Targeting the Tie2/Tek Receptor in Astrocytomas

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Tie2 is an endothelial cell-specific receptor tyrosine kinase, whose activation is positively and negatively modulated by angiopoietin-1 and angiopoietin-2, respectively. Angiopoietin-mediated modulation of Tie2 activation contributes to normal vessel development and stability, however, its role in tumor angiogenesis is not well known. We investigated the role of Tie2 activation in malignant astrocytomas, a common and highly vascularized primary human brain tumor. We found that Tie2 expression and activation increases with increasing malignancy grade of astrocytomas. Inhibition of Tie2, using a kinase-deficient Tie2 construct, decreases growth of malignant human astrocytoma subcutaneous and intracranial xenografts. Tie2 inactivation disrupted the tumor vascularity, with a decrease in microvascular density, increased presence of abnormally dilated vessels, and loss of interaction between endothelial cells and surrounding smooth muscle cells, all collectively resulting in increased tumor cell apoptosis. Overall, these findings strongly suggest that Tie2 activation contributes significantly to astrocytoma tumor angiogenesis and growth. We postulate that targeting Tie2 activation, either independently or in conjunction with other anti-angiogenic therapies, such as against vascular endothelial growth factor, is of potential clinical interest. (Am J Pathol 2004; 164:467–476)

Tumor angiogenesis is essential for the growth and metastasis of solid human cancers. Since the introduction of the “angiogenic switch” by Folkman,^{1–3} various pro- and anti-angiogenic factors that contribute to tumor angiogenesis have been identified, and has led to an accumulating interest and attempts at targeting various angiogenic pathways in hopes of improving cancer therapeutics. Two families of angiogenic-specific cytokines, vascular endothelial growth factor (VEGF) and angiopoietins, have attracted special attention because of expression of their cognate receptor tyrosine kinases almost exclusively by endothelial cells (ECs).⁴ VEGF,

through activation of VEGFR1 and VEGFR2, promotes EC differentiation, proliferation, migration, and formation of primitive tubules.^{3,4} VEGF is known to be a potent mediator of embryonal and adult vascular development, in addition to being a key regulator of tumor angiogenesis, especially malignant human astrocytomas.^{5–10} Angiopoietins, by modulating activation of their EC-specific receptor tyrosine kinase Tie2 (also known as Tek), plays a crucial role in embryonal and adult vessel development, however, their role in tumor angiogenesis remains relatively unknown.^{4,11–15}

Tie2 is the first known receptor tyrosine kinase to be dually regulated *in vivo* by both an activator angiopoietin-1 (Ang1) and an inhibitor angiopoietin-2 (Ang2). Ang1 activation of Tie2 induces vessel stability, by increasing the interaction of ECs with the surrounding smooth muscle cells (SMCs) and pericytes of the extracellular matrix.^{4,16} In contrast, Ang2 antagonizes Ang1, thereby destabilizing the vessel and exposing ECs to angiogenic factors such as VEGF that facilitate neo-angiogenesis.^{4,16} Furthermore, with accumulating knowledge of the downstream signal transduction pathways activated by Tie2,^{17–19} the biological role of this receptor is proving to be quite complex. In addition to vessel stability, activation of Tie2 by angiopoietins regulates various aspects of EC biology such as survival and migration.^{20–23} There is also accumulating evidence suggesting that Tie2 activation regulates angiogenesis in a highly context and tissue-dependent manner, with close collaboration with VEGF and potentially other angiogenesis regulators.^{24–28}

The molecular mechanism(s) by which Tie2 regulates tumor angiogenesis, especially malignant human astrocytomas, also known as glioblastoma multiforme (GBM) are not well known. Our report is the first to address the functional role of Tie2 receptor signaling in human astrocytomas. Our findings suggest that Tie2 activation contributes significantly to astrocytoma angiogenesis and overall growth. This provides evidence that Tie2 can act as a potential novel therapeutic target in human GBMs, which can be used either independently or in conjunction with other anti-angiogenic therapies.

