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From Cell Biology to Tissue Engineering

Abnormal elastin and collagen deposition is present in extracranial arteriovenous malformations: A comparison to intracranial disease

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Summary. Background. Vascular malformations are characterized by anomalous vascular channels with fragile walls and a propensity to bleed. Arteriovenous malformations (AVMs) in particular have disorganized vascular spaces with intervening fibrosis. Characterization of the structural abnormalities of these vessels has not been comprehensively evaluated. We hypothesize that AVMs are likely to demonstrate altered elastic and collagen fiber organization and distribution, reflecting their fragility, vascular instability, and abnormal development.

Methods. Fifteen AVMs were histologically evaluated by H&E, elastin and trichrome staining. To identify potential differences between extracranial and intracranial AVMs, 5 AVMs were harvested from the brain (n=5) and 10 from extracranial sites involving the skin and deep soft tissue (n=10).

Results. The elastin staining demonstrated reduplication, fragmentation and disruption of internal elastic lamina as well as irregular thickness, and inconsistent vascular density of all AVM specimens. Trichrome staining revealed thickening of the intimal layers of AVM arteries and demonstrated an irregular thickness of venous walls within the malformation and some areas of medial degeneration. Intracranial AVMs are characterized by more intramural inflammation with predominant neutrophil and lymphocyte infiltration. In contrast, extracranial AVMs display more extravascular inflammation with mast cell and neutrophil infiltration. Microvascular proliferations intervening between larger blood vessels were also noted in both types of AVMs, but more obvious in extracranial AVMs.

Conclusion. These observed histologic anomalies of AVMs demonstrate disorganized deposition of elastin and collagen that point to the clinically observed vascular instability and fragility of these lesions.

Key words: Arteriovenous malformation, Elastin, Collagen

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Introduction

Vascular anomalies are composed of a heterogeneous group of vascular disorders which are categorized into vascular tumors and malformations based on the updated official ISSVA (International Society for the Study of Vascular Anomalies) classification of vascular anomalies in 2014 (Wassef et al., 2015). Most vascular malformations present at birth and grow proportionally with patients, demonstrating an error of vascular morphogenesis but with a normal rate of cell turnover and mitosis, a characteristic which distinguishes them from vascular tumors (Mulliken and Glowacki, 1982). Arteriovenous malformations (AVMs) are relatively uncommon but represent one of the most complicated vascular malformations, resulting in

considerable morbidity. AVMs are characterized clinically by progressive growth, soft tissue destruction, fragility and a bleeding propensity that is difficult to control. Histologically, they demonstrate both arterial and venous architecture, variable communication between disparate vascular channels, lacking of intervening capillaries, and often extensive fibrosis. However, little is known about the components of the vascular channels and extravascular spaces in AVMs that contribute to their dysfunction.

Elastic and collagen fibers are essential supportive and stabilizing components of blood vessel walls and surrounding structures. Abnormal architecture, dysregulated production or degradation of elastin and collagen may contribute to vascular fragility, vessel rupture and hemorrhage. This is evident in conditions such as Marfan syndrome (Wanga et al., 2017), Ehlers-Danlos syndrome (Abayazeed et al., 2014), and Williams-Beuren syndrome (Heinz et al., 2016). We hypothesized that AVMs likely demonstrate disrupted elastic and collagen fiber organization and distribution, reflecting their clinically observed fragility, vascular instability, and propensity to hemorrhage. Herein, we present a pilot study on the specific examination of collagen and elastic fiber framework of AVMs in both intracranial and extracranial lesions. To our knowledge, this is the first study to investigate the dedicate structure of blood vessel walls in AVMs.

Materials and methods

This study was conducted under Institutional Review Board approval granted by the University of Arkansas for Medical Sciences (IRB 202187). Archival, formalin-fixed, paraffin embedded slides of AVMs were retrieved. Slides were stained by hematoxylin and eosin (H&E), Masson trichrome (TRI), and modified Verhoeff von Gieson (VVG) elastic tissue stain using commercially available kits from Sigma (HT-25A and HT15, respectively) and following manufacturer's instructions. With the VVG elastic stain, elastic fibers stain black, collagen fibers red and muscle fibers yellow. With the trichrome stain, collagen fibers are blue and muscle fibers are red. In all, 15 AVMs were examined, including samples from brain (n=5) and extracranial skin and soft tissue (n=10). The diagnosis of all lesions was made by clinical presentation, radiographic imaging, and intraoperative findings. Diagnosis was confirmed following resection by a pathologist experienced with vascular pathology evaluation (Dr. SS).

