Involvement of Neutrophil Extracellular Traps in Cerebral Arteriovenous Malformations

Kenji Shimada, Izumi Yamaguchi, Manabu Ishihara, Takeshi Miyamoto, Shu Sogabe, Kazuhisa Miyake, Yoshiteru Tada, Keiko T. Kitazato, Yasuhisa Kanematsu, Yasushi Takagi

BACKGROUND: **Cerebral arteriovenous malformations (cAVMs) represent tangles of abnormal vasculature without intervening capillaries. High-pressure vascular channels due to abnormal arterial and venous shunts can lead to rupture. Multiple pathways are involved in the pathobiology of cAVMs including inflammation and genetic factors such as *KRAS* mutations. Neutrophil release of nuclear chromatin, known as neutrophil extracellular traps (NETs), plays a multifunctional role in infection, inflammation, thrombosis, intracranial aneurysms, and tumor progression. However, the relationship between NETs and the pathobiology of cAVMs remains unknown. We tested whether NETs play a role in the pathobiology of cAVMs.**

METHODS: **We analyzed samples from patients who had undergone surgery for cAVM and immunohistochemically investigated expression of citrullinated histone H3 (CitH3) as a marker of NETs. CitH3 expression was compared among samples from cAVM patients, epilepsy patients, and normal human brain tissue. Expressions of thrombotic and inflammatory markers were also examined immunohistochemically in samples from cAVM patients.**

RESULTS: **Expression of CitH3 derived from neutrophils was observed intravascularly in all cAVM samples but not other samples. Nidi of AVMs showed migration of many Iba-I-positive cells adjacent to the endothelium and endothelial COX2 expression, accompanied by expression of IL-6 and IL-8 in the endothelium and intravascular neutrophils. Unexpectedly, expression of CitH3 was not necessarily localized to the vascular wall and thrombus.**

CONCLUSIONS: **Our results offer the first evidence of intravascular expression of NETs, which might be associated with vascular inflammation in cAVMs.**

INTRODUCTION

Cerebral arteriovenous malformations (cAVMs) are tortuous, morphologically abnormal vascular channels without intervening capillaries.[1] These channels shunt high-pressure arterial blood from feeding arteries directly into the venous outflow system and thus risk catastrophic hemorrhagic stroke, particularly in young patients.[2] The pathobiology of cAVMs has been reported to involve abnormal angiogenesis, inflammation, and certain genetic factors including KRAS mutations, which are strong drivers of tumorigenesis.[3-8] However, the detailed pathobiology of cAVM remains elusive and the involvement of multiple pathways has been suggested.

Neutrophils release chromatin fibers with modified histone and intracytoplasmic antimicrobial proteases such as myeloperoxidase via cell death after phagocytic activation against invading microbes. These fibers are called neutrophil extracellular traps (NETs).[9,10] Citrullinated histone-H3 (CitH3) has been shown to be involved in the formation of NETs.[11] Although NETs appear important for innate immune defense, they are a double-edged sword that can also potentially cause damage to the host.[12] Recent evidence suggests that NETs might play a role in noninfectious diseases including thrombosis, atherosclerosis, intracranial aneurysm, and cancer.[9,10,13,14] However, little is known about NET expression and the functional roles in cAVM. Considering the multiple and complex pathways of cAVM pathobiology including inflammation and cancer-related gene

Key words
- Cerebral arteriovenous malformation
- Citrullinated histone-H3
- Neutrophil extracellular traps

Abbreviations and Acronyms
cAVM: Cerebral arteriovenous malformation
CitH3: Citrullinated histone-H3
fXa: Factor Xa
H3: Histone 3
NET: Neutrophil extracellular trap

Department of Neurosurgery, Tokushima University Hospital, Tokushima, Tokushima, Japan

To whom correspondence should be addressed: Kenji Shimada, M.D., Ph.D. [E-mail: s_kenji1032@yahoo.co.jp]

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Table 1. Characteristics of Patients with Arteriovenous Malformation

