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Evidence of endothelial progenitor cells in the human brain and spinal cord arteriovenous malformations

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Abstract

Objective—Brain and spinal cord arteriovenous malformations (AVMs) are characterized by aberrant angiogenesis and vascular remodeling. Endothelial progenitor cells (EPCs) can be recruited by stromal cell-derived factor-1 (SDF-1), and participate in vascular remodeling in both physiological and pathological settings. We hypothesized that there was increased EPC levels in the brain and spinal cord AVM nidus.

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The authors have no personal, financial or institutional interest in any of the drugs, materials, or devices described in this article.

Authorship

There were many collaborators in this interdisciplinary project. In addition to reviewing the final manuscript, the authors' participation is noted below.

- **Peng Gao**, MD, PhD, drafted the manuscript, performed the laboratory assays in tissue, and participated in the study design and analysis.
- **Yongmei Chen**, MD, PhD, performed the laboratory assays in tissue and participated in the study design and analysis.
- **Michael T. Lawton**, MD, was responsible for the harvest and selection of surgical specimens, and participated in the histopathological screening and study design.
- **Nicholas M. Barbaro**, MD, was responsible for harvest and selection of surgical specimens from epilepsy patients, and participated in the study design.
- **Guo-Yuan Yang**, MD, PhD, participated in the study design.
- **Hua Su**, MD, participated in the study design.
- **Feng Ling**, MD, provided the human spinal cord AVM tissue, and participated in the study design.
- **William L. Young**, MD, was involved in all aspects of the study.
- **UCSF BAVM Study Project** members participated in other aspects of the work and are acknowledged.

Methods—Microsurgical specimens without endovascular embolization and radiosurgery from the brain (n=12) and spinal cord (n=5) AVMs were examined. Hemangioblastoma, meningioma, cerebral cortex obtained from epilepsy surgery, and the basilar artery (BA) from the autopsy were chosen for control comparisons. EPCs were identified as cells that were double-positive for the stem cell marker CD133 and the endothelial cell marker VEGFR-2 (vascular endothelial growth factor receptor-2 or KDR). In addition, SDF-1 was characterized by immunohistochemistry.

Results—Both brain and spinal AVM tissues displayed more CD133, SDF-1, and CD68-positive signals than epilepsy and basilar artery control tissues. The level of EPCs was increased in the brain and spinal cord AVM nidus, mainly at the edge of the vessel wall. The expression of SDF-1 was co-localized with CD31-positive and α -smooth muscle cells, and was predominantly found within the vessel wall.

Conclusion—Our data demonstrate that EPCs are present in the nidus of the brain and spinal cord AVMs, which may mediate pathological vascular remodeling and impact the clinical course of AVMs.

Keywords

Angiogenesis; Endothelial progenitor cell; Stromal cell-derived factor-1; Vascular malformation

INTRODUCTION

Brain arteriovenous malformations (BAVMs) are characterized by aberrant angiogenesis and vascular remodeling, and are an important cause of hemorrhagic stroke in young adults. The etiology and pathogenesis are unknown, and better understanding of the molecular events influencing disease susceptibility and clinical progression is needed to optimize patient care. Data from our group and other investigators have shown that BAVM nidus has higher levels of angiogenic factors, including vascular endothelial growth factor (VEGF) and matrix metalloproteinase-9 (MMP-9).^{1–5} Reports on SCAVMs (spinal cord AVMs) are relatively rare.

Bone marrow-derived endothelial progenitor cells (EPCs) have been shown to play a critical role in postnatal vasculogenesis and vascular homeostasis by secreting paracrine factors or by direct incorporation into the vasculature.^{6,7} Other studies indicate that EPCs also potentially initiate and promote tumor neovascularization.^{8–10} In addition, they have been extensively investigated as a risk biomarker and outcome predictor in cardiovascular diseases as well as in stroke.^{11,12} Stromal cell-derived factor-1 (SDF-1) is a pleiotropic chemokine that has been demonstrated to play a central role in EPC recruitment.¹³ So far, little is known about the number and function of EPCs in patients with brain AVMs. In this study, we investigated whether AVM nidus harbors increased number of EPCs, which, in turn, mediates pathological vascular remodeling and impacts the clinical course of brain and spinal cord AVMs. Furthermore, we used immunohistochemical staining to determine whether SDF-1 is expressed in the brain and spinal cord AVM nidus, and to further investigate the cellular source of SDF-1 through double-fluorescent staining with specific cellular markers.