Results

15 AVMs were collected, including 10 extracranial and 5 intracranial, from June 2010 to Aug 2014. The diagnosis of all the cases were clinically made based on their symptoms, physical examinations and radiologic studies and confirmed by histologic exanimations. Although they were de-identified patient samples, all the patients were categorized as stage III according to Schobinger classification. Stage III implies that there is progressive disease/bleeding and growth, and surgical excision is performed. Internal or external hemorrhage was occurring in the population we studied in this report. All lesions were re-reviewed with H&E microscopy. VVG elastic tissue and Masson trichrome staining were

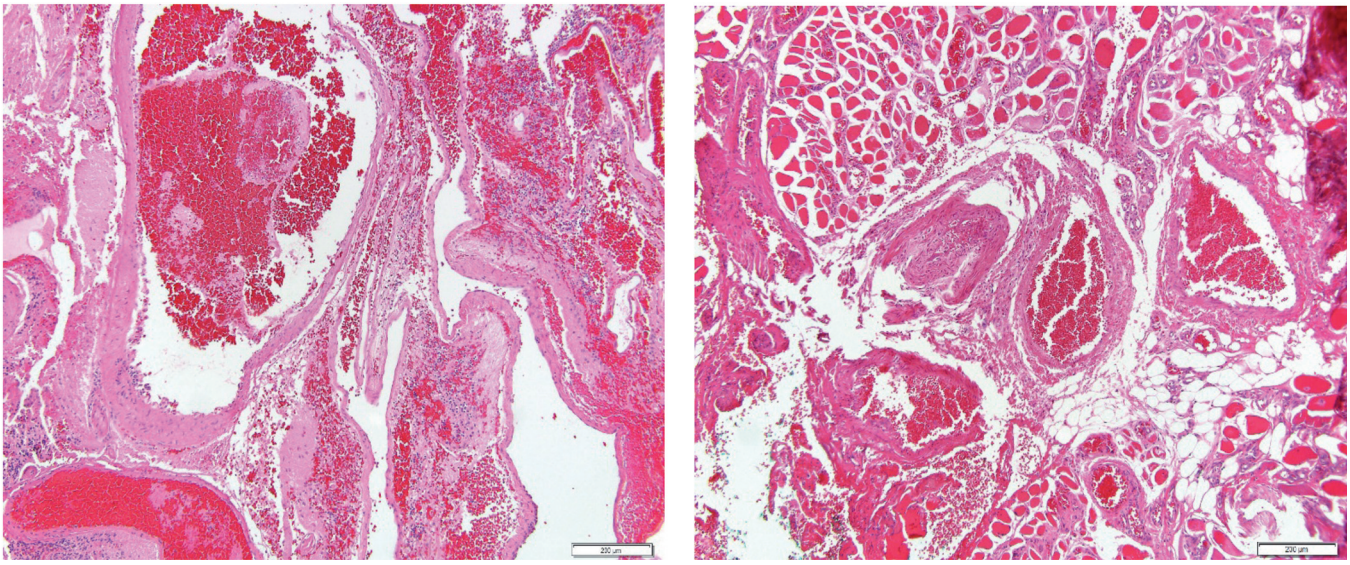

Fig. 1. Histology of brain and extracranial AVMs. Brain AVMs presented with large, dilated, thin-walled vascular structures (left). Extracranial AVMs more often showed small to medium sized, thicker-walled vascular structures and smaller lumens (right). x 100.

performed on all samples.

Histologic features best evaluated by H&E staining included vascular lumen size, wall thickness, inflammation (intraluminal, intramural, and extravascular), presence or absence of microvascular changes, and the composition of the extravascular space. Attributes of the vascular walls (such as changes to the internal elastic laminae and vascular fibrosis) were appreciated on H&E but were made more apparent with VVG elastic and trichrome staining. VVG and trichrome stains highlighted subtle abnormalities not appreciated on routine histopathologic evaluation and added support to the concept of altered collagen and elastic tissue frameworks within these vascular malformations.

By light microscopy, intracranial AVMs were characterized more often by large, dilated, thin-walled vascular structures (Fig. 1, left), possibly due to looser and softer cerebral glial tissue with lower restrictive strength on the expanding malformed blood vessels compared to the denser connective tissues in extracranial area. Consequently, extracranial AVMs more often showed small to medium sized, thicker-walled vascular structures and smaller lumens (Fig. 1, right).

