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 neutralizing antibody bevacizumab, which is in clinical use for cancer therapy. Furthermore, bevacizumab displayed potent antiangiogenic efficacy *in vivo* and suppressed growth of xenografts derived from SCLGC but limited efficacy against xenografts derived from a matched non-SCLGC population. Together these data indicate that stem cell–like tumor cells can be a crucial source of key angiogenic factors in cancers and that targeting proangiogenic factors from stem cell–like tumor populations may be critical for patient therapy. (Cancer Res 2006; 66(16): 7843-8)

Introduction

Angiogenesis is critical to tumor formation and maintenance (1). Regulation of angiogenesis represents a complex process involving autocrine and paracrine growth factor loops with an interplay between tumor cells and the neovasculature (2). Malignant gliomas are highly lethal neoplasms that exhibit striking angiogenesis with elevated expression of vascular endothelial growth factors (VEGFs), which promote blood vessel formation by endothelial precursors (3, 4). VEGFs are secreted or cell surface–bound proteins expressed by tumor cells that bind to specific high-affinity transmembrane receptors primarily expressed on endothelial cells (5) to promote endothelial cell proliferation, migration, and survival. We recently showed that a novel antiangiogenic receptor kinase inhibitor (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 the Duke Hospital clinical pharmacy (Durham, NC). All other chemicals were 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

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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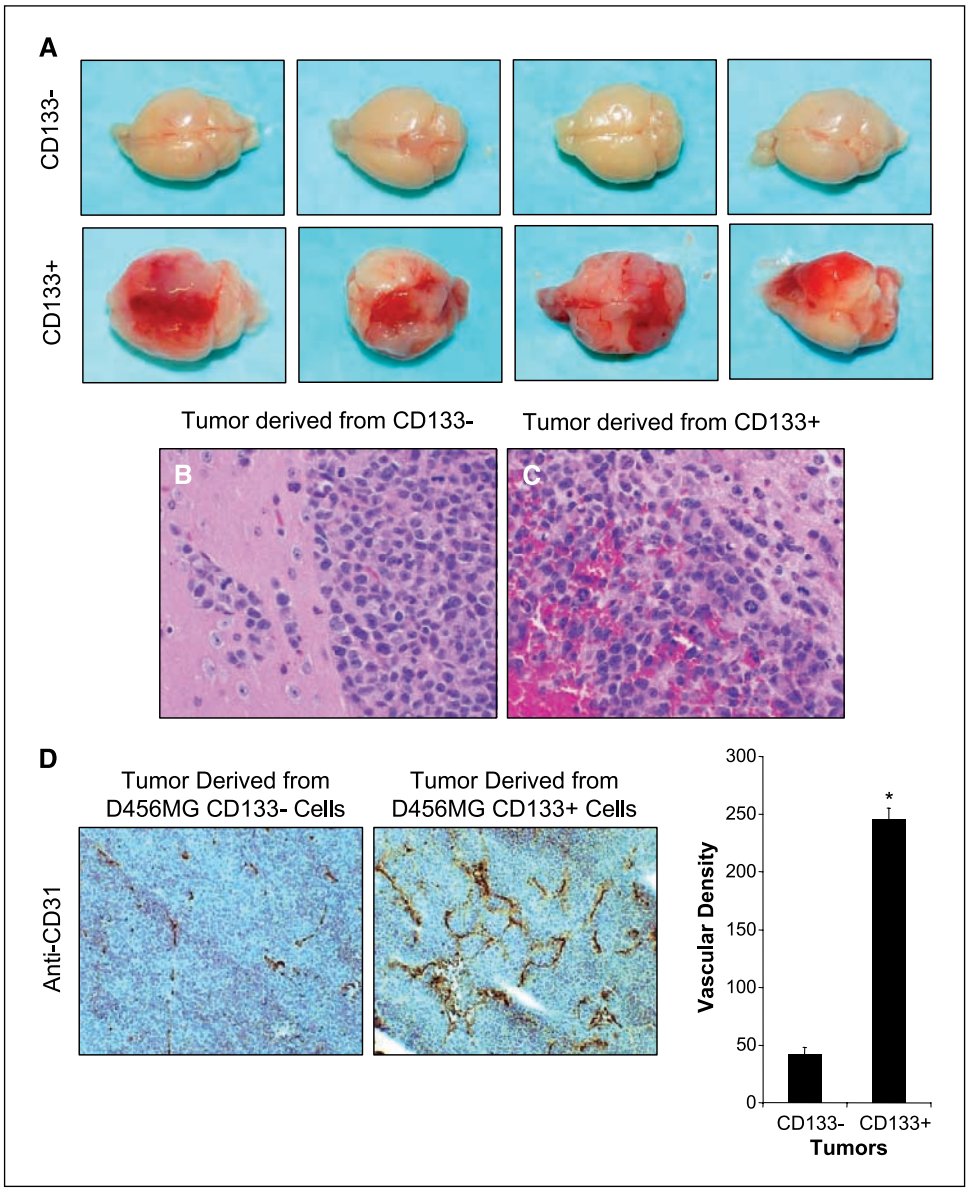

