MicroRNA-18a Improves Human Cerebral Arteriovenous Malformation Endothelial Cell Function

Raquel Ferreira, PhD; Tiago Santos, MSc; Arun Amar, MD, PhD; Stanley M. Tahara, PhD; Thomas C. Chen, MD, PhD; Steven L. Giannotta, MD; Florence M. Hofman, PhD

Background and Purpose—Cerebral arteriovenous malformations (AVMs) are a vascular disease that disrupts normal blood flow and leads to serious neurological impairment or death. Aberrant functions of AVM-derived brain endothelial cells (AVM-BECs) are a disease hallmark. Our aim was to use microRNA-18a (miR-18a) as a therapeutic agent to improve AVM-BEC function.

Methods—Human AVM-BECs were tested for growth factor production and proliferation under different shear flow conditions and evaluated for tubule formation. Thrombospondin-1, inhibitor of DNA-binding protein 1, and vascular endothelial growth factor (VEGF) isotype mRNA levels were quantified by quantitative real-time polymerase chain reaction. Thrombospondin-1, VEGF-A, and VEGF-D protein expression was measured using enzyme-linked immunosorbent assay. Proliferation and tubule formation were evaluated using bromodeoxyuridine incorporation and growth factor–reduced Matrigel assays, respectively.

Results—miR-18a increased thrombospondin-1 production but decreased inhibitor of DNA-binding protein 1, a transcriptional repressor of thrombospondin-1. miR-18a reduced VEGF-A and VEGF-D levels, both overexpressed in untreated AVM-BECs. This is the first study reporting VEGF-D overexpression in AVM. These effects were most prominent under arterial shear flow conditions. miR-18a also reduced AVM-BEC proliferation, improved tubule formation, and was effectively internalized by AVM-BECs in the absence of extraneous transfection reagents.

Conclusions—We report VEGF-D overexpression in AVM and the capacity of miR-18a to induce AVM-BECs to function more normally. This highlights the clinical potential of microRNA as a treatment for AVM and other vascular diseases. (Stroke. 2014;45:00-00.)

Key Words: arteriovenous malformations ■ endothelial cells ■ microRNAs ■ thrombospondin 1 ■ vascular endothelial growth factor D

Cerebral arteriovenous malformations (AVMs) are vascular lesions defined by an anomalous tangle of vessels (nidus), in which arteries and veins are directly connected without an intervening capillary system. Current medical treatment involves highly invasive procedures because surgical access to the AVM nidus presents significant risk to adjacent brain areas, underlining the importance of developing more efficient and safer therapies.

AVM-derived brain endothelial cells (AVM-BECs) have distinct characteristics compared with normal control BECs. AVM-BECs also express low levels of thrombospondin-1 (TSP-1), a key regulator of angiogenesis. TSP-1 treatment or silencing of one of its transcriptional repressors, inhibitor of DNA-binding protein 1 (Id-1), brings AVM-BECs to more normal rates of proliferation, migration, and tubule formation efficacy. Moreover, TSP-1 antagonizes a key proangiogenic factor, vascular endothelial growth factor A (VEGF-A), which is expressed at higher levels in AVM-BECs as compared with control BECs.

MicroRNAs (miRNAs) are small noncoding RNAs that inhibit gene expression by targeting messenger RNA for cleavage or translational repression. In this study, we used microRNA-18a (miR-18a) to inhibit Id-1 expression, thereby increasing TSP-1 release to normalize several of the reported aberrant features of AVM-BECs. We also showed that miR-18a enters endothelial cells without traditional transfection reagents, thus opening new therapeutic perspectives for the use of miRNA. In our study, miR-18a improved the aberrant angiogenic features of AVM-BECs by increasing TSP-1 and decreasing both VEGF-A and VEGF-D secretion.

Materials and Methods

Human surgical specimens were obtained in accordance with the Institutional Review Board (HS-04B053), Keck School of Medicine,
University of Southern California, and in accordance with Animal Research: Reporting In Vivo Experiments (ARRIVE) guidelines. AVM-BECs were obtained from brain tissues of 6 patients after AVM resection. Control BECs were derived from structurally normal cortex of 4 patients who underwent surgery for epilepsy (Table I in the online-only Data Supplement). Cells were isolated as described previously and used only until passage 5. Cells were treated for 24 hours with lipofectamine-delivered (2 µg/mL) miR-18a (20–80 nmol/L; Thermo Scientific), naked miR-18a mimic (20–80 nmol/L), scrambled miRNA sequence (20–80 nmol/L; Thermo Scientific) and small interfering RNA for green fluorescent protein (siGFP) (20–80 nmol/L; Life Technologies) as indicated.

