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Progression of Arteriovenous Malformation: Possible Role of Vasculogenesis

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Abstract

Introduction—Arteriovenous malformations (AVMs) cause significant morbidity, primarily because they expand over time and recur after treatment. We hypothesized that neovascularization might contribute to AVM progression.

Methods—AVM tissue was prospectively collected from 12 patients after resection. Schobinger stage was determined by clinical history. Neovascularization in Stage II lesions ($n=7$) was compared to Stage III AVMs ($n=5$) that had progressed. Specimens were analyzed using immunohistochemistry for CD31, Ki67, and CD34/CD133. Quantitative real-time RT-PCR (qRT-PCR) was used to determine mRNA expression of factors that recruit endothelial progenitor cells (EPCs): vascular endothelial growth factor (VEGF), stromal derived factor-1 α (SDF-1 α), and hypoxia-inducible factor-1 α (HIF-1 α). VEGF receptors (VEGFR1, VEGFR2, neuropilin 1, 2) also were quantified using qRT-PCR.

Results—Stage III AVMs showed greater microvessel density (5.8%) than Stage II lesions (1.3%) ($p=0.004$); no difference in proliferating endothelial cells was noted ($p=0.67$). CD133⁺CD34⁺ EPCs were elevated in Stage III (0.53%) compared to Stage II AVMs (0.25%) ($p=0.03$). HIF-1 α and SDF-1 α were increased 7.6 and 7.9 fold in Stage III, compared to Stage II lesions (1.7 fold and 3.3 fold), respectively ($p=0.02$). Neuropilin 1 and neuropilin 2 expression was greater in Stage III (5.8 fold and 4.6 fold) than Stage II AVMs (3.0 fold and 2.4 fold) ($p=0.03$).

Conclusion—Higher-staged AVMs exhibit increased expression of EPCs and factors that stimulate their recruitment. Neovascularization by vasculogenesis may be involved in progression of AVM.

Keywords

angiogenesis; arteriovenous malformation; endothelial; endothelial progenitor cell; vasculogenesis

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INTRODUCTION

Arteriovenous malformation (AVM) is believed to result from an error in vascular development between the fourth and sixth weeks of gestation.¹ Arteriovenous channels in the primitive retiform plexus may fail to regress; AVMs are 20 times more common in the central nervous system where apoptosis is rare.^{2,3} Genetic abnormalities cause certain types of familial AVM. *Hereditary hemorrhagic telangiectasia* (HHT) results from mutations in endoglin and activin receptor-like kinase 1, which affect transforming growth factor-beta signaling.^{4,5} *Capillary malformation-arteriovenous malformation* (CM-AVM) is caused by mutations in *RASA1*.⁶

AVM has an absent capillary bed that shunts blood directly from the arterial-to-venous circulation, through a fistula (direct connection of an artery to a vein) or nidus (abnormal channels bridging the feeding artery to the draining veins). Although the presence of AVM may be problematic, it is the *expansion* of the lesion which is the primary cause of morbidity. Eighty-two percent of children will have enlargement of their malformation prior to adulthood, and most AVM will recur following treatment.⁷ AVM expansion can cause disfigurement, bleeding, infection, pain, ulceration, and congestive heart failure.⁷

The mechanism for AVM enlargement is unknown. Although AVM has been considered a quiescent lesion, as evidenced by endothelial turnover,⁸ the endothelium of different staged lesions has not been studied. Because increasing tissue mass requires neovascularization,^{9–14} we hypothesized that *expansion* of AVM may be dependent on angiogenesis and/or vasculogenesis. The purpose of this study was to determine if more severe AVMs exhibit increased angiogenic/vasculogenic factors compared to lower-staged lesions. If expanding AVMs are associated with elevated angiogenic/vasculogenic factors, then targeted pharmacotherapy against these factors might prevent AVM enlargement and reduce recurrence following embolization or resection.

