Tumor Stromal-Derived Factor-1 Recruits Vascular Progenitors to Mitotic Neovasculature, where Microenvironment Influences Their Differentiated Phenotypes

Manish Aghi,[1] Kenneth S. Cohen,[2] Rachael J. Klein,[2] David T. Scadden,[2] and E. Antonio Chiocca[3]

[1]Program in Neurosurgery, Massachusetts General Hospital and Harvard Medical School; [2]Center for Regenerative Medicine and Cancer Center, Massachusetts General Hospital, Boston, Massachusetts and [3]Department of Neurological Surgery, Dardinger Laboratory for Neuro-Oncology and Neurosciences, Comprehensive Cancer Center, James Cancer Hospital and Solove Research Institute, The Ohio State University Medical Center, Columbus, Ohio

Abstract

Mechanisms underlying tumor vasculogenesis, the homing and engraftment of bone marrow–derived vascular progenitors, remain undefined. We hypothesized that tumor cell–secreted factors regulate vasculogenesis. We studied vasculogenic and nonvasculogenic intracranial murine gliomas. A PCR screen identified stromal-derived factor-1 (SDF-1/CXCL12) and vascular endothelial growth factor (VEGF) expression by vasculogenic glioma cells and spontaneously arising vasculogenic tumors in $NF1^{+/-}$:$Trp53^{+/-}$ mice, but not by nonvasculogenic glioma cells. Enforced SDF-1, not VEGF, expression in nonvasculogenic cells caused vasculogenesis. Combined SDF-1 and VEGF expression augmented vasculogenesis over SDF-1 expression alone. Blocking SDF-1 receptor CXCR4 reduced short-term homing and long-term engraftment of vascular progenitors. Implanting tumor cells secreting SDF-1 was therefore necessary and sufficient to incorporate marrow-derived precursors into tumor endothelium. SDF-1 seemed to exert these effects by acting locally intratumorally and did not cause an efflux of marrow-derived progenitors into circulation. Tumor microenvironment determined additional fates of marrow-derived cells. Hypoxia, observed with ectopic s.c. murine tumors at levels approximating that of intracranial human glioblastoma, interacted with tumor-secreted SDF-1 to expand engrafted vascular progenitor differentiated phenotypes to include pericytes as well as endothelium. In contrast, less hypoxic orthotopic intracranial murine gliomas contained only marrow-derived endothelium without marrow-derived pericytes. Furthermore, we found that vasculogenesis is significant for tumors because it generates endothelium with a higher mitotic index than endothelium derived from local sources. Although CXCR4 blockade selectively targeted endothelium generated by vasculogenesis, completely inhibiting vessel formation may require combination therapy targeting locally derived and marrow-derived endothelium. (Cancer Res 2006; 66(18): 9054-64)

Introduction

During development, new blood vessels form through angiogenesis, defined as the sprouting of endothelium from preexisting vasculature, or vasculogenesis, defined as the recruitment and differentiation of circulating bone marrow–derived precursors into mature endothelium (1). Until recently, it was thought that postnatal new blood vessel generation, as occurs during tumor growth, was due solely to angiogenesis (2). However, recent studies have raised controversy about the role of vasculogenesis in postnatal blood vessel development. Several studies identified bone marrow–derived endothelial precursor cells (EPC) in blood (3, 4) and bone marrow (5). Other studies found an EPC contribution to endothelium in ischemia (6–12) and murine (2, 4, 6, 13–16) and human (17) tumors. However, other studies failed to detect marrow-derived tumor endothelium (18–23). Some studies that failed to detect marrow-derived tumor endothelium used ectopic s.c. tumors and found intratumoral bone marrow–derived cells to include $CD45^- CD11b^+ NG2^+$ pericytes, periendothelial cells at the abluminal aspect of microvessels that secrete endothelium-supportive factors (21, 24). Therefore, the controversy elicited by these studies is whether bone marrow–derived vascular progenitors contribute to tumor blood vessels and, if they do, whether they contribute to tumor endothelial or periendothelial cells.

Some of these discordant findings likely reflect different experimental tumors used in these studies, varying in their recruitment of circulating vascular progenitor cells into tumor vessels. This suggests that tumor-secreted factors, such as chemokines or growth factors, promote vasculogenesis. Identification of these mediators is essential to design therapies that completely inhibit tumor vessel formation. Factors regulating tumor recruitment and differentiation of bone marrow–derived cells into pericytes must also be identified because pericytes are essential for microvascular stability and function particularly in late-stage tumors (25). An additional unresolved question is whether bone marrow–derived endothelium occupies a unique niche in tumor endothelium with distinct phenotypic properties that must be acknowledged when pharmacologically targeting tumor vasculature.

Herein, we report three findings that provide insight into the role of vasculogenesis in tumor blood vessel formation and help explain discordant findings reported elsewhere about whether vasculogenesis occurs. First, we studied two murine glioma cell lines with different vasculogenic potential (i.e., one forms intracranial gliomas that incorporate marrow-derived vascular progenitors into their blood vessels, whereas the other does not). A screen of secreted factors identified elevated expression of stromal-derived factor-1 (SDF-1/CXCL12) and vascular endothelial growth factor (VEGF) by vasculogenic cells relative to nonvasculogenic cells. By expressing

SDF-1 and VEGF in nonvasculogenic tumor cells, we show that the former, but not the latter, factor is sufficient to mediate engraftment of bone marrow–derived vascular progenitors into tumor blood vessels. Pharmacologic blockade of the SDF-1 receptor inhibits engraftment, indicating that SDF-1 is necessary for vasculogenesis. Second, endothelial proliferation, a defining glioblastoma feature, is more common in marrow-derived than in locally derived endothelium, suggesting that tumor vasculogenesis has functional significance, generating a distinct endothelium subset essential to glioblastoma biology. Third, we report that tumor SDF-1 secretion caused bone marrow–derived cells to differentiate into endothelium and pericytes in ectopic s.c. locations but only endothelium in orthotopic intracranial locations. These marrow-derived pericytes, which have been described in other reports using s.c. tumors (21), occurred predominantly in hypoxic areas of s.c. tumors, indicating that SDF-1 secreted by cells in tumors interacted with the tumor microenvironment to determine the full range of differentiated phenotypes of vascular progenitors recruited into developing tumor blood vessels.

Materials and Methods

Cells. KR158 (provided by Tyler Jacks, Massachusetts Institute of Technology, Cambridge, MA) derives from an astrocytoma arising in a $NF1^{-/-}$:$p53^{-/-}$ C57BL/6-derived mouse (26). GL261 derives from a glioma induced by i.c. 3-methylcholanthrene inoculation into C57BL/6 mice (27). GL261 was engineered to express SDF-1 in two ways (plasmids provided by Mark Poznansky, Massachusetts General Hospital, Boston, MA). First, GL261 was infected with retrovirus expressing murine SDF-1 and green fluorescent protein (GFP). GFP-expressing GL261/mSDF clones were assayed by SDF-1 ELISA. Second, GL261 was transfected by Geneporter (Cambridge BioScience, Cambridge, United Kingdom) with plasmid pCDNA3.1 (Invitrogen, Carlsbad, CA) expressing murine SDF-1. GL261/pSDF clones selected in 1 mg/mL G418 (Life Technologies, Inc., Carlsbad, CA) were assayed by SDF-1 ELISA. Empty vectors transduced or transfected into GL261 generated GL261/m and GL261/p, respectively. KR158/ΔEGFR, GL261/ΔEGFR, and GL261/pSDF-ΔEGFR resulted from transducing KR158, GL261, and GL261/pSDF with retrovirus expressing constitutively active epidermal growth factor receptor (EGFR) mutation ΔEGFR/EGFRvIII, whose expression was confirmed with anti-EGFRvIII antibody L8A4 (28). KR158/ΔEGFR, not KR158, formed gliomas on intracranial implantation. KR158 secreted 8.3 ng/mL SDF-1 plus 0.4 ng/mL VEGF; KR158/ΔEGFR secreted 8.5 ng/mL SDF-1 plus 3.8 ng/mL VEGF.

Real-time reverse transcription-PCR. RNA extracted from cells or tumors with Trizol (Invitrogen) was converted to cDNA with cDNA archive (Applied Biosystems, Foster City, CA) or reverse transcription kits (Promega, Madison, WI). Real-time reverse transcription-PCR (RT-PCR) used (*a*) a 13 chemokine primer panel (29) and SYBR Green in the Mx3000P (Stratagene, La Jolla, CA) provided by Andrew Luster (Massachusetts General Hospital), (*b*) murine VEGF (sense, 5′-GGAGATCCTCGAGGAG-CACTT-3′ and antisense, 5′-GGCGATTTAGCAGCAGATATAAGAA-3′) and CXCR4 (sense, 5′-TCAGTGGCTGACCTCCTCTT-3′ and antisense, 5′-TTTCAGCCAGCAGTTTCCTT-3′) primers (Invitrogen) plus SYBR Green in the Prism 7000 (Applied Biosystems), or (*c*) primer-probe combination for murine SDF-1/CXCL12 (Applied Biosystems) plus Taqman (Applied Biosystems) in the Prism 7000. Reactions involved 10 minutes at 95°C, 40 cycles at 95°C for 15 seconds, and then 60°C for 1 minute. VEGF and SDF-1 absolute quantification used plasmid-generated standard curves.

ELISAs. VEGF plus SDF-1 concentration measurements used ELISA kits (R&D Systems, Minneapolis, MN). Tumor-conditioned medium was harvested after 2 days from confluent cultured cells. Mouse plasma generated by centrifuging blood in EDTA tubes (2,000 × *g*, 20 minutes) was diluted with 1:1 (SDF-1) or 1:5 (VEGF) PBS.

Animals. Animal studies were approved by the Massachusetts General Hospital Animal Care Subcommittee. Bone marrow was isolated from

C57/BL6 mice constitutively expressing β-galactosidase (B6.129S7-*Gtrosa26*, The Jackson Laboratory, Bar Harbor, ME) or GFP [C57BL/6-Tg(ACTB-EGFP) 1Osb/J, The Jackson Laboratory] by flushing femurs and tibiae with DMEM. Isogenic wild-type (C57BL/6, The Jackson Laboratory) or transgenic mice expressing endothelial nitric oxide synthetase (eNOS) promoter-driven β-galactosidase (provided by William Aird, Beth Israel Deaconess Medical Center, Boston, MA; ref. 30) were lethally irradiated ([137]Cs Irradiator, JL Shepherd, San Fernando, CA). Marrow nucleated cells (1×10^6 to 4×10^6) were injected into recipient lateral tail veins. Successful transplants displayed >70% β-galactosidase[+] or GFP^{+} cells by blood flow cytometry. $NF1^{+/-}$:$Trp53^{+/-}$ mice, provided by Tyler Jacks, were sublethally irradiated (due to incompatibility) followed by tail vein injection of 10^6 β-galactosidase[+] marrow nucleated cells. Flow cytometry 8 weeks after transplantation identified 50% β-galactosidase^{+} cells. Mice were then followed for spontaneously arising tumors.

