Abnormal Balance in the Angiopoietin-Tie2 System in Human Brain Arteriovenous Malformations

Tomoki Hashimoto, Tiffany Lam, Nancy J. Boudreau, Andrew W. Bollen, Michael T. Lawton, William L. Young

Brain arteriovenous malformations (BAVMs) are congenital vascular lesions that often present as cerebral hemorrhage in young adults. The variable nature of the clinical course, especially with respect to spontaneous hemorrhage, recurrence, growth, and regression, suggests that BAVMs are lesions with active angiogenesis and vascular remodeling. We examined mRNA and protein expression of angiopoietin 1 (Ang1) and Ang2 by semiquantitative reverse transcriptase–polymerase chain reaction, in situ hybridization, and Western blot in BAVMs and control brains obtained from temporal lobectomy for medically intractable seizures. Although Ang1 mRNA levels were similar in BAVMs and controls, Ang1 protein levels were 30% lower in BAVMs than in controls. Ang2 mRNA levels were 40% higher and Ang2 protein levels were 8-fold higher in BAVMs than in controls. In situ hybridization showed that the Ang2 mRNA was localized to the perivascular area in BAVMs. This abnormal balance in the Ang-Tie2 system may, in part, explain the aberrant vascular phenotype in BAVMs.

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rain arteriovenous malformations (BAVMs) are presumed to be sporadic congenital lesions resulting from abnormal vascular development. The variable nature of the clinical course, especially with respect to spontaneous hemorrhage, recurrence, growth, and regression, strongly suggests active vascular changes in most lesions. Such changes have been speculated to involve angiogenesis and vascular remodeling.

Angiopoietins (Ang) and their receptor, Tie2, play a critical role in angiogenesis and vascular stability. Ang1, an agonist for the Tie2 receptor, promotes interaction between endothelial cells (ECs) and peri-EC support cells to stabilize vessels. Ang2 is an antagonist for the Tie2 receptor that acts to destabilize these attachments by preventing Ang1 stimulation of Tie2. The vascular phenotypes observed by overexpression of Ang2 or homozygous disruption of the Tie2 gene are strikingly similar to BAVM vessels. They display abnormally dilated vessels that lack mature peri-EC support structure. Similarly, loss-of-function mutations in the Tie2 gene are found in patients with familial mucocutaneous venous malformations, vascular lesions resembling BAVMs. Previously, we demonstrated decreased Tie2 expression in BAVMs. In this study, we describe an abnormal balance between Ang1 and Ang2 expression at both protein and mRNA levels.

Materials and Methods

We collected BAVM specimens from 7 women and 9 men (32±14 years of age), as previously described. All patients received embolization therapy before BAVM resection. Control subjects consisted of 2 women and 2 men (30±11 years of age). The protocol of this study complies with the guidelines for the conduct of research involving human subjects by the National Institutes of Health and the Committee on Human Research at the University of California San Francisco and Columbia University College of Physicians and Surgeons.

Reverse Transcriptase–Polymerase Chain Reaction, Northern Blot, In Situ Hybridization, and Western Blot

Semiquantitative reverse transcriptase–polymerase chain reaction (RT-PCR), in situ hybridization, Western blot, and Northern blot were performed as previously described. Primer sets for Ang1, Ang2, and Tie2 were from R&D Systems. Primary antibodies against Ang1 and Ang2 were provided by Regeneron Pharmaceutical (Tarrytown, NY).

Statistical Analysis

mRNA levels are normalized by 18S rRNA. Data (mean±SD) are presented as a relative expression, with mean control as 100%, and compared by the Mann-Whitney test.

An expanded Materials and Methods section can be found in the online data supplement available at http://www.circresaha.org.

Results

Using semiquantitative RT-PCR, we observed that Ang1 mRNA levels were similar in the BAVMs (n=5) and controls (n=3) (105±6% versus 100±4%, P=0.46) (Figure 1A). Western blot analysis, however, revealed that Ang1 protein levels were lower in the BAVMs (n=10) compared with controls (n=3) (66±15% versus 100±14%, P=0.01) (Figure 1B). Using semiquantitative RT-PCR, we found that Ang2 mRNA levels were higher in the BAVMs (n=5) compared with the controls (n=3) (139±17% versus 100±12%, P=0.03) (Figure 1C). Northern blot analysis confirmed high levels of Ang2 mRNA in the BAVMs, whereas Ang2 mRNA was not detectable in the two controls (see the online data supplement, available at http://www.circresaha.org). Western blot analysis showed that Ang2 protein levels were markedly higher in the BAVMs (n=10) compared with controls (n=3) (754±147% versus 100±40%, P=0.01) (Figure 1D). Anal-
Figure 1. Expression of Ang1 mRNA, Ang1 protein, Ang2 mRNA, and Ang2 protein in BAVMs and the control brain. In each panel (A, Ang1 mRNA; B, Ang1 protein; C, Ang2 mRNA; and D, Ang2 protein), top shows densitometric intensity of mRNA or protein levels expressed as a percentage of values obtained in control brain samples (mean±SD). (Ang mRNA levels are normalized by 18S rRNA.) Bottom shows representative gel from semiquantitative RT-PCR or Western blot. †, ‡Different sets of primer for 18S rRNA yielding different product sizes were used. Ang1 protein appeared at ~150 kDa under nonreduced conditions (B). Ang2 protein appeared at ~75 kDa under reduced conditions (D). CB indicates control brain; PC, positive control. *P<0.05.