Figure 1. SCLGC form more vascular and necrotic tumors when grown in the brains of immunocompromised mice. Ten thousand cells derived from $CD133^-$ or $CD133^+$ tumor cell populations cultured from D456MG xenografts were implanted into the right frontal lobes of athymic nude BALB/c mice. Mice were sacrificed after 28 days. *A,* four pairs of representative brains bearing tumors derived from $CD133^-$ and $CD133^+$ cells. *B* and *C,* pathologic morphology of brain tumors derived from $CD133^-$ and $CD133^+$ 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 $CD133^-$ tumor cells implanted had tumor formation (×400). *C,* in contrast, all brains implanted with $CD133^+$ tumor cells displayed large tumors with widespread necrosis, hemorrhage, and neoangiogenesis (×400). *D,* the blood vessel density in tumors derived from $CD133^-$ and $CD133^+$ 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 $CD133^-$ cells (Student's *t* test).

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 *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 $CD133^-$ 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 *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 $CD133^-$ 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. TranSignal 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/.

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 cellular 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.

Statistical analysis. Descriptive statistics were generated for all quantitative data with presentation of means ± SEs. Significance was tested by one-way ANOVA using the SAS Enterprise Guide 3.0 (Cary, NC).

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. S1*A*). 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. S1*B*), and underwent multilineage differentiation (neuronal, astrocytic, and oligodendrocytic) under