Number	Age (year)/Sex	Onset	S-M Grade	Location	Nidus Size (cm)	Hemorrhage	Embolization
1	23/Male	Consciousness disturbance	II	Frontal	3	Yes	No
2	52/Male	Headache	I	Frontal	3	No	No
3	23/Female	Headache	I	Parietal	2	Yes	No
4	28/Female	Headache	I	Parietal	2	Yes	Yes
5	29/Female	Seizure	III	Occipital	4	No	No
6	37/Male	Hemiparesis	II	Frontal	3	No	No
7	10/Female	Seizure	II	Frontal	1	Yes	Yes
8	23/Male	Seizure	II	Temporal	3	No	No
9	20/Female	Seizure	I	Frontal	2	No	No

S-M, Spetzler-Martin.

mutations, NETs may be associated directly or indirectly with cerebral AVM pathobiology. The present study examined expressions of CitH3 as a central player in NETs in tissue samples of AVM nidi extracted from cAVM patients and compared with normal brain tissues and brain tissue samples from epileptic foci extracted from epilepsy patients resistant to medical therapy as surgical controls. Here, we provide the first documentation of an association between expression of intravascular CitH3 and vascular inflammation in cAVM.

MATERIALS AND METHODS

Patient Recruitment and Characteristics

This study was approved by the Clinical Research Review Board at our institution. Human tissue samples were obtained during routine clinical procedures. Informed consent including for the use of tissue samples was obtained from patients with cAVM and epilepsy. We analyzed samples from 9 patients who underwent neurosurgery at our institution (Table 1) after digital subtraction

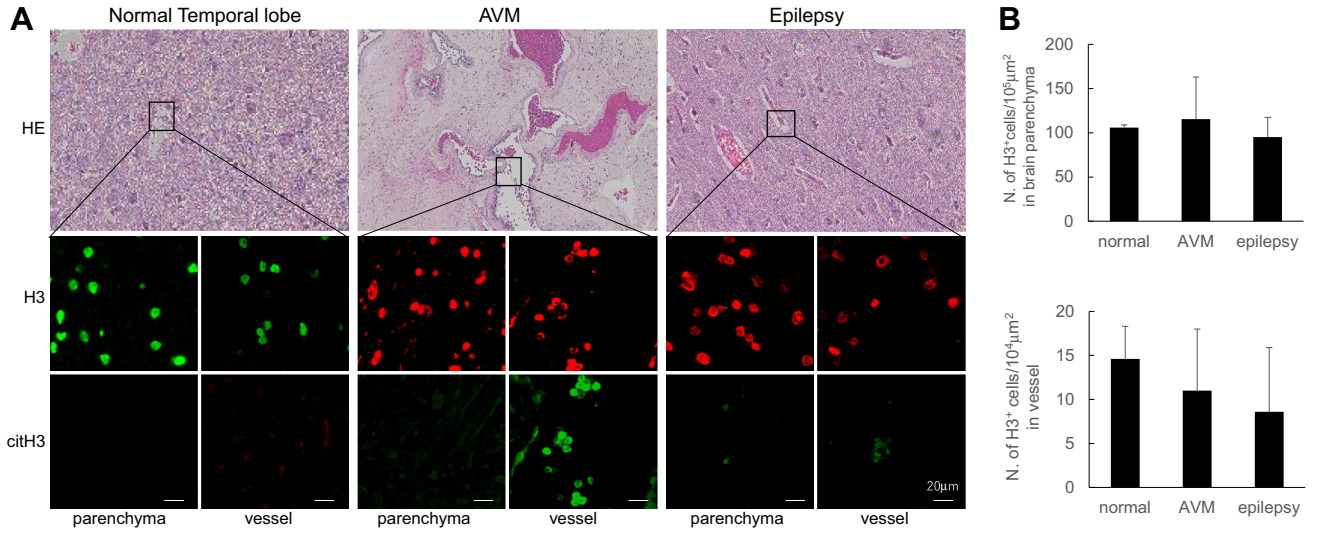

Figure 1. Expression of H3- and CitH3-positive cells in brain tissue samples from patients with cerebral arteriovenous malformations (cAVMs) ($n = 9$), epilepsy ($n = 3$), and human cadavers (normal control, $n = 3$). (**A**) H3-positive cells were detected in brain parenchyma and the intravascular space in all cAVM, epilepsy, and normal samples. CitH3-positive cells were observed only in the intravascular space of all cAVM samples. (**B**) Expression levels of H3-positive cells in brain parenchyma and blood vessels of cAVM, epilepsy, and normal samples show no significant intergroup differences. Data are given as mean ± standard deviation.

