Extracellular Vimentin/VWF (von Willebrand Factor) Interaction Contributes to VWF String Formation and Stroke Pathology

Titilope A. Fasipe, MD*; Sung-Ha Hong, PhD*; Qi Da, PhD; Christian Valladolid, BS; Matthew T. Lahey, BS; Lisa M. Richards, PhD; Andrew K. Dunn, PhD; Miguel A. Cruz, PhD; Sean P. Marrelli, PhD

Background and Purpose—VWF (von Willebrand factor) strings mediate spontaneous platelet adhesion in the vascular lumen, which may lead to microthrombi formation and contribute to stroke pathology. However, the mechanism of VWF string attachment at the endothelial surface is unknown. We tested the novel hypothesis that VWF strings are tethered to the endothelial surface through an interaction between extracellular vimentin and the A2 domain of VWF. We further explored the translational value of blocking this interaction in a model of ischemic stroke.

Methods—Human endothelial cells and pressurized cerebral arteries were stimulated with histamine to elicit VWF string formation. Recombinant proteins and antibodies were used to block VWF string formation. Mice underwent transient middle cerebral artery occlusion with reperfusion. Just before recanalization, mice were given either vehicle or A2 protein (recombinant VWF A2 domain) to disrupt the vimentin/VWF interaction. Laser speckle contrast imaging was used to monitor cortical perfusion.

Results—Pressurized cerebral arteries produced VWF strings following histamine stimulation, which were reduced in arteries from Vim KO (vimentin knockout) mice. VWF string formation was significantly reduced in endothelial cells incubated with A2 protein or antivimentin antibodies. Lastly, A2 protein treatment significantly improved cortical reperfusion after middle cerebral artery occlusion.

Conclusions—We provide the first direct evidence of cerebral VWF strings and demonstrate that extracellular vimentin significantly contributes to VWF string formation via A2 domain binding. Lastly, we show that pharmacologically targeting the vimentin/VWF interaction through the A2 domain can promote improved reperfusion after ischemic stroke. Together, these studies demonstrate the critical role of VWF strings in stroke pathology and offer new therapeutic targets for treatment of ischemic stroke.

Visual Overview—An online visual overview is available for this article. **(*Stroke*. 2018;49:2536-2540. DOI: 10.1161/STROKEAHA.118.022888.)**

Key Words: endothelium ■ reperfusion ■ stroke ■ thrombosis ■ vimentin ■ von Willebrand factor

Ischemic stroke is the foremost cause of long-term disability, as well as the fifth leading cause of death in the United States.[1] VWF (von Willebrand factor)—a multimeric glycoprotein—has long been implicated in ischemic stroke pathology.[2,3] Stimulated endothelial cells secrete VWF multimers as long hyper-adhesive strings[4] that, when anchored along the luminal surface, can adhere to circulating platelets. These VWF strings are thought to contribute to microthrombi formation.[4] ADAMTS13 (a disintegrin and metalloprotease with thrombospondin type-1 repeats, member 13) cleaves the VWF strings and has been proposed as a potential therapeutic target for stroke.[5,6] However, clinical studies using recombinant ADAMTS13 for stroke in humans have not been conducted. As a novel alternate approach, we propose a switch of emphasis from promoting more rapid string degradation to one of preventing and reducing string formation in the vascular lumen.

Vimentin—a cytoplasmic intermediate filament protein—is also found on the cell surface of multiple cell types, including endothelial cells.[7,8] We previously demonstrated that vimentin on the platelet surface binds to the A2 domain within the

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From the Section of Hematology-Oncology, Department of Pediatrics (T.A.F.), Department of Molecular Physiology and Biophysics (C.V.), and Department of Medicine (Q.D., M.A.C.), Baylor College of Medicine Houston, TX; Center for Translational Research on Inflammatory Diseases, Michael E. DeBakey Veterans Affairs Medical Center, Houston, TX (T.A.F., S.-H.H., Q.D., C.V., M.A.C., S.P.M.); Department of Biomedical Engineering, University of Texas at Austin (L.M.R., A.K.D.); and Department of Neurology, McGovern Medical School at UTHealth, Houston, TX (S.-H.H., M.T.L., S.P.M.).

*Drs Fasipe and Hong contributed equally.

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Correspondence to Sean P. Marrelli, PhD, Department of Neurology, McGovern Medical School at UTHealth, 6431 Fannin St, MSE R344, Houston, TX 77030, email sean.p.marrelli@uth.tmc.edu or Miguel A. Cruz, PhD, Section of Cardiovascular Research, Department of Medicine, Baylor College of Medicine, 2002 Holcombe, Bldg 109, R-146, Houston, TX 77030, email miguelc@bcm.edu

activated form of VWF.[9] In circulating or plasma VWF, the A2 domain is buried within the globular morphology of the protein. However, the A2 domain is exposed in the newly released VWF molecules or VWF strings from stimulated endothelium.[10] In the present study, we examined the novel hypothesis that cell-surface vimentin on endothelium binds the released VWF strings via the A2 domain, thereby contributing to the formation of intraluminal VWF strings. We further performed a proof-of-concept experiment to demonstrate the potential of pharmacologically targeting this A2/vimentin interaction to facilitate improved reperfusion after ischemic stroke.

Methods

The data that support the findings of this study are available from the corresponding author on reasonable request.