Intracranial and extracranial AVMs were marked by inflammatory mediators

Inflammation was commonly observed in all AVMs, whether within the lumens of the abnormal vessels, within the walls, or within the extravascular space. Intramural inflammation was seen in 4 out of 5 brain AVMs (80%), and consisted of vessel wall infiltration by neutrophils, lymphocytes or eosinophils (Fig. 2, left). In contrast, only 3 out of 10 (30%) extracranial AVM

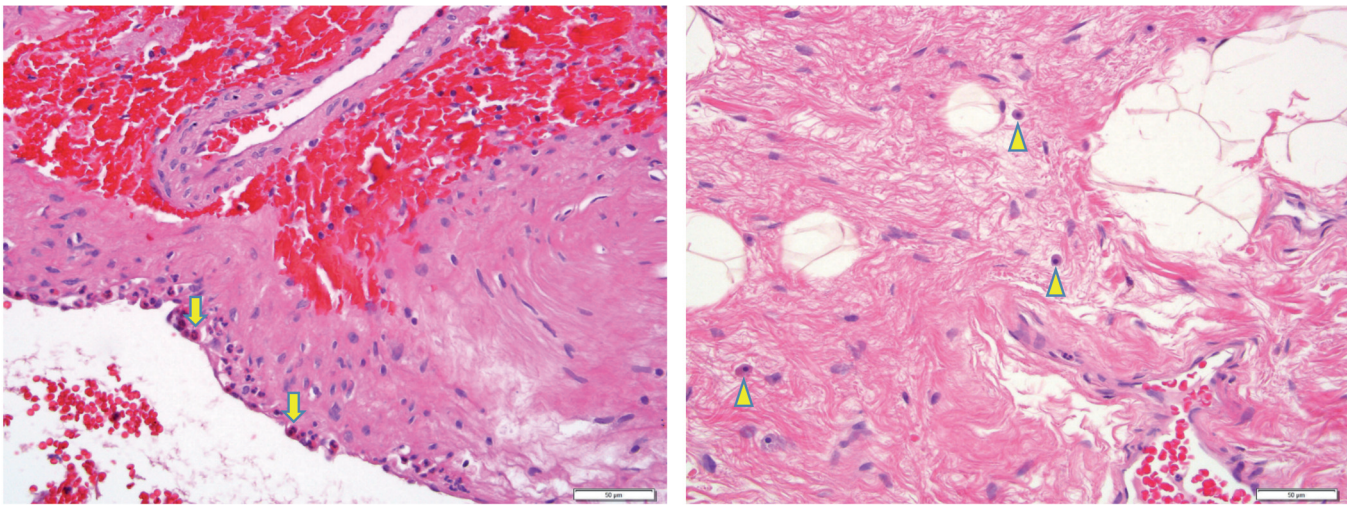

Fig. 2. Inflammation of AVMs. Intramural neutrophil infiltration was more often seen in brain AVMs (left, arrows). Extravascular inflammation was more commonly seen in extracranial AVMs, consisting mainly of mast cells (right, arrowheads). x 400.

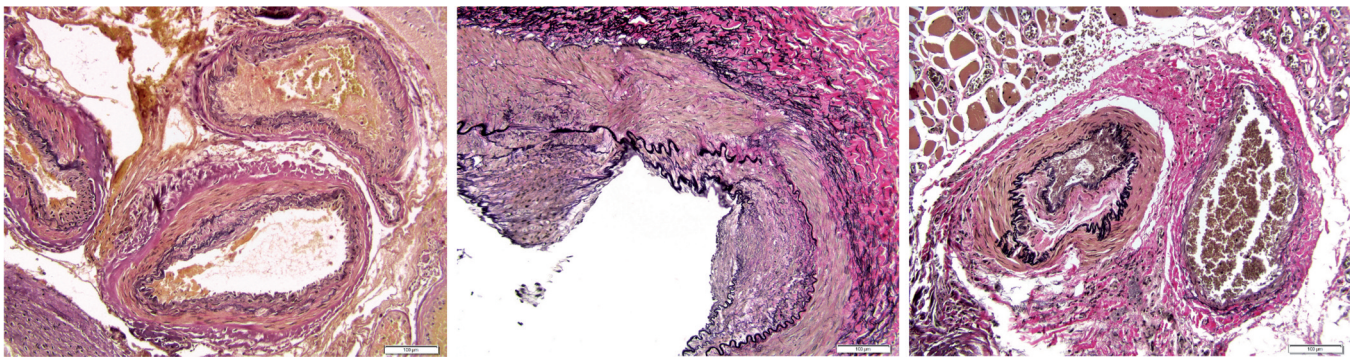

Fig. 3. VVG staining of AVMs. Disruption, fragmentation and reduplication of internal elastic laminae were found in brain (left) and extracranial (middle and right) AVMs. The irregular thickness of elastic laminae was also a common finding in both types. x 200.

lesions showed intramural inflammation (neutrophil and eosinophil infiltration). Extravascular inflammation, on the other hand, was more commonly seen in extracranial AVMs, and was present in 80% (8 of 10) of cases. Extravascular inflammation consisted mainly of mast cells, neutrophils, eosinophils, and lymphocytes (Fig. 2, right). Extravascular inflammation was seen in 2 of 5 (40%) of brain AVMs and consisted of macrophages in 2 cases, with additional neutrophils and lymphocytes in one case.