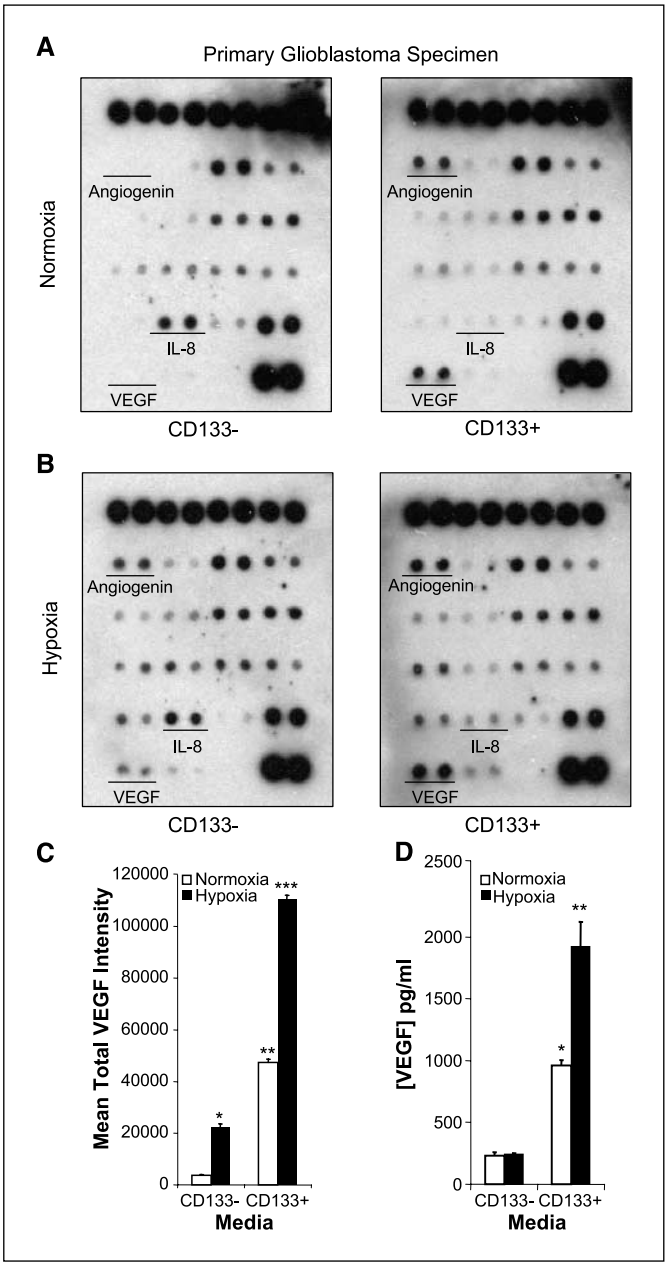

Figure 2. SCLGC express elevated level of VEGF and hypoxia induces increased VEGF expression. *A* and *B,* to determine if SCLGC differentially express proangiogenic factors, we used commercial antibody arrays (Panomics TranSignal Human Angiogenesis Antibody Array) to compare the expression levels of regulators of angiogenesis in conditioned media from matched $CD133^+$ and $CD133^-$ glioma cultures. Media conditioned for 24 hours from short-term $CD133^-$ or $CD133^+$ glioma cultures derived from a patient biopsy specimen under normoxic (*A*) or hypoxic (*B*) conditions were collected. Cells were maintained under hypoxic conditions for 24 hours. Membranes were processed based on kit instructions. The intensity of VEGF expression was quantified with ImageJ software. *C,* total mean intensity of VEGF blot was calculated based on the product of area and mean intensity. *, $P = 0.0036$, hypoxic versus normoxic conditions of $CD133^-$ conditioned medium (ANOVA); **, $P = 0.001$, $CD133^+$ conditioned medium versus $CD133^-$ conditioned medium (ANOVA); ***, $P = 0.005$, hypoxic versus normoxic conditions of $CD133^+$ conditioned medium (ANOVA). *D,* VEGF expression levels in media conditioned for 24 hours from glioblastoma patient specimen cell populations grown in 24-well plates were measured using a VEGF Quantikine ELISA (R&D Systems) according to the directions of the manufacturer. Triplicate samples were collected for each sample. *, $P = 0.0002$, normoxic $CD133^+$ medium versus $CD133^-$ medium (ANOVA); **, $P = 0.0094$, hypoxic versus normoxic conditions of $CD133^+$ conditioned medium (ANOVA); $P = 0.011$, hypoxic $CD133^+$ medium versus hypoxic $CD133^-$ medium (ANOVA).

