Alternatively Activated Macrophages Play an Important Role in Vascular Remodeling and Hemorrhaging in Patients with Brain Arteriovenous Malformation

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Background: Angiogenic and immunoactive lesions in brain arteriovenous malformation (BAVM) contribute to hemorrhagic events and the growth of BAVMs. However, the detailed mechanism is unclear. Our objective is to clarify the relationship between hemorrhagic events of BAVM and alternatively activated macrophages in the perinidal dilated capillary network (PDCN). *Methods:* We examined microsurgical specimens of BVMs (n = 29) and focused on the PDCN area. Ten autopsied brains without intracranial disease were the controls. We performed immunostaining of the inflammatory and endothelial cell markers, macrophage markers (CD163 and CD68), and vascular endothelial growth factor A (VEGF-A). We evaluated each cell's density and the vessel density in the PDCN and analyzed the relationship to hemorrhagic events of BAVM. *Results:* The PDCN was involved in all the resected arteriovenous malformations, and these vessels showed a high rate of CD105 expression (72.0 ± 10.64%), indicating newly proliferating vessels. Alternatively activated macrophages were found, with a high rate (85.6%) for all macrophages (controls, 56.6%). In the hemorrhagic cases, the cell density was significantly higher than that in the nonhemorrhagic cases and controls (hemorrhagic group, 290 ± 44 cells/mm^2; nonhemorrhagic group, 180 ± 59 cells/mm^2; and control, 19 ± 8 cells/mm^2). The cell density of alternatively activated macrophages showed a positive correlation with the vessel density of the PDCN. Double immunostaining showed that VEGF-A was secreted by alternatively activated macrophages. *Conclusion:* Our data suggest that alternatively activated macrophages may have some relationships with angiogenesis of PDCN and hemorrhagic event of BAVM. **Key Words:** Arteriovenous malformation—alternatively activated macrophage—perinidal dilated capillary network—vascular remodeling—VEGF.

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Introduction

Brain arteriovenous malformation (BAVM) is a major cause of hemorrhagic stroke in young patients. Therefore, a key therapeutic strategy is the prevention of bleeding and rebleeding. Multimodal therapies such as gamma knife, endovascular embolization, surgical resection, and a combination of these therapies are available.[1] However, some patients are not able to receive these treatments because of untreatable lesions (e.g., a large size, eloquent area, and deep drainer).[2] In addition, even if BAVM appears to be resolved, it sometimes recurs or rebleeds.[3] Thus, it is essential to clarify the pathogenesis and mechanism of rupture in BAVM. Although a satisfactory sporadic BAVM animal model does not exist, some recent reports have described the pathogenesis of BAVM.

Recently, some investigators demonstrated that areas containing dilated vessels existed in the perinidal space (i.e., the perinidal dilated capillary network [PDCN]). In a 3-dimensional anatomical study, Sato et al[4] reported that the PDCN is connected to the nidus, feeding arteries, and draining veins via the arterioles and venules, as well as to the normal capillaries, arterioles, and venules. Tu et al[5] reported that the perinidal capillaries demonstrated an abnormal ultrastructure of the blood–brain barrier with no basement membranes or astrocytic foot processes. These vessels are related to intraoperative and postoperative hemorrhages.[4,5] Some studies have reported that angiogenic or cell proliferating factors and signals (vascular endothelial growth factor [VEGF], platelet-derived growth factor, notch, and nuclear factor-κB) were detected in human BAVM samples.[6-9] Furthermore, endothelial cells of arteriovenous malformation (AVM) showed more frequent apoptosis and proliferation than normal controls in a culture study.[10] Therefore, BAVM and perinidal dilated capillaries are not considered static congenital lesions; instead, they are dynamically growing (or changing) lesions of their own.[11]

Chen et al[12] described that an aberrant immune response was an important factor of vascular remodeling in the BAVM microenvironment. They also found that macrophages and neutrophils infiltrate more frequently than lymphocytes in BAVM tissue. Recent studies showed that the macrophages act as cellular chaperones for vascular anastomosis and remodeling of the extracellular matrix.[13,14] However, it is unclear whether these macrophages are associated with vascular remodeling in BAVM tissue.

Macrophage activation is clearly divided into 2 pathways: classical activation (changing to M1 phenotype) and alternative activation (changing to M2 phenotype).[15-18] Classical activation is induced by bacterial products (e.g., lipopolysaccharides) and interferon-γ. In contrast, alternative activation is induced by Th2-type cytokines such as interleukin (IL)-4, IL-10, IL-13, vitamin D3, the macrophage colony stimulation factor, or a glucocorticoid.[15-18] Alternatively activated macrophages/microglia are associated with a high degree of vascularization, which influences wound repair.[19,20] In some malignant tumors, alternatively activated macrophages are tumor-associated macrophages (TAMs), which are associated with poor prognosis in breast, colon, prostate, glioma, and cervical cancers. TAMs promote invasion, growth, angiogenesis, and metastasis of malignant tumors.[21-25]

Our study focused on alternatively activated macrophages (i.e., M2 macrophage or TAMs) in BAVM. We used the antibody (Ab) for CD163, a member of the scavenger receptor cysteine-rich superfamily restricted to monocyte/macrophage lineage, which is a useful marker for anti-inflammatory or alternatively activated macrophages.[26] We aimed to investigate the origin of the dilated capillary network, analyze the macrophage phenotype and function, and clarify the relationship between macrophages and dilated capillary vessel formation in surgically resected BAVM samples.

