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Contribution of bone marrow-derived cells associated with brain angiogenesis is primarily through leukocytes and macrophages

Qi Hao[1], **Jianrong Liu**[1], **Rajita Pappu**[2], **Hua Su**[1], **Radoslaw Rola**[2], **Rodney A. Gabriel**[1], **Chanhung Z. Lee**[1], **William L. Young**[1,2,3], and **Guo-Yuan Yang**[1,2]

1*Center for Cerebrovascular Research, Department of Anesthesia and Perioperative Care, University of California, San Francisco, San Francisco, CA.*

2*Department of Neurological Surgery, University of California, San Francisco, San Francisco, CA.*

3*Department of Neurology, University of California, San Francisco, San Francisco, CA.*

Abstract

Objective—We investigated the role of bone marrow-derived cells (BMDCs) in an angiogenic focus, induced by VEGF stimulation.

Methods and Results—BM from GFP donor mice was isolated and transplanted into lethally irradiated recipients. Four weeks after transplantation, groups of mice received adeno-associated viral vector (AAV)-VEGF or AAV-lacZ gene (control) injection and were sacrificed at 1 to 24 weeks. BMDCs were characterized by double-labeled immunostaining. BMDCs' function was further examined through matrix metalloproteinase-2, -9 (MMP-2, -9) activity. We found that capillary density increased after 2 weeks, peaked at 4 weeks ($p<0.01$), and sustained up to 24 weeks after gene transfer. GFP-positive BMDCs' infiltration in the angiogenic focus began 1 week, peaked at 2 weeks, and decreased thereafter. The GFP-positive BMDCs were co-localized with CD45 (94%), CD68 (71%), 5% Vimentin (5%), CD31/vWF (1%), and alpha-smooth muscle actin (α-SMA, 0.5%). Infiltrated BMDCs expressed MMP-9. MMP-9 KO mice confirmed the dependence of the angiogenic response on MMP-9 availability.

Conclusions—Nearly all BMDCs in the angiogenic focus showed expression for leukocytes/macrophages, indicating that BMDCs minimally incorporated into the neovasculature. Co-localization of MMPs with GFP suggests that BMDCs play a critical role in VEGF-induced angiogenic response through up-regulation of MMPs.

Keywords

adeno-associated virus; angiogenesis; BMDCs; MMPs; VEGF

Angiogenesis plays an essential role during ischemic brain injury[1] or cerebrovascular disorders such as brain arteriovenous malformations.[2] Vascular endothelial growth factor (VEGF) and matrix metalloproteinases (MMPs) are the key factors in activating angiogenesis in adults. VEGF is increased in brain diseases such as stroke, Alzheimer's disease and brain AVM.[3, 4] MMPs are responsible for the degradation of extracellular matrix component and are necessary during the angiogenesis process in both *in vivo* and *in vitro* experiments.[5] MMP-9 is especially important because it plays a central role in angiogenesis.[6, 7] We have previously demonstrated that MMP-9 activity is increased after VEGF stimulation in the adult mouse brain, accompanied

Address correspondence to: Guo-Yuan Yang, MD, PhD UCSF Department of Anesthesia 1001 Potrero Ave, Room 3C-38, San Francisco, CA 94110 Tel: 415-206-8916; Fax: 415-206-8907 E-mail: ccr@anesthesia.ucsf.edu.

by increased focal angiogenesis.[8] Inhibition of MMP-9[9] or neutrophil depletion[8] can decrease the MMP response to VEGF stimulation.

Although the mechanisms of angiogenesis have been extensively studied, the extent to which BM-derived cells (BMDCs) contribute to angiogenesis in the adult brain is unclear. BMDCs are recruited to the sites of physiological and pathological angiogenesis.[10, 11] Transplantation of BMDCs benefits the recovery of ischemic tissue injury.[12, 13] Further, whether BMDCs directly incorporate into the vascular structures or play other functions in the brain remain unclear.

Recently, BMDCs have been recognized as an important source for storing and activating MMPs. Neutrophil, macrophage and mast cells are the critical suppliers of MMPs, especially MMP-9.[14, 15] In the present study, we used an adeno-associated viral vector delivery of human $VEGF_{165}$ cDNA (AAVVEGF) into the mouse brain to induce reproducible focal cerebral angiogenesis. We harvested BM from EGFP transgenic donor mice and transplanted them into lethally irradiated recipients to track BMDCs. Our aim was to demonstrate the function of BMDCs in VEGF-induced angiogenesis in the adult mouse brain.

Methods

Experimental Design

All experimental procedures for using laboratory animals were approved by the Institutional Animal Care and Use Committee, University of California, San Francisco.

Experiment 1—Sixty adult C57BL/6 male mice at age 8-10 weeks (Charles River, Wilmington, MA) were lethally irradiated and subsequently transplanted with BM cells collected from C57BL/6-TgN (ACTβGFP) mice (Jackson Laboratory, Bar Harbor, ME). Following 4 weeks of BM transplantation, circulating blood was collected for FACS analysis to confirm the full hematopoietic recovery. The mice then underwent AAVVEGF or AAVlacZ injection into the right brain, and were sacrificed 1, 2, 4, 12, and 24 weeks after the gene transfer. The brains were harvested for further analysis (See Supplemental Figure IA at http://atvb.ahajournals.org.).

