CCD camera (Sony DXC 970 MD), mounted on a transmitted-light microscope (Zeiss Axiioskop).

Statistical Analysis

All analyses were completed using StatView 4.1 for the Macintosh (Abacus Concepts). All errors were calculated as the standard error of the mean (SEM). The 1-tailed Student t-test was used to compare means (2 sample, unequal variance), with p < 0.05 considered statistically significant.

RESULTS

Over-Expression of Ang1 in Established Human GBM Cell Lines

Parental U87 cells express the highest amount of endogenous Ang1 and VEGF-A, while U373 express moderate amounts and U343 cells express none (Fig. 1A). Over-expression of Ang1 in U87, U373, and U343 cells did not alter their in vitro proliferation rate, morphology, or baseline VEGF-A expression compared to parental controls (data not shown). For each of the 3 human GBM cell lines (U87, U373, U343), 2 single clones with the highest expression of Ang1 above baseline parental levels, as well as 1 pooled clone generated from pooling 5 individual clones, were selected for in vivo studies (Fig. 1B). Potential clonal variability was accounted for by using multiple clones, pooled clone, and empty vector transfectants.

The biological activity of the exogenously secreted Ang1 in the CM of the selected stable clones was confirmed using Tie2 phosphorylation (Fig. 1C). Tie2 phosphorylation was detected at 5 min, when 3T3-Tie2 cells were stimulated with the CM of Ang1 over-expressing clones, in contrast to stimulation with CM of control empty vector transfectants (Fig. 1C). To ensure that Tie2 phosphorylation was specifically due to Ang1 present in the CM of stable clones, we used the Tie2 dominant-negative mutant protein, ExTek, previously shown to inhibit Ang1 activation of Tie2 (34, 37). ExTek at 500 ng was able to inhibit Tie2 phosphorylation induced by CM of U87:Ang1, U373:Ang1, and U343:Ang1, confirming that Tie2 activation is directly related to Ang1 in the CM (Fig. 1C).

Effect of Ang1 on In Vivo Astrocytoma Growth: Ang1 Promotes GBM Growth in a Context-Dependent Manner

We assessed the impact of Ang1 on growth of GBM xenografts as both subcutaneous and intracranial tumor
Fig. 2. Effect of Ang1 on growth of subcutaneous (A, C) and intracranial (B, D) GBM xenograft models. A: Subcutaneously injected U87:Ang1 stable and pooled clones grew faster and to a significantly larger overall tumor size \( (p = 6 \times 10^{-5}) \), compared to control empty vector transfected clones. B: Intracranial xenografts of U87:Ang1 stable clones generated tumors with a faster growth rate, as reflected by a significant decrease in overall survival, compared to U87:Control \( (p = 4.5 \times 10^{-5}) \). C: U373:Ang1 clones grew slower but with no statistically significant change compared to controls. D: Stereotactically injected intracranial U373:Ang1 xenografts had a slightly longer survival compared to U373:Control mice, although with no statistical significance.

models, using stable cell lines of U87, U373, and U343 over-expressing Ang1, described above (Fig. 1B). We, along with others, have previously demonstrated that the in vivo growth potential of parental U87 cell lines in NOD-SCID mice to be high, followed by U373 cells, with U343 cells not growing as xenografts at all. Interestingly, the growth potential of these 3 cell lines corresponds closely with their endogenous levels of Ang1 and VEGF-A expression (Fig. 1A) \( (33) \).