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Materials and Methods

Cells and Reagents

Established human GBM cells, U-87 MG, were obtained from the American Type Culture Collection (Rockville, MD) and maintained in Dulbecco's minimal essential medium (Cellgro, Herndon, VA), supplemented with 10% fetal bovine serum and penicillin-streptomycin antibiotics. Human umbilical vein endothelial cells (HUVECs) were obtained from the American Type Culture Collection and maintained in HAM's media (Clontech, Palo Alto, CA). 3T3-Tie2 cells were generated by transfecting NIH-3T3 cells to stably express Tie2, which were maintained in Dulbecco's minimal essential medium plus 250 μ g/ml of G418. All cell lines were grown at 37°C in a 5% CO₂ incubator. Sf9 insect cells were cultured as monolayers in Sf9 media (Life Technologies, Inc., Grand Island, NY) at 27°C. Mouse monoclonal anti-ExTek antibody (Ab33) was generated by using human ExTek.6His as an antigen.²⁹

Purification of ExTek.6His Protein

ExTek is a soluble protein that contains the extracellular portion of the Tie2 receptor and is produced using a baculovirus expression system.²⁹ Briefly, baculovirus-ExTek.6His at ~1 pfu/cell was used to infect Sf9 cells for 72 hours at 27°C and the supernatant collected and dialyzed against 8 L of phosphate-buffered saline (PBS) (pH 8.0) for 48 hours. Using the 6HIS tag on the ExTek protein, ExTek was purified using a Talon cobalt-based resin-kit (Talon Kit; Clontech, Palo Alto, CA). Mock control solution was generated from the supernatant of uninfected SF9 cells following the same purification procedure. Aliquots of purified ExTek.6His protein and mock control solution were analyzed on an 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel with Coomassie stain (~90 kd). Western blot analysis with an ExTek-specific antibody (Ab33) in 5% skim milk-TBST was used to demonstrate the specificity of the protein (Figure 1A). ExTek was stored at -80°C and used within 2 weeks of purification.

Tie2 Phosphorylation

Tie2 phosphorylation was tested in 3T3 cells stably transfected to express Tie2 receptor (3T3-Tie2) because the receptor density and phosphorylation status of Tie2 can be detected much more clearly than if HUVECs were used (according to our experience and personal communication with other labs). 3T3-Tie2 cells (10⁴) were grown in a six-well plate to confluence, media removed, and cells washed with PBS and pretreated with 1 mmol/L sodium orthovanadate, pH 8.0, for 10 minutes at 37°C.¹⁷ To induce Tie2 phosphorylation, the receptor was stimulated with Ang1 present in the conditioned media (CM) of stably transfected malignant human astrocytoma cell lines overexpressing Ang1, established in our lab.³⁰ To test whether ExTek can inhibit Tie2 phosphorylation, 0,

Figure 1. Characterization of ExTek. **A:** Western blot analysis of 1 μ l of purified ExTek protein using Ab33 that specifically recognizes ExTek. **B:** Tie2 phosphorylation assay using 3T3-Tie2 cells stimulated by Ang1 (CM of stable cell lines overexpressing Ang1 was used as the source of Ang1). Activation/phosphorylation of Tie2, as determined by anti-phosphotyrosine Tie2 immunoblotting (**top**), is inhibited by the addition of ExTek (500 ng/ml). ExTek prevents phosphorylation of Tie2 and maintains the phosphorylation of the receptor at baseline control or orthovanadate control levels. An equal amount of Tie2 receptor density was ensured in each lane (**bottom**), as confirmed by stripping and reprobing the membrane with Tie2 antibody (**bottom**). **C:** Tubule HUVEC bioassay. HUVECs formed capillary-like tubes after 48 hours of exposure to Ang1. ExTek (500 ng/ml) inhibited this Ang1-induced tube formation. Original magnification, $\times 100$ (**C**).

100, 300, or 500 ng of ExTek was added to the 3T3-Tie2 cells, followed by stimulation with Ang1 CM for varying times. After stimulation, cells were washed three times with PBS containing phosphatase inhibitors (1 mmol/L orthovanadate, 10 μ g/ml aprotinin, 1 mmol/L phenylmethyl sulfonyl fluoride, 10 μ g/ml leupeptin) and lysed with phosphorylation lysis buffer. Lysates were immunoprecipitated with 3 μ g of Tie2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and protein A-Sepharose (Invitrogen, Carlsbad, CA) and run on an sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel. The membranes were blocked in 2% bovine serum albumin in TBST buffer for 4 hours before Western blotting with anti-phosphotyrosine antibody (mouse monoclonal 4G10, UBI, 1:1000 dilution) and bands detected using enhanced chemiluminescence. Membranes were exposed and developed under the same conditions and using the same exposure times when two or more membranes were to be compared, as in Figure 7. Membranes were stripped and reprobed with anti-Tie2 antibody in 5% skim milk-TBST to ensure equal loading and receptor density in each lane (Figure 1B). Tie2 phosphorylation was also analyzed *in vivo* in the GBM xenografts after treatment with ExTek (Figure 7), using similar conditions and reagents to the *in vitro* experiments outlined above. Tie2 receptor phosphorylation status was semiquantitatively measured using a computer image analysis to digitize the Western blot and calculate band intensity. A ratio was calculated of the band intensity seen on phospho-Tie2 blot to the band intensity seen on Tie2 receptor blot.