Experiment 2—Twenty-four adult male MMP-$9^{-/-}$ mice and their wild type littermates at age 8-10 weeks received either AAVVEGF or AAVlacZ injection into the right brain. The capillary counts and focal inflammation were examined 2 and 4 weeks after injection to explore the relationship between angiogenesis and MMP expression.

BM Transplantation

Donor BM was harvested from 8 to 10-week old male C57BL/6-TgN mice expressing GFP under a β-actin transcriptional promoter in all tissues. The mice were sacrificed to remove tibias and femurs. BM was flushed out of the tibias and femurs with Iscove's modified Eagle medium (IMDM, Invitrogen, Carlsbad, CA) containing 1% fetal serum, dispersed by gentle aspiration, then centrifuged at 1200 rpm for 10 minutes. Cells were resuspended in PBS at a concentration of 1×10^7/ml.

Recipient mice received lethal irradiation with a total dose of 9.5 Gy using a Gammacell 40 irradiator (MDS-Nordion, Ottawa, Canada). 2×10^6 BMDCs in 0.2 ml PBS were immediately injected into the irradiated recipient mice via the tail vein. To prevent infection, recipients routinely drank water with polymyxin (10 mg/L) and neomycin (100 mg/L) for 4 weeks after irradiation.

FACS Analysis

To detect the level of hematopoietic engraftment, peripheral blood was obtained from the retro-orbital plexus of the recipient mice following 4 weeks of BM injection. Blood from non-transplanted C57BL/6 mice was collected as a control. The cells were suspended in PBS and processed with FACSCalibur.

AAVVEGF Gene Transfer in the Mouse Brain

Following 4 weeks of irradiation, the recipient mice were anesthetized using ketamine/xylazine (100/10 mg/kg body weight). The animals were then placed in a stereotactic frame with a mouth holder (David Kopf Instruments, Tujunga, CA). A burr hole was drilled in the pericranium 2 mm lateral to the sagittal suture and 1 mm posterior to the coronal suture. A 10 μl Hamilton syringe was inserted into the right parenchyma at a depth of 3.0 mm under the cortex. Two μl of viral suspension containing 2×10^9 genome copies of AAVVEGF or AAVlacZ were injected into the right lateral ventricle and basal ganglia at a rate of 0.2 μl per minute.

Quantification of Capillary Density

We have established a relatively simple and reliable method to quantify the number of capillaries in a given brain region using lectin-stained imaging, referred to as “capillary density.” This method gives consistent mean values and variance.[16, 17] (Supplemental Figure IIA and IIB) Two brain coronal sections from the lectin-stained brain were chosen, 1 mm anterior and 1 mm posterior to the needle track. Three areas (left, right, and bottom of the track) were examined using a low power objective lens (10X). Two investigators without knowledge of treatment conditions assessed vessel counts separately. If there was a discrepancy, both investigators recounted together under a monitor’s supervision. Capillary density for each animal was calculated as the mean of the vessel counts obtained from the 6 pictures (3 per reader).[18]

Double-labeled Fluorescent Staining

Coronal sections (20 μm in thickness) were cut, air-dried and fixed in 4% paraformaldehyde for 20 minutes, and incubated in 5% blocking serum for 30 minutes. The sections were incubated overnight in primary antibodies: vWF and Vimentin (Chemicon, Temecula, CA), α-smooth muscle actin (Sigma, St. Louis, MO), CD68 (Serotec, Kidlington, UK), MMP-12 (Santa Cruz Biotechnology, Santa Cruz, CA), CD45 (Lab Vision, Fremont, CA), ICAM-1 (Biolegend, San Diego, CA), and MMP-9 (a gift from Dr. Robert Senior of Washington University, St. Louis, MO). The secondary antibodies were Alexa 594 red. Negative controls were performed by omitting primary antibodies. BrdU was injected i.p. daily at a dose of 100 mg per mouse per day for 5 days.[19] After sacrificing the animals, the brain was removed and brain sections were rinsed with 0.1M boric acid pH 8.5 at 25°C for 10 minutes, blocked by using a MOM solution (MOM Kit, Vector Laboratories) at 25°C for 1 hour, and incubated using a BrdU antibody (Sigma) at room temperature overnight. The secondary antibody was Alexa 594 red anti-mouse IgG. Double-labeled immunostaining sections were evaluated using a fluorescence microscope (Nikon Microphoto-SA, Melville, NY).

