Stem Cell–like Glioma Cells Promote Tumor Angiogenesis through Vascular Endothelial Growth Factor

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Abstract

Malignant gliomas are highly lethal cancers dependent on angiogenesis. Critical tumor subpopulations within gliomas share characteristics with neural stem cells. We examined the potential of stem cell–like glioma cells (SCLGC) to support tumor angiogenesis. SCLGC isolated from human glioblastoma biopsy specimens and xenografts potently generated tumors when implanted into the brains of immunocompromised mice, whereas non-SCLGC tumor cells isolated from only a few tumors formed secondary tumors when xenotransplanted. Tumors derived from SCLGC were morphologically distinguishable from non-SCLGC tumor populations by widespread tumor angiogenesis, necrosis, and hemorrhage. To determine a potential molecular mechanism for SCLGC in angiogenesis, we measured the expression of a panel of angiogenic factors secreted by SCLGC. In comparison with matched non-SCLGC populations, SCLGC consistently secreted markedly elevated levels of vascular endothelial growth factor (VEGF), which were further induced by hypoxia. In an in vitro model of angiogenesis, SCLGC-conditioned medium significantly increased endothelial cell migration and tube formation compared with non-SCLGC tumor cell–conditioned medium. The proangiogenic effects of glioma SCLGC on endothelial cells were specifically abolished by the anti-VEGF mab (Avastin, Genentech, South San Francisco, CA), was purchased from Sigma (St. Louis, MO) unless otherwise specified.

Chemicals and reagents. Neutralizing anti-VEGF antibody, bevacizumab (Avastin), in combination with the topoisomerase I inhibitor irinotecan (ZD6474) was highly effective against multiple central nervous system cancer xenografts, including gliomas (6). Receptor kinase inhibitors had limited benefit in clinical trials in gliomas (7, 8), but frequent glioblastoma patient tumor responses were observed using a neutralizing anti-VEGF monoclonal antibody, bevacizumab (Avastin), in combination with the topoisomerase I inhibitor irinotecan (9). Improvements in the efficacy of these treatments will require advances in our understanding of the molecular mechanisms promoting glioblastoma angiogenesis. Recent studies of glioblastomas and other brain cancers identified tumor subpopulations that share characteristics with normal neural stem cells (10–14). Stem cell–like glioma cells (SCLGC) express neural stem cell markers (including the cell-surface antigen prominin-1/CD133), are capable of self-renewal, form neurosphere-like spheroids, and differentiate into multiple nervous system lineages (neurons, astrocytes, and oligodendrocytes; refs. 10–13). When xenotransplanted into the brains of immunocompromised rodents, human SCLGC show greater potential than non-SCLGC cancer cells to initiate tumor formation (12, 13). The mechanisms responsible for the differential tumorigenic capacity of SCLGC remain unknown, but non-SCLGC do survive when transplanted intracranially (13). As angiogenesis represents a critical step in tumor formation (the “angiogenic switch”), we examined whether SCLGC contribute to tumor formation through promoting angiogenesis. We now report that SCLGC enhance glioma angiogenesis through a VEGF-dependent mechanism.

Materials and Methods

Chemicals and reagents. Neutralizing anti-VEGF antibody, bevacizumab (Avastin, Genentech, South San Francisco, CA), was purchased from Sigma (St. Louis, MO) unless otherwise specified.

Patient biopsy specimens. Specimens from patients undergoing biopsy for newly diagnosed or recurrent glioblastoma were collected under a Duke University Institutional Review Board–approved protocol. Pathologic diagnosis was confirmed by R.E.M.

Human glioma xenografts. D456MG xenograft was derived from a biopsy specimen from a 4-year-old child with glioblastoma. D54MG xenografts.
xenograft is the Duke subline of A-172, which was derived from a 54-year-old male with glioblastoma. Xenografts were maintained in the flanks of athymic BALB/c \( \text{nu/nu} \) mice and early passages were used to minimize genetic drift.

