RESEARCH ARTICLE

# Minor Contribution of Bone Marrow-Derived Endothelial Progenitors to the Vascularization of Murine Gliomas

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**Until recently, it was generally accepted that the vascularization of solid tumors occurred exclusively through the sprouting and co-option from pre-existing blood vessels. Growing evidence now suggests that bone marrow-derived endothelial progenitor cells (EP) circulate in the blood and may play an important role in the formation of new blood vessels in certain tumors. Whether endothelial progenitors participate in the vascularization of brain tumors has not yet been evaluated. In this study, we examined the contribution of EP to tumor angiogenesis in a murine glioma tumor model. Donor bone marrow cells obtained from transgenic mice constitutively expressing β-galactosidase or GFP either ubiquitously or transcriptionally regulated by an endothelial specific promotor Tie-2 were injected into lethally irradiated adult mice. After bone marrow reconstitution by donor cells, mice were implanted with syngeneic GL261 murine glioma cells. Morphological and confocal 3-dimensional analysis showed that the majority of the engrafted donor marrow cells were expressing hematopoietic and/or microglia markers, but did not appreciably contribute to the tumor vasculature. Implantation of glioma cells genetically engineered to overexpress VEGF produced highly vascularized tumors. However, the number of endothelial progenitors incorporated in the tumor vasculature did not increase. These data strongly suggest that neovascularization in the brain might fundamentally be regulated by the sprouting of pre-existing vessels and implicate that circulating endothelial progenitors do not play a significant role in this process.**

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## Introduction

In humans, gliomas constitute about 30 to 45% of all intracranial tumors. Malignant gliomas, the most common primary brain tumors, are characterized by very rapid local growth and invasiveness. One of the events that accompanies glioma progression is the induction of an angiogenic phenotype. The vasculature of low-grade gliomas resembles that of normal brains, whereas high-grade gliomas show prominent microvascular proliferation with areas of very high vascular density compared to low grade gliomas and normal brain (37, 38).

Similar to brain vascular development during embryonic life, brain tumor vessels develop by the sprouting and co-option of neighboring preexisting vessels, a process termed angiogenesis (35). Vasculogenesis, defined as the in situ differentiation of vascular endothelial cells from primitive precursor cells (angioblasts) was thought to occur only in the early phases of vascular development (18, 44). Recent evidence in the literature suggests that putative angioblasts, also known as endothelial progenitors (EP), might persist in adult life and contribute to the vascularization of tissues in physiological and pathological settings (2, 13, 20, 25, 48). Endothelial progenitors have been isolated from peripheral blood and the bone marrow (3, 34). Similar to embryonic angioblasts, circulating endothelial progenitors have the capacity to proliferate and differentiate into mature endothelial cells. However, they neither acquire markers characteristic of mature endothelium nor the capacity to form a lumen (40). Recent data indicate that certain cytokines, such as vascular endothelial growth factor released by tumor cells, contribute to tumor neovascularization not only by inducing proliferation and migration from mature endothelial cells but also by the mobilization of endothelial progenitors and hematopoietic cells to the tumor vascular bed (41). Despite the finding that EP contribute to the neovascularization in the early phases of tumor growth little is known about the tumor types that are most dependent on the recruitment of EP and hematopoietic cells for their growth. Based on evidence reported in the literature, mobilization and incorporation

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of EP into the tumor vasculature is essential for the growth of lymphomas, Lewis lung carcinoma and colon cancers (2, 29). So far, the role of EP in gliomas has not yet been elucidated. The blood-brain barrier, which restricts the passage of macromolecules, viruses and cells, the absence of a lymphatic drainage and the inadequate expression of MHC molecules and adhesion molecules in brain endothelium might affect the ability of circulating progenitors to interact with and to become incorporated into the brain endothelium (43). In this study, we have used tagged bone marrow cells in order to characterize the contribution of bone marrow-derived EP to the vasculature of a murine glioma model. We further examined whether overexpression of VEGF in glioma cells would increase endothelial progenitor engraftment to the tumor vasculature. We demonstrate here that, although bone marrow cells contribute substantially to the microglial brain population, the overall contribution of EP to the tumor vasculature is not significant.

## Material and Methods

***Generation of chimeric animals.*** All animal experimental procedures were performed in accordance with protocols approved by the local institutional animal care and use committee.

Recipient mice, athymic nude mice (n = 21) and wild-type C57BL/6 (n = 41) aged between 6 to 8 weeks were lethally irradiated with 8.5 Gray of a 137Cs gamma source.

The following transgenic strains were used as donors: *i)* Rosa 26, which expresses the LacZ gene encoding for β-galactosidase in all tissues (TgR[Rosa26]26 Sor) (52); *ii)* Tie-2 LacZ, which expresses LacZ under the transcriptional regulation of the Tie-2 promotor (FVB/N-TgN[Tie2LacZ]182Sato (46); and *iii)* C57BL/6-GFP, a strain carries the GFP driven by chicken β-actin promoter and cytomegolovirus intermediate early enhancer. All cell types in this animal express GFP (BL6 βActin-EGFP) (32). Bone marrow cells were harvested aseptically by flushing the tibias and femurs. One day after lethal irradiation, recipient mice received $5 \times 10^6$ BM cells injected into the lateral tail vein. Immune-deficient athymic nude mice received bone marrow transplantation from Tie-2 LacZ donor cells; C57BL/6 received Rosa 26 or GFP syngeneic donor cells. For control studies, athymic nude mice (n = 2) and C57BL/6 mice (n = 3) received BM cells from wild-type FVB/N or C57BL/6, respectively.

***Hematopoietic engraftment analysis.*** Donor cell engraftment efficiency was determined by analysis of bone marrow and peripheral blood. Bone marrow samples were collected as described above. Blood samples were obtained from the heart immediately before mice were sacrificed and separated by a Ficoll (Pharmacia) gradient density. Light density mononuclear cells were washed in saline (PBS, no calcium or magnesium). Cells were labeled with lineage-specific antibodies conjugated to PE (BD PharMingen, San Diego, Calif) (Mac-1, B220, CD45, Gr-1, TER119). For the detection of β-galactosidase in samples obtained from Tie-2 and Rosa chimeras, cells were stained with fluorescent β-gal substrate fluorescein di-β-D-galactopyranoside (FDG, Molecular Probes, Eugene, Ore) (31) according to the manufacturer's instructions. Samples were examined in 2-color FACS using Cellquest software (Becton Dickinson, Heildelberg, Germany).

***Cell lines.*** The GL261 mouse glioma tumor was originally induced by intracerebral injection of chemical carcinogen into the C57BL/6 and maintained on syngeneic host by alternating intracranial and subcutaneous implantation of small pieces of tumor. Detailed characterization of the cell line has been published elsewhere (5, 47).

The cells were cultured in Dulbecco's modified Eagle Medium (DMEM, Life Technologies) supplemented by 10% fetal calf serum. GP+E86 virus producer cells encoding for the full length mouse $VEGF_{164}$ cDNA (45) were kindly provided by Dr Wizigmann-Voss (MPI, Bad Nauheim, Germany) and were grown in DMEM containing 10% FCS (Life Technologies) and 0.2 mg/ml of the neomycin analog G418 (Calbiotech).

***Generation of VEGF overexpressing GL261.*** To generated GL261 overexpressing $VEGF_{164}$, virus supernatant from GP+E86 VEGF cells were applied to subconfluent GL261 overnight. Twenty-four hours after infection, cells were incubated with medium containing 0.4 mg/mg G418 for the selection of positive clones. Colonies of neomycin-resistant clones were harvested after 14 days selection and assayed for VEGF recombinant expression.

***Characterization of VEGF overexpressing GL261.*** Subconfluent cells were washed 3 times in PBS and incubated for 24 hours with DMEM-containing 1% serum. For hypoxia experiments GL261 WT and VEGF transfected cells were cultured in an anaerobic (oxygen content approximately 0.2%) gas chamber (Becton

Dickinson) as described previously (36). After removal from the gas chamber, cells were immediately processed for RNA extraction. Conditioned medium was collected, centrifuged and frozen in aliquots.

Mouse VEGF protein levels were measured in conditioned medium using the Quantikine TM immunoassay kit (R&D system, Mineapolis, Minn), following the manufacturer's instructions in 2 independent experiments.

Total RNA was collected from confluent cells and electrophoresed through an agarose-formaldehyde denaturing gel and transferred onto nylon membranes. The filters were hybridized for 2 hours at 68°C with 32P-labeled cDNA encoding for the full length mouse $VEGF_{164}$ (10) in hybridization solution (QuickHyb, Stratagene, La Jolla, Calif). Loading control was performed with $^{32}P$ cDNA encoding 18S. Densitometry was performed using TINA 2.0 software.