Tube Formation Bioassay

Fibrinogen (Calbiochem, La Jolla, CA) was dissolved in serum-free media and polymerized using a low concentration of thrombin (Sigma-Aldrich, St. Louis, MO) and

Figure 2. Expression profile of Tie2 in human GBM specimens and explant xenografts. **A:** Immunohistochemical analysis of normal brain (NB), low-grade astrocytoma (LGA), and GBM for Tie2 demonstrates increasing Tie2 expression in the ECs with increasing grade of astrocytoma (arrowheads). **B:** Immunohistochemical characterization of human GBM explants grown as subcutaneous xenografts in NODSCID mice. Similar to human GBM specimens, the explants were GFAP-positive [GFAP (glial fibrillary acidic protein), which is an intermediate filament present in glial cells and used to characterize the astrocytic cell type origin of GBM tumors]. The explants expressed high levels of Tie2 on the vessel ECs, high levels of Ang1 in the astrocytoma cells and Ang2 on the vessel ECs. **C:** Tie2 anti-phosphotyrosine immunoblot assay demonstrating increased Tie2 phosphorylation in both operative GBM specimens and tumor explants grown in NODSCID mice compared to control normal brain.

soaked in Dulbecco's minimal essential medium media for 2 hours at 37°C to inactivate thrombin.³¹ HUVECs at $\sim 3 \times 10^4$ cells were plated in each well of a six-well plate and allowed to adhere for 3 hours. Media was removed and replaced with control media or combined with CM from the Ang1-overexpressing stable cell lines (Zadeh and colleagues, submitted). To verify that ExTek could inhibit Tie2 activation and tube formation, ExTek was added at concentrations of 0, 100, 300, or 500 ng per well of six-well plates containing Ang1-CM. Presence of endothelial tubes was assessed after 48 hours using the inverted microscope at high power ($\times 100$) (Figure 1C).

In Vivo Xenograft Models

Subcutaneous Human GBM Explants

Human operative samples, of pathologically confirmed GBM specimens, were transplanted in the subcutaneous space of NODSCID mice. A palpable tumor grew within 80 days of transplant, with the GBM explants in repeat passages found to retain their astrocyte-specific GFAP (glial fibrillary acidic protein) staining and other pathological characteristics similar to the original GBM, including increased vascularity and necrosis (Figure 2, A and B). Additionally, the angiogenic profile of elevated VEGF, VEGFR, Tie2, and angiopoietin expression, remained similar to human GBM tumors that we have previously characterized.³² Animals were sacrificed by cervical dis-

location when tumor size reached 2.5 cm $\times$ 2.5 cm, according to animal protocols. Two hours before sacrifice, animals were injected with BrdU to detect the extent of proliferating cells. Tumors were dissected and collected for both paraffin section in formalin and frozen samples in liquid nitrogen.

Intracranial Xenografts

For orthotopic GBM models, human U87 GBM cells (10^6) were stereotactically injected 3-mm deep into the frontal cortex of NODSCID mice. U87 cells express high levels of ANG1 and VEGF as we have previously shown.³² To achieve repeated delivery of ExTek into the tumor center, a modified guide-screw technique was used.³³ The animals were followed clinically and when they exhibited symptoms consistent with failure to thrive as per animal care protocols, the mice were sacrificed by perfusion fixation after BrdU injection. The brains were dissected out and placed in formalin.

ExTek Treatment of GBM Xenografts

Subcutaneous Human GBM Explants

ExTek treatment was started when explants reached a palpable size of ~ 0.1 cm $\times$ 0.5 cm (tumor volume = 0.025 cm³), with bi-weekly direct intratumoral injection of 0.1 mg of ExTek suspended in 100 μ l of PBS, pH 7.6, and mock vehicle solution treatment for control tumors. Twenty explants were randomized to ExTek treatment, five to no treatment and five to vehicle treatment groups. Tumor volume [using the formula (length $\times$ width²)/2] was measured bi-weekly in a blinded manner by two independent observers and the animals sacrificed when tumor size reached beyond 2.5 cm $\times$ 2.5 cm.