Gelatin Zymogram for MMP-9 Activity Detection

MMP-$9^{-/-}$ and wild-type mice were sacrificed at 2 and 4 weeks after AAVVEGF gene transfer. The brain tissues from treated animals were collected and homogenized with 100 μl prepared lysis buffer. Equal amounts of protein were loaded onto a gel and analyzed by gelatin zymogram to characterize gelatinase activity. After electrophoresis, the gel was washed and incubated overnight at 37°C in a developing buffer. The gels were stained with Coomassie Blue R-250 (Bio-Rad, Hercules, CA) and subsequently washed in destaining solution. Standard MMP-9, -2 (Chemicon, Temecula, CA) was examined on the gel to identify corresponding

MMP-9, -2 bands. Gelatinolytic bands were quantified by scanning densitometry and analyzed using NIH Image 1.63 software.

Statistical Analysis

Parametric data in the different groups were compared using one-way ANOVA followed by Fisher's protected least significant difference test. All data are presented as mean ± standard deviation. A $p<0.05$ was considered statistically significant.

Results

BM Transplantation and Hematopoietic Recovery

To evaluate the efficacy of BM transplantation into the lethally irradiated mice, we performed flow cytometric analysis of recipient blood 4 weeks after BM transplantation. As measured by fluorescent intensity, 93% nucleated cells from recipient mice expressed GFP, indicating that most of the host stem cells were replaced by donor cells (Supplemental Figure IB). No visible abnormal behavioral changes were noted following irradiation and BM transplantation; the animals moved freely and ate and drank normally.

VEGF-induced Focal Angiogenesis is Associated with Infiltrating BMDCs

To determine the kinetics of angiogenic response induced by VEGF in the brain, we injected AAVVEGF or AAVlacZ into the mouse brain and further analyzed the tissue at 1, 2, 4, 12, and 24 weeks after gene transfer. The number of capillaries increased as early as 1 week after AAVVEGF injection and peaked at 4 weeks (Figure 1). Although the magnitude of capillary density gradually decreased thereafter, capillary density in the AAVVEGF group remained higher than in the AAVlacZ group for up to 6 months. BMDCs were found within the VEGF-induced angiogenic region as early as 1 week after transplantation, peaking at 2 weeks, and progressively decreasing afterwards (Figure 1). It should be noted that the peak of BMDC accumulation occurred before the peak of increased capillary density.

CD45- or CD68-positive Cells are the Major BMDCs Recruited to the Angiogenic Region

BM has the capacity to differentiate into a spectrum of cell types, including leukocytes, endothelial cells, pericytes, and smooth muscle cells. To identify the cell types that BM cells differentiated into in the brain angiogenic focus after AAVVEGF injection, we used CD45 and CD68 immunostaining to characterize infiltrated BMDCs. We found that the majority of GFP-positive cells co-localized with CD45 and CD68 (Figure 2). CD45 and CD68-positive cells comprised the majority of the GFP-positive cells at 94% and 71%, respectively. We further identified that the GFP-positive BMDCs co-localized with CD31, vWF, Vimentin, and alpha-smooth muscle actin (α-SMA), indicating that BMDCs could differentiate into endothelial cells, pericytes, and smooth muscle cells; these were recruited to the angiogenic region although they only occupied a small percentage (1±1%, 5±3%, 0.5±0.2%, respectively). In addition, BrdU-positive GFP cells were associated with blood vessels, suggesting that GFP-positive cells were actively proliferating. Notably, there was no positive double-staining associated with GFAP, a marker for astrocytes, or NeuN, a marker for neurons.

BMDC Homing is Associated with ICAM-1 Expression in the Angiogenic Focus

ICAM-1 is the main endothelial surface receptor responsible for recruitment of BMDCs into the brain after VEGF stimulation.[20] ICAM-1 deficiency impairs VEGF-induced angiogenesis.[21] To explore ICAM-1's function during angiogenesis in brain tissue, we examined ICAM-1 expression. ICAM-1 expression was undetectable in the AAVlacZ-transduced mouse brain and the contralateral hemisphere of the AAVVEGF-transduced mouse brain. Interestingly, ICAM-1 positive staining was observed in the ipsilateral hemisphere in the angiogenic region

where BMDCs were homing (Figure 3). Both GFP-positive BMDCs and up-regulated ICAM-1 expression were observed in the same region but not in the contralateral hemisphere, suggesting that BMDCS homing in the angiogenic region is associated with focal overexpression of ICAM-1.

BMDCs--A Source of MMPs in the Angiogenic Focus

To identify the source of MMPs in the angiogenic focus, we examined MMP-9 and MMP-12 expression. MMP-9 positive cells were only detected in the angiogenic focus in the AAVVEGF-transduced mouse brain, corresponding to the homing BMDCs. No MMP-9 positive cells were detected in the contralateral hemisphere (data not shown). MMP-9 positive staining co-localized well with GFP-positive cells, suggesting that the homing BMDCs were the source of MMP-9 (Figure 4). Since MMP12 is macrophage elastase, we further examined MMP12 expression. We detected well co-localized MMP-12 and GFP-positive cells in the angiogenic focus although they were much less than MMP-9 and GFP-positive cells.