Elastin deposition in AVMs was disorganized

In the AVMs we examined, both intracranial and extracranial types, distortions of normal blood vessel structures were evident, particularly of the internal elastic laminae (IEL) of arterial components. An arterial elastic component was identified by VVG staining in all but one of the examined AVMs (an extracranial AVM). Evidence of abnormal elastic fiber organization included disruption, fragmentation, and re-duplication of the IEL. IEL were re-duplicated within the abnormal vessels in all brain AVMs and 7 extracranial AVMs; and they were also noted to be fragmented and disrupted in all brain AVMs and 9 extracranial AVMs (Fig. 3, left and middle). Another common finding was an irregular thickness of elastic laminae (Fig. 3, right), which could support the idea of abnormal vascular morphogenesis.

AVM fibrocollagen deposition was abnormal

Eccentrically oriented intimal fibrosis was noticed in 4 out 5 brain AVMs and 7 out 10 extracranial AVMs (Fig. 4, upper left and upper right). Medial degeneration, characterized by disorganization and expansion of the normal media by loose fibrosis, was present in all of brain AVMs and 5 out of 10 extracranial AVMs (Fig. 4, lower left and lower right). The findings were summarized in Table 1 and Table 2.

The extravascular space of the AVMs was notable for the presence of gliosis (in brain AVMs) and fibrosis (in extracranial AVMs). All brain AVMs showed gliosis in the extravascular space in association with hemorrhage and hemosiderin deposition free or within macrophages. The trichrome stain identified wispy collagen deposition in 3 of 5 cases and zonal fibrosis in one case (Fig. 5, left and middle). In extracranial AVMs, fibrosis was a near constant feature, present in 90% (9 of 10) cases, but ranging from slight to prominent in degree (Fig. 5, right).

Table 1. Brain arteriovenous malformations: histologic features.

Case number	Vascular wall thickness (range)	Lumen size	Disrupted IEL (VVG)	Reduplication of IEL (VVG)	Eccentric Intimal fibrosis (TRI)	Medial degeneration (TRI)	Inflammation (location, type)	Extravascular changes	Microvascular proliferation
1	0.01-0.2 mm	Large, dilated	Present (smaller vessels)	Present (smaller vessels)	Present (>larger vessels)	Present, focal	Intraluminal: neutrophils (rare) Intramural: neutrophils and lymphocytes (subendothelial) Extravascular: absent	Pigmented macrophages, hemorrhage, Hemosiderin, gliosis with wispy collagen	Absent
2	<0.01-0.2 mm	Large, dilated	Present	present	Present (>larger vessels)	present	Intraluminal: neutrophils Intramural: neutrophils and eosinophils Extravascular: absent	Pigmented macrophages, hemorrhage, hemosiderin, gliosis with wispy collagen	Very focal (may be normal vasculature or part of AVM)
3	<0.01-0.14 mm	Large, collapsed	Present	present	Present, focal	Present, focal	Intraluminal: neutrophils Intramural: neutrophils and eosinophils (subendothelial) Extravascular: absent	Hemorrhage, hemosiderin, gliosis	Absent
4	0.01-0.18 mm	Large, dilated	present	present	Present, focal and minimal	present	Intraluminal: neutrophils (rare) Intramural: neutrophils, lymphocytes, and eosinophils Extravascular: macrophages, neutrophils, and rare lymphocytes	Pigmented macrophages, hemosiderin, hemorrhage, gliosis with focal zone of fibrosis	Very focal (may be normal vasculature or part of AVM)
5	0.01-0.15 mm	Medium to large, dilated	Present, focal	Present	Not identified	Present, focal	Intraluminal: neutrophils Intramural: absent Extravascular: macrophages	Pigmented macrophages, hemorrhage, hemosiderin, gliosis with wispy collagen	Present, focal

Extravascular hemorrhage and hemosiderin was less frequently observed in extracranial AVMs than brain AVMs, being present in only half of cases.

Microvascular proliferation was noted in AVMs

Small, well-formed, and tightly clustered capillary-

Table 2. Extracranial arteriovenous malformations: histologic features.