Methods

Patients

We surgically removed 28 BAVMs at the Kurume University Hospital between 1996 and 2010. The clinical data are summarized in Table 1; 19 patients were hemorrhagic cases and 9 were nonhemorrhagic cases. Ten autopsied brains without intracranial disease were the controls. The patients' mean age was 43.1 ± 12.4 years, and 11 were female. In the nonhemorrhagic cases, 3 patients had AVM at seizure onset, while the other AVMs were discovered incidentally. The nidus size ranged from 8 to 55 mm (mean, 25.3 mm). Four patients' AVMs were Spetzler–Martin grade I, 15 were grade II, and 10 were grade III. Of all 19 hemorrhagic cases, 15 (78.9%) cases underwent acute phase removal surgery (within 48 hours after the hemorrhagic event).

Abs

The following Abs were used in the present study: anti-CD3 Ab as a T-lymphocyte marker (1:100; Novocastra, Leica Biosystems, Newcastle, UK), anti-CD20 Ab as a B-lymphocyte marker (1:1000; Dako Corporation, Carpinteria, CA), antimyeloperoxidase Ab as a neutrophil marker (1:4000, Dako Corporation), rabbit and mouse anti-CD68 Abs as a macrophage marker (1:200; Dako, Grostrup, Denmark), anti-CD163 Ab (1:200, Novocastra) for recognizing macrophage-restricted membrane protein, anti-CD34 as a vascular endothelial cell marker (1:200; Immunotech, Marseille, France), antihuman CD105 Ab (antiendoglin, 1:200; Dako Corporation), and anti-VEGF (A20, sc-120) and antivascular endothelial growth factor A (VEGF-A) Abs (1:200; Santa Cruz Biotechnology, Santa Cruz, CA). In the present study, alternatively activated macrophages were recognized as

Table 1. *Summary of patients with brain arteriovenous malformations*

Characteristics	Hemorrhage group	Nonhemorrhage group
No. of cases	n = 19	n = 9
Age		
Mean ± SD	49.42 ± 20.91	43.89 ± 12.42
Range	8-64	8-40
Sex (male)	12 (63.2%)	5 (55.6%)
BAVM size (mm)		
Mean ± SD	23.32 ± 11.11	21.22 ± 10.06
Range	5-50	8-40
Spetzler–Martin grade		
1	1	1
2	11	6
3	7	2
4	0	0
5	0	0
AVM localization		
Superficial	11	8
Deep	8	1

Abbreviations: AVM, arteriovenous malformation; BAVM, brain arteriovenous malformation; no., number; SD, standard deviation.

the cells that showed positive immunostaining for both CD68 and CD163.[26,27]

Immunohistochemistry

All the specimens were fixed in 10% formalin for 12 hours, were embedded in paraffin (formalin-fixed paraffin-embedded), and were stored at room temperature. All the formalin-fixed paraffin-embedded specimens were cut into 5–μm-thick tissue sections. For single-label immunohistochemistry, the sections were deparaffinized and rehydrated with graded ethanol. Antigen unmasking was performed using ethylenediamine tetraacetic acid for 10 minutes at 95°C. After removing the blocking solution, 100 μL of primary diluted Ab was added to each section and incubated for 30 minutes at room temperature. After the reactions of each antigen-specific Ab was obtained, the samples were incubated with horseradish peroxidase-labeled goat antimouse (or rabbit) Ab (Nichirei, Tokyo, Japan). After rinsing with .01 mol/L of phosphate-buffered saline 3 times for 5 minutes, the reaction was visualized by means of the diaminobenzidine substrate system (Nichirei).

Next, for double immunostaining, antigen unmasking was performed using ethylenediamine tetraacetic acid for 5 minutes at 95°C. Diluted primary Abs were added to each section and were incubated for 30 minutes at room temperature. After rinsing with phosphate-buffered saline (3 times for 5 minutes), the second reaction was visualized by using the Vector VIP substrate kit for peroxidase (SK-4600; Vector Laboratories, Burlingame, CA). Some frozen samples were selected for double fluorescent staining, and after being incubated at 4°C for 24 hours with the primary Abs, the sections were incubated with Alexa Flour 488-conjugated goat antirabbit IgG or Alexa Flour 594-conjugated goat antimouth (Molecular Probes, Eugene, OR).

PDCN

The PDCNs were defined as follows: more than 50 μm in diameter and located in the perinidal brain parenchyma within 1-10 mm from the AVM border (Fig 1, A,B). These vessels were counted in the perinidal area of all the specimens, and the vessel density (count/cm^2) was calculated.