MMP-9 Required for VEGF-induced Angiogenesis

To confirm that MMP-9 is required for VEGF-induced angiogenesis, we used VEGF stimulation in the MMP-$9^{-/-}$ mice. The result showed that the number of capillaries in the AAVVEGF-transduced MMP-$9^{-/-}$ mouse brain was greatly reduced compared to AAVVEGF-transduced WT mice (Figure 5A and 5B). Furthermore, zymographic analysis showed that MMP-9 activity was highly up-regulated in the AAVVEGF-transduced WT mice but absent in the MMP-$9^{-/-}$ mice, while MMP-2 had no changes (5C). Additionally, the MMP-9 activity after 2 weeks was higher than after 4 weeks of VEGF hyper-stimulation (5D). We also noted that the peak of MMP-9 activity occurred before the peak of capillary increase.

Discussion

In the present study, we demonstrated that: 1) BMDCs were recruited into the ipsilateral hemisphere of the mouse brain after AAVVEGF transduction with corresponding ICAM-1 expression; 2) BMDCs infiltrating the angiogenic region displayed a leukocyte (94%) or macrophage (71%) phenotype, while only a minority incorporated into the vascular wall; 3) the majority (80%) of the BMDC population co-localized with MMP-9; and 4) the increase of capillaries induced by VEGF transduction was attenuated 80% (2 weeks) and 66% (4 weeks) in MMP-$9^{-/-}$ mice. Taken together, our results demonstrate that BMDCs are recruited into the VEGF-induced angiogenic focus and are involved in angiogenesis by activating MMP-9.

BMDC incorporation into endothelial cells of growing vessels varies widely, from almost no incorporation up to 56% based on different tissues and types of stimulation.[22, 23] We found that only 1% GFP donor cells were positive for endothelial cell (CD31 or vWF), 0.5% for smooth muscle cell (α-SMA), and 5% for pericyte (Vimentin). Our results indicate that BMDCs in the brain tissue only occasionally incorporate into VEGF-induced angiogenesis, although other groups found that up to 42% of cells incorporate into brain vasculature in other models.[24, 25]

We found that most of infiltrated BMDCs in the activated angiogenic region display leukocyte and macrophage markers. BMDCs are able to differentiate into astrocytes,[26] neurons,[24] and oligodendrocytes;[27] however, we did not detect GFP-positive astrocytes, neurons and oligodendrocyte under VEGF stimulation conditions, suggesting that differentiation of BMDCs may be based on specific tissues and varying stimulation. We detected GFP-positive BMDCs only in VEGF-induced angiogenic focus, and their homing in the angiogenic focus as early as 7 days after AAVVEGF transduction, peaking at 2 weeks, and gradually decreasing afterwards. This suggests that focal VEGF overexpression could recruit BMDCs. Since the

peak of BMDC homing was 2 weeks earlier than that of increased capillaries, BMDCs were likely the activator for focal angiogenesis.

The insertion of the needle to introduce the viral vector causes minimal injury and may elicit a short-lived focal inflammatory response of several days, which would be visible in both experimental and control groups. However, in a previous study, we injected AAVVEGF, AAVlacZ (as viral control), and saline (as injection control) into the mouse brain and found that there was a paucity of inflammatory responses occurring in the three groups of mice, indicating that AAV vector or injection was unlikely to cause focal inflammation.[28] In addition, we found that capillary density increased in the AAVVEGF-transduced mice but not in AAVlacZ and saline-injected mice, confirming that the increased capillaries were induced by VEGF but not AAV or injection. Generally, inflammatory-induced angiogenesis occurs in the first few days and does not persist after several weeks (Supplemental Figure IIC).

Prior to infiltration, BMDCs need to adhere to the vascular wall in a process mediated by adhesion molecules. VEGF could up-regulate ICAM-1 expression both *in vitro* and *in vivo*.[29, 30] We examined ICAM-1 and other intercellular adhesion proteins such as VCAM-1 and E-selectin. We found that ICAM-1, but not other intercellular adhesion proteins, was extensively expressed in the ipsilateral hemisphere of the VEGF-treated mouse brain which parallels with infiltrated BMDCs, suggesting that ICAM-1 plays a significant role in BMDC infiltration.[21]

VEGF enhances focal angiogenesis and promotes BBB leakage in the ischemic brain.[31] Studies have noted that it induces BBB leakage only in the ischemic brain because BBB disruption has not been detected in the non-ischemic rat brain following VEGF treatment.[31] We believe that VEGF on BBB permeability may be based on the administered dose. AdVEGF (1×10^9 genome copies) or AAVVEGF (2×10^9 genome copies) or VEGF protein 0.6-1.2 ng/day are appropriate because these doses induce maximum angiogenesis with less BBB leakage.[18, 32] The inflammatory cell invasion could be due to the increased BBB permeability in the early stage of angiogenesis.

Recently, BMDCs have been recognized as an important source for storing and activating MMPs. Macrophage, neutrophils and mast cells are the critical suppliers of MMP-9.[15] BMDCs contributing to tumor vasculature is MMP-9-dependent.[33] We showed that BMDCs were recruited into the angiogenic focus and expressed MMP-9, and that majority (80%) of the BMDCs population co-localized with MMP-9. Although MMP-9 positive staining was not co-localized with BMDCs that are possibly local endothelial cells or smooth muscle cells, we speculate that one of the most critical functions of BMDCs infiltrating into an angiogenic focus is to supply MMP-9.