***Tumor implantation.*** Bone marrow-transplanted mice were injected subcutaneously (s.c.) with $10^6$ syngeneic GL261 murine glioma cells resuspended in 100 μl of iced Matrigel (Becton-Dickinson, San Jose, Calif). Intracranial brain tumors were induced by stereotactic (Kopf stereotactic frame) injection of $2 \times 10^4$ GL261 cells in 5 μl of phosphate buffer (PBS). Cells were implanted into the right cerebral hemisphere of anesthetized mice (isofluorane) at a depth of 4 mm below surface of the skull using a 10 μl Hamilton syringe.

***β-galactosidase staining and immunohistochemistry.*** Tie-2-LacZ and Rosa 26 bone marrow chimeric animals were perfused transcardially with PBS to minimize blood cell contamination. Subcutaneous tumors and brain coronal sections were embedded in Tissue-Tek and frozen in pre-cooled Methylbutane. Cryosections (12-14 μm) were fixed in 2% paraformaldehyde (PFA) in PIPES buffer (pH 6.9), rinsed 3 times in PBS and incubated overnight in PBS-containing 5-bromo-4-chloro-3-indolyl-β-D-galactosidase (X-Gal) at 0.5 mg/ml, 20mM potassium ferricyanide, 20mM potassium ferrocyanide and 2mM $MgCl_2$. X-gal staining was performed together with positive control tissue obtained from Rosa 26 and negative control tumors obtained from animals transplanted with FVB/N or C57BL6 wild-type. After the X-gal reaction, sections were stained immunohistochemically with the following antibodies: Anti-mouse CD31 (rat, BD PharMingen), anti-mouse CD105 (rat, Southern Biotechnology Associates, Inc., Birmingham, Ala), anti-mouse CD45 (rat, Cymbus Biotechnology LTD), anti-mouse macrophage marker F4/80 (Serotec Ltd, Oxford, United Kingdom), anti-Mac-1 (rat, BD PharMingen), and anti-human von Willebrand Factor (rabbit, Dako, Glostrup, Denmark). Sections were blocked for non-specific antibody binding with 2% normal goat serum, followed by incubation with primary antibodies. Following three washes with PBS, sections were incubated with biotinylated goat anti-rabbit immunoglobulin (Vector Laboratories, Burlingame, Calif) or biotinylated goat anti-rat immunoglobulin (Southern Biotechnology Associates, Inc). After 3 PBS washes, sections were incubated with avidin-biotin complex reagent (Vector Laboratories) followed by 3,3 diaminobenzidine (Vector Laboratories) according the manufacturer's instructions. Negative controls were performed using unspecific immunoglobulins (rat IgG, BD Pharmigen; rabbit IgG, Dianova). Photomicrographs were taken using Zeiss Axioplan 2 Imaging and Axion Vision 3 Software.

***Free-floating immunofluorescence and confocal analysis.*** GFP chimeric animals were perfused with PFA 4% in PBS. Organs were post-fixated in PFA 4% for 24 hours. Coronal brain sections (50 μm) were cut using a Vibratome (Leica). Free-floating sections were incubated with the following antibodies and dilutions: rabbit anti-vWF (1:200), rat anti-mouse CD45 (1:100: Cymbus), rat anti-mouse CD31 (BD PharMingen), rat anti-mouse F4/80 (1:100; Serotec), and rabbit anti-GFP (Molecular Probe, Eugene, Ore) (1:100). Sections were then incubated with Cy5-labeled donkey anti-rabbit (Jackson ImmunoResearch, West Grove, Pa) and/or with Texas Red goat anti-rat (Southern Biotechnologies) or Texas Red goat anti-rabbit (Jackson ImmunoResearch, West Grove, Pa) mounted and analyzed with a krypton-argon laser scanning confocal imaging system (Leica). GFP were imaged with excitation at 495 nm and emission at 510 nm. Texas red was imaged with excitation at 547 nm and emission above 572 nm. Cy5 were imaged with excitation 650 nm and emission 670 nm. Microscopic data were acquired using a 40× oil-immersion objective lens.

For 3-dimensional analysis, sections were analyzed in Micro System Zeiss LSM and analyzed using Autovisualize Software (Autoquant Imaging, Inc).

***Morphometric analysis.*** The percentage of engraftment of bone marrow-derived endothelial cells was determined in mice transplanted with Tie-2LacZ BM cells. Bone marrow-derived endothelial cell engraftment was quantified by determining the number of pos-

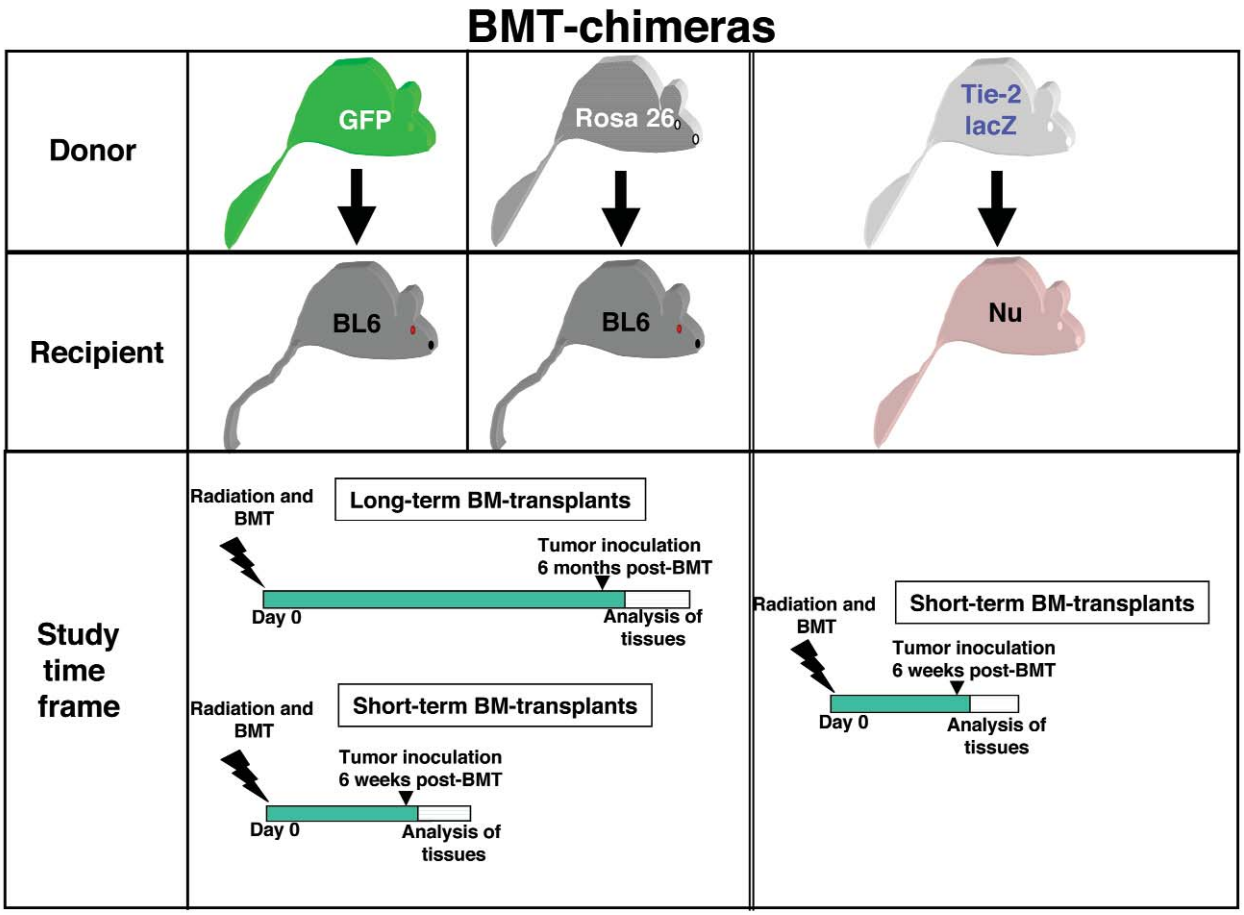



**Figure 1.** Experimental schema. Recipients C57BL/6 or nude mice were lethally irradiated and transplanted with unfractionated bone marrow cells obtained from three different transgenic mice: Rosa 26, GFP, Tie-2 LacZ. After 6 weeks (short-term transplantation) or 6 months (long-term transplantation) chimeric animals were subjected to tumor implantation. Intracranial tumors were analyzed 7 to 18 days post-implantation. Tumors growing subcutaneously were harvested 21 days after tumor inoculation.

itive vessel profiles (one or more LacZ+ cell that also co-stained with an antibody against CD31 per total vessels in a tumor section observed under the light microscope, 10× field). The mean percentage of LacZ+ vessels is based on the examination of 150 or more tumor vessels of various sizes from an average of 16 sections/each time point.