Intracranial Xenografts

ExTek treatment was started 3 weeks after intracranial injection of U87 cells. Before treatment, five animals were sacrificed at random to ensure tumor formation and confirm that screw placement was at an ideal location for drug delivery to the tumor center. Treatment with ExTek included a total of 30 to 50 μ g of ExTek suspended in 10 to 20 μ l of PBS delivered into the tumor centers every other day. A total of 20 mice received ExTek, 5 received PBS and 10 received no treatment.

Immunohistochemical Analysis of GBM Xenografts

Immunohistochemical analysis was performed on paraffin-embedded sections with antibodies to: Ki-67 (polyclonal rabbit no. A0047, 1:400; DAKO, Carpinteria, CA); Factor VIII (polyclonal rabbit no. A0082, 1:2500; DAKO); GFAP (polyclonal rabbit, 1:3000; DAKO); Ang1, Ang2, and Tie2 (polyclonal goat, 1:200, 1:400; Santa Cruz); Tie2 (polyclonal rabbit, 1:400; Santa Cruz). A goat anti-mouse antibody (1:200; Zymed, South San Francisco, CA) was

used as the secondary antibody and antigens were detected using the avidin-biotin complex method (Vector Laboratories, Burlingame, CA) and diaminobenzidine substrate. All stains were independently reviewed by a neuropathologist (PS). Double staining for ECs with BrdU, terminal dUTP nick-end labeling (TUNEL), and smooth muscle actin (SMA) were performed by Factor VIII antibody conjugated to a secondary fluorescein isothiocyanate antibody together with colorimetric detection of the second antigen.

Tumor Vascularity

Four different tumor portions were each cut at 5- μ m consecutive paraffin sections and stained with anti-Factor VIII (1:2000, DAKO) followed by detection with an avidin-biotin complex method 3,3'-diaminobenzidine (VectaStain 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 (HPF) ($\times 400$) and in five HPFs at vascular hot spots. EC-positive index was determined by measuring the proportion of EC-positive staining cells per field of view in 10 HPFs. Vessel lumen size was determined by measuring the length and width of the cross sections of Factor VIII-positive vessels and using the longest value as the diameter of the vessel. All analyses were performed using the MicroComputer Image Device (MCID-Imaging Research, Inc.) linked to a color charge-coupled device camera (Sony DXC 970 MD) mounted on a transmitted-light microscope (Zeiss Axioskop). EC apoptosis was determined by double labeling with Factor VIII and fluorescein isothiocyanate-conjugated IgG as a secondary antibody and TUNEL stain, with the proportion of vessels undergoing apoptosis calculated in 15 HPFs. EC proliferation rate was determined by double labeling with Factor VIII and BrdU. Fluorescent staining of ECs with fluorescein isothiocyanate-conjugated Factor VIII antibody and SMA was used for measuring the extent of EC interaction with SMCs. Vessel maturity was calculated using by measuring the proportion of vessels associated with SMA-positive cells in 10 HPFs.³⁴

Statistical Analysis

All analyses were completed using StatView 4.1 for the Macintosh (Abacus Concepts). All errors were calculated as the SEM. The one-tailed Student's *t*-test was used to compare means (two sample, unequal variance) and *P* < 0.05 was considered statistically significant.

Results

Biological Activity of ExTek

ExTek is a soluble protein that contains only the extracellular portion of the Tie2 receptor and acts as a dominant-negative mutant by competing for ligands to Tie2 and inhibiting its activation.^{29,35} ExTek was purified using a baculovirus expression system, with the purified product

at ~90 kd (Figure 1A). The specificity of the purified protein was determined by Western blot analysis using a specific antibody to ExTek (Ab33), which identified the single ~90-kd band observed on Coomassie stain. *In vitro* ExTek was found to bind both Ang1 and Ang2 by co-immunoprecipitation experiments and addition of purified ExTek to HUVEC and U87 cell lines *in vitro* did not alter cellular proliferation or morphology (data not shown). The ability of ExTek to inhibit Tie2 activation *in vitro* was demonstrated using two assays. First, Tie2 phosphorylation induced by Ang1 was inhibited by addition of 500 ng of purified ExTek protein (Figure 1B). Second, Ang1-induced tube formation of HUVECs grown on a fibrin gel matrix was inhibited by 500 ng of ExTek (Figure 1C).