Figure 2. Localization of Ang1 and Ang2 in BAVMs and the control brain. Photomicrographs of serial sections from BAVMs (top and middle) and the control brain (bottom) in H&E staining (A through D), CD31 immunostaining (E through H), in situ hybridization for Ang1 (I through K), and in situ hybridization for Ang2 (L through M). Middle panels show higher magnification of the insets in the top panels. Arrows indicate endothelial cell layer (top and middle) and cortical vessel (bottom). Endothelial layer was generally intact in both BAVMs (A, B, E, and F) and the CB (C, D, G, and H). BAVM vessels expressed both Ang1 (I and J) and Ang2 (L and M) in the perivascular areas. Cortical vessel in control brain expressed Ang1 (K) in the perivascular area but not Ang2 (N). Bar=100 μm.

Discussion

In this study, we found increased Ang2 mRNA and protein levels and decreased Ang1 protein levels in BAVMs. Previously, we described markedly decreased Tie2 protein in BAVMs, consistent with the decreased Tie2 mRNA observed in this study. The decreased level of Tie2 receptor expression along with the reduction in the Ang1 would be expected to reduce Tie2 signaling, thus leading to vascular instability. Furthermore, a marked increase in the expression of the Tie2 antagonist Ang2 might be expected to additionally impair vascular stability in BAVMs. This may, in part, explain the aberrant vascular phenotype in BAVMs (i.e., dilated vessels with a relative lack of mature peri-EC support).

The underlying causal mechanisms of our observed changes remain to be determined. We observed decreased Ang1 protein levels despite normal Ang1 mRNA levels, which may suggest increased proteolytic activity in BAVMs. Stimuli such as shear stress or ischemia may also affect the expression of Ang and Tie2. However, BAVM vessels are exposed to high rates of fully oxygenated blood flow and are therefore unlikely to be ischemic. Normal eNOS expression in BAVMs suggests that, at least at the time of microsurgical resection, shear stress may not be the sole cause of vascular remodeling in BAVMs. Because BAVM resection is associated with brain swelling, the decreased Ang1 may have some mechanistic involvement in vasogenic edema.

Although embolization treatment may provoke angiogenesis through the formation of organized thrombi or recanalization processes, the majority of vessels in our study were free of intraluminal inflammation or organized thrombus (Figures 2A and 2B). CD31 immunohistochemistry showed that the EC layer in the BAVMs was intact (Figures 2E and 2F). In situ hybridization showed Ang1 mRNA in the perivascular area of both BAVM and control vessels (Figures 2I through 2K). Ang2 mRNA was abundantly expressed in the perivascular area of the BAVM vessels (Figures 2L and 2M). Ang2 mRNA was not detectable in the controls (Figure 2N). No signal was observed using sense probes (see the online data supplement).

There was no correlation between expression of Ang1 or Ang2 and the interval between embolization treatment and surgery (median, 1 day; range, 1 to 49 days) or the number of embolization treatments (median, 2; range, 1 to 4 days) (see the online data supplement).
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and control specimens have a similar EC mass per unit weight using the method described by others.2,5,9

In summary, there is an abnormal balance in the Ang-Tie2 system in BAVMs that may, in part, explain the pathological vascular phenotype of BAVMs.

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Methods

Materials

After institutional review and approval, we obtained BAVM specimens after microsurgical resection as previously described.\(^1\) BAVM nidus was dissected away from any adjacent brain tissue in the operating room and a representative portion of nidus tissue was immediately frozen in liquid nitrogen and stored at -80°C until analysis. An additional piece was also placed in formalin for paraffin embedding. A structurally normal cerebral cortex was obtained from patients undergoing temporal lobectomy for medically intractable seizures; samples were collected in the same manner.

Total RNA Preparation and RT-PCR

Total RNA was isolated by the single-step guanidinium isothiocyanate method\(^2\) using Trizol reagent (Life Technologies). One μg of total RNA was reverse transcribed using Moloney-Murine leukemia virus reverse transcriptase and random decamer (RETROscript, Ambion).