METHODS

All studies involving patients were approved by the Institutional Review Board of the University of California, San Francisco (UCSF), and Xuanwu Hospital in Beijing, China. Patients gave informed consent.

Study Subjects

Patients with BAVMs evaluated at UCSF were entered into an ongoing prospective registry. We analyzed a subset of this group who underwent microsurgery and had tissue available for analysis. We selected from our tissue bank cases of unruptured (n=7) and ruptured (n=5) brain AVMs in patients who did not undergo pre-surgical embolization. Control cerebral cortex was obtained from patients undergoing surgical treatment of epilepsy as previously described. 2-15 Samples were taken from structurally normal temporal lobe remote from the epileptogenic focus. The human basilar artery (BA) from the autopsy was chosen as an additional control. Spinal cord AVM samples were kindly provided by the Department of Neurosurgery, Xuanwu Hospital, Capital University of Medical Sciences, Beijing, China. The patients did not undergo endovascular embolization before surgical resection, and medical records did not show previous history of rupture. None of the patients had a history of hereditary hemorrhagic telangiectasia (HHT). Characteristics were similar between cohorts except that more BAVM patients (33%) presented with larger BAVMs (>3cm) than the SCAVM group (0%). Also, the ethnic composition of the two groups was different: 17% of the BAVM patients were Asians, whereas 100% were Asian in the SCAVM group.

Immunohistochemistry

We used immunocytochemistry to characterize the existence of EPCs in human BAVM and SCAVM samples. Tissues from various sources (BAVM, SCAVM, basilar artery, hemangioblastoma, and meningioma) were embedded in paraffin and cut at a thickness of 10µm.

For single-label immunohistochemistry, sections were de-parafinized and rehydrated with graded ethanol. Endogenous peroxidase activity was quenched by incubating slides in 0.3% H₂O₂ in methanol for 30 min. Slides were rinsed in two changes of phosphate-buffered saline (PBS), pH 7.4, and then incubated in 5% goat serum in PBS with 0.1% Triton X-100 for 30 min at room temperature. After blocking, sections were incubated with primary antibodies diluted in PBS with 0.1% Triton X-100 at the following concentrations: rabbit polyclonal anti-human CD133, 1:100 (AbCam); rabbit polyclonal anti-human SDF-1, 1:1000 (AbCam); mouse monoclonal anti-human CD68, 1:1000 (Serotec); mouse monoclonal anti-human KDR, 1:500 (AbCam); mouse monoclonal anti-human CD31, 1:50 (Dako); mouse monoclonal anti-human α -smooth muscle actin (α -SMA), 1:400 (AbCam). After incubating at 4°C overnight and being washed in PBS, the sections were incubated with biotinylated goat anti-mouse or -rabbit (Vector Laboratories, Burlingame, CA) diluted 1:5000 for 1.5 hours at room temperature, and treated with the ABC streptavidin detection system (Vector Laboratories) for 1.5 hours after washing with PBS. The resulting horseradish peroxidase signals were detected using 3, 3'-diaminobenzidine (DAB). Sections were then dehydrated with graded alcohol (70%, 95%, and 100% alcohol, 2 min each), fixed in xylene for another 5 min, and finally counterstained with hematoxylin to show blue nuclei.

For double-fluorescent staining, after incubating at 4°C overnight with primary antibodies, sections were incubated with Alexa Fluor 594- or 488-conjugated labeling (Invitrogen) for 1.5 hours at room temperature. The sections were then mounted and photographed with fluorescent microscope. Isotype-matched host-specific antibodies or exclusion of primary antibody were used as negative controls. Immunohistochemically-stained slides were reviewed by two investigators independent of one another and blinded to all clinical data.