MMP-12, a macrophage elastase, is expressed in mononucleated cells, which is related to focal inflammation.[34] We observed that while a few BMDCs expressed MMP-12 in the angiogenic focus, the majority of BMDCs expressed MMP-9; this suggests that BMDCs mainly deliver MMP-9 and not MMP-12 during angiogenesis. The effect of MMP-12 during angiogenesis in the brain tissue needs further study.

We previously showed that neutrophil depletion[8] and tetracycline treatment[9] decrease MMP-9 activity, consequently attenuating angiogenesis. To verify the specific effect of MMP-9, we examined the number of capillaries in the AAVVEGF-transduced MMP-$9^{-/-}$ mice. We found that the number of capillaries was greatly reduced in AAVVEGF-transduced mice compared to WT mice. Zymographic results also showed that MMP-9 activity was highly up-regulated in the AAVVEGF-transduced WT mice and absent in MMP-$9^{-/-}$ mice, which parallels capillary density. Also, MMP-9 activity peaked at 2 weeks, the same with infiltrated BMDCs, ahead of

the peak of capillary proliferation. All results were consistent with the view that infiltrating BMDCs are associated with MMP-9 activity during angiogenesis.

Transplantation of WT BM cells into MMP-9$^{-/-}$ mice can restore tumor angiogenesis.[33, 35] Transplantation of MMP-9$^{-/-}$ BM cells into WT mice greatly attenuated tumor angiogenesis compared with transplanting WT BM cells into WT mice.[33] However, a study shows that non-BMDCs can compensate for the lack of MMP-9 in BM cells.[35] Future studies on MMP9$^{-/-}$ BM cells transplanted into WT mice and WT BM cells transplanted into MMP-9$^{-/-}$ mice may provide more evidence.

VEGF gene transfer produces angiogenesis in the presence of some type of vascular disease or tissue ischemia. Administration of VEGF improved recovery from ischemic injury in the heart,[36] hind limbs,[37] and the brain,[38, 39] although VEGF could also produce irregular malformed capillary networks in the heart.[40] VEGF-induced angiogenesis in the heart and limb may be tissue-specific and, in addition, depends on the duration of VEGF stimulation. Nevertheless, VEGF could induce angiogenesis in normal cells and tissues including the brain.[20, 21, 41, 42]

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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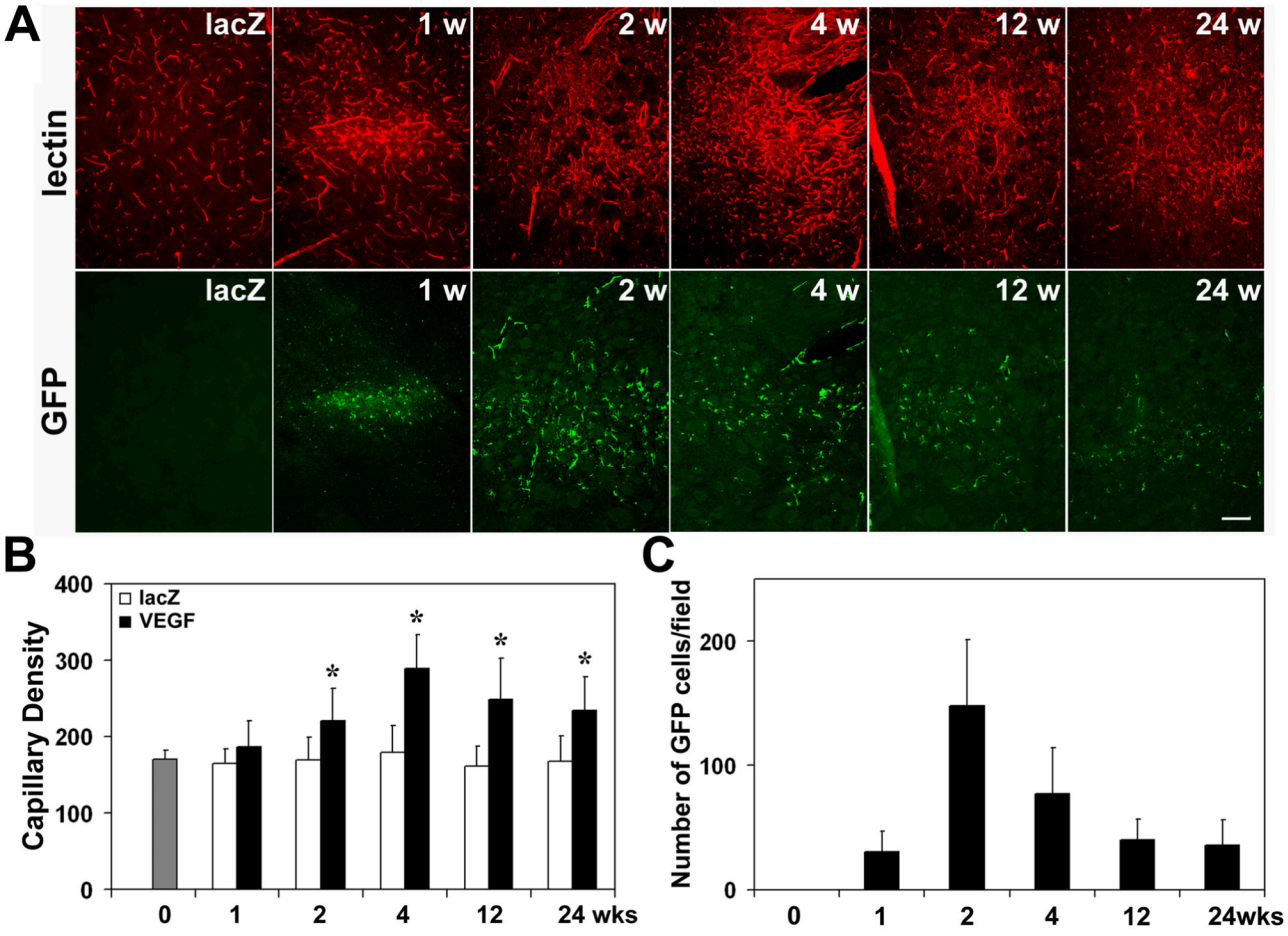