In U87 subcutaneous xenografts, which express moderately high levels of endogenous VEGF-A (Fig. 1A), over-expression of Ang1 resulted in i) a significantly faster growth rate, ii) a 3.5-fold increase in the final tumor size, and iii) a 4-fold increase in tumor cell proliferation compared to control (Fig. 2A; Table 1). Similarly, U87 intracranial xenografts over expressing Ang1 had a significantly shorter survival compared to control mice and a 2.6-fold increase in tumor proliferation, consistent with a faster tumor growth rate (Fig. 2B; Table 1). However, Ang1 over-expression in U373 cells, which express low levels of endogenous VEGF-A (Fig. 1A), resulted in subcutaneous xenografts that grew slower compared to control empty vector transfected U373 xenografts, although with no statistically significant difference (Fig. 2C; Table 1). Similarly, intracranial models of U373:Ang1 did not demonstrate a significant change in survival of mice or tumor cell proliferation compared to control U373 xenografts (Fig. 2D; Table 2). Ang1 over-expression was not sufficient to confer a growth advantage to U343 cells, which have poor tumorigenicity and express undetectable levels of both Ang1 and VEGF-A endogenously (Fig. 1A) \( (33) \).

Effect of Ang1 on Astrocytoma Vascularity: Ang1 Promotes Pathological Vascularization of GBMs

Tumor vascularity was assessed using MVD, vessel architecture, and pattern of EC distribution. In both subcutaneous and intracranial models of U87:Ang1 there was a significant (i.e. 1.7-fold) increase in MVD (Tables 1, 2). However, both subcutaneous and intracranial U373:
Ang1 xenografts had no significant change in tumor vascularity as determined by MVD, with the tumor vascular structure remaining similar to control tumor vessels (Fig. 3B; Tables 1, 2). In U87:Ang1 xenografts there was a trend towards decreased EC apoptosis, consistent with one of the biological roles of Ang1 as an EC survival factor (data not shown). An intriguing finding was the alteration of the vascular architecture in the U87:Ang1 xenografts. In addition to microvascular proliferation and increased MVD, many of the vessels take on a highly serpentine structure, with multi-layering of ECs in the vessel wall and this was more commonly observed in the intracranial U87:Ang1 models vs subcutaneous xenografts (Fig. 3A). These vascular changes are similar to vascular architectural changes seen in human GBMs and characteristically referred to as “glomerular tufting” (32, 38). The abnormal EC piling and dilated serpentine vascular structure seen in U87:Ang1 xenografts was not observed in U373:Ang1 tumors (Fig. 3A, B), suggesting that the specific molecular characteristics of U87 cell line in combination with Ang1 is required for generation of these pathological vascular changes. This data is presented as an intriguing observation, which requires further investigation involving characterization of the vascular elements under Ang1 regulation. The abnormal vascular structures seen in U87:Ang1 xenografts (Fig. 3A) were not included in the MVD count, as the vascular structures do not fit the definition of hollow-lumen vessels and their effective function as a microvessel is not clear.

**Co-Modulation of Ang1 and VEGF-A: Ang1 Requires VEGF-A to Act in a Pro-Angiogenic Capacity**

In summary, we have observed that Ang1 can have a pro-angiogenic and growth-promoting role in U87 xenografts, while having no significant impact on U373 xenografts. The above results indicate that Ang1 can play a variable impact on tumor angiogenesis, depending on the cell type investigated. The molecular basis of these differing effects of Ang1 in U87 and U373 xenografts may be explained by any number differences in their baseline molecular characteristics, of which we postulate that the endogenous VEGF-A levels of U87 vs U373 are critical factors and play pivotal roles. In order to test our hypothesis we increased VEGF-A expression in U87 cells and assessed whether the pronounced pro-angiogenic effect of Ang1 would be lost. All isoforms of VEGF-A were downregulated using an antisense construct containing the VEGF-A (189) isoform, as previously described (35). Xenografts were generated by a 1:1 mixing of U87:Antisense-VEGF-A and U87:Ang1 (“U87:Antisense-VEGF-Ang1”) cells, as described previously (28). The “U87: Antisense-VEGF + Ang1” xenografts mimic U373:Ang1 xenograft with respect to levels of VEGF-A and Ang1 expression. Western blot analysis of these mixed cell populations demonstrated high levels of Ang1 and decreased...
Effect of Ang1 on GBM vascularization stained with Factor VIII. A: Both subcutaneous and intracranial models of U87:Ang1 xenografts have a significant increase in the microvascular density (MVD) compared to controls (Subcutaneous: 3.5 vessels/HPF vs 2.12, \( p = 3.2 \times 10^{-4} \), Intracranial: 9.5 vessels/HPF vs 5.6, \( p = 0.0001 \)). Abnormal vessel architecture was present in both subcutaneous and intracranial U87:Ang1 xenografts, more prominently in intracranial models. The vessels were dilated, serpentine structures (inset lower panel), with piling of ECs (arrows) in the vessel walls compared to control vessels that are small, well-formed regular lumens, and single EC layer thick. These changes are very similar to the characteristic “glomerular tufting” seen in human GBM specimens. B: U373:Ang1 over-expressing xenografts have a moderate increase in MVD, while maintaining a vascular structure that is comparable to control tumors. The vessel lumens are small and regular in shape and extent of EC piling similar to control tumor vessels (arrows). Insets are lower power (×5) demonstrating comparable low level of microvascular proliferation in both control and U373:Ang1 xenografts.