Establishing tumors. Mice anesthetized with ketamine/xylazine (75/15 mg/kg i.p.) 4 to 8 weeks after marrow transplantation were injected with 10^6 (s.c.) or 2×10^5 (intracranial, 2 μL, 4.5-mm deep, 1-mm anterior to and 2.5-mm right of bregma) tumor cells. For short-term homing, mice with 4-week-old s.c. KR158/ΔEGFR tumors were injected i.v. with 1×10^7 to 2×10^7 carboxyfluorescein diacetate, succinimidyl ester (CFDA-SE)–labeled (Molecular Probes, Carlsbad, CA) bone marrow cells plus 30 μg AMD3100 or PBS. After 4 hourly s.c. 15 μg AMD3100 or PBS injections, tumors were excised, cut into 1 to 3 mm[3] pieces, treated with 2 mg/mL type 2 collagenase (Worthington, Lakewood, NJ) in DMEM (45 minutes, 37°C), homogenized with stomacher model 80 (Seward, Norfolk, United Kingdom) for 15 minutes (low speed), passed through a blunt needle 20 times, washed, filtered, stained with lineage markers (CD3/CD4/CD8/B220/TER119/Gr-1/Mac-1/CD49b; PharMingen, San Diego, CA), and analyzed by flow cytometry with injected progenitors identified as CFDA-SE[+]Lin[-]. For long-term SDF-1 inhibition, mice 8 weeks after GFP[+] marrow transplants underwent intracranial KR158/ΔEGFR implantation. Eleven days after tumor implantation, model 2002 Alzet (Cupertino, CA) pumps containing 200 μL of 20 mg/mL AMD3100 or PBS were implanted s.c. Euthanasia occurred 17 days after pump placement. Blood was obtained for SDF-1 ELISA, complete blood count (Hemavet 850, Drew Scientific, Dallas, TX), and flow cytometry.

Chemotaxis. Wild-type mice bone marrow cells were stained with biotinylated lineage markers (CD3/CD4/CD8/B220/TER119/Gr-1/Mac-1) followed by streptavidin microbeads (Miltenyi, Auburn, CA) and passed over a depletion column (Miltenyi). Lineage-negative cells (10^5) were placed in the top wells of Costar (Corning, NY) Transwell 5-μm plates, with unconditioned or tumor-conditioned medium (1:2 dilution with DMEM plus 10% FCS) in bottom wells. After 3 hours (37°C), cells migrating to bottom wells were counted by hemocytometer.

Immunostaining. Four weeks after tumor implantation, mice received 50 mg/kg bromodeoxyuridine (BrdUrd; Sigma, St. Louis, MO) i.p. 24 and 12 hours or 60 mg/kg pimonidazole (Chemicon, Temecula, CA) i.p. 1 hour before euthanasia. Tumors were removed, frozen in liquid nitrogen-cooled *N*-methylbutane, and cryostat sectioned coronally to 8 μm. Every fifth slide was H&E stained to identify tumor, with glioma volume estimated by the following: $(4 / 3) \times \pi \times$ (maximal width / 2) × (maximal height / 2) × (number of tumor-containing sections × 8 μm / 2). Tumor-containing slides were immunostained for β-galactosidase (mouse; Promega), von Willebrand factor (vWF; rabbit; DAKO, Carpinteria, CA), CD31 (rat; Promega), GFP (chicken; Chemicon), NG2 (rabbit; Chemicon), CD11b (rat; PharMingen), BrdUrd (mouse; Lab Vision, Fremont, CA), and Hypoxyprobe-1 adducts (mouse; Chemicon). Secondary staining used Jackson ImmunoResearch (West Grove, PA) fluorescent or DAKO peroxidase-conjugated antibodies, with the latter detected by DAKO peroxidase/3,3′-diaminobenzidine kit per manufacturers' protocol. Hoechst 33342 (Molecular Probes) provided nuclear counterstaining. Nikon (Melville, NY) Eclipse TE2000-U inverted or Zeiss (Thornwood, NY) LSM5 Pascal three-dimensional confocal laser scanning microscopes imaged five fields from five animals per tumor type. Vessels whose endothelium predominantly expressed marrow markers (>100 RGB scale) were considered to have donor-derived endothelium, whereas vessels with no or pinpoint areas of GFP[+] or β-galactosidase[+]
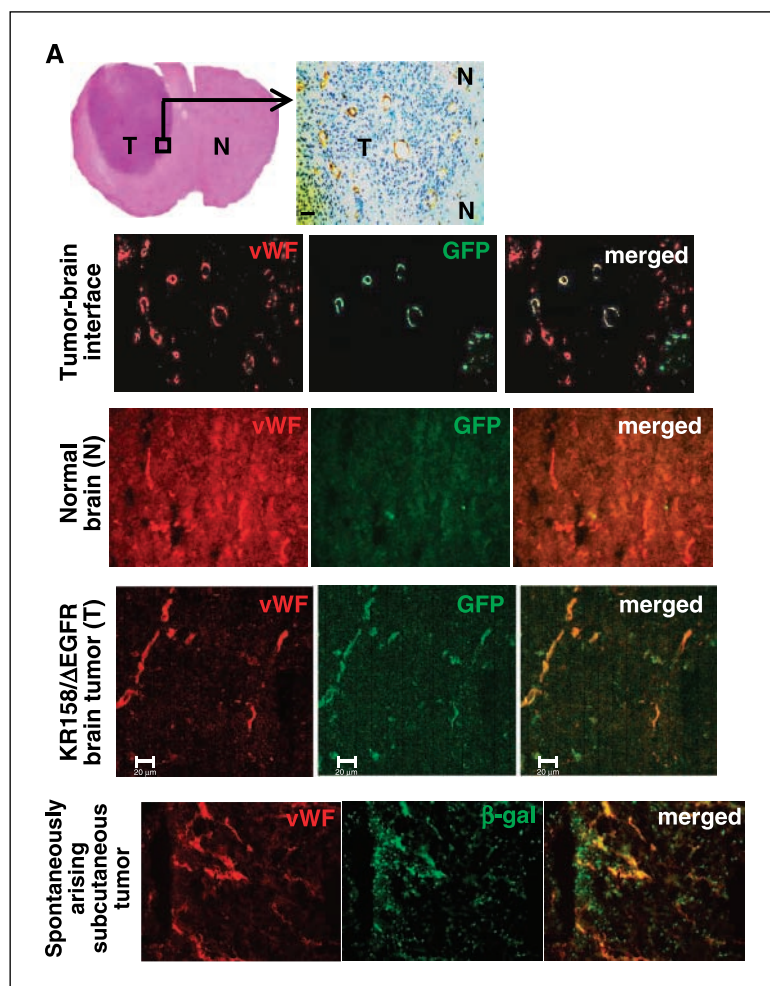
Figure 1. Bone marrow–derived cells contribute to endothelium in KR158/ΔEGFR intracranial tumors and spontaneously arising s.c. tumors in transgenic mice but not in GL261 intracranial tumors. *A, row 1, top left corner,* low-power (5×) H&E-stained cross section of KR158/ΔEGFR intracranial glioma in wild-type mice with GFP+ bone marrow transplants. *T,* tumor; *N,* normal brain. *Top right corner,* high-power (100×) peroxidase-based immunohistochemistry identified vWF+ endothelium (*brown*) at the tumor-brain interface. *Row 2,* immunofluorescence of adjacent sections identified vWF+ endothelium (*red*) and GFP+ bone marrow–derived cells (*green*), with merged images allowing identification of bone marrow–derived endothelium as discrete yellow structures resembling blood vessels, which were found only in the tumor portion of the tumor-brain interface. Marrow-derived endothelium was found in 26.2% of tumor vessels whether in the periphery or in the center of the tumor (*row 4*), but only 0.2% of vessels in normal brain in the contralateral hemisphere (*row 3*) contained marrow-derived endothelium. *Row 5,* in spontaneously arising s.c. tumors in NF1$^{-/-}$:p53$^{-/-}$ transgenic mice that received β-galactosidase+ bone marrow transplants, 19.3% of vessels had marrow-derived endothelium.

endothelium were considered to lack donor-derived endothelium. To ensure that some dual antigen-expressing structures were not two overlapping cells with different phenotypes, only cells costaining throughout optical sections measuring 200 × 0.9 μm were considered dual expressers.

Statistics. Analyses used two-tailed t tests. A Grub's law iteration censured outliers. $P < 0.05$ was considered statistically significant.

Results

KR158/ΔEGFR intracranial tumors recruit bone marrow–derived progenitors to newly formed vessels. To study the contribution of bone marrow–derived cells to intracranial tumor blood vessels, we implanted syngeneic murine KR158/ΔEGFR glioma cells intracranially into chimeric mice 8 weeks after irradiation and transplantation with either β-galactosidase+ or GFP+ bone marrow. Analysis of tumors ($n = 5$ per marrow type) 4 weeks after intracranial implantation revealed that, in the average tumor, 26.2% and 25.2% (range, 20.3-31.0%) of 76 intratumoral vessels analyzed contained β-galactosidase+ (data not shown) and GFP+ donor-derived endothelium, respectively (Fig. 1A). These vessels were equally common in the central 80% (24.5 ± 2.6%) and peripheral 20% (28.9 ± 2.7%) areas of gliomas ($P = 0.8$). In contrast, only 0.2% of vessels in the contralateral tumor-free hemisphere contained marrow-derived endothelium (Fig. 1A). Immunostaining for mature endothelium antigen vWF or immature and mature endothelium antigen CD31 yielded similar results. Many non-

endothelial marrow-derived GFP+ cells were CD45+CD11b− (data not shown), consistent with marrow-derived lymphocytes.