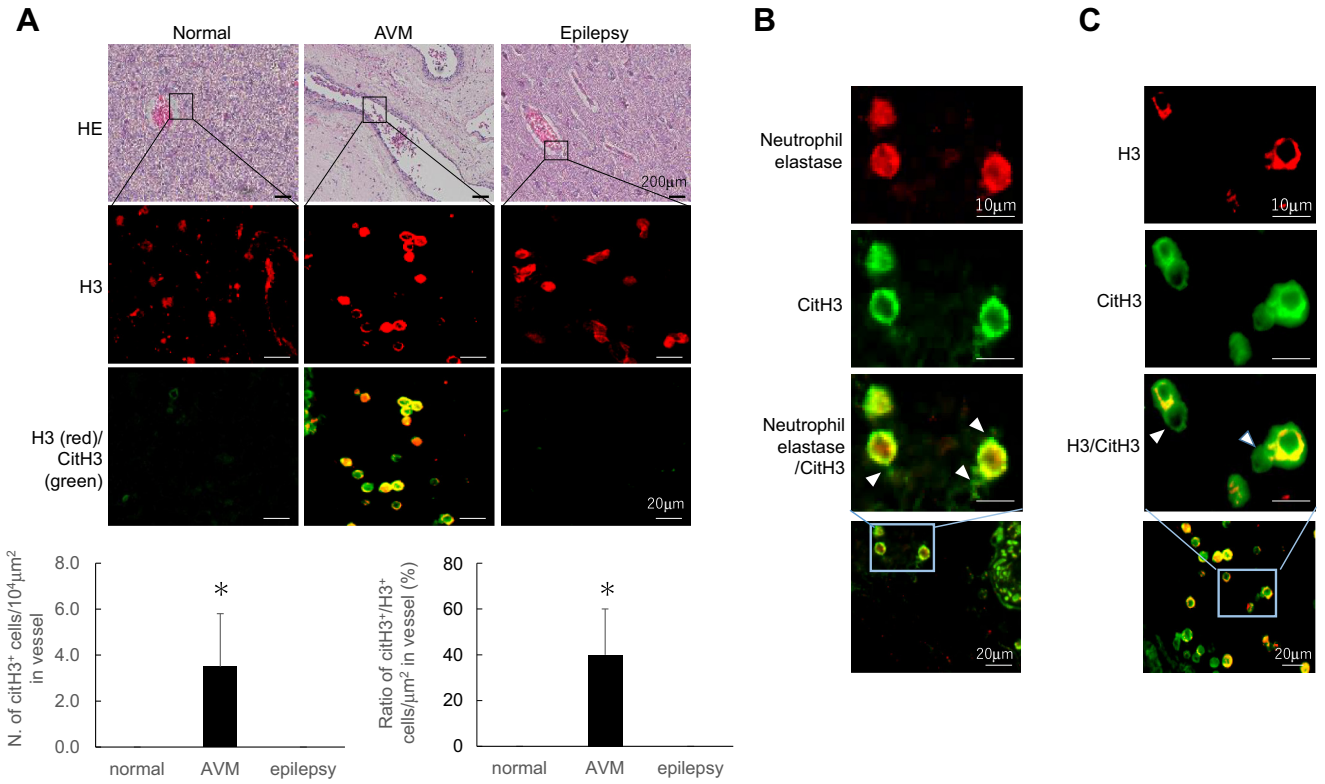

Figure 2. (**A**) H3- and CitH3-positive cells are colocalized in blood vessels of cerebral arteriovenous malformations (cAVM) samples but not in epilepsy or normal samples. Expression levels of CitH3-positive cells are positive only in blood vessels from cAVM. Of the H3- and CitH3-positive cells, 40% colocalized in blood vessels from cAVM. Data represent mean ± standard deviation. $*P < 0.05$, by Fisher exact test. (**B** and **C**) CitH3-positive cells are colocalized with neutrophil elastase-positive cells and H3-positive cells. Extracellular chromatin fibers (*arrows*) representing neutrophil extracellular traps are detected in some of both CitH3- and neutrophil elastase-positive cells and in some of both CitH3- and H3-positive cells in cAVM samples.

angiography yielded a diagnosis of cAVM. Samples were compared with 3 samples of normal human brain tissue purchased from BioChain Institute (Newark, California, USA) and samples from 3 patients with epilepsy seen at our institution.