Assessing the Cell Counts

All the tissue sections were measured using the National Institutes of Health ImageJ program (National Institutes of Health, New York City, NY) in square millimeter. Infiltrating cells were defined as the presence of a vascular wall, perivascular space, and brain parenchyma. Cell counts were performed in 5 areas that were randomly selected far from the hemorrhage site and were calculated using average counts per square millimeter (cell number/mm^2). Cell counts of the normal controls were counted in the same manner. In the CD68 and CD163 double immunostained specimens, the CD68-positive cells and CD68-/CD163-double positive cells were counted on a field with ×200 magnification.

Statistical Analysis

The counts are presented as the mean ± standard deviation, and they were analyzed using Student *t*-test for each group. The statistical analysis was performed using

JMP 9 (SAS Institute Inc., Cary, NC). *P* values less than .05 were considered significant.

Results

Perinidal Dilated Capillary Vessels Surrounding the BAVM

All the cases had the PDCN area in the perinidal brain parenchyma (Fig 1, A,B), and the mean vessel density was 99 ± 74 vessels/cm^2 (n = 28). There were no significant differences in the nidal size or hemorrhagic events. CD105-positive endothelial cells were observed in the perinidal dilated capillary vessels (mean, 72.0 ± 10.64%) (Fig 1, C), whereas only 1.4 ± .23% (mean) of CD105-positive vessels were found in normal brain parenchyma. These positive rates of CD105 immunostaining showed no significant difference between the hemorrhagic and nonhemorrhagic AVM groups. CD163-positive cells were frequently found in these areas (Fig 1, D, white arrow). The mean positive rate of CD163 (the percentage of alternative activation) in all the macrophages of all the AVMs was 85.14 ± 3.53%, which is significantly higher than that of normal brain macrophages (56.59 ± 28.74%, $P < .01$).

Quantitative Analysis of Inflammatory Cell Infiltration

First, we analyzed the inflammatory cells of the T cell, B cell, neutrophil, and general macrophages (Fig 2). All the types of inflammatory cells were observed in the perivascular space and brain parenchyma.

The cell density of CD3-positive cells (a T-lymphocyte marker) in the hemorrhagic and nonhemorrhagic cases was significantly higher than that of the controls (hemorrhagic cases: 96.7 ± 16.3 cells/mm^2, $P = .0005$; nonhemorrhagic cases: 88.4 ± 15.6 cells/mm^2, $P = .01$; controls: 6.2 ± 1.4 cells/mm^2). However, there were no significant differences between the hemorrhagic and nonhemorrhagic cases ($P = .73$; Fig 2, A).

The cell density of the CD20-positive cells (a B-lymphocyte marker) of hemorrhagic cases was significantly higher than that of the nonhemorrhagic cases ($P = .01$) and that of the controls ($P < .0001$) (hemorrhagic cases: 60.1 ± 34.6 cells/mm^2; nonhemorrhagic cases: 31.0 ± 21.3 cells/mm^2; controls: 4.4 ± 3.7 cells/mm^2; Fig 2, B).

The cell density of myeloperoxidase-positive cells (a neutrophil marker) of the hemorrhagic and nonhemorrhagic cases was significantly higher than that of the controls (hemorrhagic cases: 286.1 ± 199.4 cells/mm^2, $P \leq .0001$; nonhemorrhagic cases: 407.0 ± 275.9 cells/mm^2, $P = .011$; controls: 9.0 ± 3.2 cells/mm^2). There was no significant difference between the hemorrhagic and nonhemorrhagic cases ($P = .14$; Fig 2, C).

The CD68-positive cells (a macrophage marker) were generally observed in the vascular wall, perivascular space, and brain parenchyma. The cell density of the CD68-positive cells in the hemorrhagic cases was higher (342.3 ± 167.9 cells/mm^2) than that of the nonhemorrhagic cases (207.4 ± 94.9 cells/mm^2). The cell density of the CD68-positive cells of the controls was the lowest (19.8 ± 13.3 cells/mm^2) among the 3 groups, and there was a statistically significant difference among all the groups ($P < .01$; Fig 2, D).

Alternatively Activated Macrophages in the BAVM Tissue

The CD68-positive cells in BAVM showed a high coexpression of the CD163 antigen (Fig 3), and the cell