Characterization of Human GBM Tumor and GBM Xenografts

Expression profile of Tie2 was established across increasing malignancy grades of human operative specimens of astrocytomas using immunohistochemical analysis and Tie2 activation status determined by Tie2 phosphorylation blots (Figure 2). Similarly, human GBM explants grown as subcutaneous xenografts in NODSCID mice were characterized for Tie2 expression and activation status. We found an increase in Tie2 expression with increasing grade of human astrocytomas, with the highest levels seen in GBM operative specimens compared to low-grade astrocytoma (LGA0) and normal brain (NB) (Figure 2A). Tie2 expression was restricted exclusively to the ECs, in both human astrocytoma specimens and explant xenografts (Figure 2, A and B). Associated with increased Tie2 expression, was an increase in Tie2 phosphorylation, suggesting an increased activation of Tie2 signal transduction in GBMs (Figure 2C). Similar to human GBMs, GBM explants also demonstrated increased expression and activation of Tie2 together with marked overexpression of Ang1 by astrocytoma cells and Ang2 by ECs (Figure 2B).

Growth Restriction of GBM Explants Using ExTek

Two xenograft models of GBMs were used to test the effect of Tie2 inhibition on tumor vascularity and growth. First, the subcutaneous GBM explant xenograft model, which maintains the angiogenic profile and pathological features similar to the primary human GBMs (Figure 2B), allowed continuous measurement of tumor growth. Second, the orthotopic intracranial GBM cell line xenograft model, allowed for evaluation of the potential impact and role of the tumor microenvironment on inhibiting Tie2 in GBMs. In subcutaneous GBM explant xenografts, ExTek delivery into the tumor center twice weekly restricted the growth rate of tumors significantly compared to untreated or PBS vehicle-treated controls (Figure 3A). Final tumor size in the ExTek-treated tumors was decreased by 32% (Figure 3B). Tumor proliferation, as determined by BrdU labeling, was 55% lower in ExTek-treated tumors com-

Figure 3. Effect of ExTek on GBM explant growth. **A:** Growth curve of GBM explants treated with ExTek. There is a significant decrease in tumor growth rate compared to the control and vehicle-treated tumors. **B and C:** The final tumor volume and tumor proliferation (BrDU-positive cells) was decreased by 32% and 55%, respectively, in the ExTek-treated explants, compared to nontreated or PBS vehicle-treated controls. (For C the bar graphs lacking an error bar have a smaller than 0.1 SEM.)

pared to controls (Figure 3C), with significantly higher tumor necrosis (Table 1). In the intracranial orthotopic model, ExTek was delivered to the center of tumors three times a week via a guide-screw technique. Mice treated with ExTek survived significantly longer than control or PBS-treated animals (Figure 4, A and B, and Table 2). The 46% increase in survival correlated with a decrease in tumor proliferation rate (Figure 4C) and a threefold increase in tumor cell apoptosis (Table 2).

Effect of ExTek on Tumor Vascularity

In both the subcutaneous and intracranial GBM models, ExTek treatment resulted in a statistically significant drop in MVD (Tables 1 and 2). In addition, the vessels were abnormally dilated, with 66% larger size compared to the control tumor vessels (Figure 5, Tables 1 and 2). Furthermore, the characteristic hyperproliferation and multilay-

ering of ECs, seen in primary human GBMs and subcutaneous GBM explant xenografts, was disrupted in ExTek-treated GBM explants (Figure 5A, Tables 1 and 2). In the U87 intracranial model, ExTek treatment also resulted in dilatation of tumor vessels, both in the center and peripheral edge of the tumors (Figure 5B, Table 3). In addition to the tumor vessels, the effect of ExTek also extended to result in abnormal large vessels in the adjacent normal brain parenchyma, suggesting that the soluble ExTek has diffused outside of the tumor border and altered the extra-tumoral vessels in the normal parenchyma (Figure 5B).