**Purification and characterization of stem cell–like glioblastoma cells.** SCLGC were derived from tumors from patients or xenografts as previously described (10–13). Briefly, tumors were disaggregated, labeled with an allophycocyanin- or phycoerythrin-conjugated CD133 antibody (Miltenyi Biotec, Auburn, CA), and sorted by fluorescence-activated cell sorting (FACS). Glioma cells derived from xenografts were confirmed to be human through FACS analysis with the FITC-labeled 3B4 human specific antibody (15). Tumor spheroids were maintained and passaged as previously described (13). Immunofluorescence studies confirmed spheroid expression of stem cell markers and lineage markers when subjected to differentiation conditions as previously described (16).

**Intracranial tumor assays.** CD133\(^+\) and CD133\(^{-}/C0\) tumor cell populations were segregated by cell sorting. After short-term culture, 10,000 cells were implanted into the right frontal lobes of athymic BALB/c \( \text{nu/nu} \) mice under a Duke University Institutional Animal Care and Use Committee–approved protocol as previously described (6). Mice were maintained for 4 weeks or until the development of neurologic symptoms. Brains of euthanized mice were collected, fixed in formalin, paraffin embedded, and sectioned, or were frozen rapidly in liquid nitrogen for sections.

**Tumor vascular quantification.** Blood vessel density in frozen tumor sections was quantified as previously described (17).

**Angiogenesis antibody array analysis.** Conditioned media were harvested from matched CD133\(^+\) and CD133\(^{-}/C0\) glioblastoma tumor cell cultures grown in 60-mm plates in neural basal medium without growth factors for 24 hours. Hypoxia was induced through maintenance of cells in a closed syringe for 24 hours. TransSignal Human Angiogenesis Antibody Arrays (Panomics, Fremont, CA) were incubated with 2 mL of conditioned medium for 1.5 hours and then replaced with another 2 mL for 1.5 hours (total, 4 mL conditioned medium, 3 hours of incubation) and processed according to the instructions of the manufacturer. Intensity of VEGF expression was quantified with ImageJ software.\(^7\) Total mean intensity = area × mean intensity.

\(^7\)http://rsb.info.nih.gov/ij/.

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**Figure 1.** SCLGC form more vascular and necrotic tumors when grown in the brains of immunocompromised mice. Ten thousand cells derived from CD133\(^+\) or CD133\(^{-}/C0\) tumor cell populations cultured from D456MG xenografts were implanted into the right frontal lobes of athymic nude BALB/c mice. Mice were sacrificed after 28 days. A, four pairs of representative brains bearing tumors derived from CD133\(^+\) and CD133\(^{-}/C0\) cells. B and C, pathologic morphology of brain tumors derived from CD133\(^+\) and CD133\(^{-}/C0\) populations. The brains were fixed in formalin, paraffin embedded, cut into 5-μm sections, and then stained with H&E. B, two of six brains with CD133\(^+\) tumors implants had tumor formation (>400). C, in contrast, all brains implanted with CD133\(^{-}/C0\) tumor cells displayed large tumors with widespread necrosis, hemorrhage, and neoangiogenesis (>400). D, the blood vessel density in tumors derived from CD133\(^+\) and CD133\(^{-}/C0\) cells was measured. Tumor-bearing brains were snap frozen, cut into 6-μm frozen sections, fixed, immunostained with rat anti-mouse CD31 antibody (or IgG control antibody), and counterstained with hematoxylin. Representative images (>400). The densities of CD31 stained blood vessels were quantified. *, \( P = 0.0002 \), vascular density of tumors derived from CD133\(^+\) cells versus CD133\(^{-}/C0\) cells (Student’s t test).
VEGF ELISA. Human VEGF Quantikine ELISA Kits (R&D Systems, Minneapolis, MN) were used according to the directions of the manufacturer. Two-hundred microliters of conditioned media were collected from triplicate samples.

Endothelial migration assays. Media conditioned by CD133⁺ or CD133⁻ populations for 24 hours were added to the bottom chambers of 24-well tissue culture plates in triplicate. Human microvascular endothelial cells (20,000) were added to the upper chambers of Transwell assays (BD Biosciences, Franklin Lakes, NJ). In experiments with a VEGF neutralizing antibody, bevacizumab or immunoglobulin G (IgG) control (final, 0.5 mg/mL) was added to conditioned media 30 minutes before addition of endothelial cells. Cells were allowed to invade for 14 hours and then fixed, stained, and quantified.