Figure 1.

A. Lectin staining capillaries (red), and infiltrating BMDCs (green) in AAVlacZ and AAVVEGF groups after 1 to 24 weeks. Bar=100μm. **B.** The quantitative capillary density paralleling 1A. N=6/group, mean±SD, *=p<0.05, AAVVEGF vs. AAVlacZ group. **C**. Quantitative BMDCs at 0 to 24 weeks following AAVVEGF injection.

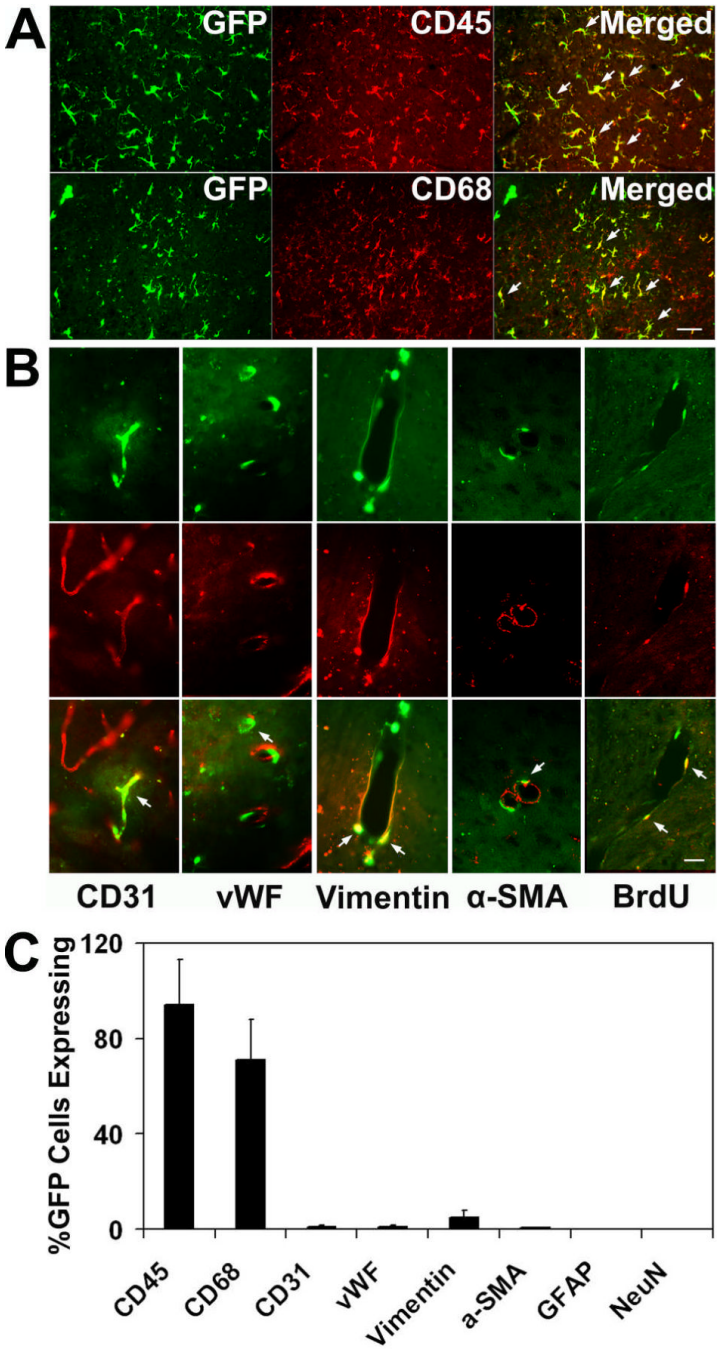

Figure 2.