Mechanisms of ExTek Action on Tumor Vasculature

We believe that the observed effects of ExTek on the tumor vasculature, was as a direct consequence of in-

Table 1. Parameters of ExTek-Treated GBM Explants

	Control <i>n</i> = 5	PBS <i>n</i> = 5	ExTek <i>n</i> = 20
Final tumor size	5.4 cm ³ (SEM = 0.9)	5.4 cm ³ (SEM = 0.8)	3.7 cm ³ * (SEM = 0.45) <i>P</i> = 8 × 10 ⁻⁴
Proliferation index (no. BrDU-positive cells/field)	2.3% (SEM = 0.05)	2.1% (SEM = 0.08)	1.0%* (SEM = 0.03) <i>P</i> = 2.6 × 10 ⁻⁶
MVD (no. vessel lumen/high-power field)	7.6 (SEM = 0.9)	7.2 (SEM = 1.2)	2.5* (SEM = 0.8) <i>P</i> = 3.5 × 10 ⁻⁵
ECP (% EC-positive cells/field)	0.12 (SEM = 0.046)	0.105 (SEM = 0.04)	0.01* (SEM = 0.013) <i>P</i> = 0.009

*, Statistical significant difference between the ExTek-treated and control/PBS-treated values.

Figure 4. Effect of ExTek on intracranial GBM growth. **A:** Kaplan-Meier survival curve of mice treated with ExTek (three times per week) versus control and PBS vehicle-treated animals. **B:** There was a significant increase in survival of mice treated with ExTek, with the overall total survival increased by 46%. **C:** Number of BrdU-positive cells (proliferating cells) in the ExTek-treated tumors was lower than in control and PBS vehicle-treated tumors. (For **C** the bar graphs lacking an error bar have a smaller than 0.1 SEM.)

hibiting Tie2-mediated signaling. In the majority of the ExTek xenografts, there was an overall decrease in Tie2 phosphorylation, compared to that seen in vehicle-treated and control tumors (Figure 7). Semiquantitative analysis of the extent of decrease in Tie2 activation shows that the Tie2-phosphorylated status/Tie2 receptor density ratio in ExTek-treated tumors was 0.49 SEM 0.07 compared to 0.3 SEM 0.01 ($P = 0.0039$) in vehicle-treated and control tumors, which is approximately a 38% decrease in Tie2 phosphorylation status with ExTek treatment. The effect of ExTek on tumor vascularity was evaluated by studying a number of biological properties of ECs that Tie2 activation has been postulated to regulate. First, EC survival was evaluated by double labeling the ECs with Factor VIII and TUNEL stain. ExTek-treated GBM xenografts had a 41% increase in the number of vessels that were undergoing apoptosis compared to vessels in control tumors (Figure 6A, Table 3). Second, EC proliferation was evaluated by double labeling the ECs with Factor VIII and BrdU stain (data not shown).

There was no significant difference in the number of BrdU-positive ECs between control and ExTek-treated tumors, which is consistent with the previously described nonmitogenic role of Tie2 activation in ECs.⁴ Third, the interaction between ECs and SMCs, postulated to be a key biological consequence of angiopoietin-mediated Tie2 activation, was examined by double labeling of the ECs with Factor VIII and surrounding SMCs with SMA (Figure 6B, Table 3). The percentage of Factor VIII-positive ECs that were juxtaposed to SMA-positive SMCs were significantly higher (64.8%) in the control PBS-treated explant xenografts, compared to ExTek-treated tumor vessels (20%) (Figure 6B, Table 3). These results taken together, suggest that inhibition of Tie2 by ExTek results in increased EC apoptosis. Furthermore, the tumor vessels are abnormally dilated because of diminished maturity index of the vasculature as evidenced by loss of interaction between ECs and surrounding SMCs. We postulate this leads to a decreased number of functional tumor vessels, which ultimately results in de-

Table 2. Parameters of ExTek-Treated Intracranial U87 Tumors

	Control <i>n</i> = 5	PBS <i>n</i> = 5	ExTek <i>n</i> = 20
Total survival (days)	58 (SEM = 3.1)	60 (SEM = 4.7)	85 (SEM = 3.7) $P = 1.5 \times 10^{-5}$
TUNEL-positive	0.9% (SEM = 0.28)	1.1% (SEM = 0.08)	3.6%* (SEM = 0.03) $P = 2.6 \times 10^{-6}$
MVD (no. vessel lumen/high-power field)	17.3 (SEM = 4.7)	17.0 (SEM = 5.2)	12.0* (SEM = 0.14) $P = 0.166$
ECP (% EC-positive cells/field)	0.109 (SEM = 0.04)	0.101 (SEM = 0.04)	0.05* (SEM = 0.004) $P = 0.009$

*Statistical significant difference between the ExTek-treated and control/PBS-treated values.