***Evaluation of VEGF plasma levels.*** Mouse VEGF plasma levels were measured in ETDA plasma of mice implanted with GL261 intracerebrally and controls by commercial ELISA kit (R&D, Minneapolis, Minn) following manufacturer's instructions.

***RNA Isolation and RT-PCR.*** Total RNA from mouse bone marrow cell suspension, from tumor and normal organs from Tie-2 LacZ BMT experimental animals was isolated as described previously (26). Reverse transcription was performed from one μg of RNA using a Superscript Preamplification kit (Life Technologies) according to the manufacturer's instructions. Primer pairs for amplification of Tie-2 LacZ and ribosomal S12 reverse-transcribed products were as follows: LacZ: 5′-GACGA AACGC CTGCC AGTAT TTAG-3′; LacZ reverse: 5′-CCGTC ACGAG CATCA TCCTC-3′; rS12: 5′-ACGTC AACAC TGCTC TACA-3′; rS12 reverse: 5′-CTTTG CCATA GTCCT TAAC-3′.

Ten microliters of each PCR mixture were electrophoresed in 1.5% agarose gel containing ethidium bromide and photographed.

## Results

***Hematopoietic engraftment in murine BM models.*** To assess the contribution of endothelial progenitors to the vascularization of a murine glioma, we employed two

| | C57BL6/Rosa | C57BL6/GFP | Nude/Tie-2 LacZ |
|---|---|---|---|
| Short term transplantation | GL261 wt<br>i.c. (n=12)<br>s.c.(n=6) | GL261 wt<br>i.c. (n=3) | s.c. (n=7)<br>GL261VEGF (n=2)<br>GL261 wt (n=5)<br><br>i.c (n=13)<br>GL261VEGF (n=4)<br>GL261 wt (n=9) |
| Long term transplantation | i.c. (n=8)<br>GL261 VEGF (n=4)<br>GL261 wt (n=4) | i.c.(n=9)<br>GL261 VEGF (n=4)<br>GL261 wt (n=5) | |

**Table 1.** *Tumor implantation in bone-marrow-chimeras.* After 6 weeks (short term transplantation) or 6 months (long-term transplantation), bone marrow chimeras were implanted either intracerebrally (i.c) or subcutaneously (s.c) with GL261 wild-type cells (wt) or GL261 genetically engineered in order to overexpress VEGF (GL261-VEGF).

different bone marrow transplantation models. Forty-one C57BL/6 mice were lethally irradiated and transplanted with unfractionated syngeneic bone marrow cells of 6- to 8-week-old Rosa26 mice, a transgenic strain in which the LacZ gene is expressed widely, or with bone marrow cells of transgenic animals that express GFP in all tissues driven by the β-actin promotor.(Figure 1) Three mice received BM from C57BL/6 wild-type mice for control purposes.

In a second bone marrow transplantation model, we used donor cells expressing LacZ under the control of a tissue-specific promotor-enhancer sequence. Immunodeficient nude mice (n=22) were transplanted with donor bone marrow cells obtained from transgenic Tie-2 LacZ mice, which express LacZ under the transcriptional control of the endothelial cell specific tyrosine-kinase Tie-2. Two mice received BM cells obtained from the FVB/N mice for control experiments (Figure 1)

After 6 weeks (short-term transplant) or 6 months (long-term transplant) mice were injected either s.c. or i.c. with GL261 murine glioma cells (Table 1).

Hematopoietic engraftment was assessed by FACS analysis when the mice were sacrificed. Chimeric animals showed multilineage hematopoietic engraftment ranging from 42 to 85% in peripheral blood of Rosa or GFP chimeras and were similar either in long-term as well as in short-term transplants. The expression of LacZ in nucleated blood cells prepared from mice transplanted with Tie-2LacZ donor cells ranged between 9 to 14% (data not shown). Bone marrow cells from Tie-2 lacZ chimeric mice showed co-expression of β-galactosidase and myeloid markers (CD11b and Gr-1), pan-hematopoietic marker CD45 and c-kit (CD117) (data not shown). X-gal staining of bone marrow and spleen confirmed the engraftment in Rosa and Tie-2 chimeras (data not shown).

***Incorporation of donor cells into murine glioma after short-term BMT.*** In short-term transplant studies, we implanted the bone marrow chimeras either subcutaneously or intracerebrally with GL261 glioma cells 6 weeks after bone marrow transplantation.

Brains were retrieved 7, 14 and 18 days after intracerebral tumor cell injection, except for brains of GFP chimeras (n=3), which were analyzed only on day 18 post-transplantation. At least 2 animals from each group were analyzed per time point. Tumors implanted subcutaneously were harvested on day 21 post-implantation. Cryosections of brain and s.c. tumors derived from Rosa 26 and Tie-2 LacZ chimeras were stained with X-gal followed by incubation with endothelial and hematopoietic cell-specific antibodies. These sections were analyzed to identify bone marrow-derived LacZ+ cells which had been incorporated into the vascular tumor bed.

In subcutaneous tumors growing in Rosa 26 chimeras, we found LacZ+ cells, particularly in areas of necrosis. LacZ+ cells did not co-stain with antibodies against endothelial cell markers (Figure 2A-C). The vast majority of LacZ+ cells in tumor tissue expressed the hematopoietic marker CD45 (Figure 2D).

In brain sections of Rosa 26 transplanted animals, very few LacZ+ cells were detected. These LacZ+ cells had a globular morphology and were mostly related to blood cells extravasated into tumor stroma or aggregated in intravascular lumens. X-gal staining of brain sections obtained from Rosa 26 transgenic mouse showed a robust β-gal staining in neural as well as non-neural brain cells and endothelial cells (data not shown).

In intracerebral tumors growing in Tie-2LacZ chimeric animals, LacZ+ cells were localized in the tumor stroma or integrated into tumor vascular structures in peripheral regions, as demonstrated by co-staining