RESULTS

EPCs Present in the BAVM and SCAVM Nidus

To determine whether EPCs were present in brain AVM tissue, we performed immunohistochemical staining with the stem cell marker CD133 and the endothelial cell marker KDR. To avoid possible confounding effects, brain and spinal cord AVM cases without embolization and radiosurgery were used for immunohistochemical staining. As shown in Fig. 1, CD133-positive signals were present in the brain and spinal cord AVM nidus, but not in the cerebral cortex from epilepsy patients or basilar artery from autopsies (serving as control). Furthermore, EPC markers were present not only in BAVM tissues with a history of rupture but also in those without, suggesting that the presence of EPC was not caused by the rupture. CD133 signals were found mainly in the vessel wall. CD68-positive signals were present in both the brain and spinal cord AVM samples, and were found mainly in the vessel wall or in the parenchymal tissue adjacent to the vascular wall. Hemangioblastoma and meningioma tissues were used as positive control. As shown in Fig. 2, hemangioblastoma was positive for CD133, CD68, and SDF-1. We also used meningioma as additional positive control and comparison (Fig 2). Consistent with the literature,^{16,17} our data showed that meningioma was positive for CD133 and SDF-1 signals. Hemangioblastoma and meningioma appeared to be roughly similar to AVM tissues in the number of EPCs.

Double immunofluorescent staining (Fig. 3) indicates that CD133 positive cells co-expressed KDR. The co-expression of KDR and CD133 demonstrates that EPCs resided mainly at the edge of the vessel wall. The data show that EPCs, identified as double-positive cells for CD133 and KDR, were present in the brain AVM nidus, and suggest that EPCs may participate in pathological vascular remodeling in AVMs.

Increased Expression of SDF-1 in the BAVM and SCAVM Nidus

We determined whether SDF-1 was expressed in brain and spinal cord AVM tissue through immunohistochemical staining. Furthermore, we investigated the cellular source of SDF-1 through double-fluorescent staining with specific cellular markers, including CD31, α -SMA, and CD68. We observed that SDF-1 was strongly expressed in both the brain and spinal cord AVM nidus. SDF-1 positive signals could be found in the lumen, or in the vessel wall of the BAVM and SCAVM nidus. SDF-1 positive signal was not found in the basilar artery obtained from autopsy or cortex from epilepsy patients (Fig. 1).

Double immunofluorescent staining (Fig. 3) indicates that SDF-1 was co-localized with CD31 and α -SMA-positive cells, but not with CD68-positive cells, suggesting that SDF-1 was expressed in endothelial cells and smooth muscle cells other than macrophage/monocytes. The data demonstrate that SDF-1 produced in the AVM nidus may mediate circulating EPC homing to the AVM nidus.

DISCUSSION

We report the first description of EPCs in the nidus of brain and spinal cord AVM tissue removed during microsurgical resection. Using immunohistochemical analysis in paraffin-embedded AVM tissues, we found that EPCs were present and increased in the brain and spinal cord AVM nidus. Minimal signals could be found in the cortex from epilepsy patients and the basilar artery from the autopsy. The co-expression of KDR and CD133 demonstrate that EPCs mainly resided in the vessel wall. We also found that increased expression of SDF-1 was present in the brain and spinal cord AVM nidus, whereas SDF-1 expression was rarely identified in the control groups. Double fluorescence staining showed that SDF-1 expression was co-localized with CD31 and α -SMA, but not CD68-positive cells. This indicated that endothelial

and smooth muscle cells were the main supplier of SDF-1. The data suggest that EPCs, recruited by increased expression of SDF-1 in the nidus of AVM, may participate in pathological vascular remodeling in brain and spinal cord AVMs.

Brain and spinal cord arteriovenous malformations (AVMs) are complexes of tortuous, tangled vessels representing fistulous connections between arteries and veins lacking a capillary bed.¹⁸ The mechanisms underlying brain and spinal cord AVMs are not clearly understood. However, for BAVM, there has been a growing body of clinical and experimental evidence suggesting that brain AVMs undergo significant vascular remodeling and angiogenesis.^{4,15,19–21} Angiographic studies that examined a total of 106 patients with mean follow-up periods of 8.4 years show that BAVM size increased in over half of the cases, whereas BAVM size decreased or disappeared in approximately one-fifth of the cases,¹⁹ indicating that most brain AVMs undergo active remodeling processes. Histopathological studies present further evidence to support the notion of active vascular remodeling and angiogenesis in BAVMs.^{19,22} They suggest that brain AVMs, or even spinal cord AVMs, harbor unstable blood vessels that continuously undergo vascular remodeling. In this regard, we hypothesized that EPCs could be a powerful angiogenic switch to initiate and sustain active vascular remodeling in brain AVMs.