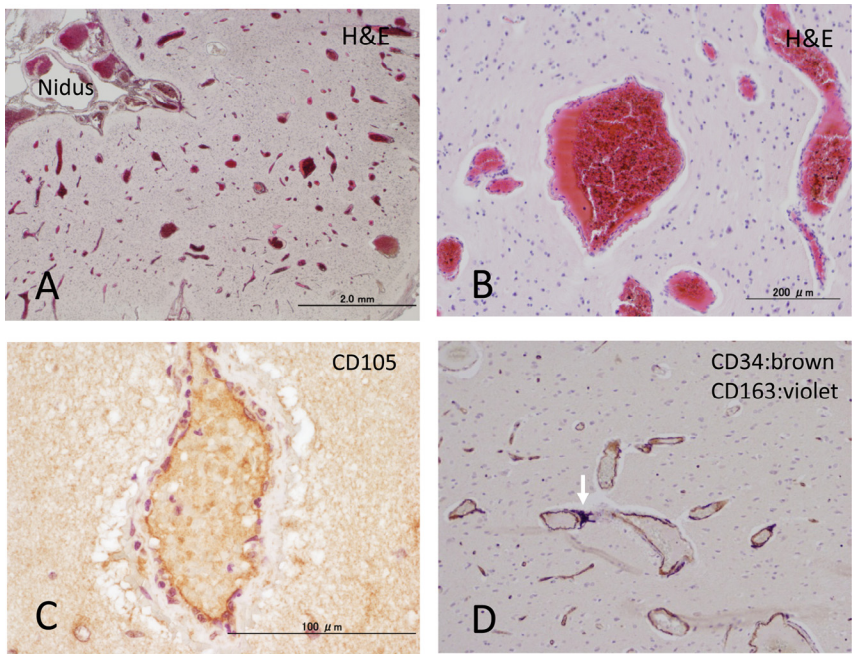

Figure 1. *H&E staining (low power magnification, A; high power magnification, B) showing perinidal dilated capillary vessels around the AVM. Dilated vessels are defined as more than 50 μm of their luminal size. The brain AVM nidus expressed CD105 in the endothelial cells of AVM (C) and CD163-positive macrophage (D, violet portion) exists near the perinidal capillary vessels (D, brown portion). Abbreviations: AVM, arteriovenous malformation; H&E, hematoxylin and eosin. (Color version of figure is available online.)*

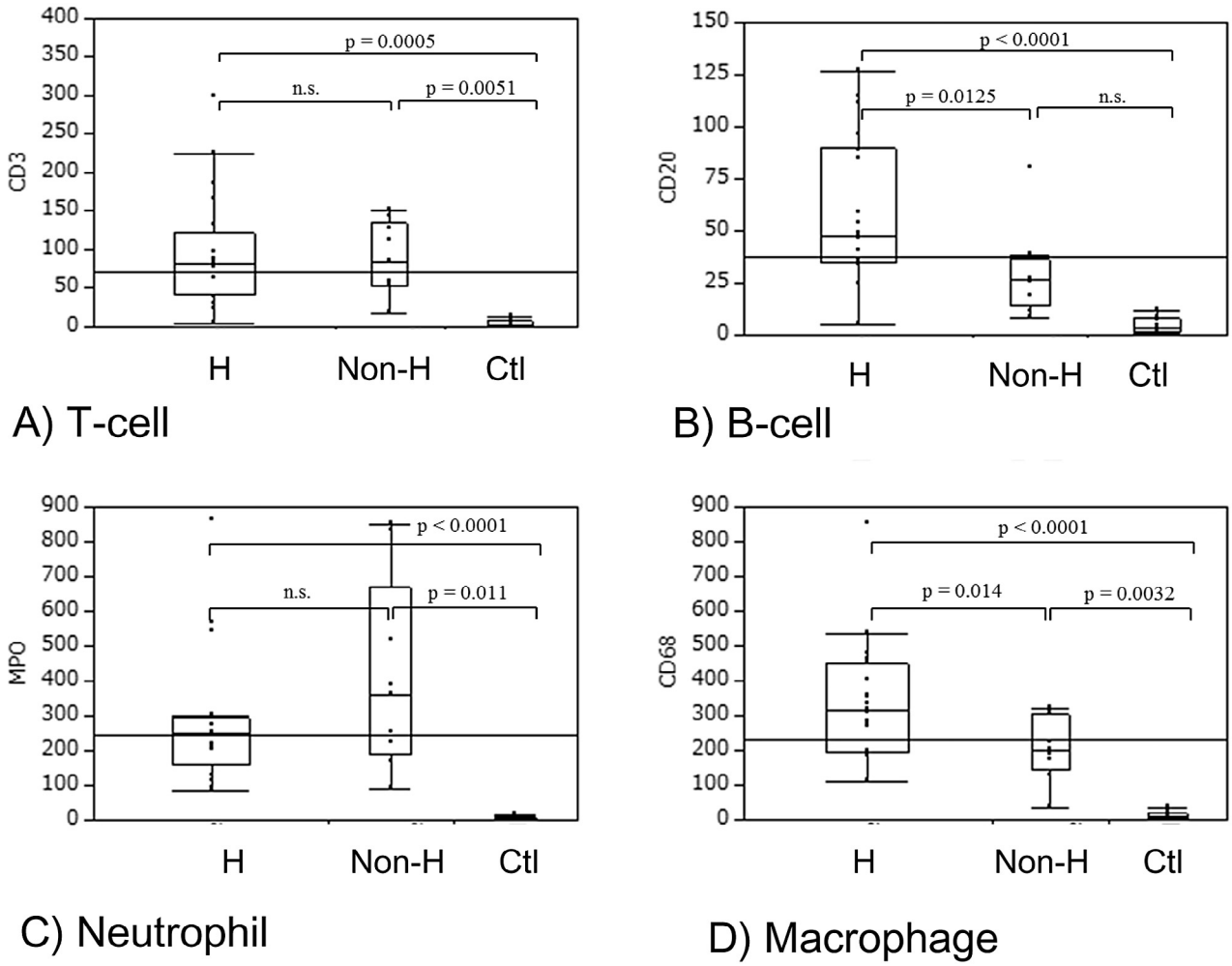

Figure 2. *Quantity analysis of the inflammatory cell infiltration. The CD3-positive T cells (A), CD20-positive B cells (B), myeloperoxidase-positive neutrophils (C), and CD68-positive macrophage (D) are analyzed among the H, non-H, and Ctl groups. The vertical bars indicate the number of cells per square millimeter. Abbreviations: AVM, arteriovenous malformation; Ctl, control; H, hemorrhagic AVM; non-H, nonhemorrhagic AVM.*