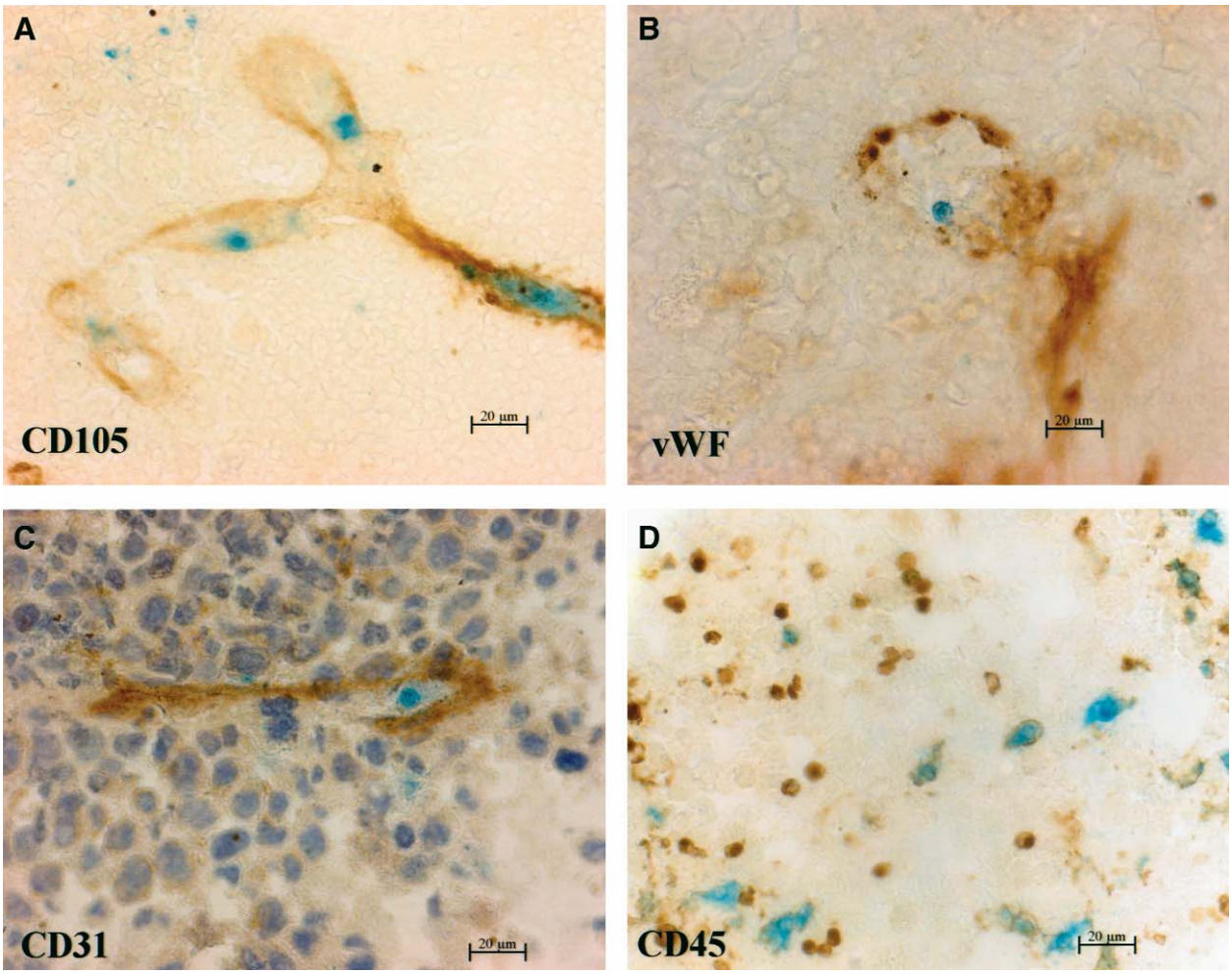



**Figure 2.** X-gal stained sections of s.c. tumor growing in Rosa-26 bone marrow chimeric mouse costained with CD 105 (**A**), vWF (**B**), CD31 (**C**) and CD45 (**D**). Several LacZ+ cells were found adherent to the vessel lumen, but not integrated into the tumor endothelium. Most LacZ+ cells stained with CD45.

with endothelial cell markers CD105 and CD31 (Figure 3A-C). Most LacZ+ cells localized in tumor stroma had a globular appearance and stained positive for CD45 (Figure 3D). Some LacZ+ cells were positive for the myeloid marker CD11b (Mac-1) (Figure 3E) and some LacZ+ cells displayed the ramified characteristic of microglia, although these cells did not express the microglia/magrophage marker F4/80 (Figure 3F). Importantly, the F4/80 antibody stained several microglial cells in the same section (Figure 3G), implying that the F4/80 negative cells might be of myeloid origin that had not completely differentiated to express the F4/80 antigen. Glioma cells were also implanted subcutaneously into Tie-2 LacZ chimeric mice. Tumor tissues stained with X-gal demonstrated that LacZ+ cells localized focally in tumor stroma, or associated with vessel profiles particularly at the tumor periphery (data not shown). In tumors obtained from control animals transplanted with wild-type FVB/N or C57BL6 donor cells, the X-gal reaction was completely absent (data not shown).

***Levels of donor contribution to tumor vascularization in Tie-2 chimeras.*** To assess the number of EP cells incorporated into tumor vessels, we quantified the number of LacZ+ vessels in i.c. and s.c. tumors retrieved from Tie-2 LacZ chimeras. The percentage of vascular engraftment is based on the ratio of LacZ+ vessels per total number of vessels in the tumor tissue. Positive criteria included vessels that contained at least one cell co-staining for LacZ and CD31. At least 16 sections/per time point containing tumor tissue were

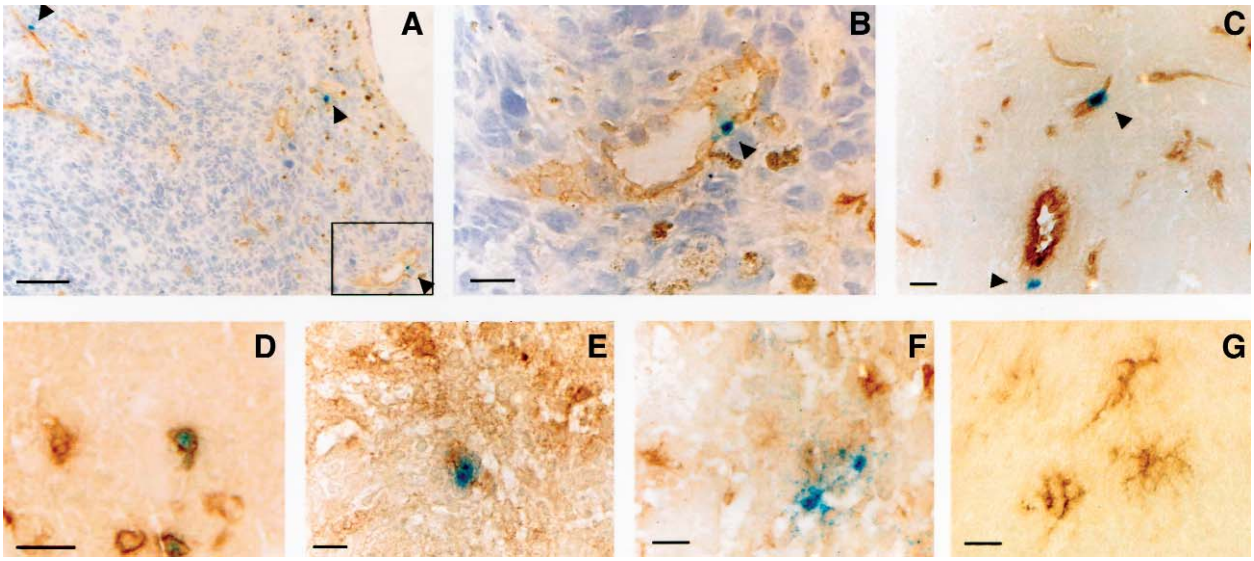



**Figure 3.** Phenotype of bone marrow-derived LacZ+ cells in intracranial murine glioma of nude Tie-2LacZ chimeric mice. **A.** X-gal histochemistry followed by immunostaining for CD105 and hematoxylin staining of tumors harvested on day 18 post-implantation demonstrates LacZ+ cells incorporated into tumor vessel or localized at the tumor stroma (arrows). **B.** High magnification of boxed area in a) showing LacZ+ cell lining the capillary lumen (arrow). **C.** Immunohistochemical analysis of day 14 tumor stained with X-gal and CD31 showing LacZ+ cell in tumor stroma and tumor vessel (arrows). **D.** Most LacZ+ cells found in tumor stroma express the pan-hematopoietic marker CD45. **E.** Some cells express the myeloid marker CD11b. **F.** Some LacZ + cells display the ramified characteristic of microglia, although they are negative for F4/80. **G.** The F4/80 antibody stains several microglial cells in the same section. Scale bars: 100 μm (**A**); 20 μm (**B-G**).

evaluated in a 10× field. Independent of the time point in which i.c. tumors were collected (on day 7, 14 and 18 post-transplantation), levels of EP incorporation into the tumor vasculature was similar with a mean prevalence of LacZ-positive vessels of 0.6% (±0.2). The average rate of vessel engraftment in s.c. tumors was 0.59%.

***Bone marrow-derived cells contribute substantially to the microglia pool but not to the tumor vascular bed.*** We further analyzed the incorporation of bone marrow-derived progenitors into the tumor vasculature of BM GFP-chimeric mice by confocal laser microscopy and 3-dimensional analysis.

Donor derived GFP+ cells primarily localized in the tumor bed and leptomeninges and were detected closely related to the tumor vasculature. In short-term chimeric animals, the unaffected contralateral hemisphere was virtually devoid of GFP-expressing cells. We did not find any GFP cells that fulfilled the criteria for transdifferentiation into endothelial cell lineage: GFP+ cells did not colocalized with endothelial cell markers such as CD31 or vWF, instead most GFP+ cells expressed the pan-hematopoietic marker CD45 (Figure 4A-C). Moreover, the vast majority of GFP+ cells that had engrafted in tumors expressed the monocyte/macrophage specific plasma membrane antigen F4/80 (Figure 4D-F). These cells had the typical ramified appearance of brain microglia, providing further evidence for microglial identity (Figure 4G-I). Importantly, immunofluorescence for localization of GFP using a rabbit anti-GFP antibody did not enhance the detection of GFP compared with the detection of GFP native fluorescence (data not shown).