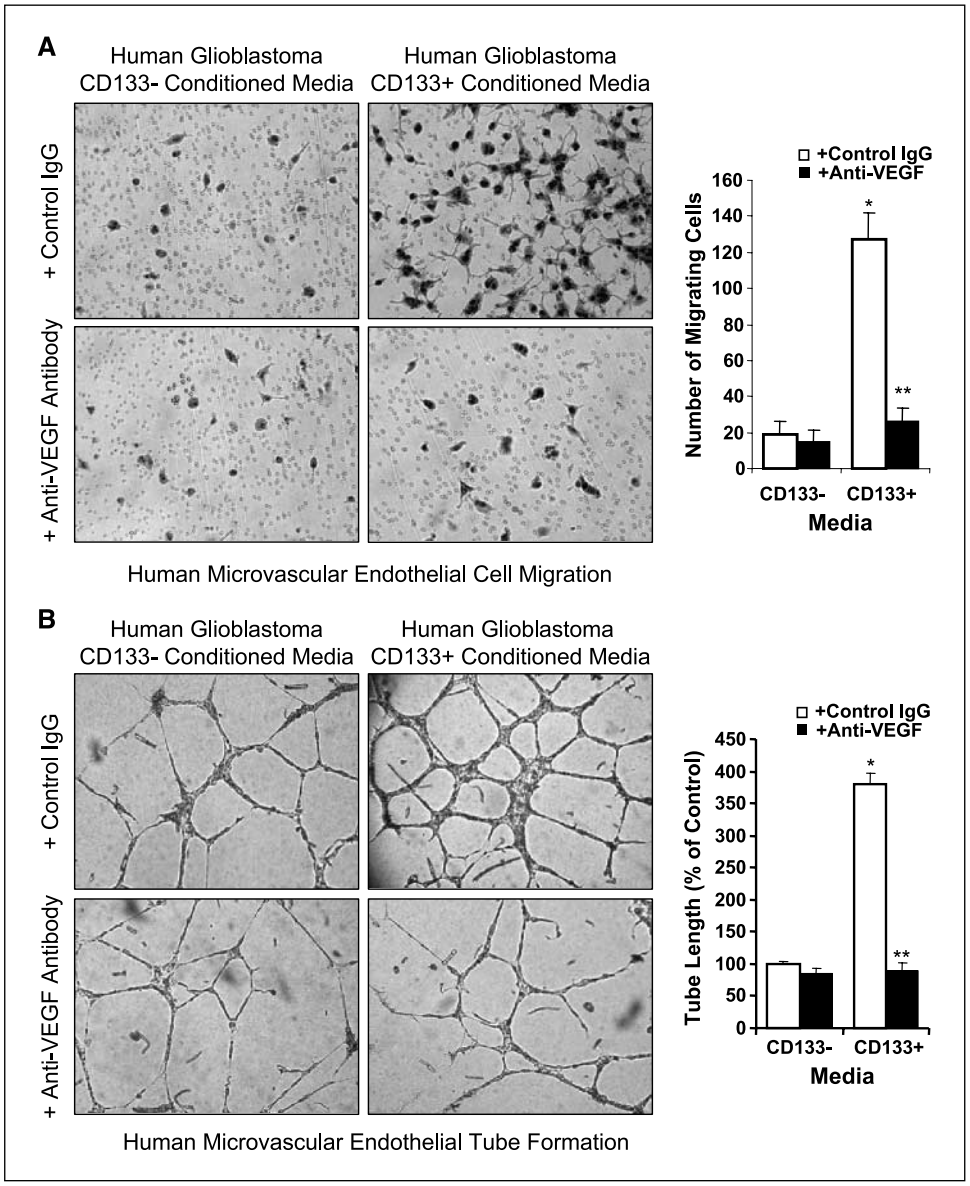

Figure 3. VEGF neutralizing antibody blocks SCLGC-induced endothelial cell migration and tube formation. *A,* a VEGF neutralizing antibody, bevacizumab, blocks SCLGC-induced endothelial cell migration. Media conditioned for 24 hours by $CD133^-$ or $CD133^+$ cultures isolated from a patient glioblastoma patient specimen were added to the bottom chambers of 24-well tissue culture plates. Bevacizumab or an IgG antibody control (antibody final concentration, 0.5 mg/mL) was added to conditioned media 30 minutes before addition to endothelial cell cultures. Human microvascular endothelial cells (20,000) were added to the upper chambers of Transwell assays (BD Biosciences). Cells were allowed to invade for 14 hours, fixed, stained, imaged, and quantified. Representative images. *, $P = 0.0003$, $CD133^+$ conditioned medium versus $CD133^-$ conditioned medium (ANOVA); **, $P = 0.0005$, bevacizumab-treated $CD133^+$ conditioned medium versus IgG control (ANOVA). *B,* VEGF neutralizing antibody blocks SCLGC-induced endothelial cell tube formation. Cultures of $CD133^-$ or $CD133^+$ glioma cells derived from a patient glioblastoma patient specimen were used to condition media for 24 hours. To these media were added human microvascular endothelial cells grown at a density of 40,000 cells/mL with 50 μL of cells added to sextuplicate wells of Matrigel-coated 96-well plates (BD Biosciences). Bevacizumab or an IgG antibody control (final concentration, 0.5 mg/mL) was added to conditioned media 30 minutes before addition to endothelial cell cultures. The endothelial cells were incubated in the conditioned media for 16 hours and then imaged. Representative images. *, $P < 0.0001$, $CD133^+$ conditioned medium versus $CD133^-$ conditioned medium (ANOVA); **, $P < 0.0001$ bevacizumab-treated $CD133^+$ conditioned medium versus IgG control (ANOVA).

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. 1*A* 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. 1*C* 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 $CD133^-$ tumor cells formed small tumors (in two of six mice in replicate experiments; Fig. 1*B*), permitting a comparison of vascularity between brains bearing $CD133^+$ and $CD133^-$ xenografts. With CD31 immunostaining, we observed that tumors derived from $CD133^+$ cells were markedly more vascular than tumors from $CD133^-$ tumor cells (Fig. 1*D*). Therefore, angiogenic potential is a critical difference between the *in vivo* tumor phenotypes of glioma SCLGC and non-SCLGC cells.