density of CD163- and CD68-double positive cells was significantly higher than that of the controls (AVM: 283 ± 123 cells/mm^2 versus controls: 19 ± 11 cells/mm^2; $P < .0001$). In hemorrhagic cases, the cell density of alternatively activated macrophages (CD163- and CD68-double positive cells) was significantly higher than that of the nonhemorrhagic and controls (hemorrhagic group: 283 ± 123 cells/mm^2, nonhemorrhagic group: 183 ± 81 cells/mm^2, and controls: 19 ± 11 cells/mm^2; Fig 4, A).

Correlation between the Alternatively Activated Macrophage and Dilated Capillary Vessel Density

When analyzing the relationship between the vessel density and the cell density of the CD163- and CD68-double positive cells (alternatively activated macrophages), a significant positive correlation was found in the hemorrhagic AVM group; however, no correlation was found in the nonhemorrhagic AVM group (Fig 4, B,C). In VEGF-A and CD163 double staining, the CD163-positive cells contained VEGF-A and were located in the vascular sprouting and fusion site (Fig 5, A). CD163-positive cell infiltrations were frequently found near small vessels in the hemorrhagic and nonhemorrhagic cases, and these cells contained VEGF (Fig 5, B,C).

Discussion

In the present study, all the BAVM specimens had areas containing the PDCN,[4,5] which is clearly distinguished from the BAVM nidus itself. We performed immunohistochemistry of CD105 (endoglin) and obtained a high CD105-positive ratio (72.0 ± 10.64%) in the vascular endothelial cells of these areas, while there were rare CD105-positive cells in the normal brain. CD105 is a transmembrane auxiliary receptor for the transforming growth factor-β (TGF-β), which is predominantly expressed in proliferating endothelial cells.[28] Thus, these findings suggest that most of these vessels were newly proliferated. Many investigators have suggested that BAVM

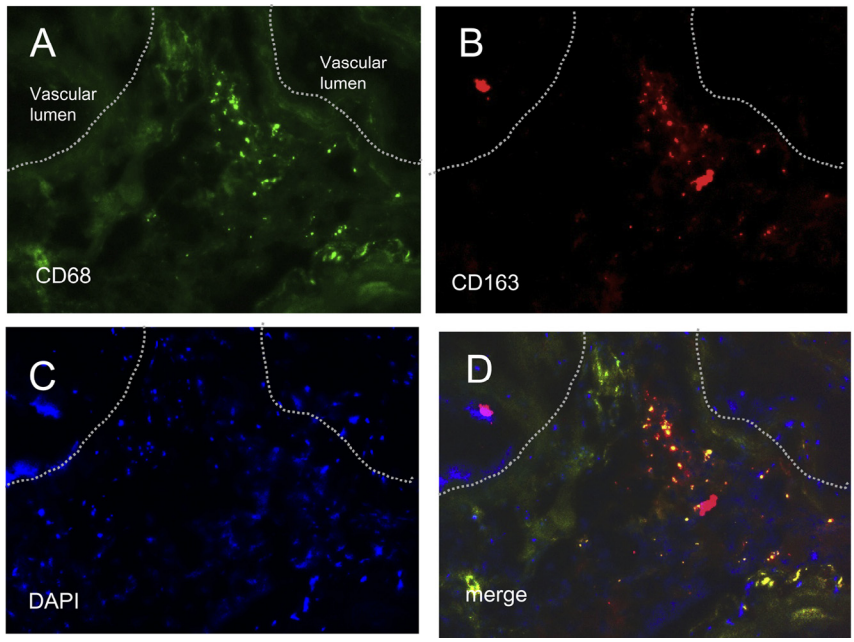

Figure 3. *CD68 (A) and CD163 (B) immnofluorescent staining and nuclear staining (C). The dotted gray lines show the endothelium layer of the large vessels. CD68- and CD163-double positive macrophage infiltration is observed in the perivascular space (D, merged images of A and B). DAPI, 4′,6-diamidino-2-phenylindole.*