Sample Preparation

Tissue samples were obtained in accordance with the tenets of the Declaration of Helsinki. Each sample was diagnosed by neuropathologists. All samples were fixed in 10% formalin in phosphate-buffered saline right after tissue resection. Formalin was sometimes exchanged to remove blood during tissue fixation for 1 week at room temperature and then processed on a tissue processor (Sakura, Tokyo, Japan). Following embedding in paraffin, each block was coded. Paraffin blocks were sectioned at 6 μm, and slides were dried at 60°C. Each tissue section from the paraffin-embedded block was dewaxed, rehydrated, and subjected to staining with hematoxylin-eosin.

Immunohistochemistry

Each section for immunohistochemistry was subjected to antigen retrieval by boiling at 100°C for 20 minutes in citrate buffer (pH 6.0). After a 30-minute, serum-free protein blockade (Dako, Carpinteria, California, USA), all primary antibodies (Ab) were diluted with Canget signal immunostaining (1:100 dilution; Toyobo, Osaka, Japan) and added for overnight incubation at 4°C. Goat polyclonal antihistone H3 Ab (Ab12079; Abcam, Cambridge, UK), rabbit polyclonal anti-CitH3 Ab (Ab5103; Abcam), mouse monoclonal antineutrophil elastase Ab (Ab254178; Abcam), rabbit polyclonal antiinterleukin (IL)-6 Ab (Ab6672; Abcam), IL-8 Ab (Ab106350; Abcam), rabbit polyclonal anticyclooxygenase-2 (COX2) Ab (Ab15191; Abcam), rabbit polyclonal anti-Iba-1 Ab (019–19741; Wako, Osaka, Japan) and an endothelial marker, mouse monoclonal ant-CD31 Ab (Ab24590; Abcam), rabbit polyclonal anti-CD42 Ab (12860-1-AP; Protein Tech, Rosemont, Illinois, USA), and rabbit polyclonal antifactor Xa Ab (Ab111171; Abcam) were the primary antibodies. Sections not treated with each primary antibody were used as negative controls. All sections were then incubated for 1 hour at room temperature with fluorescein-conjugated secondary antibodies (Alexa Fluor 594 or 488; Molecular Probes, Carlsbad, California, USA) in Canget signal immunostaining (1:1000 dilution; Toyobo), mounted with fluorescence mounting medium (Dako) and examined under a