Endothelial tube formation assays. Media conditioned by CD133⁺ or CD133⁻ populations for 24 hours were added to 2,000 human microvascular endothelial cells in sextuplicate wells of Matrigel-coated 96-well plates (BD Biosciences). In VEGF neutralizing experiments, bevacizumab or IgG control (final, 0.5 mg/mL) was added to conditioned media 30 minutes before addition to endothelial cell cultures in triplicate. Cells were incubated for 16 hours, imaged, and quantified.

Xenograft assays. Matched CD133⁺ and CD133⁻ glioma cell populations were implanted in either s.c. or intracranial locations with the specified cell number. After establishment, tumor-bearing animals were treated with either bevacizumab or a control IgG (5 mg/kg i.p. qd) for a total of 21 days (s.c.) or 19 days (intracranial) until harvest. Mice were euthanized and tumors or tumor-bearing brains were harvested, weighed, and examined.

Results and Discussion

Derivation of SCLGC. Central nervous system neoplastic stem cells were defined through their phenotypic similarities to neural stem cells (10–14). Prior reports investigated SCLGC from patient tumor biopsy specimens (10–13), but we additionally generated short-term cultures from glioblastoma xenografts derived from human patient biopsy specimens that were maintained in immunocompromised mice in either s.c. or intracranial locations. Human biopsy specimens validate biological processes directly in patient cancers, whereas human glioma xenografts permit the purification of large numbers of viable tumor cells. The CD133 cell-surface stem cell marker was used to select for a cell population enriched for SCLGC (13). Although the fraction of CD133⁺ tumor cells varied between sources, we detected between 2.9% and 4.3% of tumor cells expressed the CD133⁺ marker and formed spheroids (data not shown). We derived separate short-term cultures of CD133⁺ and CD133⁻ tumor cells through tumor disaggregation, labeling with CD133 antibody, and FACS sorting. The CD133⁺ population was >95% pure in repeated FACS analysis (data not shown). The tumor cell populations derived from human glioma xenografts grown in mice were composed nearly exclusively of human tumor cells (>99%) without significant contamination from murine cells through analysis with 3B4 antihuman antibody (data not shown).

Cultured CD133⁺ glioma cells from biopsy specimens and xenografts formed neurosphere-like spheroids when grown in stem cell medium (Supplementary Fig. S1A). These spheroids were capable of generating new spheroids under limiting passage conditions consistent with self-renewal (data not shown), expressed neural stem cell markers (CD133, Nestin, Sox 2, and Musashi 1; Supplementary Fig. S1B), and underwent multilineage differentiation (neuronal, astrocytic, and oligodendrocytic) under...
prodifferentiation conditions (Supplementary Fig. S2). In contrast, CD133⁺ tumor cells grew in an adherent fashion without spheroid formation, expressed only astrocytic markers, and underwent senescence on fulfilling the criteria of two distinct tumor cell populations: SCLGC and non-SCLGC.

Tumors generated from CD133⁺ glioma population display increased tumor vascularity, necrosis, and hemorrhage. Recent studies of stem cell–like cancer cells have suggested that tumorigenesis is dichotomized between the SCLGC and non-SCLGC populations (12, 13). To investigate the tumorigenic capacity of our glioma subpopulations, 10,000 CD133⁺ or CD133⁻ glioma cells derived from multiple human glioblastoma patient specimens or two human glioma xenografts (D456MG and D54MG) were implanted into the right frontal lobes of athymic nude mice. Regardless of tumor source, every brain implanted with the CD133⁺ tumor cells displayed gross evidence of highly angiogenic and hemorrhagic tumors, in stark contrast to the brains implanted with CD133⁻ tumor cells (Fig. 1A and data not shown). Pathologic analysis of the brains implanted with CD133⁺ glioma cells showed large, highly proliferative and vascular tumors with widespread necrosis and hemorrhage (Fig. 1C and data not shown). No mice implanted with CD133⁻ tumor cells from human biopsy specimens or the majority of xenografts displayed evidence of brain tumor formation (data not shown). D456MG xenograft-derived CD133⁻/C0 tumor cells formed small tumors (in two of six mice in replicate experiments; Fig. 1B), permitting a comparison of vascularity between brains bearing CD133⁺ and CD133⁻ xenografts (data not shown). D456MG xenograft-derived CD133⁺ conditioned medium versus CD133⁻ conditioned medium (ANOVA); **, P = 0.0005, bevacizumab-treated CD133⁺ conditioned medium versus IgG control (ANOVA).