Shear flow experiments were performed on an orbital shaker to reproduce arterial flow (12 dyn/cm²) and venous flow (4 dyn/cm²). Conditioned media were collected from cells incubated 24 hours in serum-free medium, clarified by centrifugation and used without dilution, and were analyzed for TSP-1, VEGF-A, VEGF-B, VEGF-C, and VEGF-D using commercially available enzyme-linked immunosorbent assay reagents (R&D Systems).

Cell proliferation was assayed by incubation with 5-bromo-2′-deoxyuridine (BrdU; 50 µmol/L; Sigma) for 4 hours, followed by fixation and incubation with primary mouse monoclonal anti-BrdU antibody (1:50; Molecular Probes) and secondary antibody Alexa Fluor-594 donkey anti-mouse (1:200; Molecular Probes). Hoechst-33342 (2 µg/mL; Sigma) was used for nuclear staining. All images were captured using EVOs fl AMF-4306 (AMG) microscopes.

Gene expression was confirmed by quantitative polymerase chain reaction using iQ SYBR Green Supermix (BioRad) according to the manufacturer’s instructions using a Stratagene Mx3000P Bioanalyzer (Agilent Technologies). Polymerase chain reaction products were normalized to 18S ribosomal RNA. The following forward and reverse primers were used: Id-1, 5′-GCCGAGGGCATGCGTTC-3′ and 5′-TCCTCAGATCCGGCGAGGC-3′; 18S ribosomal RNA, 5′- CGGTACTCCATCCAGGA-3′ and 5′-GCTGGAATTACC CGGCT-3′, respectively.

Statistical analysis was performed using GraphPad Prism 5.0. Statistical significance was considered relevant at P<0.05 using 1-way ANOVA followed by Bonferroni or Dunnett post hoc test. Data are presented as mean±SEM. Experimental conditions were tested in 3 independent experiments, unless stated otherwise, with duplicate samples.

Results

AVM-BECs constitutively express significantly less miR-18a than control BECs (2.94-fold decrease; data not shown) and secreted less TSP-1 than control BECs (259.76±22.93 versus 1026±82.41; Figure 1A and 1B). Lipofectamine-delivered and naked miR-18a mimic (at 40 nmol/L) significantly increased TSP-1 secretion by AVM-BECs (miR-18a(lipofectamine-delivered)=340.7±48.71; miR-18a(naked)=464.1±70.31; unTx=258.8±22.93; n=4; *P<0.05; **P<0.01; Figure 1A). Conversely, miR-18a did not change TSP-1 levels of control BECs (Figure 1B). siGFP was chosen as an additional negative control because the miR-18a mimic is a double-stranded RNA and these cells do not express green fluorescent protein.

To mimic blood flow and pressure, cells were subjected to different shear flow magnitudes: 4 dyn/cm² (venous flow); 12 dyn/cm² (arterial flow); or static conditions. Results from venous

Figure 1. The microRNA-18a (miR-18a) treatment induces thrombospondin-1 (TSP-1) release and inhibits inhibitor of DNA-binding protein 1 (Id-1) expression in arteriovenous malformation–derived brain endothelial cells (AVM-BECs) under arterial flow conditions. A, Enzyme-linked immunosorbent assay (ELISA) quantification showed that under static conditions, naked and lipofectamine-delivered (lipof.) miR-18a (40 nmol/L) significantly increased TSP-1 release in AVM-BECs (n=4; *P<0.05; **P<0.01). B, ELISA quantification showed that under static conditions, miR-18a did not affect TSP-1 release in control BECs (n=3). C, ELISA quantification showed that under arterial flow (white bars) and static conditions (black bars), naked miR-18a 40 nmol/L significantly increased TSP-1 release by AVM-BECs (n=4; *P<0.05). D, Real-time quantitative polymerase chain reaction analysis showed that under arterial flow and static conditions, naked miR-18a significantly decreased Id-1 expression (n=3; ***P<0.001; **P<0.01). Dotted line represents basal Id-1 expression in AVM-BECs. siGFP indicates small interfering RNA for green fluorescent protein.
Flow were not significantly different from static flow (data not shown). Naked miR-18a, but not lipofectamine-delivered miR-18a, significantly increased TSP-1 release by AVM-BECs under arterial flow and static conditions (unTxart=179.9±42.94; miR-18a(naked)art=335.8±73.72; unTx stat=232.5±26.03; miR-18a(naked)stat=408.2±92.61; n=4; *P<0.05; Figure 1C).