Vasculogenesis in spontaneously arising tumors. To better approximate natural tumoral growth, NF1$^{+/-}$:Trp53$^{+/-}$ transgenic mice were sublethally irradiated and transplanted with β-galactosidase+ bone marrow, causing 50% of blood cells to express β-galactosidase. At this juncture, they were observed until they spontaneously developed visible s.c. tumors 12 weeks after marrow engraftment. These s.c. tumors arose in dorsal soft tissue and were Mac-2+GFAP−CD3−CD45− (data not shown), making them likely sarcomas, which these mice frequently develop (26). In spontaneously arising s.c. tumors, 19.3% of vessels contained donor-derived β-galactosidase+vWF+ endothelium (SD, 8.2%; Fig. 1A), suggesting that vasculogenesis was not limited to implanted glioma cell lines and occurred in a distinct, unrelated tumor type, spontaneous s.c. sarcomas.

Bone marrow–derived vessels are distinct from local host-derived vessels. To further characterize the origin of glioma endothelium, transgenic mice expressing eNOS promoter-driven β-galactosidase were lethally irradiated and transplanted with GFP+ bone marrow followed by intracranial KR158/ΔEGFR implantation. In the resulting tumors, 25.6% of the vessels had donor-derived GFP+ endothelium, whereas all remaining vessels had host-derived β-galactosidase+ endothelium (Fig. 1B). Donor cells were incorporated into distinct separate vessels rather than only being

Figure 1 *Continued. B,* immunofluorescence of intracranial KR158/ΔEGFR tumors in transgenic mice expressing eNOS promoter-driven β-galactosidase that received GFP⁺ bone marrow transplants. With all endothelium (vWF⁺) stained red, host endothelium (β-galactosidase⁺) blue, and marrow-derived cells (GFP⁺) green, the merged image identified vessels with marrow-derived endothelium (*yellow*) to be distinct from vessels with host-derived endothelium (*purple*). *C,* results of absolute quantitative real-time RT-PCR on KR158/ΔEGFR cells (*left*), explanted intracranial KR158/ΔEGFR gliomas (*middle*), and spontaneously arising s.c. tumors in NF1$^{-/-}$:p53$^{-/-}$ transgenic mice (*right*). *Left,* KR158/ΔEGFR cells were screened for chemokine and VEGF expression, with only the most expressed of the 13 assessed chemokines shown (KR158/ΔEGFR expressed <10 copies/ng of chemokines MIP3-α MIP3-β, TECK, MIP1-α, MIP1-β, I-309, and SLC). Expression of SDF-1 and VEGF, the two most expressed factors identified in KR158/ΔEGFR cells, was confirmed in explanted KR158/ΔEGFR gliomas (*middle*) and in spontaneously arising s.c. tumors (*right*). *D, top, left,* immunofluorescence of intracranial GL261 tumors identified vWF⁺ endothelium (*red*) and GFP⁺ bone marrow–derived cells (*green*), with merged images allowing identification of any bone marrow–derived endothelium as discrete yellow structures resembling blood vessels, which were seen in only 0% to 0.5% of vessels (experiment repeated thrice, 0% engraftment seen twice, 0.5% once); *right,* percentage of vessels that were yellow on merged images was 26.2% in KR158/ΔEGFR intracranial tumors compared with 0% in GL261. *Bottom, left,* percentage of lineage-negative (*Lin⁻*) bone marrow (*BM*) cells exhibiting chemotaxis toward unconditioned medium (*Neg control*), conditioned medium from GL261 cells, and conditioned medium from KR158/ΔEGFR cells. *,* $P = 0.02$, compared with GL261 conditioned medium. *Right,* results of VEGF and SDF-1 ELISAs on conditioned medium from KR158/ΔEGFR and GL261 cells. KR158/ΔEGFR cells secreted considerably more VEGF and SDF-1 than GL261 cells. *Bars,* SD [*C* and *D (top)*] or SE (*D, bottom*). Bars, 20 μm (*A* and *D*) or 50 μm (*B*).
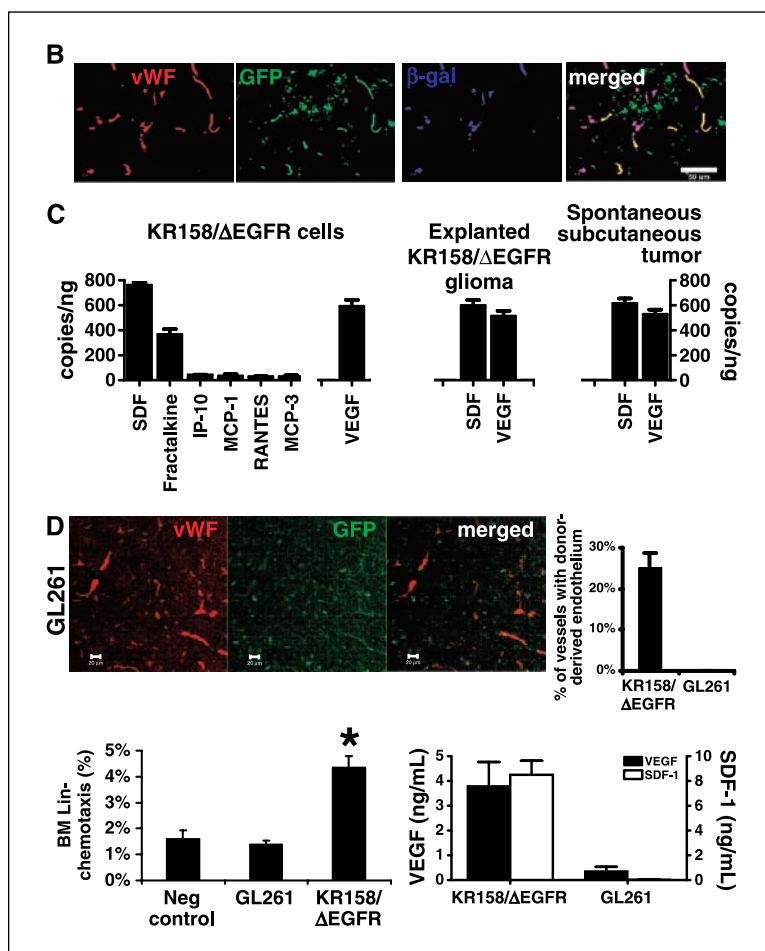
incorporated into preexisting host vessels. Furthermore, there were no GFP⁺β-galactosidase⁺ cells, making it unlikely that donor-derived endothelium resulted from fusion between bone marrow–derived nonendothelial cells and non–marrow-derived host endothelium. These findings confirmed the vasculogenic activity of KR158/ΔEGFR tumors.

SDF-1 and VEGF are highly expressed in KR158/ΔEGFR and vasculogenic spontaneously arising tumors. We hypothesized that tumor-secreted factors recruited vascular progenitors to tumor vasculature. We screened KR158/ΔEGFR cells with 13 chemokine-specific primer pairs by quantitative real-time RT-PCR, identifying several expressed chemokines, with SDF-1 most expressed (Fig. 1C). In addition, because EGFR constitutive activation found in KR158/ΔEGFR enhances secretion of growth factor VEGF (31), we measured VEGF mRNA in KR158/ΔEGFR using quantitative real-time RT-PCR and found that VEGF mRNA expression was also elevated (Fig. 1C). We then investigated SDF-1 and VEGF expression in lysates of explanted KR158/ΔEGFR intracranial gliomas and tumor and stromal cell containing spontaneously arising s.c. tumors that had marrow-derived endothelium and found that both SDF-1 and VEGF were highly expressed by these tumors also (Fig. 1C). The high SDF-1 and VEGF expression by KR158/ΔEGFR and spontaneously arising tumors, which both exhibited marrow-derived endothelium, combined with the demonstration by us (data not shown) and others (32) that SDF-1 receptor CXCR4 is the most expressed chemokine receptor by Lin⁻ progenitors and a report that human EPCs express

VEGF receptor VEGFR-2 (33), rendered both SDF-1 and VEGF possible mediators of vascular progenitor recruitment to tumor vessels.

Intracranial GL261 tumors do not incorporate bone marrow–derived progenitors in tumor vasculature; GL261 secretes less VEGF and SDF-1 than KR158/ΔEGFR. We then implanted GL261, another C57/BL6 glioma cell line, intracranially into mice with GFP⁺ bone marrow transplants. Analysis of tumors ($n = 5$) revealed that, in the average tumor, 0% to 0.5% of 78 tumor vessels analyzed contained GFP⁺ donor-derived endothelium (Fig. 1D) as reported (20). Thus, whereas KR158/ΔEGFR and GL261 intracranial gliomas exhibited comparable vessel densities (data not shown), unlike KR158/ΔEGFR gliomas, GL261 gliomas exhibited no vasculogenesis. To determine if the differing vasculogenesis between these tumors resulted from a tumor cell–secreted factor, we investigated the ability of conditioned medium from these cells to induce bone marrow–derived progenitor chemotaxis. KR158/ΔEGFR conditioned medium induced lineage-negative bone marrow progenitor chemotaxis, whereas GL261 conditioned medium lacked this chemotactic activity (Fig. 1D), consistent with a secreted factor mediating this chemotaxis. Given the elevated SDF-1 and VEGF mRNA in KR158/ΔEGFR, we used ELISA to compare the secretion of these factors by KR158/ΔEGFR and GL261 cells. ELISAs identified significantly increased VEGF/SDF-1 levels secreted by KR158/ΔEGFR (3.8/8.5 ng/mL) compared with GL261 (0.4/0.03 ng/mL; Fig. 1D).

SDF-1 expression by implanted tumor cells is sufficient to induce engraftment of tumor vessels by bone marrow–derived vascular progenitors. To determine whether tumor SDF-1 expression was responsible for vascular progenitor engraftment of tumor vessels, we engineered nonvasculogenic GL261 cells to express SDF-1 by transfection or transduction, generating GL261/pSDF (2.9 ± 0.3 ng/mL SDF-1) and GL261/mSDF (28.2 ± 3.7 ng/mL SDF-1) cells, which secreted 13- and 118-fold more SDF-1 than empty vector–expressing GL261/p (0.22 ± 0.05 ng/mL) and GL261/m (0.24 ± 0.06 ng/mL) cells, respectively (Fig. 2A). SDF-1 secretion of GL261/pSDF resembled KR158/ΔEGFR, whereas GL261/mSDF oversecreted SDF-1 relative to KR158/ΔEGFR. In intracranial tumors formed from GL261/pSDF and GL261/mSDF, 20.5% and 27.9% of vessels had donor-derived endothelium, whereas no vessels had donor-derived endothelium in control GL261/p or GL261/m tumors (Fig. 2B and C). The higher percentage of marrow-derived endothelium in higher SDF-1–expressing GL261/mSDF than in lower SDF-1–expressing GL261/pSDF indicated

augmented vasculogenesis with increasing SDF-1 secretion. However, the increase in GL261/mSDF vasculogenesis relative to GL261/pSDF was less than the differences in their SDF-1 secretion (Fig. 2), a plateau that potentially reflected a rate-limiting step in SDF-1–driven vasculogenesis, possibly the local availability of marrow-derived progenitors. Overall, these findings strongly support the hypothesis that SDF-1 secretion by tumor cells alone is sufficient to induce the recruitment of bone marrow–derived vascular progenitors to tumor neovasculature.