METHODS

Tissue Specimens

After approval by the Committee on Clinical Investigation at Children's Hospital Boston, 12 human AVM specimens were prospectively collected following the resection. All patients had embolization 24–72 hours prior to excision to reduce intraoperative bleeding; mean patient age was 22.4 years (range 10 to 48 years). Each AVM was assigned to a Schobinger Stage based on its clinical features (Table 1).¹⁵ Neovascularization of Stage II ($n=7$) specimens was compared to Stage III ($n=5$) AVMs that had progressed to a higher stage. Control tissue consisted of normal skin/subcutis.

Immunohistochemistry

Specimens were fixed in 10% formalin, embedded in paraffin or OCT (Sakura Finetek, Torrance, CA), and 5 μ m sections were cut. Paraffin slides were de-paraffinized with xylene and rehydrated with ethanol followed by antigen retrieval in Target Retrieval Solution (DAKO, Carpinteria, CA) using a pressure cooker (Biocare Medical, Concord, CA). Sections were incubated with 3% hydrogen peroxide for 10 min to block endogenous

peroxidase activity followed by 5% normal goat serum (Vector Labs, Burlingame, CA) for 1 h at room temperature. Primary antibodies were applied at 4°C overnight and then incubated with biotinylated secondary antibodies (Vector Labs, Burlingame, CA) for 1 h at room temperature. Avidin/streptavidin-based detection system (Vectastain elite ABC kit, Vector Labs, Burlingame, CA) was used. The slides were developed using 3,3'-diaminobenzidine (DAB) (Vector Labs, Burlingame, CA) as substrate and counterstained with Mayer's Hematoxylin. For double stained frozen sections, Alexa 488 or Alexa 594 labeled secondary antibodies (Invitrogen, Carlsbad, CA) were incubated for 45 min at room temperature.

To define angiogenic/vasculogenic factors in the AVM specimens, the following primary antibodies were used: CD31, an endothelial marker (DAKO, Carpinteria, CA); Ki67, a proliferation antigen (DAKO, Carpinteria, CA); CD34, located on immature endothelial cells (DAKO, Carpinteria, CA); CD133, a hematopoietic progenitor cell marker (Cell Signaling, Danvers, MA); hypoxia inducible factor-1 α (HIF-1 α) and stromal derived factor-1 α (SDF-1 α) that recruit endothelial progenitor cells (EPCs) (R & D Systems, Minneapolis, MN); vascular endothelial growth factor (VEGF), an endothelial mitogen (DAKO, Carpinteria, CA); VEGF receptor 1 (VEGFR1, Epitomics, Burlingame, CA); VEGF receptor 2 (VEGFR2, Cell Signaling, Danvers, MA); as well as the VEGF receptors neuropilin 1 (NRP1) ¹⁶ and neuropilin 2 (NRP2, Santa Cruz, Biotechnology, Santa Cruz, CA).

Quantification of immunohistochemistry was determined using image analysis. Images were obtained with a Nikon Eclipse E800 microscope (Nikon, Melville, NY) connected to a Spot 3.2 camera (Diagnostic Instruments, Sterling Heights, MI). Analysis was performed using Image-pro Analyzer 6.3 (Media Cybernetics, Silver Spring, MD) and staining was measured as percent of total area. The average of 10 random high-powered fields was used for each specimen. Proliferation was measured by counting positively-stained cells per 10 randomly selected high-power fields by two blinded investigators.