Case number	Vascular wall thickness (range)	Lumen size	Disrupted IEL (VVG)	Reduplication of IEL (VVG)	Eccentric Intimal fibrosis (TRI)	Medial degeneration (TRI)	Inflammation (location, type)	Extravascular changes	Microvascular proliferation
1	0.02-0.21 mm	Medium, dilated	Present	present	present	not identified	Intraluminal: absent Intramural: absent Extravascular: eosinophils (rare)	Slight fibrosis	Present
2	0.03-0.4 mm	Small to medium, collapsed	No identifiable elastic laminae	No identifiable elastic laminae	present	present	Intraluminal: neutrophils (rare) Intramural: absent Extravascular: lymphocytes and mast cells (rare)	Fibrosis, pigmented macrophages (few), hemorrhage	Present
3	<0.01-0.13 mm	Medium, dilated	Present	Present	Present	present	Intraluminal: neutrophils Intramural: neutrophils (rare, subendothelial) Extravascular: neutrophils, lymphocytes, mast cells, and macrophages	Fibrosis (prominent), hemorrhage	Focal
4	0.01-0.10 mm	Small to medium, collapsed	Present	Present	Not identified	not identified	Intraluminal: neutrophils (rare) Intramural: absent Extravascular: absent	Fibrosis	Present (prominent)
5	0.02-0.5 mm	Medium, collapsed	Present (on H&E, too)	Present (on H&E, too)	Present	Present, focal	Intraluminal: neutrophils, eosinophils Intramural: absent Extravascular: mast cells, rare neutrophils	Fibrosis, hemorrhage, hemosiderin	Present
6	0.01-0.15 mm	Small to medium, collapsed	Present	Present	Present, focal	Present	Intraluminal: neutrophils Intramural: eosinophils, neutrophils Extravascular: eosinophils, mast cells	Fibrosis	Present
7	<0.01-0.13 mm	Small to medium, collapsed	Present	Present	Present, focal	Not identified (no trichrome)	Intraluminal: neutrophils Intramural: absent Extravascular: mast cells	Fibrosis (wispy)	Present
8	<0.01-0.06 mm	Medium, dilated	Present	Present	Present, focal	Not identified (no trichrome)	Intraluminal: neutrophils Intramural: neutrophils and eosinophils Extravascular: mast cells	Fibrosis	Focal
9	0.01-0.12 mm	Medium, collapsed	Present	Not identified	Not identified	Present, focal	Intraluminal: neutrophils (rare) Intramural: absent Extravascular: neutrophils (rare)	Fibrosis, hemorrhage	Present
10	<0.01-0.06 mm	Small to medium, collapsed	Present, focal (minimal identifiable arterial component)	Not identified; (minimal identifiable arterial component)	Present	Not identified	Intraluminal: absent Intramural: absent Extravascular: absent	Hemosiderin, pigmented macrophages	Present (prominent)

Table 3. Comparison of extracranial and intracranial AVMs.

	Vascular wall thickness	Lumen size	Disrupted IEL	Reduplicated IEL	Eccentric Intimal fibrosis	Medial degeneration	Inflammation	Extravascular changes	Microvascular proliferation
Extracranial AVMs	0.01-0.5 mm	small to medium, dilated	9/10	7/10	7/10	5/10	Extravascular, mast cells	fibrosis	10/10
Intracranial AVMs	0.01-0.2 mm	medium to large, dilated	5/5	5/5	4/5	5/5	intramural, neutrophils	hemorrhage, hemosiderin deposition	2/5

like proliferations appeared organized and were seen in all extracranial AVMs (Fig. 6, left and middle); in two cases the microvascular proliferations were focal and in two cases they were prominent. 2 of 5 brain cases showed focal microvascular-like changes (Fig. 6, right).

Discussion

AVMs are a pathologic condition thought to be somewhat governed by inflammation and its mediators (cytokines, neutrophils, macrophages, etc.), triggered by genetic/hemodynamic factors and local hypoxia (Kim et al., 2008; Mouchtouris et al., 2015). Our previous study shows abundant inflammatory cells were found to infiltrate extracranial AVM tissues (Wei et al., 2016). These inflammatory cells can secrete large amount of matrix metalloproteinase-9 (MMP-9), including neutrophil gelatinase-associated lipocalin (NGAL) stabilized MMP-9 and tissue inhibitor of metalloproteases-1 (TIMP-1) deficient MMP-9 (Opdenakker et al., 2001; Ardi et al., 2007; Zajac et al., 2013). MMP-9 is heavily involved in tissue remodeling through the degradation of extracellular matrix substrates, such as collagen type IV and elastin (Collier et al., 1998). The observed disruption of elastin and collagen in this study could be the direct sequela of overactive enzymatic digestion of MMP-9 which is highly expressed in AVM lesions demonstrated at gene transcription and protein expression levels in our previous study (Wei et al.,