VEGF does not mediate intracranial vasculogenesis. We next asked if VEGF expression was sufficient to induce vasculogenesis. Because direct VEGF expression in GL261 minimally increases VEGF secretion (20), we increased VEGF production further by transducing glioma cells with constitutively active EGFR mutation variant III (EGFRvIII/ΔEGFR), which is present in 30% of glioblastomas and is the most common inducer of glioblastoma VEGF secretion (31, 34). ΔEGFR transduction increased GL261 VEGF secretion 10-fold (Fig. 3A). However, unlike what happened
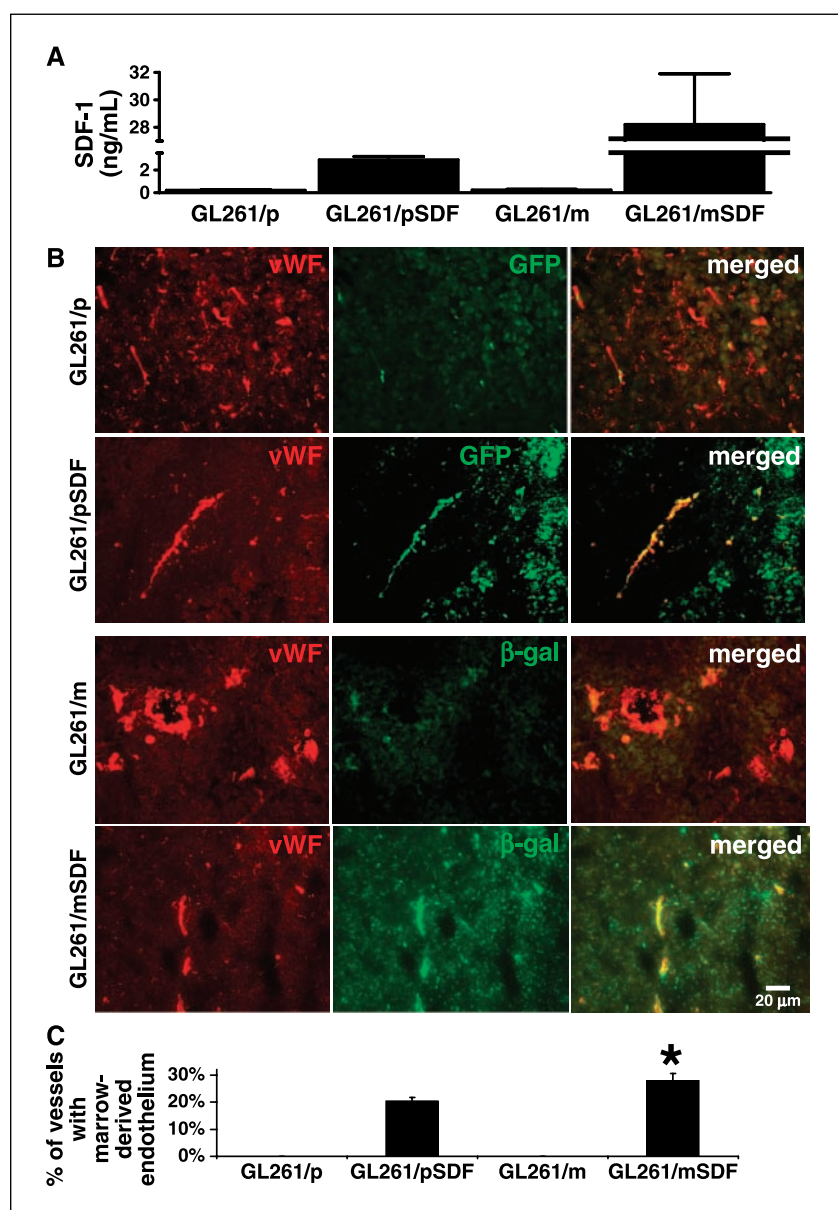
Figure 2. Transfecting GL261 to express SDF-1 leads to vasculogenesis in intracranial tumors. *A,* SDF-1 ELISA results on conditioned medium from GL261/p, GL261/pSDF, GL261/m, and GL261/mSDF cells. *Bars,* SE. *B,* immunofluorescence on intracranial GL261/p, GL261/pSDF, GL261/m, and GL261/mSDF tumors revealed marrow-derived endothelium in cells with enforced SDF-1 expression, not those expressing the empty vector. Bar, 20 μm. *C,* percentage of vessels that exhibited yellow staining on merged images was 20.5% and 27.9% in GL261/pSDF and GL261/mSDF intracranial tumors, respectively, whereas tumors derived from empty vector–expressing cell lines had no vessels with marrow-derived endothelium. *, P = 0.006, compared with GL261/pSDF, indicating increasing vasculogenesis with increasing SDF-1 secretion.

Figure 3. Enforced GL261 expression of VEGF does not lead to vasculogenesis in intracranial tumors but augments vasculogenesis when superimposed on SDF-1-expressing transfectants. *A,* VEGF ELISA results on conditioned medium from GL261, GL261/ΔEGFR, GL261/pSDF, and GL261/pSDF-ΔEGFR cells. *Bars,* SD. *B,* immunofluorescence on intracranial GL261/ΔEGFR and GL261/pSDF-ΔEGFR tumors revealed no marrow-derived endothelium in the former but augmented incorporation of marrow-derived endothelium in GL261/pSDF-ΔEGFR relative to GL261/pSDF tumors. Bar, 50 μm. *C,* although no vessels in GL261/ΔEGFR tumors exhibited yellow staining, indicative of marrow-derived endothelium, the percentage of vessels with marrow-derived endothelium in GL261/pSDF-ΔEGFR tumors was 23.6%, exceeding the 19.9% of vessels in GL261/pSDF tumors with marrow-derived endothelium. *, *P* = 0.006, compared with GL261/pSDF vasculogenesis.
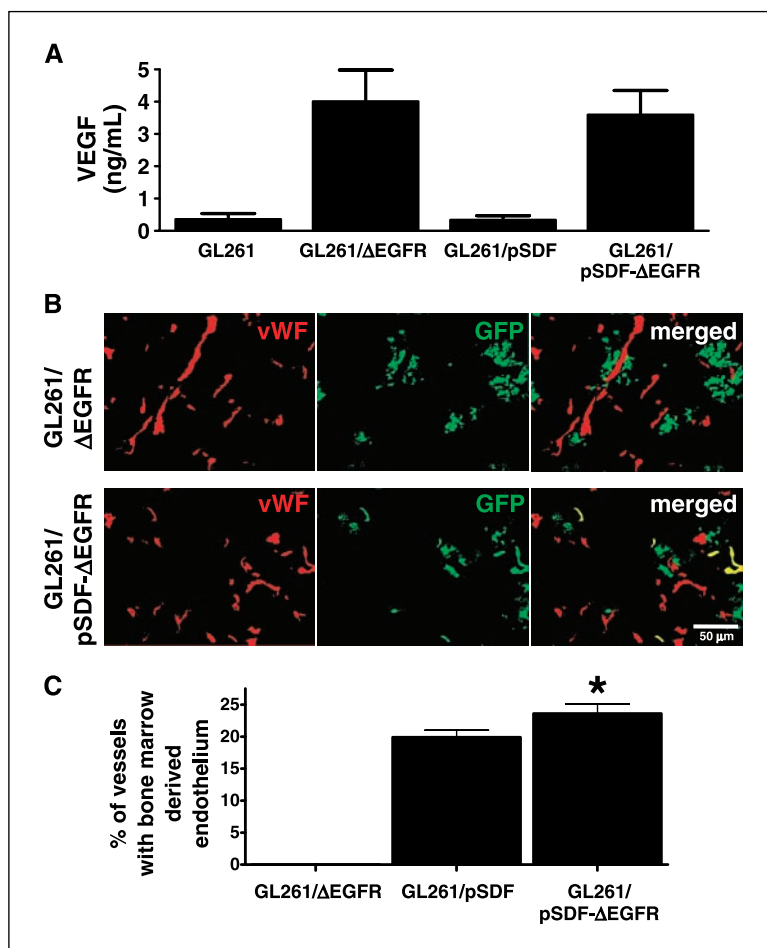
with enforced SDF-1 expression, GL261/ΔEGFR intracranial tumors lacked vessels with GFP⁺ donor-derived endothelium despite secreting nearly identical VEGF as vasculogenic KR158/ΔEGFR (Fig. 3*B* and *C*), suggesting that VEGF alone did not mediate intracranial glioma vasculogenesis.

Superimposing VEGF secretion on glioma cells secreting SDF-1 enhances vasculogenesis. Because vasculogenic KR158/ΔEGFR cells exhibit elevated SDF-1 and VEGF secretion, we asked if there was an effect of combined expression of both factors by nonvasculogenic GL261. We thus engineered GL261/pSDF to express ΔEGFR, creating GL261/pSDF-ΔEGFR cells, whose VEGF and SDF-1 secretion resembled KR158/ΔEGFR (Fig. 3*A*). In GL261/pSDF-ΔEGFR intracranial tumors, 23.6% of vessels had GFP⁺ donor-derived endothelium, significantly exceeding the percentage in intracranial GL261/pSDF tumors (Fig. 3*B* and *C*) and comparable with KR158/ΔEGFR. Taken together, these findings show that, although SDF-1/CXCL12 alone is sufficient to induce engraftment of bone marrow–derived EPCs into tumor vessels and VEGF alone fails to induce vasculogenesis, VEGF expression by SDF-1-expressing tumor cells further enhances vasculogenesis.

Glioma SDF-1 secretion does not cause an efflux of vascular progenitors from marrow to circulation. Recruiting marrow-derived vascular progenitors to tumor vasculature could be associated with an efflux of such progenitors from bone marrow into the circulation. However, the blood of animals harboring intracranial SDF-1-negative and SDF-1-positive gliomas exhibited

identical numbers of flk-1⁺CD45⁺ vascular leukocytes (0.02-0.05 K/μL = 0.5-0.6%) or Lin⁻ progenitors (0.02-0.04 K/μL = 0.5%), suggesting that tumor cell SDF-1 expression did not mobilize progenitors from the bone marrow into the bloodstream.