Quantitative Real-time Reverse Transcription-PCR (qRT-PCR)

Total RNA was extracted from each specimen using TRIzol (Invitrogen, Carlsbad, CA). RNA (0.5–1 μ g), underwent reverse-transcription using an iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). The resulting cDNA samples were diluted and analyzed in real-time PCR reactions using SYBR PCR reagents (Bio-Rad, Hercules, CA) and iQ5 real-time PCR system (Bio-Rad, Hercules, CA). Sequences of primers were: (1) VEGF, 5'-TACCTCCACCATGCCAAG-3'(sense), 5'-GGTACTCCTGGAAGATGTC-3'(antisense); (2) SDF-1 α , 5'-ACTGGGTTTGTGATTGCCTCTGAAG-3'(sense), 5'-GGAACCTGAACCCCTGCTGTG-3'(antisense); (3) Hypoxia-inducible factor 1 α (HIF-1 α), 5'-CGTTCCTTCGATCAGTTGTC-3'(sense), 5'-TCAGTGGTGGCAGTGGTAGT-3' (antisense); (4) CD133, 5'-CCTCATGTTTGGAGGATCTTG-3'(sense), 5'-TCCAAGCCTTAGGAGCATCT-3' (antisense); (5) VEGFR1, 5'-GACCTGGAGTTACCCTGATGAAA-3'(sense), 5'-GGCATGGGAATTGCTTTGG-3'(antisense); (6) VEGFR2, 5'-CACCCTCAAACGCTGACATGTA-3'(sense), 5'-GCTCGTTGGCGCACTCTT-3' (antisense); (7) NRP1, 5'-ACACCTGAGCTGCGGACTTT-3'(sense), 5'-

GGCCTGGTCGTCATCACA-3'(antisense); (8) NRP2, 5'-CCCCTGACATCCGAAGGTTTG-3'(sense), 5'-AGCGTTTTTACCGTGGGCTT-3'(antisense); (9) GAPDH, 5'-GGTGAAGGTCGGAGTCAAC-3'(sense), 5'-CCATGGGTGGAATCATATTG-3'(antisense).

Statistical Analysis

Unpaired *t* test or one way ANOVA was used to compare values between Stage II and Stage III AVMs with Prism 5 software (GraphPad Software, La Jolla, CA). *p* values <0.05 were considered significant.

RESULTS

Neovascularization

CD31 immunohistochemistry showed increased vasculature in Stage III AVMs (5.8%) compared to Stage II (1.3%) lesions ($p=0.004$); microvessel density was not different between Stage II AVM and control tissue (0.6%, $p=0.3$) (Fig. 1). Although AVMs exhibited a greater number of proliferating endothelial cells compared to control tissue (1.5/field), the total number of proliferating cells in Stage III lesions (5.5/field) was not increased compared to Stage II specimens (6.3/field) ($p=0.88$). Endothelial proliferation also was similar between Stage II (3.5/field) and Stage III (5.7/field) AVMs ($p=0.67$) (Fig. 2).

In contrast to proliferating endothelial cells, CD34⁺CD133⁺ EPCs were elevated 2.1 fold in Stage III AVMs (0.53%) compared to Stage II lesions (0.25%) ($p=0.02$); CD34⁺CD133⁺ cells were not present in normal control tissue (Fig. 3). mRNA expression of CD133 also was higher in Stage III AVM (12.3 fold increase), compared to Stage II lesions (2.7 fold).

Vasculogenic Factors

SDF-1 α expression in Stage III lesions (7.9 fold) was greater than in Stage II AVMs (3.3 fold) ($p=0.02$) (Fig. 4). HIF-1 α also was elevated in Stage III AVMs (7.6 fold) compared to Stage II lesions (1.7 fold) ($p=0.02$). Immunohistochemistry illustrated that both SDF-1 α and HIF-1 α were primarily expressed in the endothelium and perivasculature.

Although VEGF mRNA levels were similar between Stage III (1.1 fold) and Stage II (1.0 fold) AVMs ($p=0.7$), the expression of functional VEGF receptors was elevated in Stage III lesions (Fig. 5). While VEGFR2 was slightly increased in Stage III (4.8 fold) compared to Stage II AVMs (2.7 fold, $p=0.3$), NRP1 and NRP2 were significantly elevated [Stage III (5.8 and 4.6 fold, respectively), Stage II (3.0 and 2.4 fold, respectively)] ($p=0.03$). VEGFR1, an inhibitory decoy receptor, was slightly reduced in Stage III lesions (4.0 fold), compared to Stage II AVMs (4.9 fold). Immunohistochemistry showed that VEGF and its receptors were primarily localized to the endothelium and perivasculature.