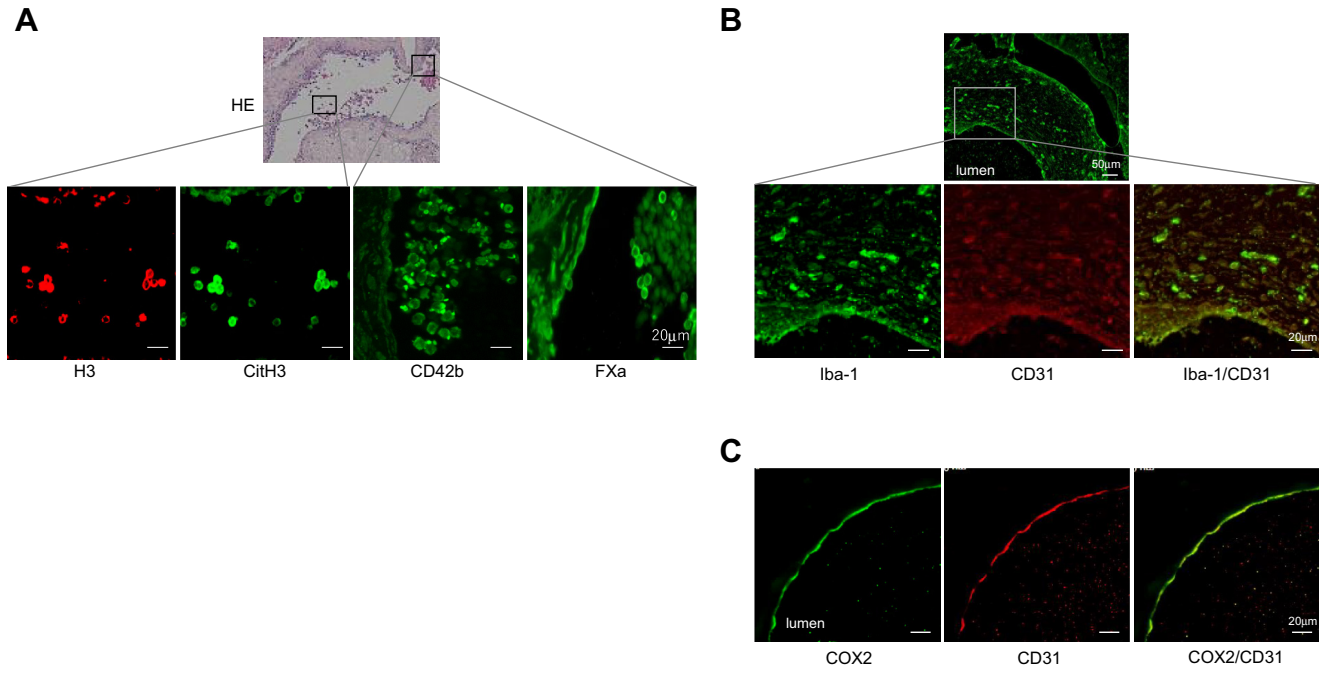

Figure 3. Representative immunohistochemistry of H3, CitH3, and CD42b^{+}-activated platelets and levels of factor Xa (fXa) in cerebral arteriovenous malformations (cAVMs). CD42b and fXa are expressed in some vascular walls and thrombi of cAVM samples. H3 and CitH3 in blood vessels of cAVMs are expressed at sites differing from expressions of CD42b and fXa (**A**). Iba-1-positive cells accumulate adjacent to the CD31-positive endothelium at the nidus of cAVM (**B**), and expression of COX2 is observed in damaged CD31-positive endothelium (**C**).

fluorescence microscope (BZ-X710; KEYENCE, Osaka, Japan) equipped with a BZ-X image analyzing system (KEYENCE). Cell count analysis was performed using the BZ-X analyzer.

Statistical Analysis

Comparisons were performed with the Fisher exact test for categorical variables. All statistical analyses used JMP 13 software (SAS Institute Inc., Cary, North Carolina, USA). Statistical significance was defined at the level of $P < 0.05$.

RESULTS

CitH3 was Expressed in Blood Vessels but not Brain Parenchyma in All cAVM Samples

Since citrullination of histones is an essential process for the formation of NETs, we immunohistochemically examined the expression of histone 3 (H3) and CitH3. The 9 cAVM nidi contained intermingled brain parenchyma and numerous blood vessels. Surgical samples from the epileptic focus of 3 epilepsy patients showed anomalous control. Normal human brain tissue samples served as normal controls ($n = 3$).

In all samples, we detected H3-positive cells in the brain parenchyma and intravascular space (Figure 1A and Supplementary Figure 1); expressions did not differ significantly among cAVMs, epilepsy samples, and normal controls (Figure 1B). However, while CitH3-positive cells were present in the intravascular space of all cAVM samples, none were observed in the intravascular space of epilepsy and normal samples (Figures 1A and 2A, Supplementary Figure 1). In cAVM samples, 40% of CitH3- and H3-positive signals were colocalized (Figure 2A). Expression of CitH3 originating from neutrophils was confirmed by double staining for neutrophil-specific protein, neutrophil elastase, and CitH3 (Figure 2B). Further, we were able to observe the extracellular chromatin fibers called NETs in some of both CitH3- and neutrophil elastase-positive cells and in some of both CitH3- and H3-positive cells in cAVM samples (Figure 2B and C).

Expressions of Intravascular CitH3 were not Necessarily Associated with Thrombotic Characteristic in cAVMs

NETs can immobilize and kill a broad range of pathogens but also elicit tissue damage and increase thrombosis.[12] We investigated expression of CD42b, an activated form of platelets, and expression of factor Xa (fXa) associated with thrombosis to examine whether NETs are associated with thrombosis in cAVMs.

CD42b and fXa were expressed in some vascular walls and thrombi of cAVMs but did not colocalize with expression of CitH3 (Figure 3A). In addition, these expressions were not detected in the vessels of normal brain tissue and in the surgical control vessels extracted from epilepsy patients (data not shown), suggesting that these expressions could not be due to handling of the pathologic tissue during resection.