To investigate if SDF-1 could even exert effects at locations remote from tumor, such as bone marrow, we assessed plasma SDF-1 in animals with SDF-1-secreting tumors. The plasma SDF-1 concentration in animals with intracranial GL261/mSDF tumors did not differ from that in animals bearing GL261/m tumors (0.8 ng/mL), making it likely that SDF-1 effects occurred locally within the intracranial tumor.

Blocking SDF-1 receptor CXCR4 inhibits intracranial KR158/ΔEGFR vasculogenesis. To determine if SDF-1 was necessary for vasculogenesis in KR158/ΔEGFR, short- and long-term effects of treating tumor-bearing mice with AMD3100, a proven antagonist of SDF-1 receptor CXCR4 (35), were assessed. First, bone marrow cells fluorescently labeled with CFDA-SE were injected i.v. into mice with established s.c. KR158/ΔEGFR tumors. After 4 hourly AMD3100 or PBS injections, harvested disaggregated tumors were stained with lineage antibodies. AMD3100 reduced the frequency of intratumoral labeled progenitors (CFDA-SE⁺Lin⁻) by 80% (Fig. 4*A*), suggesting that CXCR4 inhibition prevented the recruitment of bone marrow–derived vascular progenitors out of circulation and into the tumor. Thus, although tumor SDF-1 secretion did not alter steady state levels of circulating vascular progenitors, tumor SDF-1 secretion caused intratumoral accumulation of these progenitors.

To assess long-term effects of AMD3100 during tumor progression, osmotic pumps continuously releasing AMD3100 or PBS over 2 weeks were implanted s.c. into mice that had received GFP$^+$ bone marrow transplants followed by intracranial KR158/ΔEGFR implantation. AMD3100-induced long-term CXCR4 blockade increased plasma SDF-1 concentrations 4-fold (Fig. 4B) and increased the number of circulating flk-1$^+$CD45$^+$ or Lin$^-$ (Fig. 4C) progenitors. Interestingly, despite mobilizing progenitors into the circulation, continuous AMD3100 exposure significantly reduced vascular progenitor engraftment of intracranial tumor endothelium by ~60% (Fig. 4C). Although it is difficult to definitively prove the AMD3100-induced CXCR4 blockade confirmed *in vitro* (35) in this or any *in vivo* model, these results strongly suggest that SDF-1 was necessary for incorporation of marrow-derived cells into intracranial tumor vessels.
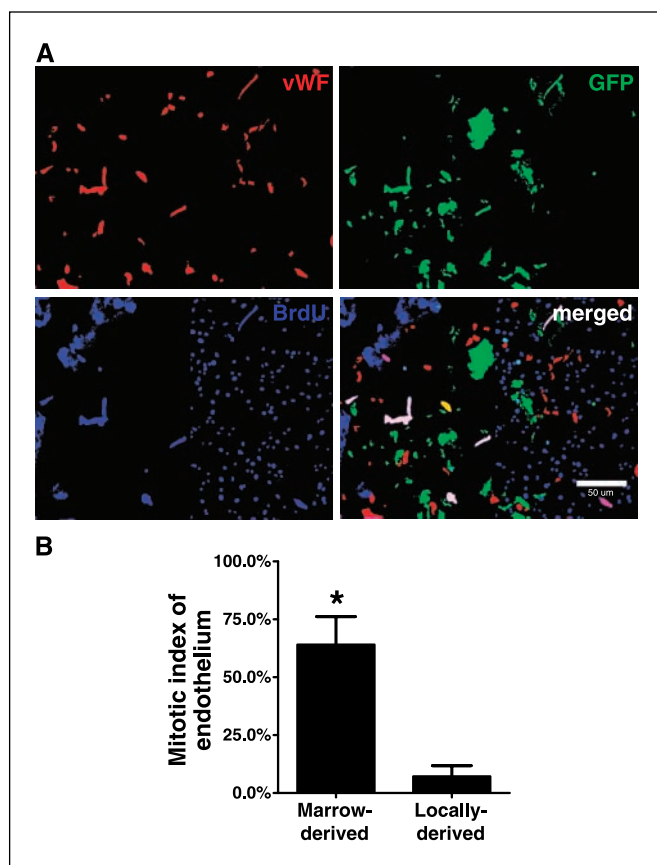
Bone marrow–derived tumor endothelium exhibits greater mitotic activity than host-derived endothelium. To date, no phenotypic features distinguishing bone marrow–derived from locally derived tumor endothelium have been reported. Because of their recent differentiation from replicating circulating EPCs, we hypothesized that marrow-derived endothelium might exhibit greater mitotic activity than host-derived endothelium. GFP-transplanted mice with KR158/ΔEGFR intracranial tumors were thus treated with BrdUrd shortly before euthanasia. By immunofluorescence, 18.2% of tumor vessels exhibited endothelial BrdUrd incorporation. The portion of tumor vessels with GFP$^+$ donor-derived endothelium exhibiting endothelial BrdUrd incorporation was 9-fold greater than the portion of tumor vessels containing all host-derived GFP$^-$ endothelium that exhibited endothelial BrdUrd incorporation (Fig. 5). Thus, endothelial proliferation, a defining glioblastoma feature, was significantly more frequent in bone marrow–derived endothelium than in locally derived endothelium, indicating a phenotypic difference between the two endothelium types.

Anatomic location alters fates of intratumoral bone marrow–derived cells. Some reports found that bone marrow–derived cells in tumors were pericytes, not endothelium (21), and that the proximity of pericytes to endothelium could lead to mistaking marrow-derived pericytes for marrow-derived endothelium. To investigate this possibility in our intracranial tumors, we analyzed intracranial KR158/ΔEGFR tumors for expression of pericyte antigen NG2, endothelial antigen CD31, and GFP by immunohistochemistry. NG2$^+$ cells were close to CD31$^+$ cells without costaining, consistent with a pericyte population. There were numerous GFP$^+$CD31$^+$ cells but no detectable GFP$^+$NG2$^+$ cells,
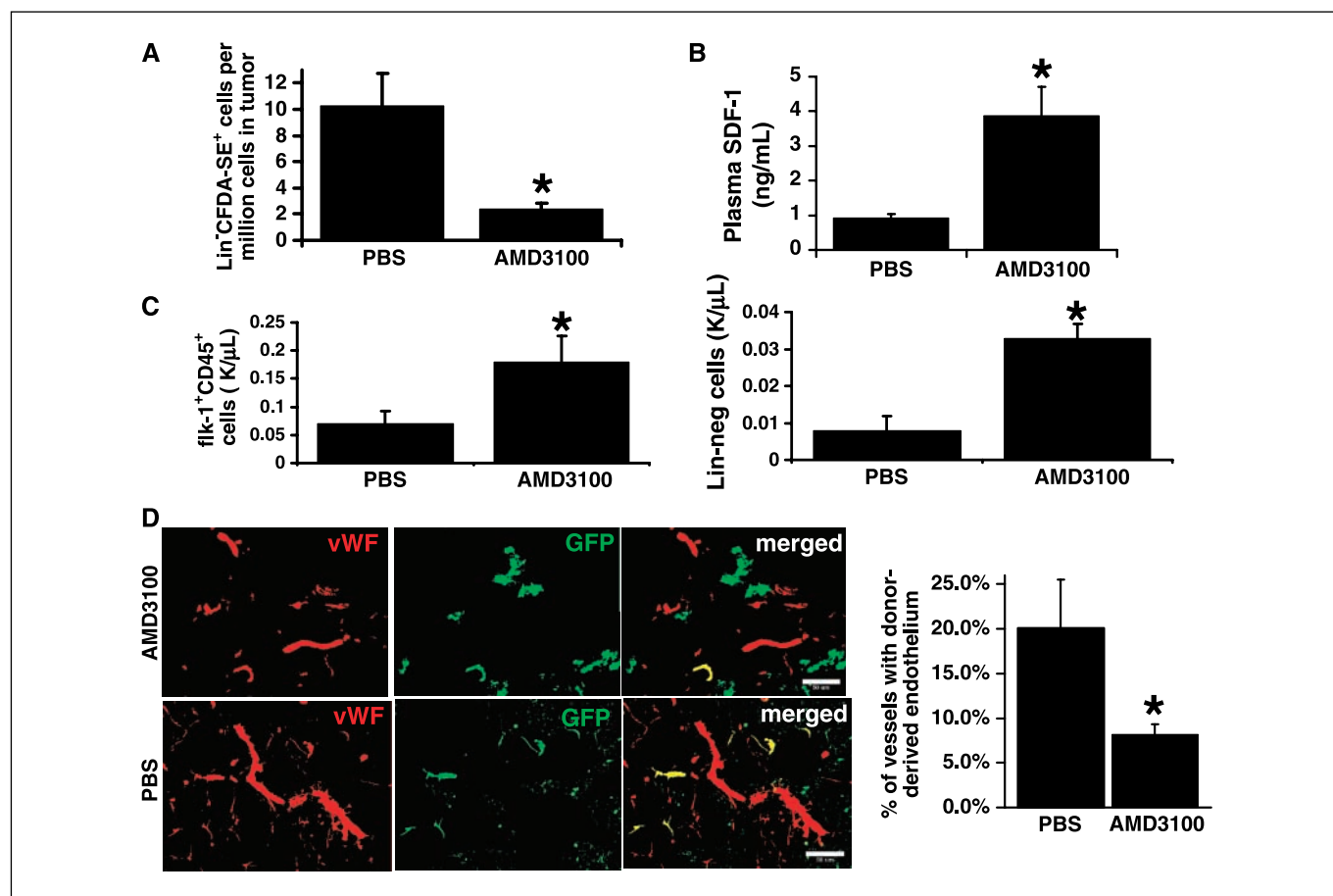
Figure 4. Short- and long-term effects of AMD3100, an antagonist of SDF-1 receptor CXCR4, on incorporation of bone marrow–derived cells into s.c. and intracranial KR158/ΔEGFR tumors. Short-term (*A*) and long-term (*B-D*) effects of CXCR4 blockade. *A,* short-term effects: AMD3100 treatment reduced by ~80% the intratumoral frequency of lineage-negative progenitor cells that had been labeled with CFDA-SE injected i.v. 4 hours before flow cytometry of homogenized established s.c. KR158/ΔEGFR tumors. *, *P* = 0.01, compared with PBS treated. *B,* long-term effects: AMD3100 blockade of CXCR4 led to increased plasma SDF-1 concentrations. *, *P* = 0.0003, compared with PBS treated. *C,* AMD3100 treatment increased absolute numbers of circulating flk-1$^+$CD45$^+$ (*left*) and Lin$^-$ (*right*) progenitor cells. *, *P* < 0.001, compared with PBS treated. *D, left,* immunofluorescence of intracranial KR158/ΔEGFR tumors in AMD3100-treated (*row 1*) and PBS-treated (*row 2*) mice. Bar, 50 μm. *Right,* continuous AMD3100 treatment over 2 weeks reduced by ~60% the presence of bone marrow–derived endothelium in intracranial KR158/ΔEGFR tumors. *, *P* = 0.007, compared with PBS treated. *Bars,* SE (*A*) or SD (*B-D*).