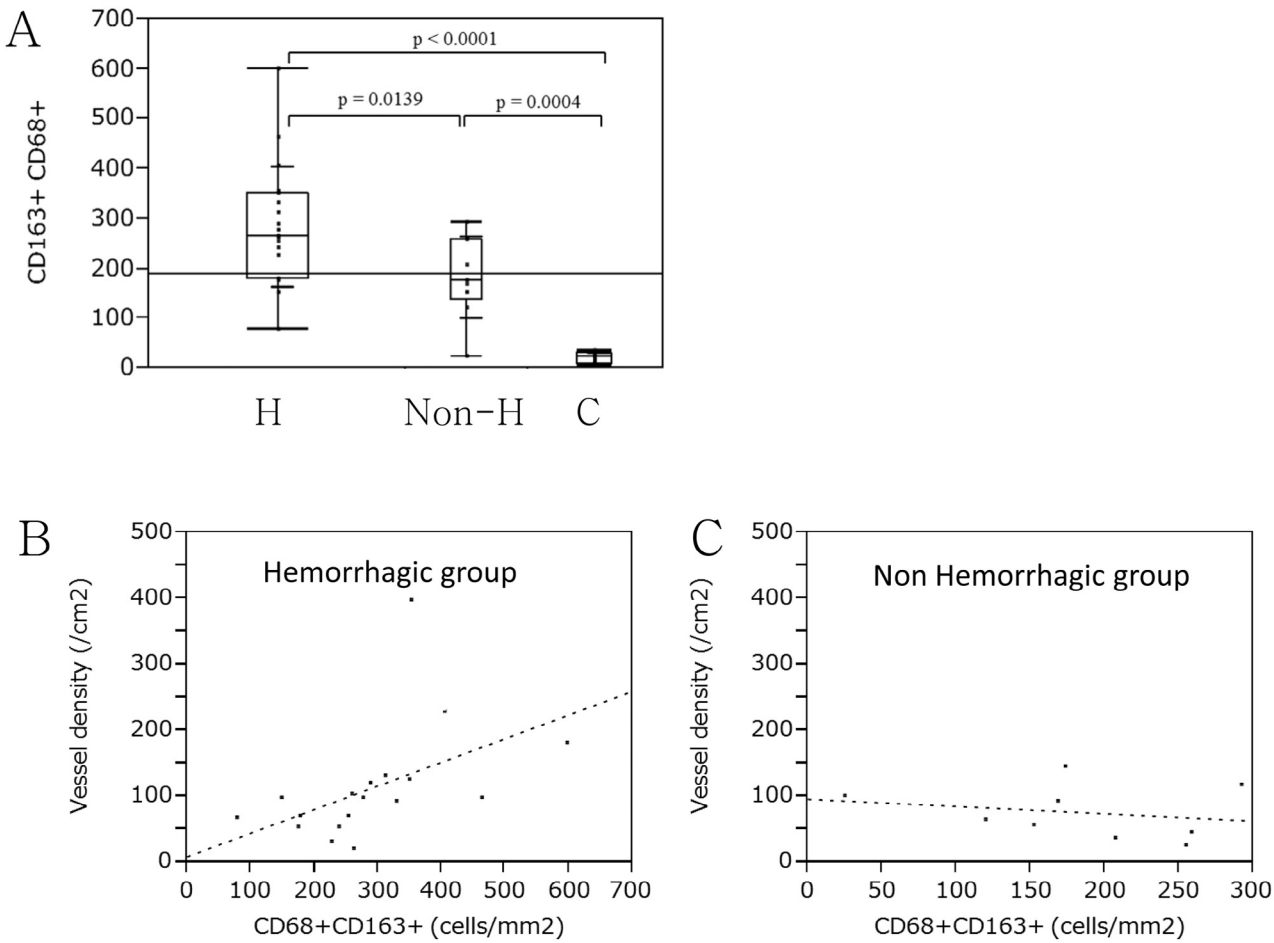

Figure 4. *Alternatively activated macrophage (CD163- and CD68-double positive cells) exist more frequently in the hemorrhagic AVM (H group) compared to the other groups (non-H, nonhemorrhagic group; C, control group) in the AVM perinidal area (A). The relationship between the vessel density in the perinidal area and the alternatively activated macrophages in hemorrhagic AVM (B) and nonhemorrhagic AVM (c). Abbreviation: AVM, arteriovenous malformation.*