***Incorporation of donor derived cells in long-term BM chimeric animals.*** We tested whether donor bone marrow cells participate in the vascularization in murine gliomas when stable and complete reconstitution of the hematopoietic system has been achieved (at least 4 months after bone marrow transplantation (11). C57BL/6 (n = 17) were bone marrow transplanted with syngeneic Rosa 26 or GFP donor cells. We performed intracranial tumor implantation 6 months after bone marrow transplantation to ensure durable, stable hematopoietic reconstitution and collected the brains on day 7 and 14 post-tumor implantation.

Confocal analysis showed GFP+ cells associated with tumor vessels, some lining the luminal vessel side. These cells did not colocalize with CD31 or vWF and were CD45+ (Figure 5A-F). In long-term chimeras,

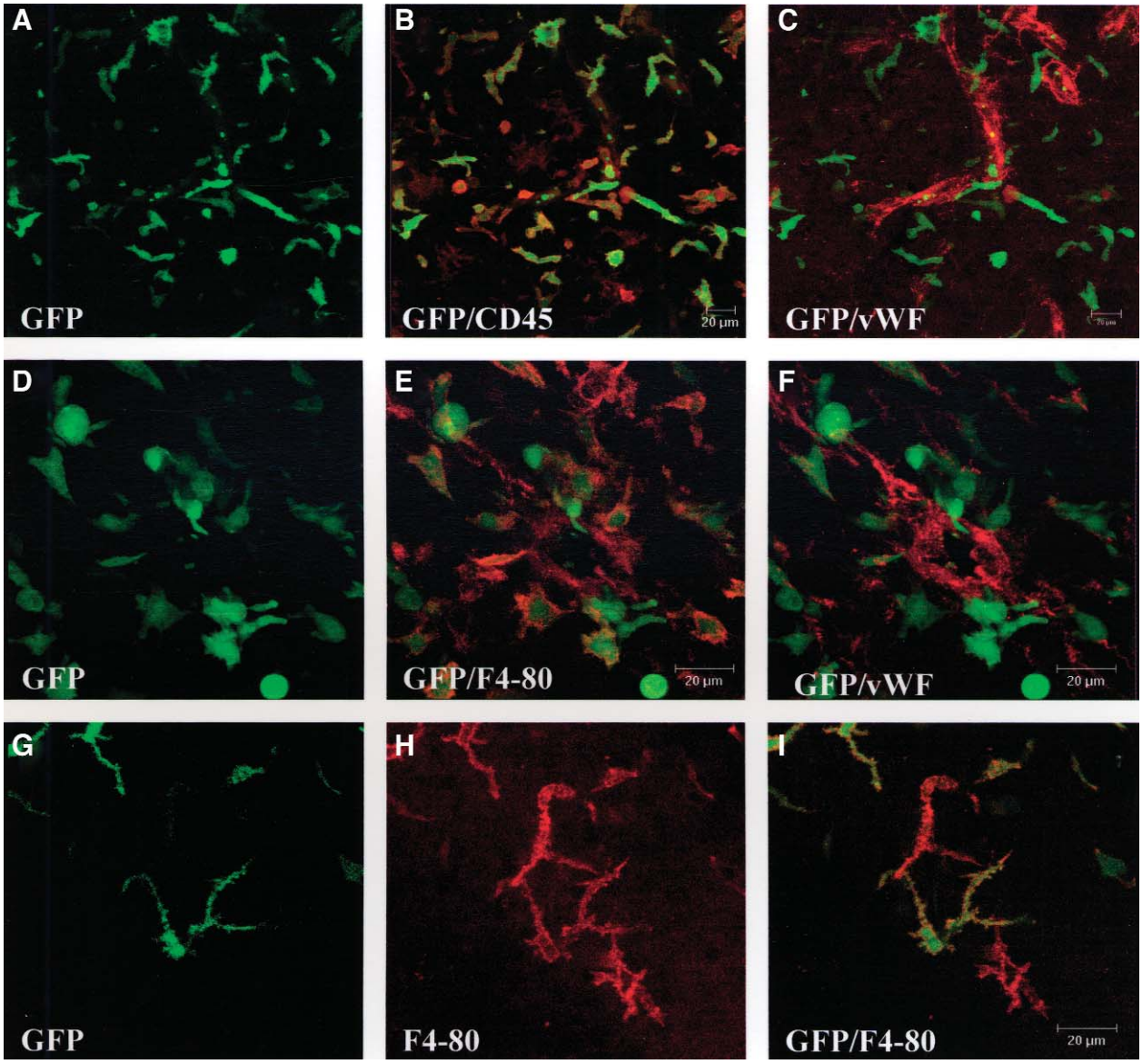



**Figure 4.** Donor cells contribute to microglia pool but not to tumor endothelium. Representative confocal micrographs of intracerebral tumors in C57BL6/GFP chimeras. GL261 cells were inoculated six weeks after bone marrow transplantation (short-term transplant). Brains were analyzed 18 days post-implantation. The left column identifies GFP+ cells (**A, D, G**). Merged confocal images of GFP (green) and CD45 (red) (**B**) and GFP (green) and vWF (red) (**C**) from the same section demonstrate that GFP+ cells associated with the tumor vascular structures are infiltrating hematopoietic cells. Merged confocal images of GFP (green) and F4/80 (red) (**E**) and GFP (green) and vWF (red) (**F**) showing that GFP expressing cells co-expressed macrophage/microglia marker F4/80 but do not colocalize with vWF. Higher magnification of GFP+ cells expressing F4/80 show the typical ramified morphology of microglia cells (**G, H, I**).

GFP-expressing cells increased significantly and were detected not only in the tumor bed but also in normal brain, predominantly in perivascular and leptomeningeal sites. Increased density of GFP+ cells was also observed in the choroid plexus (Figure 5G-H). Compared with GFP chimeras, in Rosa 26 chimeric animals, few LacZ+/CD45+/CD31- cells were found around the tumor blood vessels, at the leptomeninges and choroid plexus (data not shown).

For 3-D imaging, sections of GFP chimeras were scanned in a Zeiss microscope. The relationship of vessels (blue channel), GFP (green channel) and CD45