DISCUSSION

AVMs cause morbidity because they enlarge over time and recur following treatment.⁷ Although vascular malformations have quiescent endothelium compared to tumors,⁸ their vasculature has not been compared to normal control tissue or between static and active

lesions. Our hypothesis that neovascularization contributes to AVM progression was supported by the finding that Stage III lesions had greater microvasculature compared to Stage II AVMs. Any enlarging lesion requires neovascularization to support its increasing tissue mass through the process of *angiogenesis* (growth of new blood vessels from pre-existing vasculature),^{9–14} or *vasculogenesis* (*de novo* formation of new vasculature).^{17–21} Because all AVMs had a greater endothelial proliferative index than control tissue, angiogenesis may be contributing to its growth. However, our results suggest that vasculogenesis, rather than angiogenesis, is more important for neovascularization of AVMs.

Because the number of proliferating endothelial cells was not different between Stage II and III AVMs, the increased vasculature noted in Stage III lesions was primarily the result of EPCs recruited from the bone marrow, rather than from the division of mature endothelial cells in the lesion. This observation was supported by the findings that: (1) EPCs were not found in normal control tissue, but were expressed in all AVMs, (2) Stage III AVMs had a greater proportion of EPCs than Stage II lesions, (3) HIF-1 α , a transcription factor which stimulates EPC recruitment through upregulated VEGF transcription and SDF-1 α expression,^{22–24} was elevated in Stage III compared to Stage II lesions, (4) SDF-1 α , a cytokine produced by fibroblasts which guides EPCs to hypoxic areas,^{25,26} had greater expression in Stage III compared to Stage II AVMs, (5) VEGF signaling, which mobilizes EPCs into the circulation from the bone marrow,^{27–29} is likely increased in Stage III compared to Stage II lesions.

Although the total expression of VEGF was similar in Stage II and III AVMs, the amount of biologically active ligand may be greater in Stage III lesions because of differences in receptor expression. Stage III AVMs had a slightly increased VEGFR2, and reduced expression of VEGFR1, a decoy receptor which binds VEGF.³⁰ Consequently, Stage III lesions may have more VEGF accessible to bind VEGFR2, the biologically active receptor.³⁰ A similar paradigm occurs in infantile hemangioma; endothelial cells have constitutive activation of VEGFR2 because of reduced VEGFR1.³¹ Both NRP1 and NRP2 were greater in Stage III than Stage II AVMs, and thus more receptor was available for VEGF signaling in higher-staged lesions. NRP1 and NRP2 are co-receptors with VEGFR2 that augment VEGF-VEGFR2 binding and enhance VEGFR2 signaling.^{32–35}

The primary stimulus for AVM expansion and EPC recruitment may be ischemia, which attracts EPCs from the bone marrow.^{19,25,36} AVMs typically cause local hypoxia because arteriovenous shunting reduces capillary oxygen delivery; many lesions are complicated by ulceration.^{1,15} EPCs from the bone marrow are attracted to hypoxic sites and contribute to both physiological and pathological post-natal neovascularization.¹⁸ EPCs have been implicated in the vascularization of wounds,³⁶ malignant tumors,^{37,38} infantile hemangioma,^{39–42} the retina,⁴³ atherosclerosis,⁴⁴ as well as the brain⁴⁵ and heart⁴⁶ following infarction.