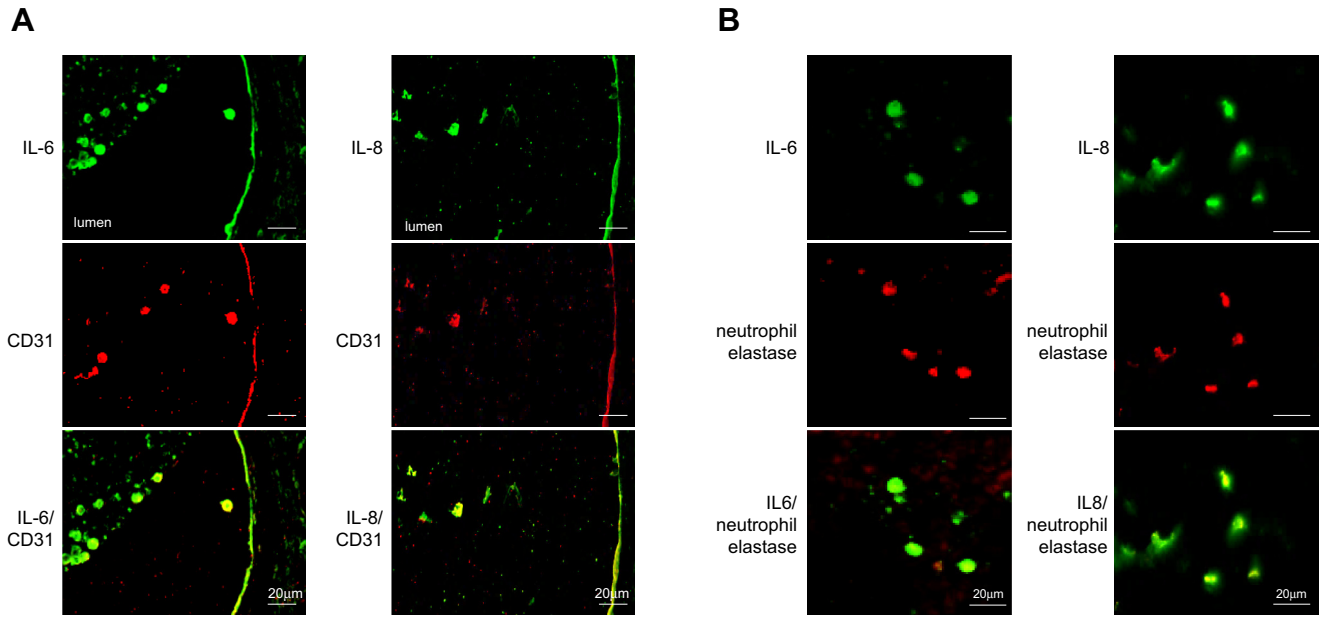

Figure 4. Inflammatory cytokines IL-6 and IL-8 are observed in CD31-positive endothelium and granule cells (**A**) and are also detected in intravascular neutrophils of cerebral arteriovenous malformations (**B**).

Formation of Neutrophil Extracellular Traps Might Be Associated with Vascular Inflammation

Finally, we examined whether NETs exert a primary effect on AVM pathobiology. Previous studies have suggested that endothelial KRAS mutation is associated with the pathobiology of cAVM patients.[7,8] In colon cancer, exosomal KRAS mutation leads to the formation of NETs via increases in IL-8 and then aggravates cancer.[15] IL-8 interacted with its receptor CXC chemokine receptor 2 on neutrophils, leading to the formation of NETs in atherosclerosis.[16] Furthermore, COX2 expressed in the cAVM is associated with vascular inflammation.[17] On the basis of these findings, we immunohistochemically assessed whether CitH3 expression was associated with inflammation. High expression of Iba-1-positive microglia adjacent to the endothelium (**Figure 3B**) and COX2 expression in damaged endothelium (**Figure 3C**) were observed in cAVMs. In addition, high expressions of IL-6 and IL-8 were also detected in the damaged endothelium (**Figure 4A**) and intravascular neutrophils (**Figure 4B**) of cAVM. Vascular inflammatory changes with expression of Iba-1-positive cells and COX2 expression in the cAVM seems to affect intravascular neutrophils, resulting in the expression of NETs.