Figure 5. Bone marrow–derived endothelium exhibits greater BrdUrd incorporation. *A,* immunofluorescence identified CD31^{+} endothelium (*red*), BrdUrd^{+} proliferating cells (*blue*), and GFP^{+} bone marrow–derived cells (*green*). This image is from the tumor margin, with significant numbers of replicating tumor cells to the *right*, progressing to fewer tumor cells and more normal tissue at the tumor periphery to the *left*. On the merged image, bone marrow–derived endothelium stains yellow (red + green), proliferating endothelium stains purple (red + blue), and proliferating marrow-derived endothelium stains white (red + green + blue). Bar, 50 μm. *B,* portion of tumor vessels with GFP[+] donor-derived endothelium that exhibited endothelial BrdUrd incorporation was significantly greater than the portion of tumor vessels containing all locally derived GFP[−] host endothelium that exhibited endothelial BrdUrd incorporation (65% versus 7%). *Bars,* SD. *, P = 0.0002, compared with locally derived endothelium.

suggesting that all bone marrow–derived vascular/perivascular cells in these tumors were endothelium and none were pericytes (Fig. 6*A*). The lack of donor-derived pericytes in intracranial KR158/ΔEGFR tumors was confirmed when no perivascular GFP^{+} cells immunostained for CD11b, another pericyte antigen (data not shown). Therefore, bone marrow–derived cells in SDF-1-secreting intracranial tumors differentiated into endothelium, not pericytes.

Most studies investigating intratumoral bone marrow–derived cells used ectopic s.c. tumors, whereas few investigated orthotopic intracranial tumors, such as described above. Therefore, we studied s.c. tumors derived from these same cell lines in animals with GFP[+] bone marrow transplants. In s.c. KR158/ΔEGFR tumor immuno-histochemistry, 19.9% of vessels contained donor-derived GFP[+] endothelium and 11.9% of NG2^{+} cells were GFP[+] (Fig. 6*A*). This finding of marrow-derived endothelium and pericytes in s.c. KR158/ΔEGFR tumors was confirmed by flow cytometry of disaggregated s.c. KR158/ΔEGFR tumors, which revealed that 19.9% of Sca-1^{+} CD31^{+}CD45^{−} endothelium and 22.3% of CD31^{−}CD45^{−}CD11b^{+} pericytes were donor-derived GFP^{+} (Fig. 6*B*). In s.c. GL261/pSDF

tumor immunohistochemistry, 19.7% of vessels had GFP^{+} endo-thelium and 12.5% of NG2^{+} cells were GFP[+]. In contrast, no endothelium and 1.2% of NG2^{+} cells in s.c. GL261/p control tumors were donor-derived GFP[+]. Thus, in s.c. tumors, unlike intracranial tumors, SDF-1 was sufficient to stimulate the differentiation of marrow-derived cells into both tumor endothelium and pericytes.

Given the different vascular progenitor cell phenotypes seen with identical tumor cells in different microenvironments, we hypoth-esized that microenvironmental factors influenced the range of differentiated vascular progenitor phenotypes. Specifically, we hypothesized that greater hypoxia in the s.c. space, which is less vascularized than the brain, represented one such factor. Indeed, s.c. KR158/ΔEGFR tumors had a greater hypoxic fraction than intracranial KR158/ΔEGFR tumors (58% versus 21%; P = 0.0009) as assessed by pimonidazole staining, consistent with previously reported greater hypoxia in gliomas implanted s.c. rather than intracranially (36). We then determined the number of bone marrow–derived endothelial cells and pericytes in hypoxic and nonhypoxic areas in intracranial and s.c. tumors. Interestingly, in s.c. KR158/ΔEGFR tumors, 13.3% of pericytes in hypoxic areas were marrow derived, whereas only 2.2% of pericytes in nonhypoxic areas were marrow derived (P = 0.00003; Fig. 6*C* and *D*). However, the percentage of vessels with marrow-derived endothelium did not differ in hypoxic versus nonhypoxic areas in s.c. (20.0% versus 24.7%) or intracranial (24.9% versus 26.4%) tumors (P > 0.05; Fig. 6*C* and *D*). Furthermore, the increased marrow-derived pericytes in hypoxic areas did not reflect hypoxia-induced SDF-1 because, unlike other cells (37), hypoxia did not increase SDF-1 secretion of these cells (data not shown).

Collectively, these findings suggest that, although tumor SDF-1/CXCL12 expression is responsible for recruiting vascular progenitors to tumors, factors in the tumor microenvironment, one of which we found to be tumor hypoxia, contribute to the final location and range of phenotypes that these progenitors differentiate into.

Discussion

Numerous recent studies have investigated whether tumor endothelium forms by angiogenesis, remodeling surrounding tissue vessels, or vasculogenesis, the recruitment of bone marrow–derived circulating endothelial progenitor cells into tumors and local differentiation of these cells into tumor endothelium. In these reports, vasculogenesis use for blood vessel formation varied considerably among tumors, suggesting that tumor-synthesized factors, such as chemokines or growth factors, regulate the process. Identifying factors mediating tumor use of bone marrow–derived cells to form endothelium as well as the marrow-derived pericytes found in some studies (21) is of biological interest and important for developing new therapies.

Herein, we have shown that tumor SDF-1/CXCL12 secretion is both necessary and sufficient to induce vasculogenesis in intracranial gliomas. VEGF alone failed to cause vasculogenesis in this model but enhanced the vasculogenesis of SDF-1-secreting tumors independent of the previously shown ability of VEGF to stimulate SDF-1 secretion (38). In addition, although VEGF up-regulates mature endothelium CXCR4 expression (39), we found that VEGF did not up-regulate EPC CXCR4 transcription (data not shown), making VEGF-induced CXCR4 expression an unlikely mechanism of interaction between VEGF and SDF-1. The finding that VEGF stimulates vasculogenesis only in the setting of tumor cell SDF-1 secretion may explain conflicting reports, in which

VEGF induced vasculogenesis in some tumors (40) but not in others (20). Plasma SDF-1 was unchanged in animals with SDF-1-secreting tumors, suggesting local SDF-1 effects. Plasma VEGF was 9-fold higher in animals with VEGF-secreting tumors (data not shown), suggesting possible remote effects of VEGF and consistent with elevated cancer patient plasma VEGF (41).

SDF-1 and VEGF both represent possible tumor-secreted mediators of vasculogenesis. SDF-1/CXCL12 is up-regulated in some hypoxic cells (37) and increases EPC recruitment from circulation into ischemic tissues (42). VEGF is also up-regulated in hypoxic cells (43) and increases the amount of circulating EPCs in some models (6, 33). SDF-1 is expressed by ovarian, pancreatic, and breast cancers, and in
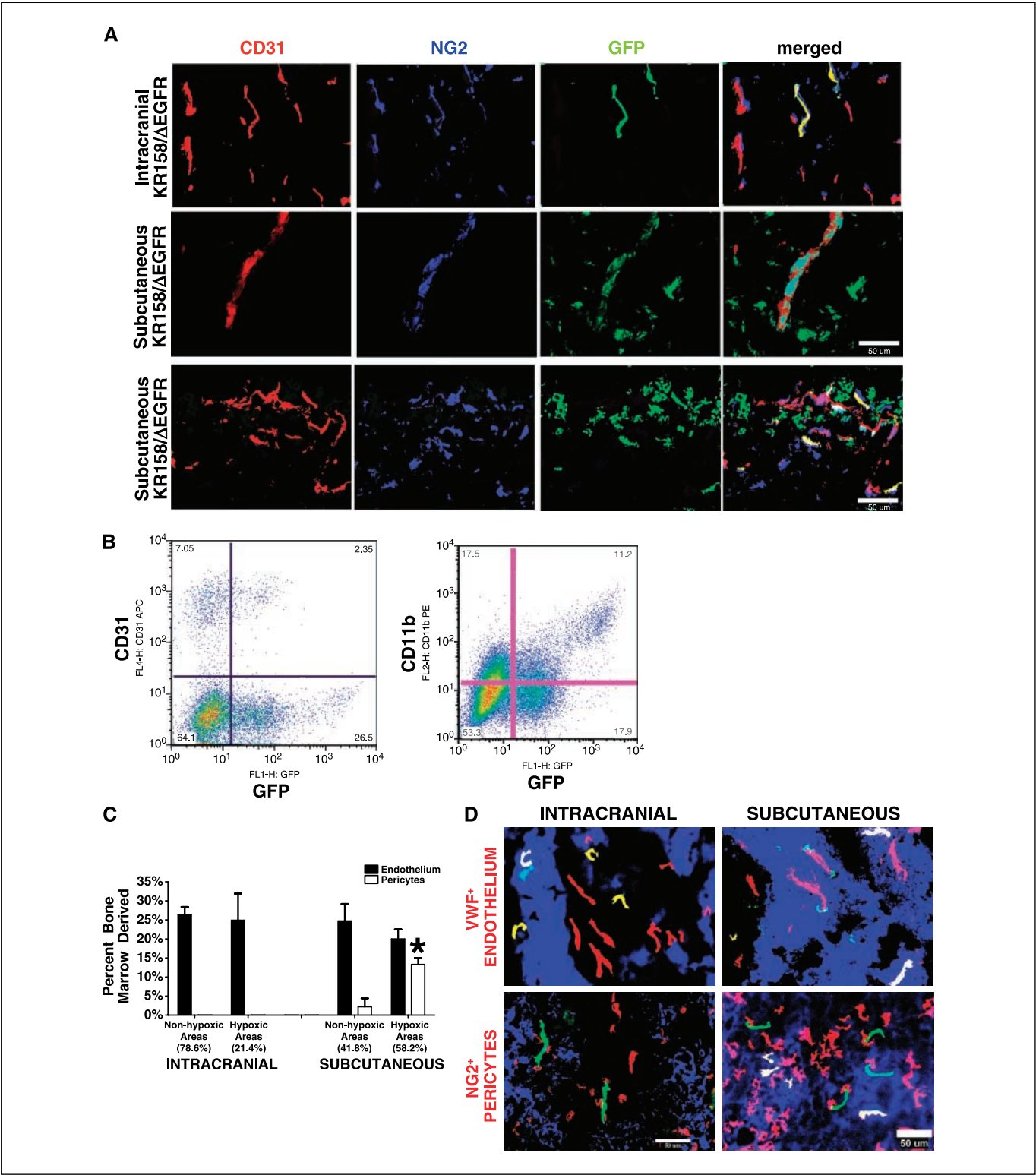
glioblastomas, SDF-1 expression correlates with tumor grade and necrosis (44). VEGF is expressed by breast, prostate, and colon cancers as well as glioblastomas, particularly the 30% of glioblastomas overexpressing EGFR, which up-regulates VEGF expression (31, 34).