levels of VEGF-A expression when compared to Ang1 and VEGF-A levels in U87:Control, U87:Ang1, U87:Control + Ang1, and U87:Antisense-VEGF cell lines (Fig. 4A). In accordance with our observations, decreasing VEGF-A expression resulted in loss of the pro-angiogenic capacity and growth-promoting effect of Ang1 that was seen in U87:Ang1 xenografts. The U87:Antisense-VEGF + Ang1 xenografts had a similar final tumor volume as U87:Controls and a considerably lower growth rate and final tumor volume compared to U87:Ang1 and U87:Control + Ang1 over-expressing tumors (Fig. 4B; Table 3). Overall, these mixing studies demonstrate that presence of VEGF-A is necessary for Ang1 to act in a pro-angiogenic manner and Ang1 synergizes the angiogenic response elicited by VEGF-A. Therefore, the variable tumor growth seen in response to Ang1 in U87 and U373 xenografts (Fig. 2A, C) can be accounted for by the difference in their baseline VEGF-A expression.

Additionally, the increased MVD seen with U87:Ang1 tumors (Table 1) was not present with U87:Antisense-VEGF + Ang1 xenografts, suggesting that growth alteration of the xenografts was due to a decrease in tumor vascularity (Table 3). However, despite a decrease in MVD, similar dilated serpentine vascular structures together with EC multi-layering that was seen prominently in U87:Ang1 xenografts (Fig. 3A) was present in the U87:Antisense-VEGF + Ang1 xenografts (Fig. 5). This observation suggests that these vascular structures, which bear similarity to “glomerular tufts” characteristically seen in human GBMs, are modulated by Ang1 and furthermore are most likely independent of VEGF-A.

DISCUSSION

The role of angiopoietins in tumor angiogenesis remains unclear, with contradictory results seen in different tumor types investigated (18–22, 24–30, 39). Ang1 upregulation in breast and squamous cell cancers inhibits tumor growth and vascularity (19, 28), while in colon, hepatic, and lung cancer models Ang1 has no effect (22, 29, 30). We have previously shown that inhibition of Tie2 restricts GBM growth by disrupting tumor angiogenesis as evidenced by a decrease in MVD and loss of mature tumor vessels due to loss of EC and SMC interaction (34). However, the distinct contribution of the individual
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Fig. 4. A: Western blot analysis for VEGF-A and Ang1 expression in the conditioned media (CM) of U87 stable clones with modulated expression of Ang1 and VEGF-A. Upper panel (A): Immunoprecipitation and Western blot analysis of CM of cell lines for all 3 isoforms of VEGF-A. Lower panel (A): immunoprecipitation-Western blot analysis of Ang1. Secretion of all 3 isoforms of VEGF-A are low by U87:Antisense-VEGF-A (lane 2), 1:1 mix of U87:Antisense-VEGF + U87:Ang1 (lane 3) and U373 parental (lane 1) cells, compared to U87:Control (lane 4), U87:Ang1 (lane 5) and a 1:1 mix of U87:Control + Ang1 (lane 6) cells. Secretion of Ang1 by U87:Antisense-VEGF-A + U87:Ang1 cells (lane 3) was similar to U87:Ang1, U87:Control + U87:Ang1 and U373:Ang1 cells. B: Final tumor volumes of xenografts demonstrates U87:Antisense-VEGF + Ang1 cells to have decreased tumorigenicity compared to U87:Ang1 cells, with their final tumor volume similar to the 2 xenografts used as controls: U87:Control and U87:Antisense-VEGF. Final tumor volumes were significantly increased, especially in the U87:Ang1 and to a lesser extent in the U87:Control + Ang1 xenografts (*p < 0.05 compared to U87:Control).