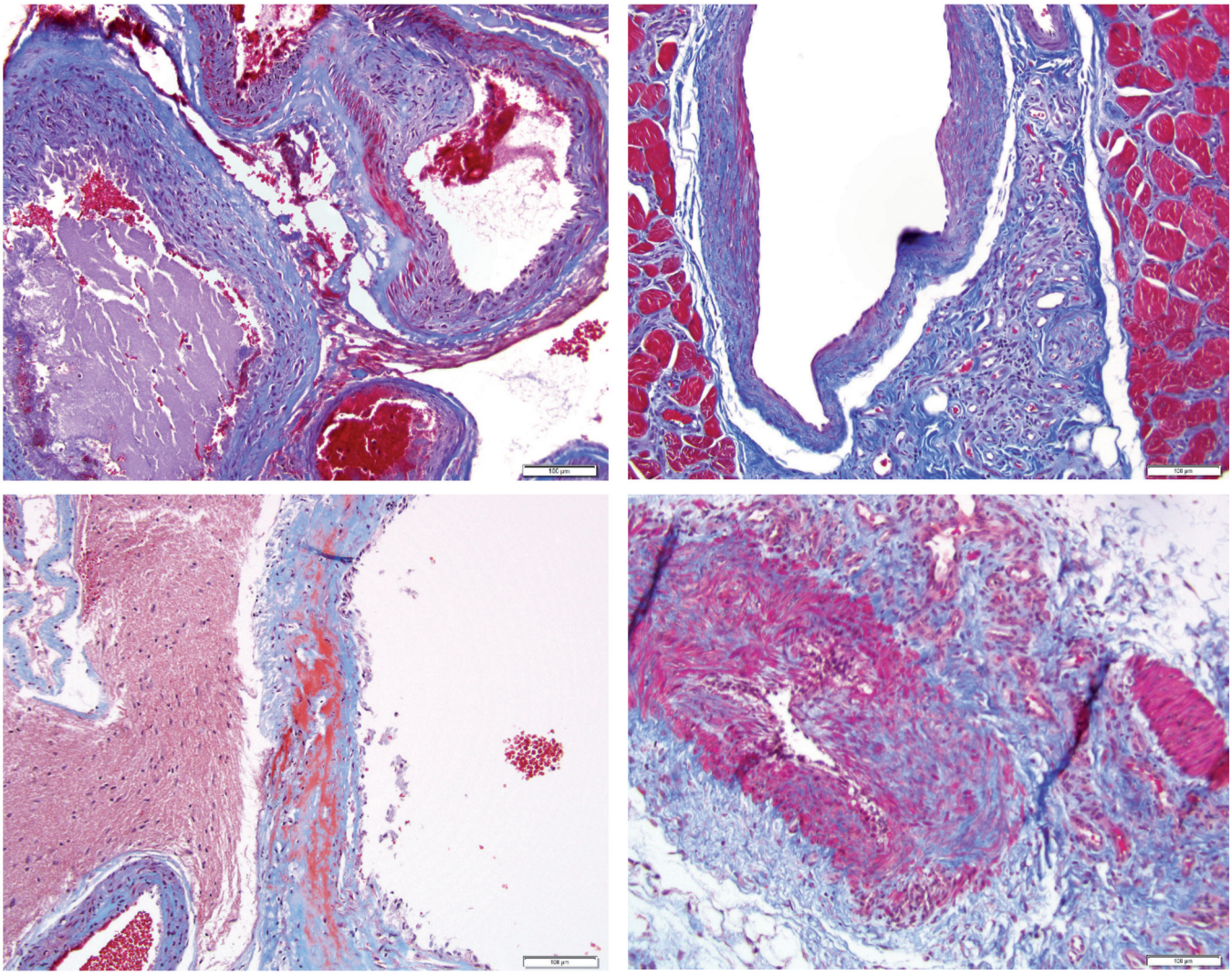

Fig. 4. Trichrome staining of AVMs. Eccentric intimal fibrosis was noticed in brain and extracranial AVMs (upper left and right). Medial degeneration, characterized by disorganization and expansion of the normal media by loose fibrosis, was present in brain and extracranial AVMs (lower left and right). x 200.

2016). Active vascular remodeling mediated by MMP-9 secreted from inflammatory cells contributes to the relentless progression of AVMs. Increased expression of VEGF-A in AVM lesions also detected in our previous study can recruit a subgroup of neutrophils and macrophages from the circulation, the former contains more MMP-9 than neutrophils recruited by regular inflammatory stimulators (Christoffersson et al., 2012), the latter can produce TIMP-1 deficient MMP-9 (Zajac et al., 2013). It has been reported that substance P (SP) and the neurokinin-1 (NK-1) receptor are expressed in vascular anomalies (Ortiz-Prieto et al., 2017). SP/NK-1 receptor system is involved in neurogenic inflammation, endothelial cell proliferation and angiogenesis and that VEGF is regulated by substance P. Other underlying angiogenic mechanisms by which inflammatory cells contribute to the expansion of AVMs may also exist. We noticed the different infiltration locations and types of inflammatory cells between intra and extra-cranial AVMs (Table 3). However, the mechanism underlying the discrepancy is not clearly understood and needs further investigation.