Figure 5. Effect of ExTek on tumor vascularity. **A:** Immunohistochemical analysis of tumor vascularity using Factor VIII (Ab:VWF) staining on subcutaneous GBM explants (i). The characteristic multilayering of ECs, seen in human GBMs is preserved in GBM explant xenografts (control + PBS). However, in ExTek-treated tumors the EC layering is absent, with abnormally dilated vessels seen throughout the tumor. Computer-assisted image analysis reveals a decrease in MVD (ii), in addition to a decrease in proportion of cells that are EC-positive per field of view (iii). **B:** Immunohistochemical analysis of tumor vascularity using Factor VIII staining on intracranial GBM cell line xenografts (i). ExTek treatment resulted in striking dilatation of vessels throughout the tumor center and periphery compared to control tumors in which the vessels are well formed. Additionally, the vessels in the normal brain parenchyma, adjacent to the tumor were dilated (arrowheads) in ExTek-treated tumors compared to control in which the vessels in the adjacent parenchyma were normal. There was a decrease in MVD (ii) and an increase in vessel diameters (iii) compared to control/PBS-treated tumors. (For Bii the bar graphs missing an error bar have a smaller than 0.1 SEM.)

creased proliferation and increased apoptosis of the tumor cells (Figures 3 and 4).

Discussion

Astrocytomas are the most common primary adult brain tumor, with the most malignant grade of astrocytomas, GBM, being characterized by florid and abnormal tumor angiogenesis. GBM vessels are abnormal in both structure and function, with multilayering of ECs and SMCs.^{36,37} Often there is intratumoral hemorrhage, arterial-venous shunting, and thrombosis, with lack of a normal blood-brain barrier and cerebrovascular autoregulatory response.^{36–39} The presence of these distinct structural and physiological aberrations makes the GBM vasculature an excellent model for studying the role of molecular regulators in tumor angiogenesis.

VEGF and VEGFRs are overexpressed in GBMs and have been shown to be functionally relevant using various inhibitory strategies in preclinical models.^{6,8,40–44} This has led to several clinical trials in GBMs targeting

Table 3. Vessel Characteristics in Control Versus ExTek-Treated Tumors

	Vessel size (μmol/L)	EC + TUNEL (% association)	EC + SMA (% association)
Ctl	11.3 (SEM = 0.9)	35%	64.8%
ExTek	19.1 (SEM = 1.6) P = 0.00058	76%	20%

*Statistical significant difference between the ExTek-treated and control/PBS-treated values.

Figure 6. Effect of ExTek on EC apoptosis and interaction with SMCs. **A:** Double staining of tumor-associated ECs (with anti-Factor VIII immunofluorescence staining) and TUNEL-positive apoptotic cells (brown). Approximately 35% of vessels in control GBMs versus 76% of the ExTek-treated GBMs are TUNEL-positive. **B:** Double staining for ECs (fluorescent green) and SMCs with smooth muscle antigen (brown). In control tumors 64.8% of Factor VIII-positive vessels are associated with SMA, whereas in ExTek-treated tumors only 20% of the ECs have SMA-positive vessels.

Figure 7. Inhibition of Tie2 phosphorylation by ExTek in GBM explants. **A:** Anti-phosphotyrosine Tie2 immunoblots of representative GBM explants treated with intratumoral injections of ExTek (**bottom**) or PBS control (**top**). ExTek-treated GBM explants tended to have variably decreased levels of phosphorylated Tie2 compared to PBS controls. Equivalent Tie2 receptor density loaded in each lane is ensured with blots being blocked with anti-Tie2 antibody.

either VEGF or VEGFR. However, clinical trials have not demonstrated a similar benefit, emphasizing that other relevant modulators of angiogenesis contribute significantly to the overall angiogenic growth of tumors. The role of Tie2 receptor activation by angiopoietins in astrocytoma angiogenesis has not been investigated. Previously we and others have reported on the expression profile of the ligands for Tie2, angiopoietins, in human astrocytomas.^{12,30,32,45–48} Compared to normal brain and lower grade astrocytomas, GBM tumor cells express the highest amount of Ang1, with the GBM-associated ECs expressing increased levels of Ang2. In this study we focused on the role of Tie2 signal transduction in astrocytomas. Our data using human operative tumor specimens demonstrates that Tie2 receptor expression and activation is markedly increased in GBMs compared to low-grade astrocytomas and normal brain (Figure 2), suggesting that activation of Tie2 is involved in regulating the vascularization of GBMs.