Tie2 ligands (Ang1 and Ang2) to GBM angiogenesis remains unknown. To date there has been 1 study demonstrating that in U87 intracranial xenograft models Ang2 increases tumor cell invasion; however, the impact of Ang2 on astrocytoma growth and angiogenesis remains unknown (21).

In this study we found that over-expression of Ang1 in both subcutaneous and intracranial GBM xenografts resulted in a variable effect on tumor angiogenesis and growth, which we believe can be explained by differences in VEGF-A expression between the 2 xenograft models studied. In U87 cells, which endogenously express moderate levels of VEGF-A (Fig. 1A) (33), Ang1 over-expression markedly enhanced tumor angiogenesis and overall tumor growth (Fig. 2A, B; Tables 1, 2). In contrast in U373 cells, which express minimal levels of VEGF-A (Fig. 1A) (33), over-expression of Ang1 made no significant change on tumor angiogenesis and growth.
TABLE 3

<table>
<thead>
<tr>
<th>Condition</th>
<th>n</th>
<th>SEM (cm³)</th>
<th>MVD</th>
</tr>
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<tbody>
<tr>
<td>U87:Ctl</td>
<td>10</td>
<td>2.6 (SEM = 0.26)</td>
<td>1.89 (SEM = 0.18)</td>
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<td>U87:Ang1</td>
<td>10</td>
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<td>2.4 (SEM = 0.19)</td>
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<tr>
<td>U87:Antisense-VEGF + Ang1</td>
<td>10</td>
<td>2.14 (SEM = 0.2)</td>
<td>1.26 (SEM = 0.1)</td>
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Abbreviations: SEM, standard error of mean; MVD, microvascular density.

In order to test whether there is a VEGF-A dependent pro-angiogenic role for Ang1 in GBMs, we decreased expression of all VEGF-A isoforms while maintaining high Ang1 expression in the U87 cells (Fig. 4A). Xenografts composed of a mixture of U87:Antisense-VEGF + U87:Ang1 lost the angiogenic growth advantage seen with U87:Ang1 xenografts alone, and tumors grew with comparable rates to control tumors (Figs. 2A, 4B). These experiments support our postulate that the initiating angiogenic signal of VEGF-A is necessary to allow for Ang1 to synergize the pro-angiogenic effect of VEGF-A and enhance tumor angiogenesis. These results can also offer an explanation for the variability seen in the literature on the response to Ang1 in different tumor models (22, 29, 30).

We have also over-expressed VEGF-A in U373 cells in an attempt to generate xenografts that mimicked U87: Ang1 xenografts. However, we observed that over-expressing VEGF-A in U373 cells also led to a spontaneous upregulation of Ang1 expression (data not shown). Therefore the appropriate and obvious control U373 xenografts that over-express VEGF-A alone (without Ang1 upregulation) was not possible. These results are in further support of a regulatory and co-modulatory interaction of VEGF-A and Ang1. The mechanism(s) of VEGF-A-induced Ang1 expression is not known at this stage and will be the subject of future investigations.