In addition to hypoxia, other mechanisms may have stimulated EPC recruitment to the AVM. For example, increased blood flow stimulates VEGF production and endothelial proliferation.⁴⁷ Because both males and females have a two-fold risk of progression in

adolescence, elevated circulating hormones during this period may promote EPC mobilization.⁷ Estrogen and testosterone stimulate VEGF production and growth hormone is pro-angiogenic.^{48–53}

Preoperative embolization may have contributed to EPC recruitment to the AVM, although all specimens underwent the same protocol and thus there were no differences between groups. Because a Stage III AVM is more ischemic than a Stage II lesion at baseline, embolization may have preferentially enhanced the hypoxic environment in Stage II lesions; this would bias the results against a difference in vasculogenesis between Stage II and III AVMs.

Not only may vasculogenesis contribute to the natural progression of AVM, it could stimulate recurrence after treatment as well. The recurrence rate of AVM following embolization or resection is high.⁷ Both of these interventions cause trauma, which is well-known to incite AVM enlargement.^{1,15} In addition, both embolization and resection cause hypoxia and stimulate VEGF and matrix metalloproteinase (MMP) production, which attract EPCs from the bone marrow.^{54–57}

The finding that EPCs are present in AVM and are more common in higher-staged lesions provides a potential target for pharmacotherapy (Fig. 6). For example, angiogenesis inhibitors have been shown to suppress mobilization of EPCs.^{58–60} If EPC recruitment to the AVM could be inhibited, neovascularization would be limited and its size might remain stable. Drugs potentially could slow the natural progression of AVM, reducing the need for intervention with embolization or excision. Similarly, if pharmacotherapy could control recurrence, the efficacy of embolization and resection would be improved. Although not a cure, pharmacotherapy might eliminate expansion, and patients would live with a “dormant” AVM, similar to the antiangiogenic strategy for controlling cancer.⁶¹

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Figure 1. Microvessel density in AVMs. CD31 immunohistochemical staining shows greater vascularity in Stage III compared to Stage II lesions. $*p < 0.05$

Figure 2. Cellular proliferation in AVMs. Stage II and Stage III AVMs have similar proliferative indexes by Ki67 immunohistochemistry, suggesting that elevated neovascularization in Stage III AVMs does not result from angiogenesis.

Figure 3. Endothelial progenitor cells (EPCs) in AVMs. Immunofluorescence using CD34 (red) and CD133 (green) antibodies shows EPCs (yellow) in AVMs but not in control tissue. Stage III lesions have greater EPCs compared to Stage II lesions, suggesting that EPCs contribute to AVM neovascularization. * $p < 0.05$

Figure 4.

Expression of factors that stimulate EPC recruitment in AVMs. qRT-PCR shows elevated HIF-1 and SDF-1α in Stage III, compared to Stage II AVMs. Immunohistochemistry illustrates HIF-1 α and SDF-1α are primarily located around the vasculature. * $p < 0.05$

Figure 5.

VEGF and its receptor expression in AVMs. Although VEGF mRNA is similar between Stage II and III AVMs, VEGF signaling may be increased in Stage III AVM because of a greater amount of biologically active receptors in Stage III lesions. Immunohistochemistry shows that VEGF receptors are primarily located around the vasculature. * $p < 0.05$

Figure 6.

Potential clinical translation of study results. Pharmacotherapy using FDA-approved drugs against neovascularization might prevent expansion of AVM. (*Above, left*) 6 year-old male with slowly enlarging Stage 2 AVM of right face. (*Above, right*) Age 25, AVM has progressed to Stage 3. (*Below, left*) 19 year-old male following embolization and resection of left cheek AVM. (*Below, right*) Age 21, AVM enlarging after treatment.

Table 1

Schobinger Clinical Staging of Arteriovenous Malformation (AVM)

Stage	Clinical Findings
I (Quiescence)	Warm, pink-blue, shunting on Doppler
II (Expansion)	Enlargement, pulsation, thrill, bruit, tortuous veins
III (Destruction)	Dystrophic skin changes, ulceration, bleeding, pain
IV (Decompensation)	Cardiac failure