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. 2*D* and Supplementary Fig. S3*C*). 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. S3*C*). 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. 3*A* and Supplementary Fig. S6*A*) and tube formation (Fig. 3*B* and Supplemental Fig. S6*B*). Addition of bevacizumab to neutralize VEGF in CD133$^+$ glioma conditioned medium blocked its proangiogenic effects on

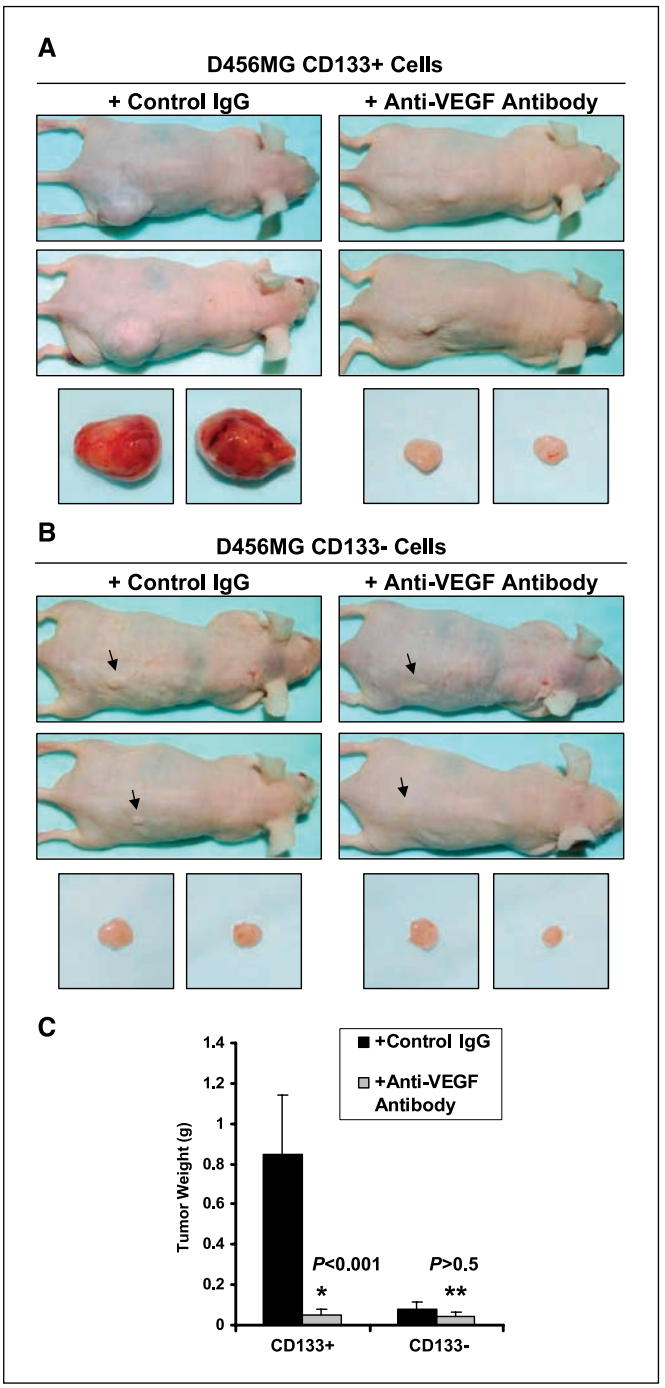

Figure 4. VEGF neutralizing antibody bevacizumab specifically suppresses SCLGC tumor growth and hemorrhage *in vivo*. *A,* treatment with VEGF-neutralizing antibody, bevacizumab, inhibits the *in vivo* tumor growth of xenografts derived from SCLGC and blocks the SCLGC-induced tumor hemorrhage *in vivo*. 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. The tumors derived from CD133$^-$ cells were small and did not display significant hemorrhage. Bevacizumab treatment did not significantly inhibit the growth of the xenografts derived from CD133$^-$ cells. *C,* the tumor weights for tumors from (*A*) and (*B*) were quantified. *, $P < 0.001$, bevacizumab treatment versus control IgG. **, $P > 0.5$, bevacizumab treatment versus control IgG.

endothelial cell migration (Fig. 3*A* and Supplementary Fig. S6*A*) and tube formation (Fig. 3*B* and Supplementary Fig. S6*B*) to levels equal to the effects of $CD133^-$ glioma conditioned medium. In contrast, bevacizumab displayed only marginal effects on human microvascular endothelial cells exposed to non-SCLGC conditioned medium. Thus, the effects of SCLGC on endothelial cell behavior are dependent on VEGF activity and bevacizumab specifically blocks the effects of SCLGC on endothelial cell behavior in cell culture assays.

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*A*).

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. S7*B*). 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.

Acknowledgments

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