Stem cell–like glioma cancer cells express elevated levels of VEGF. To determine potential molecular mechanisms underlying the differential vascular phenotypes of SCLGC and non-SCLGC derived tumors, we compared the expression of regulators of angiogenesis secreted by CD133⁺ and CD133⁻ cells. Patterns of angiogenesis regulator expression differed between specimens but...
revealed modest changes in angiogenin, interleukin-8, interleukin-6, and basic fibroblast growth factor (Fig. 2 and Supplementary Fig. S3). However, VEGF levels in CD133+ glioma cell conditioned media were consistently up-regulated 10- to 20-fold compared with CD133- media (Fig. 2 and Supplementary Fig. S3) in populations derived from both patient biopsies and human glioma xenografts. Increased VEGF secretion in CD133+ versus CD133- cells was also confirmed through VEGF ELISA (Fig. 2D and Supplementary Fig. S3C). Furthermore, both CD133+ and CD133- glioma tumor cells increased VEGF expression in response to hypoxia, but CD133+ tumor cells expressed elevated VEGF levels relative to the CD133- cells under hypoxic conditions (Fig. 2 and Supplementary Fig. S3C). In summary, SCLGC consistently expressed higher levels of VEGF than the non-SCLGC under normoxic and hypoxic conditions.

SCLGC induce endothelial cell migration and tube formation. VEGF and other angiogenic factors potently regulate endothelial behavior in new vasculature formation. In in vitro models of angiogenesis, angiogenic factors promote endothelial cell migration and/or induce formation of vascular tubular structures when cultured on Matrigel. We tested the ability of conditioned media from both CD133+ and CD133- cells to modify endothelial cell phenotypes. CD133+ glioma cell conditioned medium consistently increased migration of human microvascular endothelial cells as compared with CD133- conditioned medium (Supplementary Fig. S4). The addition of conditioned medium from CD133+ glioma cultures also promoted endothelial cell tube formation in vitro by increasing human microvascular endothelial cell tube length, number of branch points, and tube complexity (Supplementary Fig. S5). Together these results suggest that CD133+ cells produce proangiogenic factors that can directly modify endothelial cell behavior.

Targeting VEGF inhibits effects of SCLGC on endothelial cells. Methods to inhibit tumor-associated angiogenesis are under development, including anti-VEGF antibodies and low molecular weight VEGF receptor tyrosine kinase inhibitors. One neutralizing anti-VEGF antibody, bevacizumab/Avastin, binds to human VEGF-A ligand to prevent endothelial cell receptor activation and has shown combinatorial efficacy with irinotecan (CPT-11) in high-grade glioma patients (9). To determine if the increased levels of VEGF in CD133+ glioma conditioned media represent the mechanism through which CD133+ glioma cells promote in vitro measures of angiogenesis, we examined the effect of bevacizumab antibody in our endothelial cell assays. Similar to the above studies, conditioned medium from CD133+ cells derived from a human glioblastoma specimen or a human xenograft, mixed with the IgG control antibody, induced a striking increase in human microvascular endothelial cell migration (Fig. 3A and Supplementary Fig. S6A) and tube formation (Fig. 3B and Supplementary Fig. S6B). Addition of bevacizumab to neutralize VEGF in CD133+ glioma conditioned medium blocked its proangiogenic effects on