The same volume of reverse transcription (RT) product from each sample was subjected to PCR amplification. Duplex PCR was performed to simultaneously amplify the gene of interest (Ang1, Ang2, or Tie2) and 18s ribosomal RNA (rRNA), as an internal control to control for sample-to-sample variations in RT-PCR reaction as previously described by Connfield et al.\(^3\)

We used a primer set for Ang1, Ang2 and Tie2 (R&D Systems), and a primer / competimer set for 18s rRNA (Classic 18s rRNA primer and competimer, product size: 495 bp, and alternate 18s rRNA primer and competimers, product size 324 bp; QuantumRNA, Ambion). PCR conditions include initial denaturation for 4 min at 94 °C followed by 28
cycles at 94°C, 55°C, and 72°C for 30 sec each plus a final incubation for 10 minutes at 72°C. PCR products were visualized and isolated by running 2% agarose gel containing 1 μg/ml ethidium bromide. The identity of each PCR product was confirmed by its DNA sequence. Densitometry was used to assess the band intensity of PCR product. Ang1, Ang2, and Tie2 mRNA expression was normalized to 18s rRNA. The ratio of the band from the mRNA of interest to the band from 18s rRNA was compared among samples.

Preliminary experiments showed that PCR was at the exponential phase of amplification and increasing amounts of RNA resulted in a linear increase in band intensity for both 18s rRNA and the mRNA of interest (Ang1, Ang2, and Tie2) (Online Figure 1). Each reaction was repeated 3 times to confirm reproducibility. The absence of genomic contamination in the mRNA samples was confirmed by the absence of a signal for reverse transcriptase–negative controls.

**In-Situ Hybridization**

In-situ hybridization was performed as previously described. Sense and antisense digoxigenin-labeled RNA probes were generated by linearizing pCR- II-TOPO vector (Invitrogen) containing Ang1 or Ang2 cDNA (generated by RT-PCR described above) and incubating with T7 or SP6 RNA polymerase and digoxigenin-conjugated dUTP (Genius; Roche Molecular Biochemicals). In-situ hybridization was performed using paraffin embedded tissues. Sections were de-paraffinized and rehydrated. Each section was digested for 15-20 minutes by protease K. The sections were then hybridized overnight with 0.2 – 0.3 ng / ml of riboprobes at 50°C. After washing, sections were incubated overnight with a 1:500 dilution of HRP-conjugated antibodies against digoxigenin and developed using NBT/BCIP (DIG nucleic acid detection Kit; Roche Molecular Biochemicals). Non-specific hybridization was assessed using respective sense riboprobes.

**Northern blot**
One BAVM and two control specimens were large enough to perform Northern blot. 7.5 µg of total RNA was run through 1% agarose formaldehyde gel and transferred to Hybond-N membranes (Amersham) according to standard procedures. Membranes were hybridized using [32 P]dCTP-labeled Ang2 cDNA probes and exposed to Kodak X-Omat film. Ang2 cDNA probe was generated using the primer as described above.

**Western blot**

Western blot for Ang1 was performed in the non-reduced condition, while the western blot for Ang2 was done in the reduced condition. For immunoblot, the specimens were homogenized in a buffer containing 50 mM hydroxymethyl aminomethane (Tris) and 1 mM ethylenedinitrilo tetraacetic acid (EDTA) with pH of 7.4 with protease inhibitor cocktail (4-(2-aminoethyl)benzenesulfonyl fluoride, pepstatin A, trans-epoxysuccinyl-L-leucylamido(4-guanidino)butane, bestatin, leupeptin, and aprotinin) (Sigma-Aldrich). The protein concentrations were measured by colorimetric assay using bovine serum albumin as a control. Electrophoresis was done using 4-12 % gradient gel (NuPAGE BIS-TRIS electrophoresis system, Invitrogen). Samples for Ang1 were mixed with the sample buffer (Invitrogen) without reducing agent. Samples for Ang2 were mixed with the sample buffer with 1,4-Dithio-L-threitol (0.2 M final concentration) and incubated at 70°C for 10 minutes. An equal amount of protein was loaded and electrophoresed on 4-12 % gradient gels. Subsequently, proteins were electroblotted onto polyvinylidene difluoride (PVDF) membranes. The PVDF membranes were blocked at room temperature with 5% dried milk in Tris buffered saline (TBS) with 1% polyoxyethylene-sorbitan monolaurate (Tween 20) for 1 hour. The membranes were then probed with primary antibody for 1 hour, followed by appropriate species-specific horseradish-peroxidase (HRP)-conjugated secondary antibody. Protein expression was detected with an enhanced chemiluminescence detection system (ECL+Plus, Amersham Pharmacia Biotech). Bands were visualized on film and a
densitometric scanner was used to estimate the relative amounts of proteins. We used primary antibodies against Ang1 (rabbit polyclonal, 1:10,000) and Ang2 (rabbit polyclonal, 1:10,000) (Regeneron Pharmaceutical). In preliminary experiments, controls with increasing amounts of protein verified that quantitative band intensities fell within a linear range. We used recombinant Ang1 and Ang2 from Regeneron as positive control.
Results