DISCUSSION

This study demonstrated for the first time the presence of intravascular NETs by detecting expression of CitH3 in all samples from cAVMs but in no samples from normal brain tissue or in surgical control samples from foci of epilepsy. These findings indicate CitH3 expression is specific to samples from cAVMs and independent of the tissue resection procedure. Next, we revealed expression of Iba-1-positive cells adjacent to the endothelium and endothelial COX2 expression in cAVMs, reflecting vascular inflammation. In accordance with these findings, we observed expression of inflammatory cytokines IL-6 and IL-8 in not only the endothelium but also some intravascular neutrophils of cAVMs. Expression of intravascular NETs may thus reflect the effects mediated by the vascular inflammation of cAVMs. Earlier investigations of NETs focused on their presence in infectious disease like sepsis, and their critical role in noninfectious diseases such as inflammation, atherosclerosis, thrombosis, cancer, and intracranial aneurysm has also been reported.[9,10,12-14] Multifunctional NETs are therefore presumed to be related to the main pathologic conditions that lead to cAVM formation.

Vascular inflammation plays a fundamental role in the progression and rupture of cAVM.[5,17] Consistent with our previous study showing inflammatory changes in the nidi of cAVMs,[18] the present study demonstrated high expression of Iba-1 around the endothelium and endothelial COX-2, reflecting inflammatory changes in the cAVM nidus. Consistent with another study,[7] our previous study demonstrated the detection of endothelial KRAS mutations in cAVM.[8] KRAS mutations are important factors in tumorigenesis that are associated with increased gene expressions related to angiogenesis and Notch signaling that leads to the development of cAVMs.[7] In addition, another recent study reported that exosomal KRAS mutation promotes the formation of tumor-associated NETs via IL-8 upregulation and deteriorates colorectal cancer.[15] IL-8 interacted with its receptor CXC chemokine receptor 2 on neutrophils, leading to the formation of NETs in atherosclerosis.[16] In a study assessing plasma levels of CitH3 as a novel prognostic blood biomarker,

patients with advanced cancer showed high plasma levels of IL-6 and IL-8 in correlation with plasma levels of CitH3.[14] On the basis of these studies, we immunohistochemically confirmed colocalized expressions of IL-6 and IL-8 with damaged endothelium and some intravascular neutrophils of cAVM (see **Figure 4B** and **C**). Intravascular expression of NETs may thus be associated with elevated cytokine levels through endothelial KRAS mutation.

Since histones induce structural changes in fibrin, rendering it stronger and more resistant to fibrinolysis, NETs are causally implicated in the pathogenesis of arterial, venous, and microvascular thrombosis by promoting coagulation and enhancing clot stability.[12] However, CitH3 did not colocalize with thrombus expressing activated platelet $Cd42^{+}$ cells in the present study. The presence of CitH3 in blood vessels may thus be independent of thrombus formation in the vascular walls of cAVM nidi.

Our study has some limitations. First, the sample size was small and some samples were derived from ruptured AVMs, which may have reflected inflammation. However, we could not identify any difference between ruptured and unruptured samples regarding expression of CitH3. Second, as ethical antemortem acquisition of normal brain tissues and cerebral vessels is not possible, the normal brain tissue samples we purchased for use as controls were obtained from human cadavers. Therefore for comparative purposes, we included brain tissue samples containing normal cerebral vessels around the epileptic foci extracted from epilepsy patients. Additional studies are needed to clarify the significance of the link between CitH3 and the pathobiology of cAVMs.

CONCLUSIONS

Our results offer the first evidence of intravascular CitH3 expression in tissue samples from patients with cAVMs. CitH3 expression might be associated with vascular inflammation.

CRediT AUTHORSHIP CONTRIBUTION STATEMENT

Kenji Shimada: Conceptualization, Methodology, Writing – original draft. **Izumi Yamaguchi:** Data curation. **Manabu Ishihara:** Data curation. **Takeshi Miyamoto:** Data curation. **Shu Sogabe:** Data curation. **Kazuhisa Miyake:** Data curation. **Yoshiteru Tada:** Data curation. **Keiko T. Kitazato:** Conceptualization, Methodology. **Yasuhisa Kanematsu:** Data curation. **Yasushi Takagi:** Supervision.