In s.c. tumors formed from glioma cells, SDF-1/CXCL12 secretion was sufficient to expand the fate of locally recruited bone marrow–derived progenitors to include endothelium and pericytes, whereas intracranial glioma SDF-1/CXCL12 secretion led to marrow-derived endothelium but not pericytes. Given recent demonstrations that brain and pancreatic tumors exhibit greater hypoxia in ectopic s.c. locations than in orthotopic intracranial (36) or pancreatic (45) environments, s.c. tumors used to study tumor vessel formation may enable the study of hypoxia-mediated events essential to tumor growth, which may be difficult to identify in orthotopic murine environments. One of these events may be the incorporation of marrow-derived pericytes, which, in our study and in another (23), were not detected in orthotopic tumors. Furthermore, we found that pimonidazole, which binds cells with pO_2 <10 mm Hg, bound 21% of the average intracranial murine tumor but 58% of the average murine s.c. tumor. Given the low pO_2 in human tumors, including glioblastomas (5 mm Hg), pancreatic cancers (2.7 mm Hg), and prostate cancers (2.4 mm Hg; ref. 46), hypoxic ectopic s.c. murine tumors, which contained marrow-derived pericytes in this study, may be better than less hypoxic orthotopic murine tumors at mimicking the extreme hypoxia of human tumors, such as glioblastoma.

We confirmed that the microenvironment in s.c. tumors is more hypoxic than in intracranial tumors and found that bone marrow–derived pericytes tended to localize to hypoxic areas of s.c. tumors. Because pericytes, like endothelium, originate from bone marrow–derived progenitors, our findings suggest that tumor cell SDF-1/CXCL12 recruits bone marrow–derived vascular progenitors, whereas tumor microenvironment controls the range of differentiated phenotypes and location of these cells. The effects of tumor microenvironment may involve hypoxia in the microenvironment, stimulating the release of mediators that interact with SDF-1/CXCL12 to expand the fate of marrow-derived progenitors to include pericytes. These factors might include angiopoietins, which are regulated by hypoxia and influence pericyte biology, and could recruit angiopoietin 1 receptor (Tie2)-expressing marrow-derived proangiogenic monocytes (18, 23).

Tumor growth requires communication between tumor cells and cells in the microenvironment. It was recently reported that tumor fibroblast SDF-1 expression increases incorporation of injected Sca-1$^+$CD31$^+$ EPCs obtained from donor marrow (47). Sca-1 and CD31 are both mature endothelial and T lymphocyte markers, making it unclear if tumor fibroblast SDF-1 expression regulates vasculogenesis or angiogenesis involving local mature endothelium. Indeed, SDF-1 secretion from s.c. tumor perivascular extracellular stroma was shown to promote local angiogenesis and recruit nonendothelial marrow-derived CXCR4$^+$ cells (22). We defined progenitor engraftment as tumor endothelium (CD31$^+$vWF$^+$) expressing donor marrow markers, ensuring distinguishing of vasculogenesis from angiogenesis. Furthermore, glioblastomas in their orthotopic intracranial environment lack fibroblasts, suggesting that, in our model, tumor cells themselves regulate vascular progenitor cell engraftment through SDF-1 secretion. The lack and presence of vasculogenesis in GL261/p and Gl261/pSDF tumors, respectively, independent of location further suggest that tumor cell SDF-1, rather than stromal cell SDF-1, stimulates vasculogenesis. Nonetheless, it remains possible that additional cell types within gliomas, such as astrocytes or microglia, could up-regulate SDF-1.

Just as we found varied SDF-1 secretion by murine glioma cells caused differences in intracranial glioma vasculogenesis, heterogeneous SDF-1 secretion by cell lines may explain discordant data about whether vasculogenesis occurs in experimental animal tumors. Although our findings in murine gliomas may not reflect the biology of human gliomas, whose reliance on SDF-1 for vasculogenesis is difficult to verify, it is worth noting that SDF-1 expression is heterogeneous within human gliomas (44) and ovarian carcinomas (39). Thus, a study in which six human tumors developing after bone marrow transplants contained 1% to 12% bone marrow–derived tumor endothelium versus 2% in normal endothelium (17) may reflect heterogeneous tumor SDF-1 secretion.

Glioma SDF-1 expression increases with tumor grade and colocalizes with necrosis, angiogenesis, and invasiveness (44). This may reflect a need for blood vessels formed from circulating marrow-derived vascular progenitors in necrotic or invasive areas because, in these areas, the tumor might be unable to rely solely on remodeling preexisting vessels. Indeed, invasive tumors, such as glioblastomas, may require generating blood vessels from circulating vascular progenitors more than nodular malignancies. Glioblastomas originate from tumor stem cells, which resemble neural stem cells (48). Given that neural stem cells express CXCR4 and migrate along a SDF-1 gradient, SDF-1 secretion in the infiltrative glioblastoma edge may provide a gradient for CXCR4$^+$ cancer stem cells to migrate along. In fact, glioblastoma SDF-1 may be multifunctional. Besides this potential contribution to invasiveness and as described here, vasculogenesis, glioblastoma SDF-1 secretion may also stimulate autocrine growth (49) and regulate T lymphocyte infiltration (50).

We found that endothelial proliferation, a defining glioblastoma feature, was more prevalent in bone marrow–derived endothelium than in locally derived endothelium, suggesting that these two tumor endothelium types not only have different lineages but also have distinct differentiated phenotypes. Furthermore, continuous blockade of SDF-1 receptor CXCR4 blocked recruitment of bone marrow–derived EPCs to tumors without affecting local endothelium, suggesting that the phenotypically distinct subset of

Figure 6. Microenvironment hypoxia influences the location and phenotype range of differentiated vascular progenitors that home to tumors. *A,* immunofluorescence identified CD31$^+$ endothelium (*red*), NG2$^+$ pericytes (*blue*), and GFP$^+$ marrow-derived cells (*green*). Marrow-derived endothelium exhibited yellow staining resembling vessels in merged images seen in intracranial (*row 1*) and s.c. (*row 3*) KR158/ΔEGFR tumors. Marrow-derived pericytes exhibiting cyan (blue + green) staining occurred only in s.c. (*rows 2* and *3*) KR158/ΔEGFR tumors. *B,* flow cytometry of s.c. KR158/ΔEGFR tumors revealed marrow-derived endothelium and pericytes. *Left plot,* GFP$^+$CD31$^+$CD45$^-$ marrow-derived endothelium (*top right quadrant*), which, in this example, represented 25.0% (19.9% average; *n* = 5) of tumor CD31$^+$CD45$^-$ endothelial cells (*top left* and *top right quadrants*). *Bottom right quadrant,* contains nonendothelial marrow-derived cells, which were analyzed in the *right plot. Right plot,* done after gating for CD31$^-$CD45$^-$ cells, shows that 39.0% (22.3% average; *n* = 5) of CD11b$^+$ pericytes (*top left* and *top right quadrants*) were marrow-derived GFP$^+$ (*top right quadrant*). *C,* s.c. KR158/ΔEGFR tumors exhibited greater hypoxia than intracranial KR158/ΔEGFR. *X axis,* hypoxic versus nonhypoxic fractions. The percentage of marrow-derived endothelium did not vary between hypoxic and nonhypoxic areas in either location. Unlike intracranial tumors, s.c. tumors contained marrow-derived pericytes, significantly more in hypoxic than in nonhypoxic areas. *, *P* = 0.00003, compared with percentage of pericytes that were marrow-derived in nonhypoxic areas. *D,* immunofluorescence of intracranial (*left*) and s.c. (*right*) KR158/ΔEGFR tumors: vWF$^+$ endothelium (*row 1*) or NG2$^+$ pericytes (*row 2*) were stained red, marrow-derived GFP$^+$ cells were stained green, and hypoxic areas were stained blue using pimonidazole staining. Marrow-derived endothelium or pericytes stained yellow in nonhypoxic areas and white in hypoxic areas. Bar, 50 μm (*A* and *D*).

tumor endothelium derived from circulating EPCs can be pharmacologically targeted.

AMD3100 reduced tumor vessel density and growth, but the difference lacked significance (data not shown), suggesting that tumors compensate for pharmacologic vasculogenesis inhibition by increasing their reliance on angiogenesis, the sprouting of vessels from local endothelium. However, pharmacologically inhibiting vasculogenesis might impair tumor growth in settings, where local tumor endothelium has already been targeted by radiation or antiangiogenic therapy. Indeed, in one experimental model, wild-type bone marrow–derived EPCs rescued tumor growth when local angiogenesis was genetically impaired (13), validating the need for therapies combining vascular progenitor blockade with the targeting of local endothelium.