EPCs are a unique subtype of circulating bone marrow-derived cells that have the ability to proliferate and differentiate into mature endothelial cells.²³ These cells are identified as those positive for the stem cell marker CD133, the hematopoietic marker CD34, as well as the endothelial cell markers KDR, CD31 or VE-cadherin.^{24–26} EPCs are identified as the cells positive for the stem cell marker CD133, the hematopoietic marker CD34, as well as the endothelial cell markers: vascular endothelial growth factor receptor-2 (KDR), CD31 or VE-cadherin.^{24–26} Although the exact phenotype of EPCs is still controversial, the combination of unique surface markers of hematopoietic and endothelial lineages, such as CD133, CD34 and KDR, have been used to identify EPCs in many studies.^{27,28} CD133 has been commonly used as a marker for the isolation and identification of hematopoietic and endothelial progenitor cells. However, CD133 is also expressed by several types of adult cells including epithelial cells and reactive astrocytes.^{29,30} In this study, we used CD133 and KDR double fluorescent staining to define EPCs. Endogenous mobilization of EPCs from the bone marrow into the peripheral blood occurs in various physiological and pathological conditions, including ischemia, trauma, inflammation, and angiogenic stimulations. Circulating EPCs can migrate and localize to sites of active angiogenesis and vascular remodeling where they accelerate neovascularization.³¹ In addition, EPCs act as a risk marker and an outcome predictor. Experimental results obtained from both animal studies and recent clinical trials suggest that EPC plays a critical role in various human diseases.^{11,32–35} Circulating levels of EPCs have been reported to be increased in patients with lung cancer and correlate with clinical behavior.³⁶ Elevated levels of EPCs have been found in circulation and tumor tissues in patients with multiple myeloma and breast cancer.^{37,38} Their presence correlated with disease activity. Furthermore, EPCs have been found in infantile hemangioma contributing to early growth.^{39,40} The number and function of EPCs have been found to be decreased in patients with coronary artery disease, and inversely correlated with a number of risk factors.³² Both the number and the function of EPCs are decreased in patients with diabetes mellitus.^{34,35} The degree of impaired EPC function is correlated to the severity of diabetic vasculopathy. EPCs have also been reported to play a role in choroidal neovascularization.^{41,42} Stroke patients have lower baseline EPC levels compared with control subjects.^{43,44}

While EPCs have been extensively investigated as a risk biomarker and outcome predictor in cardiovascular diseases and cancer, nothing is known about the number and function of EPCs in patients with brain and spinal cord AVMs. In this study, we characterized EPCs with CD133 and KDR double-positive, and demonstrated the existence of EPC in AVM tissues. Our

findings show that EPCs mainly reside in the vessel wall. AVMs are characteristic of high-flow arteriovenous shunting. Increased EPC levels might allow for more efficient adaptive responses, e.g., an increased number might mitigate the effects of high flow through a nidus and decrease the risk of rupture. On the other hand, increased vascular remodeling promoted by EPC availability might allow more rapid growth leading to symptoms. Our study represents a first step in developing a novel paradigm to explain the clinical behavior of AVMs. Future work is needed to determine whether the number of circulating EPCs is altered in the peripheral blood of brain and spinal cord AVM patients.

SDF-1 (also known as CXCL12), a pleiotropic chemokine, has been identified as a specific mediator of circulating EPC mobilization,^{13,45,46} and plays a central role in recruiting EPCs from the peripheral blood to the target tissue.^{13,47} SDF-1 is constitutively expressed in various cell types including endothelial cells, and its expression is strongly elevated in hypoxia and inflammation.^{48–50} By activating its cognate receptor CXCR4, SDF-1 signaling pathway is involved in various physiological and pathological processes and exerts important functions in angiogenesis and vascular remodeling.⁵¹ In this study, we found that brain and spinal cord AVM displayed more SDF-1 positive signals than the control, suggesting that SDF-1 expression is upregulated in brain and spinal cord AVMs. Double fluorescence staining showed that endothelial and smooth muscle cells are the main supplier of SDF-1. The SDF-1 expressed in the vessel may trap and mediate the retention of circulating EPCs within the nidus of brain and spinal cord AVM.