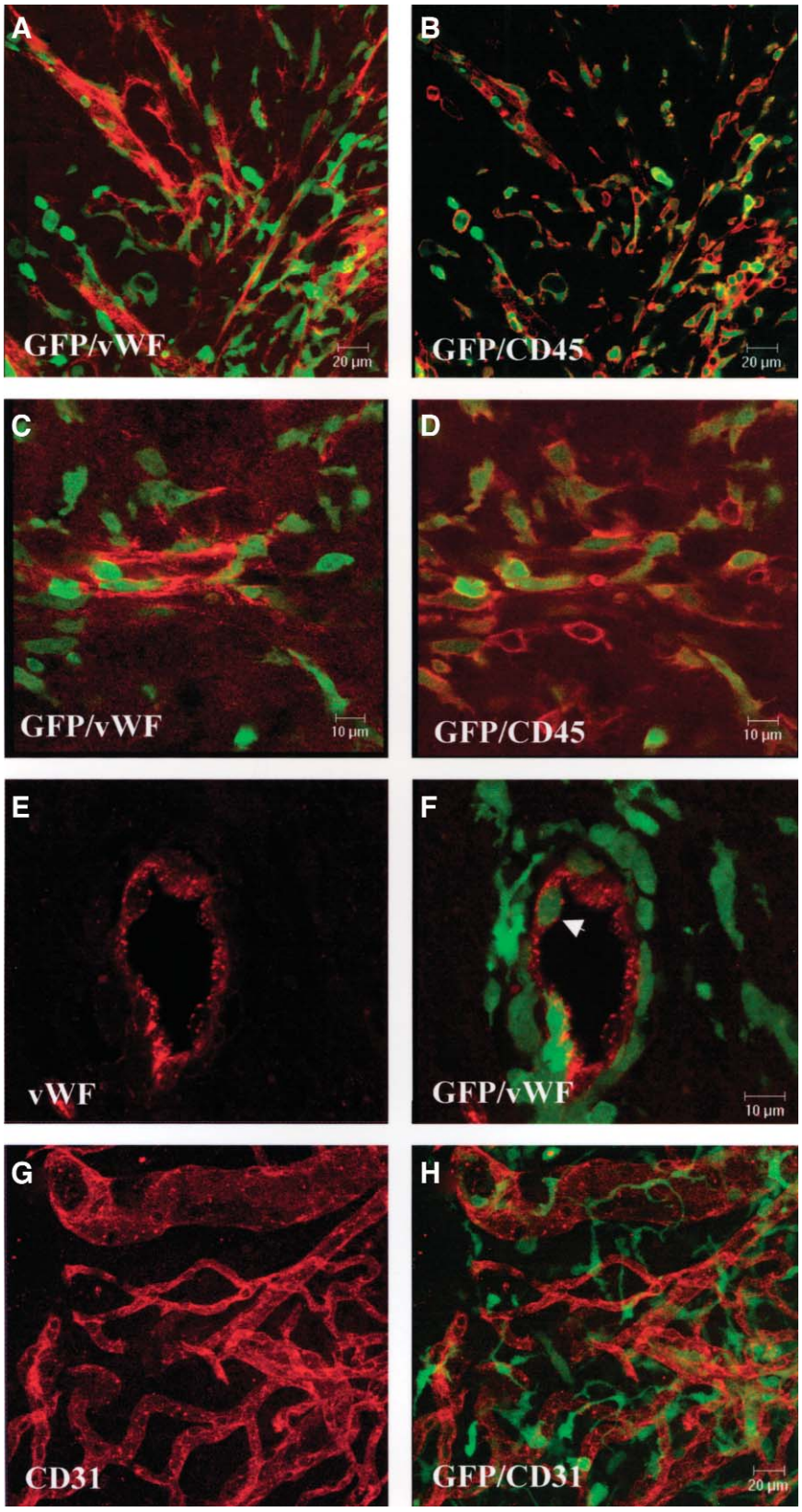



**Figure 5.** Brain sections of C57BL6/GFP long-term chimeric animals collected 7 days post i.c. inoculation of GL261. **A.** Confocal micrograph of tumor area discloses several GFP+ cells (green) associated with tumor vessels (vWF, red). **B.** Colocalization of GFP (green) and CD45 (red) in the same section showed in A. **C.** Higher magnification of tumor vessel stained with vWF (red) shows several GFP+ cells (green) infiltrating the endothelium. **D.** Same section stained with CD45 (red) demonstrates the close association of GFP+CD45+ cells with the tumor endothelium. **E-F.** Confocal microscopy of tumor vessel stained with an antibody to vWF (red) shows GFP+ cell (arrow) lining the tumor capillary lumen (z-axis only 2 μm). Note that in the merged image there is no colocalization of vWF and GFP. **H-I.** Several GFP-expressing cells (green) are detected in choroid plexus (stained with CD31, red). Merged images show no colocalization of CD31 and GFP in choroid plexus.

(red channel) delineated in 3-D images confirmed that bone marrow-derived GFP cells adherent to tumor vessels were trafficking leukocytes (data not shown).

***VEGF overexpression did not increase the incorporation of EP into glioma blood vessels.*** Because VEGF is known to increase circulating bone marrow-derived endothelial progenitors, we assessed the impact of increasing VEGF expression in glioma cells on their ability to induce recruitment and integration of EP in the tumor vascular bed. In vitro under normoxic conditions the average levels of VEGF protein in a conditioned medium of GL261 cells is low (28.5 ± 12.6 pg/ml). GL261 stably transfected with murine $VEGF_{164}$ showed a significant increase in VEGF levels in conditioned serum (1441 ± 65pg/ml)

as compared to the parental cell line (Figure 6A). We also investigated VEGF mRNA levels in GL261 cells under hypoxic conditions. A 10-fold upregulation of VEGF mRNA was observed after 16 hours of hypoxia in GL261 (Figure 6B). Compared with wild-type cells, a VEGF-overexpressing clone showed a 20-fold upregulation, which further increased under hypoxia (Figure 6B). In vitro growing curves of the VEGF-overexpressing cells and the parental cells were similar (data not shown).

To investigate whether VEGF local levels have an effect on the number of donor derived cells engrafted in the tumor vasculature, GL261 (wild-type) or VEGF overexpressing GL261 were intracerebrally implanted in long-term bone marrow chimeric animals (GFP and Rosa) and in Tie-2 LacZ chimeras. Tumor endothelium was visualized by CD31 and vWF immunostaining. Intracranial tumors were analyzed on day 7 and 14 post implantation (Figure 7A). The morphometric analysis was performed in tumor sections retrieved from Tie-2-lacZ chimeric animals. VEGF-overexpressing cells originated highly vascularized rapidly growing tumors which induced symptoms related to intracranial tumors around day 14 (wild-type GL261 tumors induce symptoms around day 24) and showed an increased capillary density on day 7 ($217 \pm 21$ capillaries/mm$^2$) compared to parental control ($121 \pm 16$ vessels/mm$^2$). On day 14 capillary density in VEGF overexpressing tumors was lower ($113 \pm 20$ capillaries/mm$^2$) compared to capillary density evaluated in tumors generated with wild-type cells ($154 \pm 20$ capillaries/mm$^2$). These results are most probably due to the increase in intratumoral edema found in VEGF-overexpressing tumors (Figure 7B). Analysis of s.c. tumors growing in Tie-2 chimeras harvested on day 21 showed an increased capillary density in VEGF-overexpressing tumors compared to tumors originated from wild-type cells ($96.5 \pm 36 \times 39.3 \pm 22.5$ capillaries/mm$^2$, respectively) (Figure 7B).

Although VEGF overexpression had profound effects on glioma growth and vascularization, we could not detect higher numbers of bone marrow-derived EP cells that incorporated into neovessels. The mean of vascular engraftment was 0.33% in i.c. tumors and 0.6% in s.c. tumors (Figure 7C).

In Rosa and GFP long-term chimeras, double staining analysis showed that in the VEGF- overexpressing tumors many vessels were surrounded by several layers of mononuclear CD45+ cells, but donor-derived cells did not coexpress endothelial cell markers. This finding further indicates that although VEGF induced chemotaxis of microglia cells, local levels of VEGF had no effect on the bone marrow-derived endothelial progenitor engraftment in mouse glioma (data not shown).

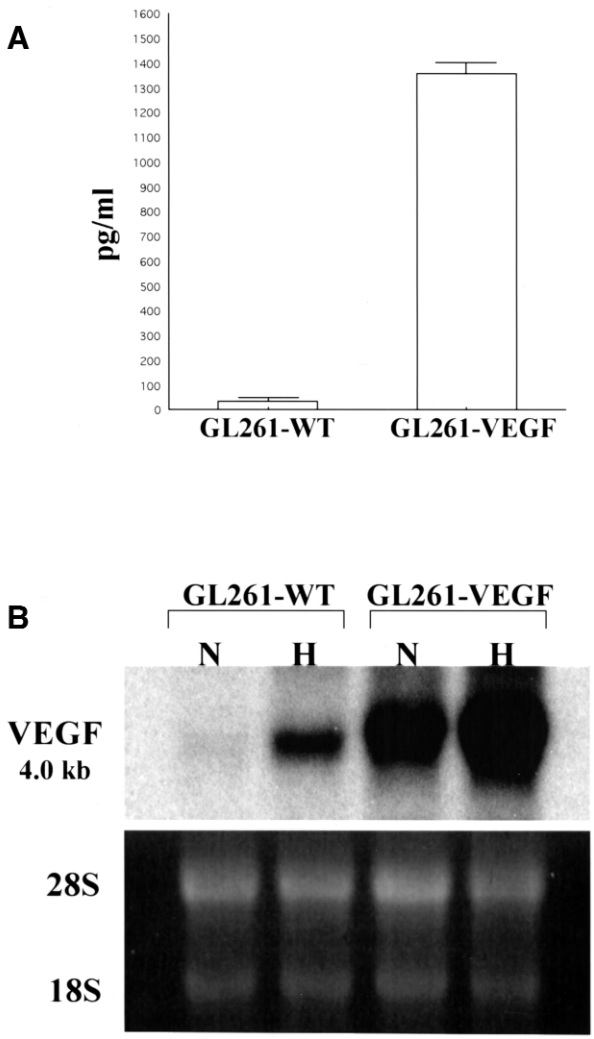
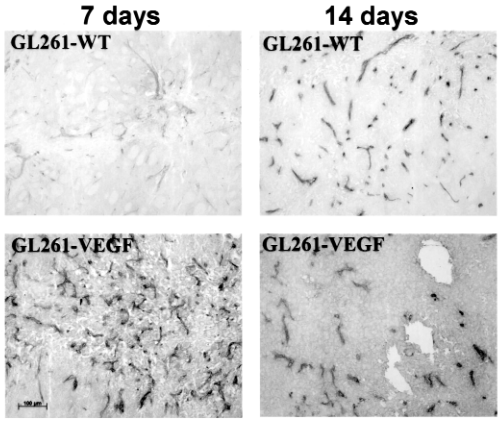



**Figure 6.** Characterization of cells overexpressing VEGF **A.** In vitro levels of VEGF protein (pg/ml) in conditioned medium of GL261-WT and GL261-VEGF (clone#1) under normoxic conditions determined by VEGF Elisa. Data expressed in pg/ml represent the mean and S.D. mean of two preparations of conditioned medium. **B.** Northern blot analysis of VEGF expression in GL261-WT and GL261-VEGF. Ten micrograms of total RNA from confluent monolayers of GL261-WT or GL261-VEGF cells incubated for 16 hours under normoxic conditions (N) or under hypoxia (H). Ethidium bromide-stained gel indicates amount of total RNA in each lane.