Acknowledgments

References

1. Aghi M, Chiocca EA. Contribution of bone marrow-derived cells to blood vessels in ischemic tissues and tumors. Mol Ther 2005;12:994–1005.
2. Conway EM, Collen D, Carmeliet P. Molecular mechanisms of blood vessel growth. Cardiovasc Res 2001;49:507–21.
3. Asahara T, Murohara T, Sullivan A, et al. Isolation of putative progenitor endothelial cells for angiogenesis. Science 1997;275:964–7.
4. Gehling UM, Ergun S, Schumacher U, et al. *In vitro* differentiation of endothelial cells from AC133-positive progenitor cells. Blood 2000;95:3106–12.
5. Shi Q, Rafii S, Wu MH, et al. Evidence for circulating bone marrow-derived endothelial cells. Blood 1998;92:362–7.
6. Asahara T, Takahashi T, Masuda H, et al. VEGF contributes to postnatal neovascularization by mobilizing bone marrow-derived endothelial progenitor cells. EMBO J 1999;18:3964–72.
7. Murohara T, Ikeda H, Duan J, et al. Transplanted cord blood-derived endothelial precursor cells augment postnatal neovascularization. J Clin Invest 2000;105:1527–36.
8. Takahashi T, Kalka C, Masuda H, et al. Ischemia- and cytokine-induced mobilization of bone marrow-derived endothelial progenitor cells for neovascularization. Nat Med 1999;5:434–8.
9. Jackson KA, Majka SM, Wang H, et al. Regeneration of ischemic cardiac muscle and vascular endothelium by adult stem cells. J Clin Invest 2001;107:1395–402.
10. Kocher AA, Schuster MD, Szabolcs MJ, et al. Neovascularization of ischemic myocardium by human bone-marrow-derived angioblasts prevents cardiomyocyte apoptosis, reduces remodeling, and improves cardiac function. Nat Med 2001;7:430–6.
11. Crisa L, Cirulli V, Smith KA, et al. Human cord blood progenitors sustain thymic T-cell development and a novel form of angiogenesis. Blood 1999;94:3928–40.
12. Asahara T, Masuda H, Takahashi T, et al. Bone marrow origin of endothelial progenitor cells responsible for postnatal vasculogenesis in physiological and pathological neovascularization. Circ Res 1999;85:221–8.
13. Lyden D, Hattori K, Dias S, et al. Impaired recruitment of bone-marrow-derived endothelial and hematopoietic precursor cells blocks tumor angiogenesis and growth. Nat Med 2001;7:1194–201.
14. Garcia-Barros M, Paris F, Cordon-Cardo C, et al. Tumor response to radiotherapy regulated by endothelial cell apoptosis. Science 2003;300:1155–9.
15. Davidoff AM, Ng CY, Brown P, et al. Bone marrow-derived cells contribute to tumor neovasculature and, when modified to express an angiogenesis inhibitor, can restrict tumor growth in mice. Clin Cancer Res 2001;7:2870–9.
16. Ruzinova MB, Schoer RA, Gerald W, et al. Effect of angiogenesis inhibition by Id loss and the contribution of bone-marrow-derived endothelial cells in spontaneous murine tumors. Cancer Cell 2003;4:277–89.
17. Peters BA, Diaz LA, Polyak K, et al. Contribution of bone marrow-derived endothelial cells to human tumor vasculature. Nat Med 2005;11:261–2.
18. De Palma M, Venneri MA, Roca C, et al. Targeting exogenous genes to tumor angiogenesis by transplantation of genetically modified hematopoietic stem cells. Nat Med 2003;9:789–95.
19. Gothert JR, Gustin SE, van Eekelen JA, et al. Genetically tagging endothelial cells *in vivo*: bone marrow-derived cells do not contribute to tumor endothelium. Blood 2004;104:1769–77.
20. Machein MR, Renninger S, de Lima-Hahn E, et al. Minor contribution of bone marrow-derived endothelial progenitors to the vascularization of murine gliomas. Brain Pathol 2003;13:582–97.
21. Rajantie I, Ilmonen M, Alminaite A, et al. Adult bone marrow-derived cells recruited during angiogenesis comprise precursors for periendothelial vascular mural cells. Blood 2004;104:2084–6.
22. Guleng B, Tateishi K, Ohta M, et al. Blockade of the stromal cell-derived factor-1/CXCR4 axis attenuates *in vivo* tumor growth by inhibiting angiogenesis in a vascular endothelial growth factor-independent manner. Cancer Res 2005;65:5864–71.
23. De Palma M, Venneri MA, Galli R, et al. Tie2 identifies a hematopoietic lineage of proangiogenic monocytes required for tumor vessel formation and a mesenchymal population of pericyte progenitors. Cancer Cell 2005;8:211–26.
24. Ziegelhoeffer T, Fernandez B, Kostin S, et al. Bone marrow-derived cells do not incorporate into the adult growing vasculature. Circ Res 2004;94:230–8.
25. Bergers G, Song S, Meyer-Morse N, et al. Benefits of targeting both pericytes and endothelial cells in the tumor vasculature with kinase inhibitors. J Clin Invest 2003;111:1287–95.
26. Reilly KM, Loisel DA, Bronson RT, et al. Nf1;Trp53 mutant mice develop glioblastoma with evidence of strain-specific effects. Nat Genet 2000;26:109–13.
27. Ausman JI, Shapiro WR, Rall DP. Studies on the chemotherapy of experimental brain tumors: development of an experimental model. Cancer Res 1970;30:2394–400.
28. Reist CJ, Archer GE, Kurpad SN, et al. Tumor-specific anti-epidermal growth factor receptor variant III monoclonal antibodies: use of the tyramine-cellobiose radioiodination method enhances cellular retention and uptake in tumor xenografts. Cancer Res 1995;55:4375–82.
29. Means TK, Hayashi F, Smith KD, et al. The Toll-like receptor 5 stimulus bacterial flagellin induces maturation and chemokine production in human dendritic cells. J Immunol 2003;170:5165–75.
30. Guillot PV, Liu L, Kuivenhoven JA, et al. Targeting of human eNOS promoter to the Hprt locus of mice leads to tissue-restricted transgene expression. Physiol Genomics 2000;2:77–83.
31. Maity A, Pore N, Lee J, et al. Epidermal growth factor receptor transcriptionally up-regulates vascular endothelial growth factor expression in human glioblastoma cells via a pathway involving phosphatidylinositol 3′-kinase and distinct from that induced by hypoxia. Cancer Res 2000;60:5879–86.
32. Wright DE, Bowman EP, Wagers AJ, et al. Hematopoietic stem cells are uniquely selective in their migratory response to chemokines. J Exp Med 2002;195:1145–54.
33. Peichev M, Naiyer AJ, Pereira D, et al. Expression of VEGFR-2 and AC133 by circulating human CD34(+) cells identifies a population of functional endothelial precursors. Blood 2000;95:952–8.
34. Abe T, Terada K, Wakimoto H, et al. PTEN decreases *in vivo* vascularization of experimental gliomas in spite of proangiogenic stimuli. Cancer Res 2003;63:2300–5.
35. Schols D, Este JA, Henson G, et al. Bicyclams, a class of potent anti-HIV agents, are targeted at the HIV coreceptor fusin/CXCR-4. Antiviral Res 1997;35:147–56.
36. Blouw B, Song H, Tihan T, et al. The hypoxic response of tumors is dependent on their microenvironment. Cancer Cell 2003;4:133–46.
37. Ceradini DJ, Kulkarni AR, Callaghan MJ, et al. Progenitor cell trafficking is regulated by hypoxic gradients through HIF-1 induction of SDF-1. Nat Med 2004;10:858–64.
38. Hong X, Jiang F, Kalkanis SN, et al. SDF-1 and CXCR4 are up-regulated by VEGF and contribute to glioma cell invasion. Cancer Lett 2006;236:39–45.
39. Kryczek I, Lange A, Mottram P, et al. CXCL12 and vascular endothelial growth factor synergistically induce neoangiogenesis in human ovarian cancers. Cancer Res 2005;65:465–72.
40. Conejo-Garcia JR, Benencia F, Courreges MC, et al. Tumor-infiltrating dendritic cell precursors recruited by a β-defensin contribute to vasculogenesis under the influence of Vegf-A. Nat Med 2004;10:950–8.
41. Pelletier F, Bermont L, Puzenat E, et al. Circulating vascular endothelial growth factor in cutaneous malignant melanoma. Br J Dermatol 2005;152:685–9.
42. De Falco E, Porcelli D, Torella AR, et al. SDF-1 involvement in endothelial phenotype and ischemia-induced recruitment of bone marrow progenitor cells. Blood 2004;104:3472–82.
43. Feldkamp MM, Lau N, Rak J, et al. Normoxic and hypoxic regulation of vascular endothelial growth factor (VEGF) by astrocytoma cells is mediated by Ras. Int J Cancer 1999;81:118–24.
44. Rempel SA, Dudas S, Ge S, et al. Identification and localization of the cytokine SDF1 and its receptor, CXC chemokine receptor 4, to regions of necrosis and angiogenesis in human glioblastoma. Clin Cancer Res 2000;6:102–11.
45. Buchler P, Reber HA, Lavey RS, et al. Tumor hypoxia correlates with metastatic tumor growth of pancreatic cancer in an orthotopic murine model. J Surg Res 2004;120:295–303.
46. Brown JM, Wilson WR. Exploiting tumour hypoxia in cancer treatment. Nat Rev Cancer 2004;4:437–47.
47. Orimo A, Gupta PB, Sgroi DC, et al. Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion. Cell 2005;121:335–48.
48. Singh SK, Hawkins C, Clarke ID, et al. Identification of human brain tumour initiating cells. Nature 2004;432:396–401.
49. Barbero S, Bonavia R, Bajetto A, et al. Stromal cell-derived factor 1α stimulates human glioblastoma cell growth through the activation of both extracellular signal-regulated kinases 1/2 and Akt. Cancer Res 2003;63:1969–74.
50. Dunussi-Joannopoulos K, Zuberek K, Runyon K, et al. Efficacious immunomodulatory activity of the chemokine stromal cell-derived factor 1 (SDF-1): local secretion of SDF-1 at the tumor site serves as T-cell chemoattractant and mediates T-cell-dependent antitumor responses. Blood 2002;100:1551–8.

Cancer Research

The Journal of Cancer Research (1916–1930) | The American Journal of Cancer (1931–1940)

AACR American Association for Cancer Research

Tumor Stromal-Derived Factor-1 Recruits Vascular Progenitors to Mitotic Neovasculature, where Microenvironment Influences Their Differentiated Phenotypes

Manish Aghi, Kenneth S. Cohen, Rachael J. Klein, et al.

Cancer Res 2006;66:9054-9064.

Updated version	Access the most recent version of this article at: http://cancerres.aacrjournals.org/content/66/18/9054

Cited articles	This article cites 49 articles, 24 of which you can access for free at: http://cancerres.aacrjournals.org/content/66/18/9054.full#ref-list-1
Citing articles	This article has been cited by 16 HighWire-hosted articles. Access the articles at: http://cancerres.aacrjournals.org/content/66/18/9054.full#related-urls

E-mail alerts	Sign up to receive free email-alerts related to this article or journal.
Reprints and Subscriptions	To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.
Permissions	To request permission to re-use all or part of this article, use this link http://cancerres.aacrjournals.org/content/66/18/9054. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.