A. CD45 (upper) and CD68 (bottom) immunostaining in AAVVEGF-transduced brain. Arrows indicate CD45/GFP or CD68/GFP positive cells. Bar=50μm. **B.** GFP cells (green) and different cell markers (red). Yellow shows merged signals. Bar=25μm. **C.** Semi-quantitation of the percentage of BMDCs expressing different cell markers in AAVVEGF-transduced mice.

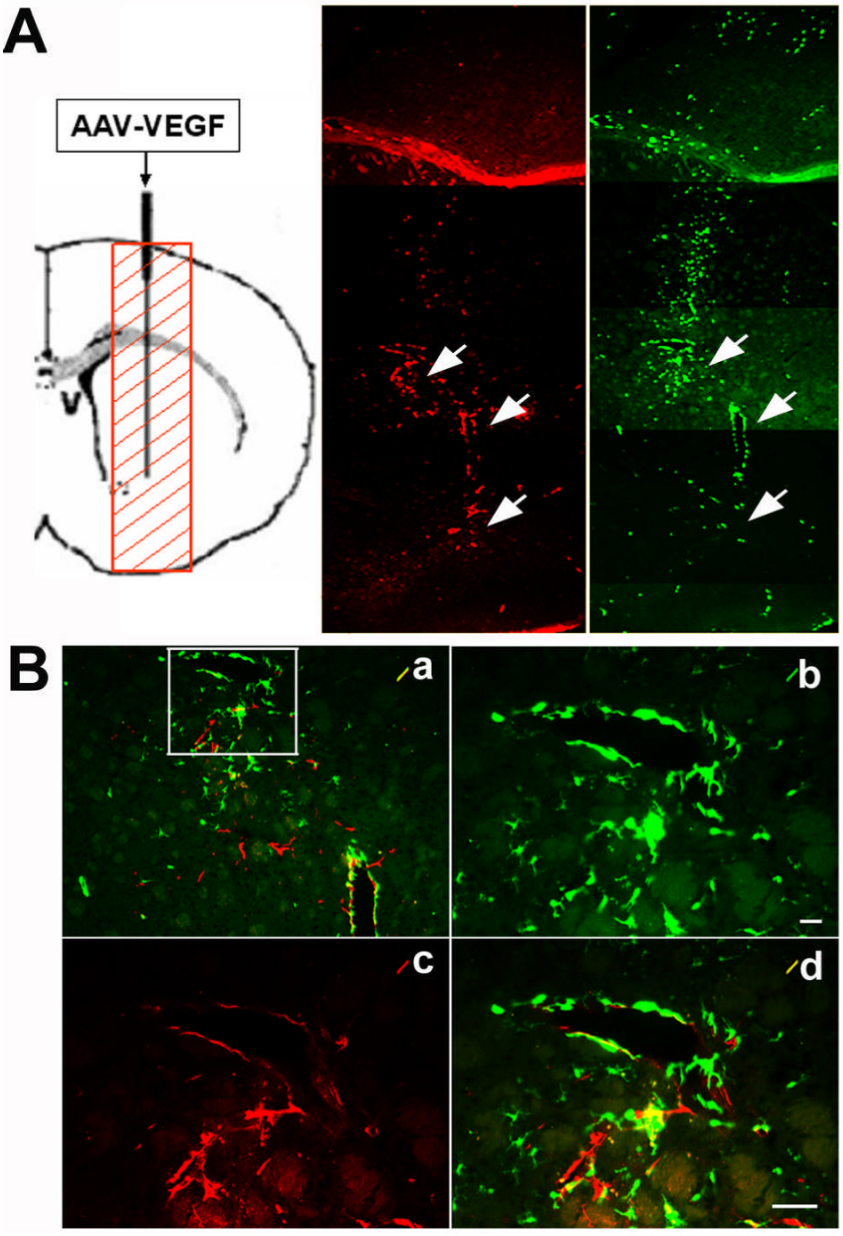

Figure 3.

A. Diagram shows BMDCs (green) associated with ICAM-1 (red) expression in the ipsilateral side of VEGF-transduced brain. **B**. High magnification images (b, c, d) from boxed region in (a) indicate that BMDCs (b) associated with ICAM-1 (c), as shown in d. Bar=50μm.