To date the functional role of Tie2 activation in tumor angiogenesis has been examined only in breast and melanoma cancer xenograft models,^{29,35,44} but not in astrocytomas. In this study we have shown that inhibition of Tie2 activation resulted in a significant alteration in tumor vascularity and decreased growth, of both subcutaneous and intracranial xenograft models of GBMs. ExTek treatment of subcutaneous GBM explant xenografts reduced overall tumor growth by 32% and prolonged survival of intracranial orthotopic GBM cell line xenografts by 46% (Figures 3 and 4). The restricted tumor growth and decreased tumor cell proliferation in both GBM models can be attributed to disrupted tumor vascularization, as evidenced by dilated vessels suggesting lack of maturation of the neovasculature formed by the tumor and inefficient blood flow to the tumor cells. In both xenograft models significant vascular abnormalities, secondary to inhibition of Tie2 phosphorylation by ExTek (Figure 7), resulted in increased EC apoptosis, a significant decrease in MVD and overall disruption of the tumor vasculature. A striking feature present in both the subcutaneous and intracranial GBM xenografts (Figure 5), was the abnormally dilated and immature vessels. We propose this is secondary to a decrease in the maturation index of the vessels, as evidenced by a loss of interaction

between ECs and perivascular supportive SMCs (Figure 6). We postulate that inhibition of Tie2 activation by ExTek prevents formation of new tumor vessels (decreased MVD) and dampens the response and sensitivity to angiogenic stimuli that are critical for neoangiogenesis and growth of solid tumors such as GBMs.

Soluble receptor tyrosine kinases are known to inhibit activation of the full-length receptor by binding available ligands, and this approach has been established for Tie2.^{35,44,49} However, a question that remains is whether the inhibition of angiogenesis by ExTek is primarily because of its effects on Ang1 or Ang2. Studies on colon and squamous cell carcinoma xenograft models^{50,51} and our own observations of Ang2 overexpression in GBM xenograft models (Zadeh and colleagues, submitted), indicate that Ang2 overexpression results in dilated and abnormal tumor vessels. In ExTek-treated GBM xenografts, the vessel morphologies are similar to Ang2-overexpressing tumors (Figure 5), implying that the effects of ExTek are primarily by inhibiting Ang1. Furthermore, Ang1 has been demonstrated in both physiological and tumor angiogenesis to promote vessel stability by recruiting SMCs and increasing EC survival.^{12,20,21,50,52} Consistent with this, the current study with ExTek exhibited many of the vascular morphological abnormalities that would be predicted by inhibiting the angiogenic role of Ang1, such as decreased SMC recruitment and increased EC apoptosis (Figure 6). Inhibition of Ang1 by ExTek would be predicted to result in dilated vessels as we observed, because of lack of smooth muscle and other supportive perivascular cells. We therefore postulate, through these ExTek-mediated Tie2 inhibition studies, Ang1 to be the principal and dominant ligand to activate Tie2 and contribute to the characteristic pathological angiogenesis seen in human GBMs. An interesting observation that we are pursuing, is the role of Ang1 in the characteristic EC hyperproliferation in GBMs (Figure 5A), an angiogenic phenotype that is not observed in other solid human cancers.^{36,37} However, it is possible that the relative contributions of Ang1 and Ang2 to tumor angiogenesis is tumor-type- and tumor-stage-dependent. To answer these questions precisely, to decipher the mechanisms and contribution of each of the angiopoietins, will require development of specific inhibitors of each ligand, an area of future experimentation.

In conclusion, this study for the first time has shown that Tie2 signal transduction plays a significant regulatory role in the pathological vascular growth of GBMs. Furthermore it demonstrates that inhibition and prevention of the Tie2 kinase activity in GBMs leads to decreased tumor growth by disrupting tumor vascularity, both in subcutaneous and orthotopic xenograft models. This study, using ExTek as proof-of-principal, serves to provide the rationale for testing future strategies targeting Tie2 activation. This may include small molecule inhibitors of Tie2 or the angiopoietins that can be used either alone or in conjunction with other anti-angiogenic strategies, in hopes of improving our current anti-angiogenic treatment of GBMs.

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