In keeping with our results from this study in a GBM model are recent reports emphasizing the highly VEGF-A- and context-dependent manner in which Ang1 modulates EC biology and angiogenesis in physiological
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Fig. 5. Tumor vascularity was decreased in U87:Antisense-VEGF + Ang1 tumors compared to U87:Ang1 as measured by Factor VIII staining for microvascular density (MVD). U87:Antisense-VEGF + Ang1 had similar MVD to that of U87:Control. However, the vascular architecture in U87:Antisense-VEGF + Ang1 demonstrated similar alterations seen in U87:Ang1 xenografts, resembling “glomerular tufting” (arrows). These vascular alterations, primarily marked by dilated, serpentine structures that have piling of EC in the vessel wall, were not seen in U87:Control or U87:Antisense-VEGF tumors, where the vessels were small, regular in shape and vessel walls are lined with 1 layer of EC.

models (13, 15–17, 40, 41). In a cutaneous mouse model, Ang1 acts in a pro-angiogenic manner and synergizes the VEGF-A-induced angiogenesis (40). However, in cardiac tissue Ang1 acts in an anti-angiogenic capacity, antagonizing the angiogenic response elicited by VEGF-A (17). Additionally, the ability of Ang1 to induce tubule structures in HUVECs was shown to be dependent on the presence of VEGF-A (15). To date, the potential interactive and combinatorial impact of VEGF-A and Ang1 has not been investigated in tumor angiogenesis, and our study illustrates that the pro-angiogenic role of Ang1 is VEGF-A dependent and that their combinatorial impact has a cumulative increase in tumor angiogenesis.

Associated with Ang1-induced alterations in GBM growth are corresponding alterations in GBM vascularity. Ang1 upregulation in U87 xenografts resulted in increased tumor microvascular proliferation and MVD, suggesting that the growth advantage is conferred by increased tumor vascularity (Tables 1, 2). The vascular architectural changes observed in U87:Ang1 xenografts are noteworthy. Ang1 over-expression led to multi-layering of vessel EC and dilated serpentine vessels that are reminiscent of the “glomerular tufting” seen characteristically in human GBMs (Fig. 3). These structural changes, resembling “glomerular tufting” are also seen in the U87:Antisense-VEGF + Ang1 mixed xenografts (Fig. 5),...
suggesting that Ang1-induced “glomerular tufting” is independent of VEGF-A. These findings propose Ang1 to be a main molecular regulator of the characteristic vascular alterations seen in human GBMs. These findings are in accordance with our previous observation that inhibition of Tie2 in human GBM xenograft models significantly decreased the extent of EC piling or “glomerular tufting” seen in GBMs (34). Therefore, collectively, these observations imply that Ang1 is the principle responsible ligand to activate Tie2 and regulate the pathological vascular structures seen in human GBMs.

In addition to the differences in VEGF-A levels, a number of other genetic and molecular differences between the U87 and U373 xenograft models are recognized as factors that can explain the variable response to Ang1 seen in this study. For example, the relative contribution of Ang2 and the combined effects of VEGF-A, Ang1, and Ang2 are also critical factors that require further investigation. Similarly, the p53 status (U87-wt, U373, U343-mut) (42, 43), neuropillin expression (a coreceptor for Ang1, and Ang2 are also critical factors that require further investigation. Significantly decreased the extent of EC piling or “glomerular tufting” seen in GBMs (34). Therefore, collectively, these observations imply that Ang1 is the principle responsible ligand to activate Tie2 and regulate the pathological vascular structures seen in human GBMs.

In conclusion, this study demonstrates that Ang1 regulates GBM angiogenic growth in a VEGF-A dependent manner. Ang1 synergizes the potent tumor angiogenic response triggered by VEGF-A by increasing MVD and generating more stable vascular structures, thereby increasing GBM growth; whereas in the absence of the initial angiogenic trigger signaled by VEGF-A, Ang1 cannot alter tumor vascularity. These results have significant therapeutic implications as they point to the need for “angiogenic profiling” of individual GBMs. This might be helpful in identifying angiogenic pathways of the tumor for the purpose of combinatorial therapeutic strategies.

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