***VEGF plasma levels were not increased in i.c.-bearing animals.*** Plasma levels of VEGF were measured in 19 intracranial bearing animals when they were sacrificed: 9 on day 7 post implantation; 8 on day 14 post-implantation; 2 on day 18 post-implantation and 3




B

| Capillary density – Quantification by CD31 immunohistochemistry (capillaries/mm$^2$) | | |
|---|---|---|
| | GL261-WT | GL261-VEGF |
| 7days (i.c.) | 121 ±16 | 217 ±21 |
| 14 days (i.c.) | 154 ±20 | 113 ±20 |
| 21 days (s.c.) | 39.3 ±22.5 | 96.5 ±36 |

C

| | Days of testing | LacZ+ vessels number per total | Mean % of endothelial cell engraftment |
|---|---|---|---|
| GL261-WT | 7 days (i.c.) | 1/156 | 0.60 |
| | 14 days (i.c.) | 3/473 | 0.60 |
| | 21 days (s.c.) | 4/670 | 0.59 |
| GL261-VEGF | 7 days (i.c.) | 2/526 | 0.38 |
| | 14 days (i.c.) | 1/342 | 0.29 |
| | 21 days (s .c.) | 3/616 | 0.48 |

**Figure 7. A.** Comparison of the vascularity of GL261-WT and GL261-VEGF tumors grow i.c. in nude mice transplanted with Tie-2Lacz bone marrow cells assessed by immunohistochemical staining with an anti-CD31 antibody. Representative micrographs of tumors collected on day 7 and day 14 post i.c. inoculation. **B.** Morphometric analysis of intracranial (day 7 and day 14) and subcutaneous (day 21). Capillary density (capillaries/mm2) is calculated based on the examination of 16 or more cryosections per time point. **C.** Prevalence of LacZ+ positive cells in tumor vessels. Bone-marrow derived endothelial cell engraftment was quantified by determining the number of positive vessel profiles (one or more LacZ+ cells that also co-stained with an antibody against CD31 per total vessel in a tumor section observed under the light microscope (10× field). The mean percentage of LacZ+ vessels is based on the examination of 150 or more tumor vessels of various sizes from an average of 16 sections/each time point.

tumor-free mice as controls. In all plasma samples obtained from tumor-bearing mice, similar to the case in control mice, VEGF was undetectable (data not shown).

***RT detection of LacZ transcripts in Tie-2 Lacz chimeras.*** Finally, the expression of LacZ transcripts in peripheral organs was investigated using RT-PCR in animals transplanted with Tie-2 LacZ BM cells. Detection of Tie-2 LacZ transcripts in bone marrow and spleen confirmed the reconstitution of bone marrow. LacZ transcripts were found in the lungs and to a lesser extent in subcutaneous tumors. Barely detectable levels were found in the heart and liver. LacZ transcripts were absent in normal brain and kidneys (Figure 8).

## Discussion

Our results suggest that bone marrow-derived endothelial progenitors do not contribute substantially to the vascularization of GL261 gliomas. Using syngeneic bone marrow transplantation from transgenic mice in which GFP or LacZ are expressed ubiquitously, we found that bone marrow cells migrated into the brain and were closely associated with tumor vessels. However, using multiple staining and confocal microscopy 3-D analysis, we found that the majority of tagged bone marrow cells in tumor tissues expressed the macrophage/microglia marker F4/80 and/or the pan-hematopoietic marker CD45, confirming previous findings that bone marrow cells contribute substantially to the microglial population in the central nervous system (9, 16, 39).

We further studied the contribution of bone marrow-derived endothelial progenitors to glioma vascularization using donor cells that express LacZ under the control of the Tie-2 promotor. Tie-2 is a tyrosine kinase receptor, which is expressed in activated endothelial cells, but also in a subset of hematopoietic cells (24). Using Tie-2LacZ donor cells, we identified LacZ+ vessels at a prevalence around 0.6% in gliomas. Most of the Tie-2 lacZ cells were localized at the tumor periphery either in the stroma or closely associated with tumor blood vessels. Surprisingly, we found that some Tie-LacZ+ cells expressed CD45 and some exhibited a microglial morphology, although they did not express the F4/80 antigen. It has been shown that primitive marrow-derived cells, which express Tie-2, migrate into the brain and differentiate into microglia (33) but only fully differentiated microglia will be recognized by the antibody F4/80 (16). Furthermore, in the Tie-2 LacZ transgenic mice, the loss of negative-regulatory elements might be responsible for a strong and durable β-galactosidase expression, in which even small transcriptional activity of Tie-2 may be sufficient for accumulation of significant amounts of stable protein. Thus, it is plausible to speculate that LacZ+ cells with a microglia appearance are derived from primitive hematopoietic Tie-2+ stem cells

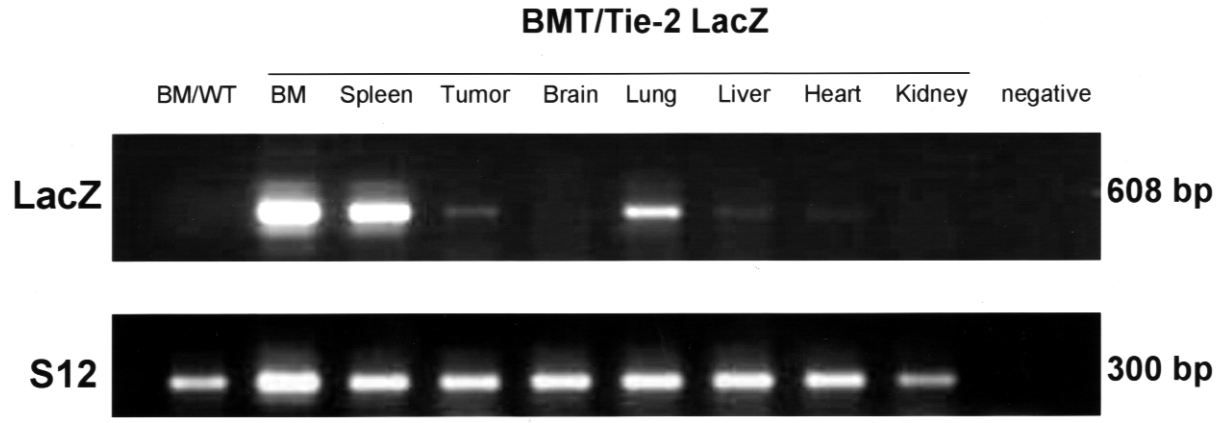



**Figure 8.** Representative reverse transcriptase-PCR detection of LacZ transcripts in tissues obtained from mouse submitted to BMT with Tie-2 LacZ donor cells. Amplified LacZ cDNA fragments comprise 608 base pairs (bp). BMT/WT indicates bone marrow from recipient transplanted with FVB/WT. Abundant expression of LacZ transcripts was detected in bone marrow and spleen. Lung and SC tumor demonstrated LacZ transcript expression to a lesser extent. In the liver or heart, LacZ transcripts were barely detectable. Negative: no template control. S12 was used as a loading control (amplified cDNA are 300 bp).

which neither completely differentiate to express F4/80 antigen, nor lose LacZ expression under Tie- 2 promotor. Confirming the findings of double labeling immunohistochemistry in the Tie-2 chimeras, in the FACS analysis we found expression of β-galactosidase in primitive and myeloid hematopoietic cells (c-kit+, CD11b+ and Gr-1+) obtained from Tie-2 transgenic donor and transplanted recipient mice (data not shown). In line with these results, de De Palma et al (15) found that a population of Tie-2-expressing bone marrow-derived cells which preferentially localized to the tumor periphery expressed a distinctive combination of surface markers like CD45, CD11b, CD34 and Sca-1. These cells were found in close association with the host endothelium, suggesting a possible role in the angiogenesis.