VVG staining was utilized to define the elastic fiber deposition within the AVMs. Elastic tissue staining is useful in confirming an arterial component of the lesions by highlighting the IEL and external elastic lamina (EEL), which sometimes are not well-visualized by light microscopy alone. Normal arteries are lined by flattened endothelial cells with a thin underlying fibrocollagenous tissue (the intima). The media of normal muscular arteries is composed of smooth muscle cells and bounded on either side by a well-defined IEL (near the lumen) or a less distinct, often incompletely formed EEL (near the extravascular space). The elastic laminae of veins, in contrast, are normally less well developed and often discontinuous. We noticed disruption, fragmentation and re-duplication of elastic fibers in our AVM specimens, especially in the IEL. Similar abnormalities in the elastic framework of arteries may be seen in disorders such as vasculitis and cardio- or peripheral vascular diseases (Subhashree et al., 2006; Ting et al., 2016). Whether the identifiable elastic tissue changes in our series of AVMs were due to innately

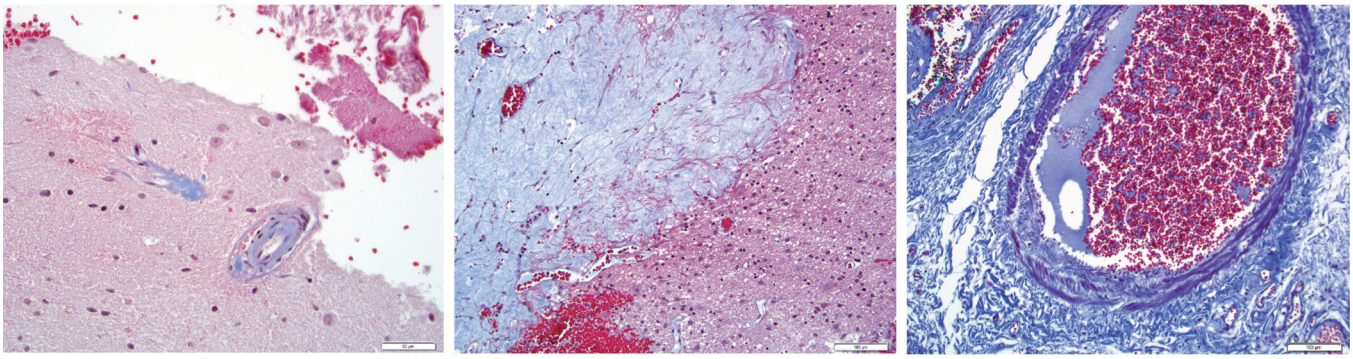

Fig. 5. Extravascular fibrosis of AVMs by trichrome staining. The trichrome stain identified wispy collagen deposition or zonal fibrosis in brain AVMs (left and middle). In extracranial AVMs, fibrosis was a near constant feature, present in most of cases, ranging from slight to prominent in degree (right). Left, x 400; middle, right, x 200.

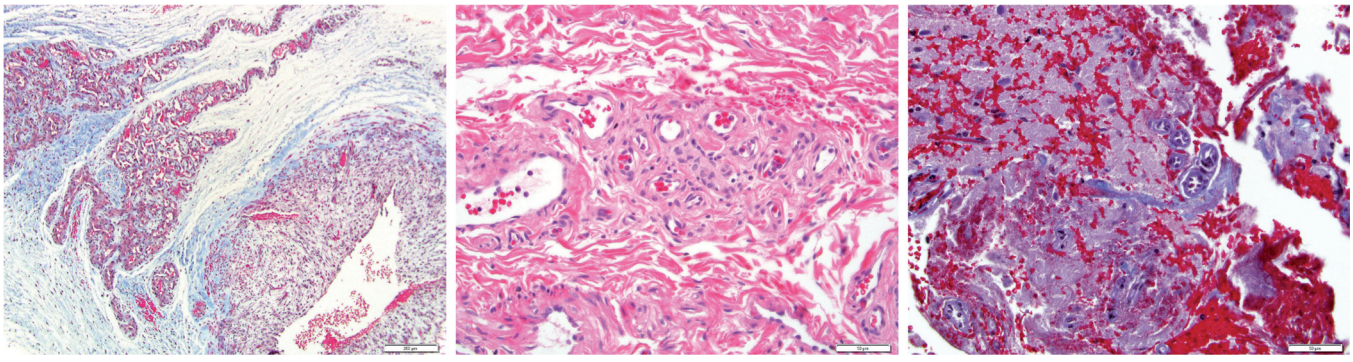

Fig. 6. Microvascular proliferation in AVMs. Presence of small, well-formed, and tightly clustered capillary-like microvascular proliferations was seen more commonly in extracranial AVMs (left, trichrome) and (middle, H&E) compared to brain AVMs (right, trichrome). Left, x 100; middle, right, x 400.