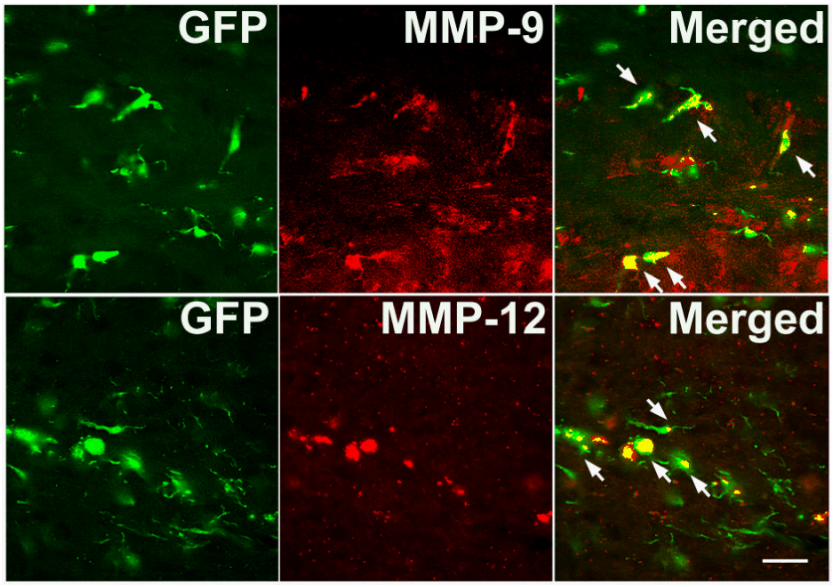

Figure 4.
Photomicrographs show MMP-9 (upper) and MMP-12 (bottom) immunostaining in the AAVVEGF-transduced brain. BMDCs (green) and MMPs (red) are well merged (arrows), suggesting that BMDCs express MMP-9 and MMP-12. Bar=25μm.

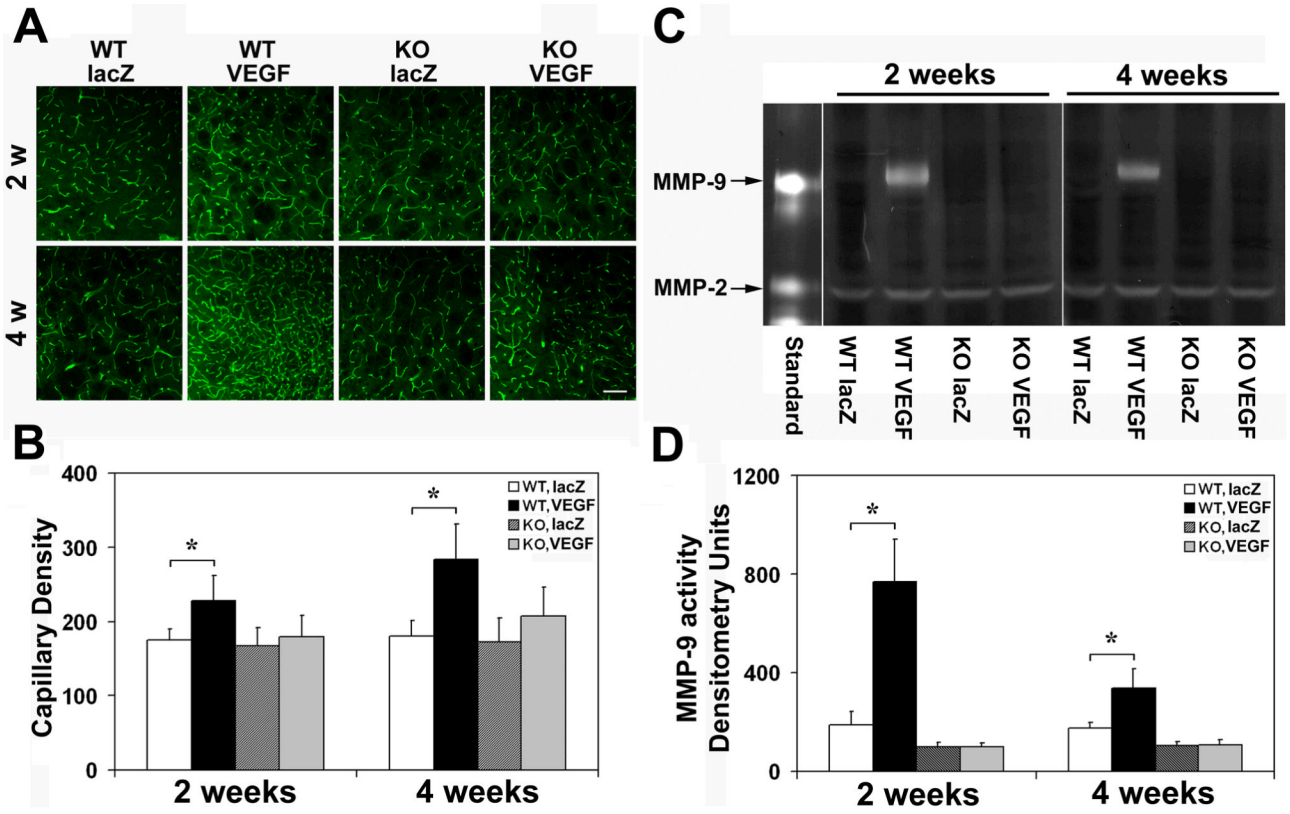

Figure 5.

A. Lectin-stained capillaries in WT and MMP-9$^{-/-}$ mice at 2 and 4 weeks after gene transfer. Bar=100μm. **B.** Quantitative capillary density parallels 5A. **C.** MMP-9 and MMP-2 activity (zymogram gel) in WT and MMP-9$^{-/-}$ mice. **D.** Quantitative MMP-9 activity parallels 5C. N=6/group, mean±SD. *=p<0.05, AAVVEGF-transduced vs. AAVlacZ-transduced group (**B, D**).