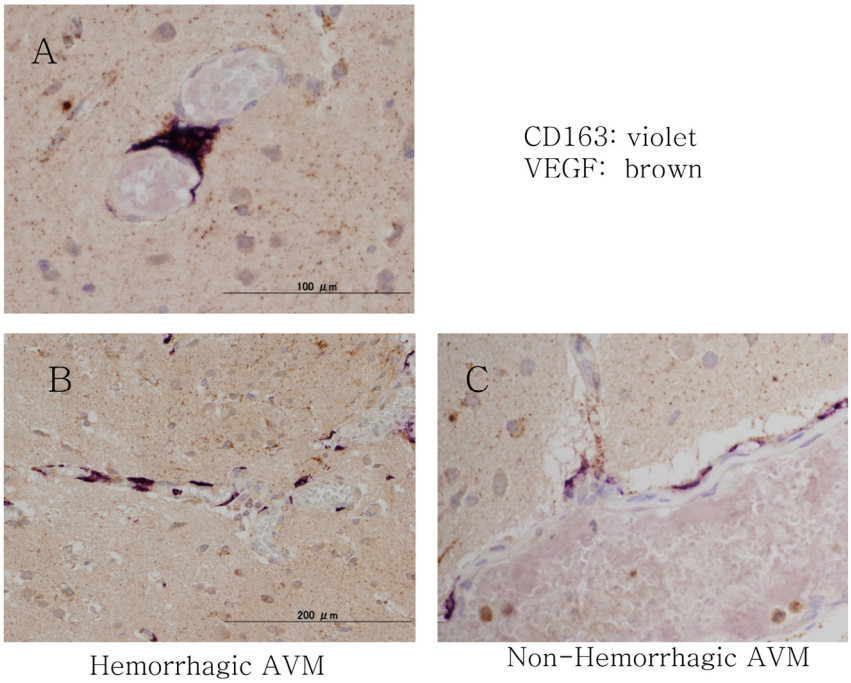

Figure 5. *Double immunostaining showing vascular endothelial growth factor secretion (brown) and CD163-positive macrophages (violet). (A) High-power magnification; (B) hemorrhagic AVM; (C) nonhemorrhagic AVM. Abbreviation: AVM, arteriovenous malformation. (Color version of figure is available online.)*

has angiogenic activity of its own, and these capillaries may originate from AVM. Alternatively, other angiogenic factors (cells) may act to proliferate normal capillary vessels, which are connected to the BAVM nidus and other dilated capillary vessels.

At first, we performed a quantitative analysis of the inflammatory cells in hemorrhagic and nonhemorrhagic AVMs, and in normal controls. In the AVM samples, the number of all the inflammatory cells was significantly higher than that in the controls; particularly, the neutrophils and macrophages were obvious in contrast to the T and B lymphocytes. Our data correspond to a previous study by Chen et al,[12] who focused on the immune response in BAVM. Chen et al showed that infiltrating neutrophils and macrophages are observed in nonhemorrhagic BAVMs, and neutrophil-derived MMP-9 is significantly higher in hemorrhagic cases. Similarly, our data showed the significant increase of neutrophil infiltration in both hemorrhagic and nonhemorrhagic AVMs, but there was no statistically significant difference between these 2 groups. Alternatively, our data showed that only macrophage infiltration was correlated with a hemorrhagic event. Therefore, we analyzed the macrophage phenotype and function.

CD163- and CD68-double positive cells (alternatively activates macrophage) were observed in the vascular wall, perivascular space, and brain parenchyma. Previous studies reported that CD163-positive macrophages infiltrate into some malignancies (e.g., T-cell lymphoma and glioma).[27,29] Furthermore, it was reported that the parenchymal CD163-positive macrophages/microglia have the capacity for antigen ingestion and presentation, which may contribute to the resolution of inflammation in multiple sclerosis.[30,31] In our study, about 85% of the macrophages coexpressed the CD163 antigen. However, we cannot explain the reason for this high rate. Mahmoud et al[32] reported that TGF-β can polarize macrophage M1 to the M2 phenotype. Furthermore, it was reported that TGF-β signaling plays a key role in hereditary hemorrhagic telangiectasia and sporadic AVM.[33] TGF-β may also contribute to the alternative activation of macrophages in the BAVM microenvironment.

Our data showed that alternative activated macrophages were positively associated with the number of dilated vessels in hemorrhagic AVM. Tu et al[5] reported that dilated capillary vessels lack the BBB, so they may be associated with intraoperative and postoperative hemorrhage, growth, and recurrence of surgically treated BAVM. Our findings suggest that alternatively activated macrophages have an angiogenic potential and play a key role in dilated capillary vessel formation. A previous study proved that alternatively activated macrophages had a higher angiogenic activity in poor prognostic follicular lymphoma, and other studies demonstrated an association between the vascular sprouting and numbers of infiltrating CD163-positive macrophages.[34] According to our double immunostaining of VEGF-A and CD163, double-positive macrophages were localized in the nidal vascular wall and perivascular space (outside of the CD34 positive layer); furthermore, they were localized in the dilated vessels sprouting and fusion sites. Some studies have suggested that BAVM endothelium has a potential