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Conflict of interest statement: The authors declare that the article content was composed in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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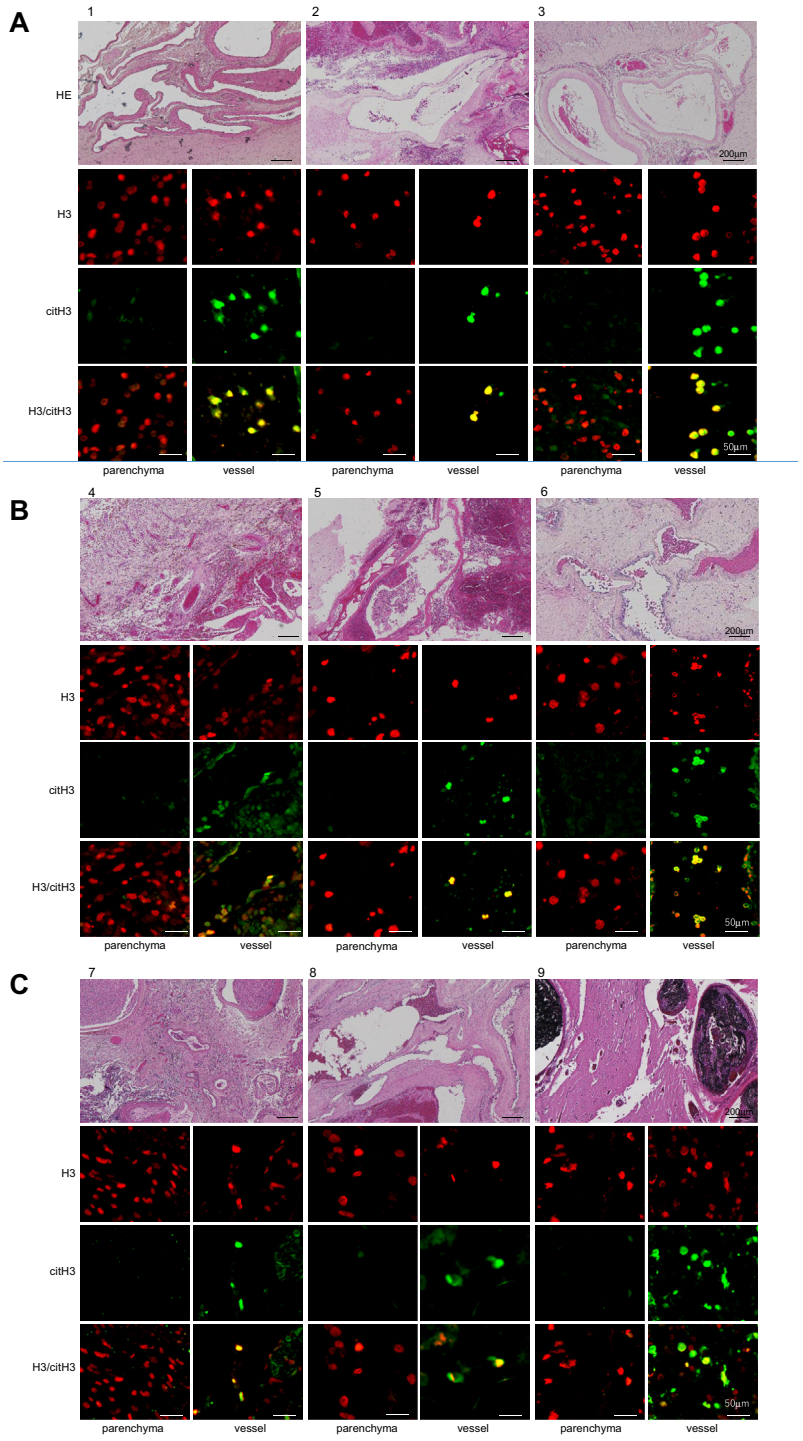
SUPPLEMENTARY DATA



Supplementary Figure 1. Histone 3–positive cells were expressed in the brain parenchyma and intravascular space in all of the samples from patients with cerebral arteriovenous malformation (AVM) (**A**–**C**). Citrullinated histone 3–positive cells were present in the intravascular space, but not in the parenchyma, in all of the samples from patients with cerebral AVM (**A**–**C**). HE, hematoxylin and eosin.