Furthermore, only naked miR-18a significantly reduced Id-1 mRNA levels under arterial flow and static conditions (miR-18a(naked)art=0.81±0.05; miR-18a(naked)stat=0.89±0.02; n=3; ***P<0.001; **P<0.01; Figure 1D). Thus, naked miR-18a, independent of flow conditions, decreased the repressor of TSP-1, Id-1, resulting in increased TSP-1 production.

VEGF-A and VEGF-D are the major VEGF isoforms expressed by AVM-BECs (data not shown). Naked miR-18a significantly reduced VEGF-A release by AVM-BECs under arterial flow and static conditions (unTxart=179.9±42.94; miR-18a(naked)art=335.8±73.72; unTx stat=232.5±26.03; miR-18a(naked)stat=408.2±92.61; n=4; *P<0.05; Figure 1C). Similarly, naked miR-18a significantly decreased VEGF-D release by AVM-BECs under arterial flow (unTxart=1453±396.8; miR-18a(naked)art=566.1±161.9; n=3–4; *P<0.05), but not under static conditions (Figure 2C). miR-18a did not affect VEGF-A or VEGF-D release from control BECs (Figure 2B and 2D). Thus, both VEGF-D and VEGF-A can be relevant targets to inhibit angiogenesis.

The effects of naked miR-18a on AVM-BECs were analyzed. miR-18a decreased AVM-BEC proliferation under arterial, but not under static, flow conditions (unTxart=19.83±2.18; miR-18a(naked)art=11.66±2.15; unTx stat=8.51±1.53; miR-18a(naked)stat=12.06±1.45; n=4–5; **P<0.01). miR-18a had no effect on control BEC proliferation (Figure 3A). Naked miR-18a significantly increased overall tubule formation (unTxart=0.45±0.06; miR-18a(naked)=0.79±0.11) and branching (unTxart=0.67±0.04; miR-18a(naked)=0.95±0.08; Figure 3B); tubule length (unTxart=0.50±0.04; miR-18a(naked)=0.80±0.11) and branch length (unTxart=0.74±0.03; miR-18a(naked)=0.95±0.07) of AVM-BECs (n=3; ***P<0.001; **P<0.01) were also increased (Figure 3C). Representative images of untreated and miR-18a–treated AVM-BECs show increased tubule formation with miRNA treatment (Figure 3D). Tubule morphology of control BECs was not altered by miR-18a. These results demonstrated that miR-18a can improve AVM-BEC structure and function.

**Discussion**

miRNAs are active regulators of angiogenesis, suggesting their use as therapeutic agents or targets for the treatment of vascular diseases. miR-18a is part of the miR-17 to miR-92 cluster, which has been linked to tumor angiogenesis. However, when evaluated individually, miR-17, miR-18a, miR-19a, and miR-20a have antiangiogenic activity. In fact, miRNA can have different and opposing roles depending on cell type and physiological/pathological context. miR-18a...
inhibits Dicer expression, a key enzyme involved in miRNA biogenesis. Dicer silencing induces the expression of TSP-1, a potent inhibitor of endothelial cell migration, proliferation, and survival. We show that increased TSP-1 induced by miR-18a treatment correlated with the inhibition of Id-1 expression. Id-1 is widely overexpressed in most human cancers, thereby representing a promising target for anticancer therapies.

Cerebral AVM is mainly characterized by vessels lacking an intervening capillary bed, thus exhibiting an inability to regulate blood flow and pressure. Hence, the AVM nidus and more specifically AVM-BECs are fundamentally affected by arterial flow and susceptible to the formation of aneurisms and hemorrhage. In fact, patients with AVM with acute symptoms (hemorrhage, severe headaches/seizures, or neurological deficits) have higher wall shear stress in feeding blood vessels than contralateral vessels. Our studies showed that the strongest effects of miR-18a were on cells subjected to arterial flow. However, in vivo, shear flow in AVM vessels may attain levels higher than 12 dyn/cm²; future studies will be performed to identify maximum shear flow values supported by this system. Although some patients were preoperatively embolized, we found no correlation between any of the parameters listed and the results obtained.

Angiogenesis is tightly regulated by several endogenous growth factors, particularly VEGF-A. TSP-1 antagonizes VEGF-A bioavailability and activity by inactivating matrix metalloproteinase-9, which suppresses the release of VEGF-A from the extracellular matrix. Endothelial cells treated with the 3 TSP-1 type 1 repeats (the active domain of TSP-1) have decreased VEGF receptor-2 phosphorylation and activation of serine/threonine protein kinase Akt, essential in VEGF-A–mediated angiogenesis. Here, we showed that not only VEGF-A but also VEGF-D, a key lymphangiogenic and proangiogenic factor, were upregulated in AVM. miR-18a significantly decreased both VEGF-A and VEGF-D release from AVM-BECs, particularly under arterial flow conditions. Although current strategies developed for human antiangiogenic therapy have primarily focused on VEGF-A (eg, bevacizumab), our results suggest that both isotypes should be...
targeted because both growth factors can function in a proangiogenic mode. VEGF-A and VEGF-D can be produced independently because macrophages treated with TSP-1 showed decreased VEGF-D expression, whereas VEGF-A secretion was unaffected. Thus, the effects of TSP-1 on VEGF-D release by endothelial cells are novel and have significant therapeutic value.