Preliminary study for semi-quantitative RT-PCR

Increasing amounts of total RNA resulted in a linear increase in band intensity for both 18s rRNA and the mRNA of interest (A representative result of duplex RT-PCR for Ang2 mRNA and 18s rRNA is shown in Online Figure 1). (18s rRNA: y = 1427 x +173, R² = 0.96, Ang2: y = 2321 x -69, R² = 0.95)

Semi-quantitative RT-PCR for Tie2

Using semi-quantitative RT-PCR, we observed that Tie2 mRNA levels were lower in the BAVMs (n=6) than in the controls (n=3) (65 ± 15 versus 100 ± 16 %, P = 0.02) (Online Figure 2).

Northern blot

Total RNA electrophoresed on a formaldehyde agarose gel showed that our RNA samples were generally intact and that an equal amount of total RNA was loaded for each sample (Online Figure 3, lower panel). Northern blot for Ang2 detected abundant Ang2 mRNA in the BAVM sample, but not in the two control brain samples (Online Figure 3, upper panel).

In-situ hybridization with sense probe

In-situ hybridization with Ang1 (Online Figure 4B) or Ang2 (Online Figure 4C) sense probe showed no signal, while in-situ hybridization with Ang1 (Online Figure 4D) and Ang2 (Online Figure 4E) antisense probes showed abundant signals in the perivascular area.

Embolization treatment and expression of angiopoietins
The specimens obtained at Columbia Presbyterian Medical Center were used for semi-quantitative RT-PCR and Northern blot. These patients received embolization treatment using n-butyl cyanoacrylate prior to microsurgical resection. The specimens obtained at University of California, San Francisco received embolization treatment with polyvinyl alcohol. There were no associations between the expression of Ang1 or Ang2 mRNA, and: (1) the interval between last embolization and surgery (14 ± 20 days; range 2-49) and (2) the number of embolization treatments (3 ± 1; range 1-4) (Online Figure 5A, B). There were no associations between the expression of Ang1 or Ang2 protein, and: (1) the interval between last embolization and surgery (2± 2 days; range 1-7) and (2) the number of embolization treatments (1.4 ± 0.5; range 1-2) (Online Figure 5C, D).
Legends for figures

**Online Figure 1. Linear relationship between total RNA and band intensity.**

Increasing amounts of total RNA resulted in a linear increase in band intensity for both 18s rRNA and Ang2 mRNA. The upper panel shows agarose gel for duplex semi-quantitative RT-PCR for Ang2 (lower bands) and 18s rRNA (upper bands). The lower panel shows the graph for an increasing amount of total RNA input and densitometric readings for Ang2 and 18s rRNA. (18s rRNA: \( y = 1427 \times +173 \), \( R^2 = 0.96 \), Ang2: \( y = 2321 \times -69 \), \( R^2 = 0.95 \))

**Online Figure 2. Expression of Tie2 mRNA in BAVMs and control brain samples.**

The upper panel shows the band intensity of Tie2 mRNA (presented as %-control) in the BAVMs and the control brain (CB). Tie2 mRNA levels are expressed as the Ang mRNA / 18s rRNA. The lower panel shows representative RT-PCR for Tie2 and 18s rRNA. * = \( P<0.05 \).

**Online Figure 3. Northern blot for Ang2.**

Total RNA electrophoresed on a formaldehyde agarose gel showed that our RNA samples were generally intact and that an equal amount of total RNA was loaded for each sample (lower panel). Northern blot for Ang2 detected abundant Ang2 mRNA in the BAVM sample, but not in the two control brain sample (CB) (upper panel).

**Online Figure 4. Microphotographs of serial sections from BAVMs**

A: hematoxylin and eosin staining of BAVM, B: in-situ hybridization with Ang1 sense probe, C: in-situ hybridization with Ang2 sense probe, D: in-situ hybridization with Ang1 antisense probe, E: in-situ hybridization with Ang2 antisense probe. Arrow indicates the endothelial layer. In-situ hybridization with Ang1 (B) or Ang2 (C) sense probe showed no
signal, while in-situ hybridization with Ang1 (D) and Ang2 (E) antisense probe showed abundant signals in the perivascular area. Bar = 100μm.

Online Figure 5. Ang expression and embolization treatment.

Left upper panel: Ang mRNA expression and the interval between last embolization and surgery. Right upper panel: Ang mRNA expression and the number of embolization treatments. Left lower panel: Ang protein expression and the interval between last embolization and surgery. Right lower panel: Ang protein expression and the number of embolization treatments.
References:


Online Figure 1
Online Figure 2
Online Figure 3
Online Figure 4