There are several points to consider regarding the different sources of tagged donor cells used in this study. First, although GFP and Rosa chimeras showed similar levels of hematopoietic engraftment, differences in the number of GFP or Rosa cells in the normal brain and tumor tissue were found. This most probably reflects differences in the detection sensitivity of these marker systems. Second, by the use of markers that are present ubiquitously, we cannot exclude the possibility that the high number of infiltrating GFP or Rosa cells present in tumors might mask rarely incorporated BM-derived endothelial progenitors, which could be detected by using the Tie-2-specific promotor. Third, the finding that several Tie-2 LacZ+ cells were expressing CD45 or myeloid marker indicates that the expression of LacZ under the Tie-2 promotor is not a reliable indicator of an endothelial or endothelial progenitor phenotype, hence determining donor cell differentiation into tumors requires colocalization with other markers. Finally, we cannot exclude the possibility that LacZ+ cells within CD31 tumor vessels in Tie-2 LacZ chimeras were trafficking leukocytes, since we did not perform double staining for CD45. Nevertheless, independent of the bone marrow source used, incorporation of bone marrow-derived endothelial progenitors into glioma vasculature appears to be rare.

Recent studies suggest that bone marrow-derived derived cells can contribute to neovascularization accompanying tumor growth. EP have been shown to contribute about 90% to tumor neoangiogenesis in lymphomas grown in angiogenesis-defective Id-mutant mice (29). This high contribution in the tumor vasculature is most probably due to the fact that recipient Id-deficient mice are unable to sustain endothelial sprouting to support tumor growth, and therefore alternative pathways will be activated. Davidoff et al (14) found that approximately 5% of GFP expression colocalizes with endothelial cell markers in neuroblastomas growing in mice transplanted with GFP transduced bone marrow cells. Reyes et al (42) demonstrated that multipotent adult progenitor cells purified from postnatal human bone marrow contribute up to 35% to neoangiogenesis in vivo in the early phases of tumor growth. Differences in experimental design such as the source of progenitors,

tumor histology, gene markers, time points of evaluation and techniques used to identify bone marrow-derived endothelial cells (confocal microscopy x X-gal staining and immunohistochemistry) could certainly reflect discrepancies found in the amount of contribution of EP to tumor vascularization. Certainly, more studies using similar models are warranted to compare the individual contribution of EP to different tumor types.

Furthermore, all animal studies performed up to now used subcutaneously implanted tumor models. We studied the contribution of EP either in tumors growing orthotopically and subcutaneously. Although i.c. inoculation of tumor cells does not necessarily mimic the normal growth and progression of human glioma, this particular syngeneic model recapitulates many of the histopathological and biological features of human gliomas, including necrosis with pseudopalisading and invasion (51) and has been used in several experimental settings (1, 22, 23). Tumors growing orthotopically present the advantage of reproducing the growing particularities which may also affect the ability to recruit EP such as differences in blood supply, functionally and morphologically characteristics of the pre-existing vascular bed, extracellular matrix and cytokine milieu. We found that EP incorporation into the tumor vasculature was very low either in gliomas growing heterotopically as well as orthotopically

Previous models showing incorporation of EP into tumor vessels are based on short-term bone marrow transplantation models. In short-term transplant models, the rescue of the bone marrow might be due to committed hematopoietic progenitors, which do not have the ability to transdifferentiate into other lineages. Moreover, recent experiments suggested that endothelial progenitors capable of contributing to capillary formation are derived from primitive hematopoietic stem cells (21). Thus, we allowed the chimeras sufficient time to recover normal levels of hematopoiesis (>3 months) and for most hematopoietic stem cell clonal instability to subside into equilibrium (>3-6 months) in order to enable a durable reconstitution of BM by donor cells. Six months after BMT we implanted GFP and Rosa 26 chimeric animals with murine glioma cells and inspected the brains for BM-derived cells. We found several vessel-associated GFP or LacZ+/CD45+ cells, which most probably represented leukocytes in diapedesis or bound to the abluminal side of host endothelium. Regardless of the levels and stability of hematopoietic engraftment, functional incorporation of BM-derived endothelial progenitors into the tumor vasculature was not significant.

It has been shown that local VEGF increases the engraftment of endothelial progenitors to the vasculature of newborn murine recipients (50). In addition, systemic application of VEGF-enhanced EP kinetics in the circulation as a result of expansion and mobilization of EP from the BM (4). VEGF signaling promotes not only the recruitment of circulating endothelial progenitors but also of hematopoietic cells to the tumor vascular bed. Colocalization of macrophages and endothelial progenitors might facilitate differentiation and incorporation of EP into tumor vasculature (27-29). We thus hypothesized that increased levels of VEGF in GL261 glioma cells should induce mobilization of BM-derived cells to the tumor vascular bed and accelerate differentiation and incorporation of EP. Although VEGF overexpression produced rapidly growing tumors with increased vascular permeability and capillary density, local levels of VEGF appeared to have no influence on the incorporation of bone marrow-derived endothelial progenitors into the tumor vasculature.

Interestingly, we observed an increase in the number of perivascular mononuclear cells in VEGF tumors. VEGF can induce both chemotaxis and proliferation of monocytes and microglia cells by interacting with VEGF receptor 1 (8, 12, 19). VEGF-activated microglia may be relevant in glioma growth since the microglial cells may account for more than 30% of cells within the glioma (6) and can release cytokines and growth factors that may further promote glioma growth, angiogenesis and vascular permeability (7).

In summary, our study strongly supports the hypothesis that neovascularization in gliomas results from stimulation of resident endothelial cells through an angiogenic process. BM-derived cells contribute substantially to the microglial population but not to the tumor vasculature. Our findings therefore suggest no significant contribution of tumor vasculogenesis (as opposed to classical tumor angiogenesis) in glioma vascularization. Why some tumors utilize marrow-derived cells for vascularization while others do not is still unclear. The odyssey from bone marrow to the vascular endothelium can be divided into three major steps. First, elevated serum levels of growth factors in tumor-bearing mice may mobilize BM-derived precursors into the circulation for the purpose of functional vessel regeneration and growth (30). These cells may be mobilized from the BM by tumor-derived VEGF by a mechanism recently described by Asahara (4) or by signaling through the Stroma Cell-Derived Factor (SDF) cytokine (49). Subsequently, additional local stimuli may promote the activation of local endothelium to

express adhesion molecules to recruit endothelial progenitors. Finally, endothelial progenitors become incorporated into the vasculature by mechanisms still not elucidated. In mice-bearing intracranial tumors we found that the levels of systemic VEGF were not increased compared to control animals. In ongoing studies, we are evaluating the number of circulating progenitors in the peripheral blood. VEGF levels in the tumor do not seem to play a relevant role in EP recruitment since the increase in both VEGF and tissue vascularity was not paralleled by an increase in EP engraftment. Finally, the brain's extracellular matrix or adhesion molecules specific to the brain endothelium do not appear to be of critical importance since the ability to recruit EP was independent of tumor localization (i.c. or s.c.). Together, these findings suggest that the capacity to mobilize and incorporate EP might be intrinsic to each tumor type. Possibly, a specific chemokine is the main determinant for recruitment of EP.

Whether the biology of this animal model recapitulates human gliomas can only be assessed by developing specific markers which recognize putative endothelial precursors in human tissues. Although we did not find a significant contribution of endothelial progenitors into the glioma vasculature, the ability of glioma tissue to incorporate bone marrow derived cells and their association with tumor vessels may have important clinical implications. Recently de Palma et al (15) showed that transplantation of genetically modified bone marrow progenitors may represent a gene therapy vehicle to tumors. Bone marrow progenitors transduced with lentiral vectors expressing a suicide gene under the transcriptional regulation of Tie2 promotor did not integrate into tumor vessels, thus corroborating our own results. However, the elimination of a subset of bone marrow-derived mononuclear cells was sufficient to inhibit angiogenesis and tumor growth. Accordingly, Davidoff et al (14) have studied the effect of bone marrow transplantation of hematopoietic cells which have been genetically modified to express a truncated, soluble form of VEGFR-2 on tumor growth. The authors found only low levels of bone marrow-derived endothelial cell engraftment (about 5%), but the growth of subcutanously implanted neuroblastomas or Willms' tumors was substantially inhibited. Together, these results suggest that inhibition of tumor angiogenesis may not be based on the impairment of endothelial cell generation by endothelial progenitors, but rather on a poorly understood paracrine mechanism dependent on pro-angiogenic hematopoietic cells. Thus, further understanding of the biology of endothelial progenitors and the role of bone marrow-derived macrophages and microglia in tumor angiogenesis is required.

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