miR-18a treatment did not affect VEGF-A or VEGF-D mRNA expression (data not shown), suggesting that the effect of miR-18a was not directly on VEGF transcription but most likely indirectly through other mediators such as TSP-1. The therapeutic potential of miR-18a was further strengthened by its ability to regulate cell proliferation and induce a structural vascular network, which requires the efficient formation of 3-dimensional tubules.

Current studies suggest several potential mechanisms by which extracellular miRNA may enter recipient cells, such as direct endocytosis, receptor-mediated endocytosis, and endosomal transport. We have shown that miR-18a can be internalized and functionally relevant without the aid of a transfection reagent, thus revealing its potential clinical application. Our study is the first to show that naked miRNA can enter nontransformed cells, particularly endothelial cells, and affect cellular function. The precise mechanism(s) of miR-18a entry into AVM-BECs is currently under investigation. From a clinical viewpoint, because endothelial cells are in direct contact with the bloodstream, intravenous miRNA delivery will exhibit high target specificity and minimal side effects. Moreover, development of modern endovascular techniques and devices to access the intracranial vasculature suggests the possibility of direct intra-arterial delivery into the AVM for more specific effects. This approach should lessen loss of the therapeutic agent by avoiding systemic circulation.

In vitro studies are necessary to understand AVM because currently available animal models are based on regional condition gene deletion plus angiogenic stimulation and do not fully resemble the human phenotype. The porcine brain AVM model successfully exhibits an abnormal tangle of nidus vessels and arteriovenous shunting; however, this model is short-lived because of the acute occurrence of spontaneous thrombosis. For these reasons, we used isolated AVM-BECs to study this vascular disease.

Our findings support the potential use of miR-18a to ameliorate the functionally aberrant AVM-BECs and emphasize the relevance of VEGF-D in this vascular dysfunction. Ultimately, the use of miRNA can be further developed to advance AVM treatment as a noninvasive, effective, and biocompatible therapeutic agent.

Sources of Funding
This work was supported by Neithart Foundation (Drs Ferreira and Giannotta).

Disclosures
None.

References
MicroRNA-18a Improves Human Cerebral Arteriovenous Malformation Endothelial Cell Function
Raquel Ferreira, Tiago Santos, Arun Amar, Stanley M. Tahara, Thomas C. Chen, Steven L. Giannotta and Florence M. Hofman

Stroke. published online November 7, 2013;
Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2013 American Heart Association, Inc. All rights reserved.
Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the
World Wide Web at:
http://stroke.ahajournals.org/content/early/2013/11/06/STROKEAHA.113.003578

Data Supplement (unedited) at:
http://stroke.ahajournals.org/content/suppl/2013/11/07/STROKEAHA.113.003578.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Stroke can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Stroke is online at:
http://stroke.ahajournals.org/subscriptions/
**Supplementary table I**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age/Sex</th>
<th>Location of pathology</th>
<th>History of hemorrhage</th>
<th>Embolization</th>
<th>Spetzler-Martin Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>AVM1</td>
<td>23/M</td>
<td>R occipital lobe</td>
<td>Yes</td>
<td>Yes</td>
<td>N/D</td>
</tr>
<tr>
<td>AVM2</td>
<td>16/F</td>
<td>R parietal lobe</td>
<td>N/D</td>
<td>Yes</td>
<td>N/D</td>
</tr>
<tr>
<td>AVM3</td>
<td>35/F</td>
<td>L frontal lobe</td>
<td>No</td>
<td>Yes</td>
<td>2</td>
</tr>
<tr>
<td>AVM4</td>
<td>22/M</td>
<td>R temporal lobe</td>
<td>Yes</td>
<td>No</td>
<td>3</td>
</tr>
<tr>
<td>AVM5</td>
<td>28/M</td>
<td>R frontal lobe</td>
<td>Yes</td>
<td>No</td>
<td>1</td>
</tr>
<tr>
<td>AVM6</td>
<td>22/F</td>
<td>R occipital lobe</td>
<td>Yes</td>
<td>Yes</td>
<td>1</td>
</tr>
</tbody>
</table>

*M, male; F, female; L, left; R, right; N/D, not determined; Age is indicated in years.*