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Figure 4. VEGF neutralizing antibody bevacizumab specifically suppresses SCLGC tumor growth and hemorrhage in vivo. A, treatment with VEGF-neutralizing antibody, bevacizumab, inhibits the in vivo tumor growth of xenografts derived from SCLGC and blocks the SCLGC-induced tumor hemorrhage in vivo. CD133+ cells (100,000) isolated from D456MG xenografts were implanted s.c. into two groups (five animals per group) of athymic nude BALB/c mice. Tumor-bearing animals were treated with either bevacizumab (5 mg/kg i.p. qd) or control IgG (5 mg/kg i.p. qd) for a total of 21 days until harvest. Mice were euthanized and tumors were harvested, weighed, and examined. Representative tumor-bearing mice and dissected tumors from the two groups. SCLGC-derived tumors from the control IgG group showed rapid tumor growth with extensive hemorrhage, but treatment with the VEGF neutralizing antibody bevacizumab significantly suppressed SCLGC tumor growth in vivo and dramatically reduced the tumor hemorrhage of xenografts derived from SCLGC. B, the VEGF neutralizing antibody bevacizumab shows limited efficacy against xenografts derived from non-SCLGC tumor cells. CD133+ cells (100,000) derived from D456MG xenografts were implanted s.c. into two groups of athymic nude BALB/c mice. Tumor-bearing animals (five animals per group) were treated with either bevacizumab (5 mg/kg i.p. qd) or control IgG (5 mg/kg i.p. qd) for a total of 21 days until harvest. Mice were euthanized and tumors were harvested, weighed, and examined. Representative tumor-bearing animals and dissected tumors from the two groups. C, tumor weight (mg) and tube complexity were compared between control IgG and bevacizumab treatment groups. **P < 0.001, bevacizumab treatment versus control IgG.
Bevacizumab specifically targets SCLGC tumor growth in vivo.

To confirm the relevance of the *in vitro* results, we examined the effects of inhibiting the VEGF axis through the use of bevacizumab. We quantified the therapeutic effects of bevacizumab on s.c. xenografts derived from matched SCLGC and non-SCLGC tumor cell populations. Bevacizumab potently inhibited the growth of SCLGC-derived xenografts with a reduction in tumor weight, vascularity, and hemorrhage (Fig. 4). In contrast, the small tumors derived from non-SCLGC tumor cells were not significantly inhibited in growth by bevacizumab. Parallel results were noted in intracranial studies (Supplementary Fig. S7). The limited fraction of stem-like cells in gliomas may be critical for tumor growth not only through their capacity for self-replication but also through a paracrine effect supporting non-stemlike tumor cells by inducing neovascularization. Although the VEGF axis is a particularly appealing molecular target in cancer, the determinants of patient response to these agents remain unclear. VEGF levels and microvascular density have not been correlated with patient response in clinical trials of bevacizumab for systemic cancers (18). Our results suggest that only a subpopulation of the tumor may be critical for driving VEGF-mediated tumor angiogenesis as the effects of a VEGF neutralizing antibody (bevacizumab) predominantly disrupt the angiogenic effects of a fraction of the tumor, the stem cell–like cancer cells.

Jain (19) has established vascular “normalization” as a potential key response to antiangiogenic therapies, in addition to the vascular pruning caused through targeting angiogenesis. Bevacizumab may be effective as a cancer therapy when combined with chemotherapy by improving chemotherapy delivery specifically to the stem cell–like tumor cells that would be expected to be proximal to the tumor vasculature due to VEGF expression. Indeed, we frequently detect CD133^+ tumor cells in locations proximal to blood vessels (Supplementary Fig. S7B). Therapies directed to target VEGF-expressing cells may exhibit specificity against cancer stem cells. VEGF receptors are not expressed by SCLGC (data not shown); thus, targeting VEGF may not have a direct effect on cancer stem cells but rather paracrine effects on endothelial cells and (possibly) other cancer cells. In conclusion, our studies suggest that the tumor subpopulation that shares characteristics with stem cells can contribute to tumor malignancy and may be a key target of antiangiogenic therapies.

References


Acknowledgments

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