Limitations

In our study, the choice of control tissue for BAVM nidus had inherent limitations. We described previously our use of brain samples taken from surgical epilepsy patients,^{2,3} but others have proposed using superficial temporal arteries.⁵² Neither of these tissues is ideal, as neither contains intracranial blood vessels of the caliber found in BAVM nidus. No positive signals were identified in both the cerebral cortex and the basilar artery. This observation-based study demonstrated increased EPC levels in brain and spinal cord AVM patients. However, it could not prove the causal role of EPCs in pathological vascular remodeling. Further research is needed to develop a more comprehensive in vitro and in vivo study that aims to analyze the roles of EPC in AVM pathophysiology.

CONCLUSION

This study reports the first observational description of EPCs in brain and spinal cord AVM patients. It characterizes the existence of EPCs and identifies the expression and localization of SDF-1 in AVM tissues. EPCs may play a role in maintaining active vascular remodeling within the AVM nidus. The data presented here suggest the potential roles of EPCs in brain and spinal cord AVM pathophysiology. This may present an opportunity for therapy development if future studies show that EPCs contribute to the natural history or treatment response of AVMs.

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FIGURE 1. H&E and immunohistochemical staining for BAVM, SCAVM and control tissues
 Panels A–B, C–D, and E–F represent BAVM, SCAVM, and control tissues, respectively. H&E staining (left column) shows the nidus of the BAVM (a1 and b1), SCAVM (c1 and d1), and the structure of the control tissues (e1 for epilepsy and f1 for basilar artery). BAVM and SCAVM tissues and control tissues were stained with the stem cell marker CD133, as well as other markers including CD68 and SDF-1. CD133, SDF-1 and CD68 columns show higher magnification of the boxed areas in the first column (H&E). In panels A–B and C–D, CD133, CD68, and SDF-1 positive signals were all observed in both BAVM and SCAVM nidus (brown color indicated by arrows), but not in control tissues (epilepsy from surgery, e2 through e4; basilar artery from autopsy, f2 through f4). CD133 was

found mainly in BAVM (a2 and b2) and SCAVM tissues (c2 and d2). Macrophages, identified as CD68+ signal, were mainly present in the vessel wall or in the parenchyma tissues adjacent to the vascular wall (a3, b3, c3, and d3). SDF-1 was found either in the lumen or in the vessel wall of the BAVM (a4 and b4) and SCAVM tissues (c4 and d4). Sections were counterstained with hematoxylin to show blue nuclei. Bar size: H&E, 500µm; CD133, CD68, and SDF-1 staining: 100µm

FIGURE 2. H&E and immunohistochemical staining for hemangioblastoma and meningioma
H&E staining shows the histology of hemangioblastoma and meningioma. CD133, CD68 and SDF-1 positive signals were observed in hemangioblastoma and meningioma (brown color indicated by arrows). Sections were counterstained with hematoxylin to show blue nuclei. Bar size: 100 μ m

FIGURE 3. Immunofluorescence staining

Panel A demonstrates the existence of EPCs in AVM nidus. H&E staining shows the AVM nidus. Boxed area designates the place where double fluorescent staining was photographed. Green shows CD133-positive signal and red shows KDR-positive signal in the vessel wall. Yellow indicates co-localization of CD133-positive cells with KDR-positive cells. Nuclei were stained with DAPI (blue). B–D, Double immunofluorescence staining of SDF-1 with specific cellular markers in AVM tissues. H&E staining shows the nidus of the AVM. Green shows SDF-1-positive signal. Red shows CD31-, α -SMA-, and CD68-positive staining. Yellow indicates co-localization. SDF-1 expression was co-localized with CD31- and α -SMA-positive cells, and was not co-localized with CD68-positive cells. Bar size: H&E, 500 μ m; dual fluorescent staining: 50 μ m. EPC, endothelial progenitor cell; AVM, arteriovenous malformation; H&E, hematoxylin and eosin; DAPI, 4,6-diamidino-2-phenylindole-2-HCl; SDF-1, stromal cell-derived factor-1; α -SMA, α -smooth muscle actin.