abnormal vessel development or as a secondary change due to the abnormal blood flow through these vascular channels cannot be ascertained with certainty. Wong and his coworkers showed arteriovenous fistula (AVF) establishment resulted in the fragmentation of the venous IEL in elastin haplodeficient mice (eln(+/-)) as well as WT mice, which indicated the increased shear stress on the venous wall induced by the AVF construction played an important role in the disruption of elastin distribution in vessel walls and was evidence to support the hypothesis that disorganized elastin was due to a secondary hemodynamic insult (Wong et al., 2015). In their study, the venous outflow tract was 21% larger in the eln (+/-) group than the WT group, which implied that elastin played a pivotal role in the outward remodeling of blood vessels after AVF surgery. A somatic mutation in the *MAP2K1* pathway was reported in sporadic extracranial AVMs (Couto el al., 2017). More recently, multiple mosaic-activating variants in RAS/MAPK pathways (KRAS, BRAF and MAP2K1) were detected in AVMs as a major etiology (Al-Olabi et al., 2018). The production of collagen and elastin is probably regulated via activating MAPK signaling pathway (Shen et al., 2018). Congenital AVMs are composed of numerous aberrant and direct arteriovenous shunts bypassing the capillary system. Theoretically, venous components are more susceptible to sheer stress damage due to increased intravascular pressure produce by the in-bursting blood flow directly from the arterial system. But intriguingly, we noticed the more prominent elastin damage in the arterial vessels instead of venous counterparts. The innately abnormal vessel development could be the explanation to what we discovered. Another explanation is the less-well developed elastin fibers in venous blood vessels were not detected as easily as in arteries even if damages existed.

Trichrome staining was useful in identifying fibrocollagenous thickening of the intimal layer of the AVMs, which generally occurs as a response to repeated vessel wall injury (Nili et al., 2012). Trichrome also highlighted irregular thickness of venous walls and areas of degeneration of the medial layer of vessels. The medial degeneration seen in these AVMs is reminiscent of the cystic medial degeneration (or cystic medial necrosis) seen in vessels from patients with genetic abnormalities of connective tissue such as Marfan syndrome (Romaniello et al., 2014). These changes are thought to result in the vascular fragility these patients have. Similar to the noted changes in elastic fiber distribution, the fibrocollagenous degeneration that can be seen histologically in AVMs may not be an intrinsic histologic feature of AVMs but more likely is a secondary response reflecting the repeated vascular injury by blood flow to innately malformed blood vessels.

In our study, one notable feature, more common in extracranial (10/10) than in brain AVMs (2/5), was the presence of microvascular proliferations intervening between the larger vascular spaces. Microvascular proliferations have been previously described in extracranial AVMs, with their development hypothesized to depend on flow rate, inflammation, and prior tissue embolization (Meijer-Jorna et al., 2013). Three mechanisms were involved in embolization induced angiogenesis, including hypoxia, inflammation and hemodynamic changes (Buell et al., 2014). Although we noticed microvascular proliferations in all of the extracranial AVMs, only some of them received the endovascular embolization prior to surgery. This may further support the note that AVMs are intrinsically angiogenic lesions. AVMs are composed of direct communicating channels between arteries and veins bypassing the capillaries which are the site of oxygen and nutrients exchange. Hypoxia is thought to play an important role in the pathogenesis of AVM progression. Microvascular changes can be regarded as the subsequence of multiple proangiogenic cytokines released under the hypoxia stress, such as VEGF-A and MMPs, which were investigated and confirmed in our previous study (Wei et al., 2016). Inflammation, as prevalent in extracranial and brain AVMs discussed as above, can also contribute to the microvascular proliferations possibly through the pro-angiogenic cascade via interleukin-6 mediated signaling pathway (Chen et al., 2006).

Conclusion

The present study suggests that abnormal patterns of elastic fiber and collagenous fiber exist in both brain and extracranial AVMs. These abnormalities in elastic and fibroconnective tissue likely contribute to the fragility of these abnormally formed blood vessels and their propensity for hemorrhage. Whether these attributes are innate characteristics of AVMs or secondary effects of high pressure blood flow, inflammation or previous treatment remains unclear. We also noticed some difference between intracranial and extracranial AVMs, as summarized in Table 3 especially as regard to the vessel wall thickness, lumen size, inflammation site, infiltrating inflammatory cell types as well as the occurrence of medial degeneration and microvascular changes. Whether they are one lesion just occurring at different locations or they are different in essence are still not clear.

Conflict of Interest. None

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