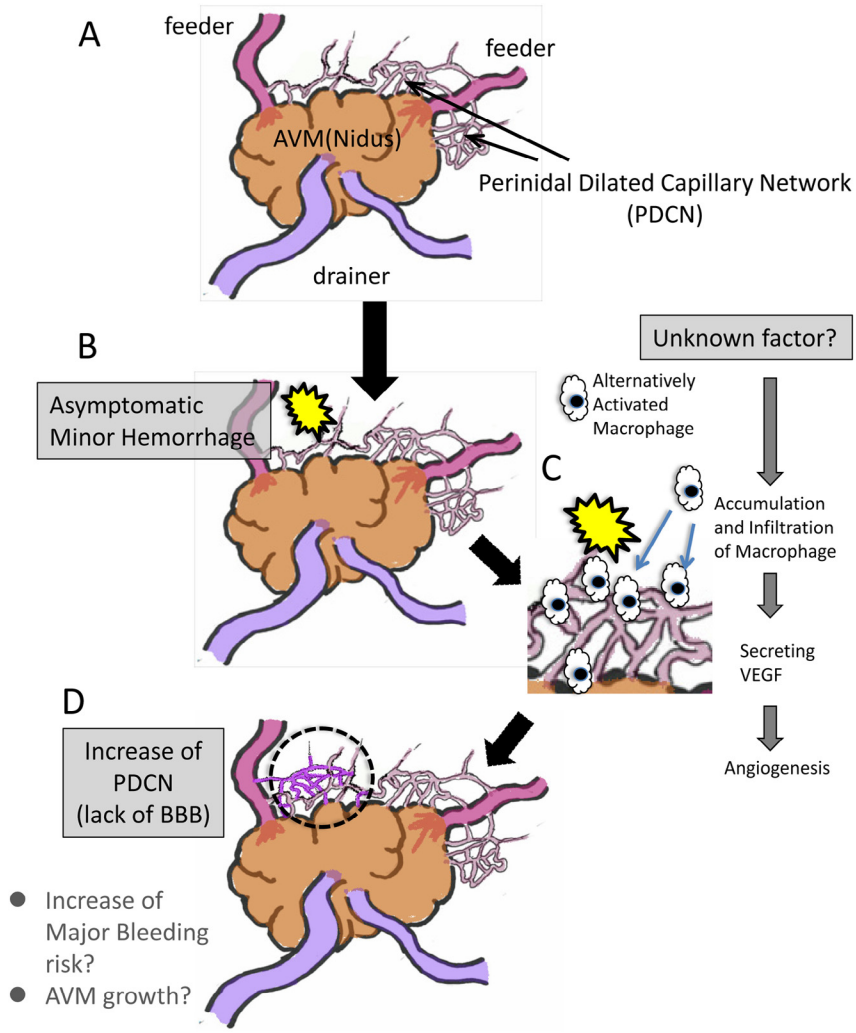

Figure 6. *Schematic drawings of the mechanism of major bleeding of AVM. The PDCN exists near the AVM nidus (A), and asymptomatic minor hemorrhage (B) triggers the accumulation and infiltration of alternatively activated macrophages to the PDCN (C). These alternatively activated macrophages have anti-inflammatory factors and VEGF, which increases the angiogenesis of PDCN. This newly grown PDCN lacks the BBB, increasing the risk of major bleeding after PDCN proliferation. Even if there is no minor hemorrhage, accumulated and infiltrated alternatively activated macrophages induce PDCN proliferation, which may cause major hemorrhage of AVM. Abbreviations: AVM, arteriovenous malformation; BBB, blood–brain barrier; PDCN, perinidal dilated capillary network; VEGF, vascular endothelial growth factor.*

for angiogenesis.[8-10] Thus, alternatively activated macrophages may also contribute to angiogenesis in BAVM by secreting VEGF.

The infiltrating cell number of alternatively activated macrophages in hemorrhagic AVM was significantly higher than that of nonhemorrhagic AVM and the normal controls. In posthemorrhage conditions, various inflammatory cells, which secrete proinflammatory cytokines and classically activated macrophages, secrete TNF-α, IL-1, and IL10 among other factors. In contrast, alternatively activated macrophages secrete anti-inflammatory cytokines such as IL-10, IL-4, and TGF-β.[35] Recently, it was reported that alternatively activated macrophages are evoked after a coronary intraplaque hemorrhage[36] and that CD163-positive cells may be recruited to hemorrhagic lesions to reduce the heme toxicity.[37] In addition, alternatively activated macrophages are regarded as nearly the spectrum of wound-healing macrophages.[33] This evidence strongly supports the response-to-injury model of the BAVM pathology, which was proposed by Kim et al.[38] Thus, there is a possibility that alternatively activated macrophages were recruited by the hemorrhagic events in our study.

In contrast, there is another possibility. Because surgical resection was performed in the acute phase (within 48 hours after the hemorrhagic event) in most cases (15 of 19 hemorrhagic cases, 78.9%), infiltration of alternatively activated macrophages may have occurred prior to the major bleeding. Guo et al[39] reported that silent intralesional microhemorrhage (i.e., asymptomatic bleeding in the nidal compartment) serves as a marker for the increased risk of symptomatic intracranial hemorrhage. The well-known function of CD163 (a hemoglobin scavenger receptor) is to bind to the hemoglobin–haptoglobin complex, which may mediate extravasal hemolysis.[28] Furthermore, CD163-positive macrophages may cause intralesional microhemorrhage through the formation of perinidal dilated capillary vessels. These fragile vessels lack the BBB, which may cause a hemorrhagic tendency, as previously mentioned (Fig 6). Thus, our data suggested that alternatively activated macrophages may

contribute to angiogenesis and hemorrhagic events, although details are still unknown and further study is necessary.

Conclusions

To clarify macrophage infiltration in BAVM tissue, we investigated the macrophage phenotype and function using the immunohistochemical method. Alternatively activated macrophages were infiltrated in surgically resected BAVM tissue, especially in hemorrhagic cases, and in PDCN. These findings suggest that alternatively activated macrophages may have a relationship with angiogenesis of PDCN and hemorrhagic event of BAVM.

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