Animals

Animal experiments were performed according to protocols approved by the Institutional Animal Care and Use Committee at Baylor College of Medicine and McGovern Medical School at UTHealth. Vim KO (vimentin deficient) and WT (wild type) mice (strain 129/SV) were obtained as we described.[9] Stroke studies were performed with male C57BL/6 N mice at 12 to 13 weeks of age from Charles River. Certain supplemental experiments were performed with C57BL/6J and CD-1 mice of both sexes.

Flow Assays

We used a microfluidic BioFlux System. Two batches of HUVECs (human umbilical vein endothelial cells) from the same donor were grown on BioFlux plates coated with fibronectin (100 µg/mL). Note that batches from different donors were used to test different inhibitory conditions. Histamine (10 or 100 µmol/L) and the fluorescein isothiocyanate-tagged VWF antibody (0.5 µg/mL) were combined with either vehicle (PBS), antivimentin antibodies (20 µg/mL), or recombinant A2 protein (50 µg/mL)[9] and perfused over the cells at shear stress 2.5 dyn/cm^2. VWF strings were visualized and analyzed as we described previously.[11]

VWF Strings in Pressurized Cerebral Arteries

Middle cerebral arteries (MCAs) and posterior cerebral arteries were isolated from WT and Vim KO mice, mounted on glass micropipettes in a vessel perfusion chamber and pressurized to 50 mm Hg in physiological salt solution (online-only Data Supplement). Histamine (100 µmol/L) was applied to the bath solution (5 minutes) to activate the endothelium and elicit VWF string formation. Strings were detected by anti-VWF antibody without permeabilization step. Further details are provided in Methods in the online-only Data Supplement.

Transient MCA Occlusion With Reperfusion Stroke Model

MCA with reperfusion was performed by introducing a silicone-coated monofilament to transiently occlude the MCA (Methods in the online-only Data Supplement). The monofilament occluder was in place for 30 minutes and reperfusion allowed for 120 minutes. Relative cerebral blood flow (rCBF) was measured from baseline to the end of the experiment. At 15 minutes before removing the occluder, either A2 protein (4 mg/kg, IV) or vehicle was injected based on predetermined randomization.

Measurement of rCBF by Laser Speckle Contrast Imaging

Laser speckle contrast imaging was performed through the intact skull over the parietal cortex to obtain uninterrupted rCBF from baseline, through occlusion, and during reperfusion (Methods in the online-only Data Supplement).

Statistical Analysis

Comparison of strings and bio-layer interferometry-binding assays were performed by Student *t* test or Welch *t* test, as appropriate. Analysis of laser speckle contrast imaging responses was performed by 2-way repeated measures ANOVA followed by Bonferroni correction. Data are presented as mean±SE. Analyses were performed with PRISM 7 (GraphPad, La Jolla, CA).

Results

We first investigated the role of vimentin in VWF string formation by utilizing intact pressurized cerebral arteries from WT and Vim KO mice. Histamine-stimulated endothelium from WT cerebral arteries demonstrated significant intraluminal strings that were identified directly by antibodies to VWF (Figure 1A). Notably, stimulated arteries from Vim KO showed significantly reduced string formation (Figure 1B). Strings were also detected in the intact brain following histamine stimulation as discrete linear accumulations of fluorescently labeled platelets (Figure I in the online-only Data Supplement). Similar to the pressurized artery preparation, stimulated mouse lung endothelial cells from Vim KO failed to form platelet-decorated VWF strings (Figure IIA in the online-only Data Supplement). Note that the expression of VWF is not affected by the lack of vimentin (Figure IIB and IIC in the online-only Data Supplement).

We examined the capacity of antivimentin antibodies or recombinant A2 domain (A2 protein) to inhibit the VWF/vimentin interaction on endothelium. Histamine-stimulated plated HUVECs demonstrated secreted VWF strings (Figure 2A) that aligned in the direction of superfusate flow. The presence of these strings was significantly diminished by incubating with either (1) antivimentin antibodies (Figure 2B) or (2) soluble A2 protein (Figure 2C). Together, these data affirm an important role for the VWF/vimentin interaction in string formation along the endothelial surface.

In a final series of experiments, we examined the role of the VWF/vimentin interaction in the reperfusion phase after ischemic stroke. Mice were randomly assigned to either vehicle (n=9) or A2 protein (n=9) treatment groups. Placement of the filament occluder within the circle of Willis produced similar reduction of blood flow (≈80% reduction) within the MCA territory between groups (Figure 3A). Laser speckle contrast imaging measurement of rCBF just before (point b in Figure 3A) or 10 minutes after vehicle or A2 protein administration (point c in Figure 3A) was not different (23.0±2.0% vs 24.6±2.2% rCBF at 15-minute occlusion, 22.9±1.9% vs 25.3±2.5% rCBF at 25-minute occlusion, respectively). On removal of the occluder, perfusion was restored in the MCA territory to peak value of 111.8±9.8% and 120.1±8.6% (*P*=0.53) in vehicle and A2 protein groups, respectively. The time to reach the peak of reperfusion was not significantly different, 14.0±2.4 vs 12.6±1.7 minutes, respectively (*P*=0.629). In both groups, perfusion declined spontaneously beginning around 20 minutes after recanalization—a phenomenon that is also noted clinically after thrombolysis or thrombectomy.[12] Notably, the A2 protein-treated group demonstrated less reduction of perfusion during the 120-minute reperfusion period

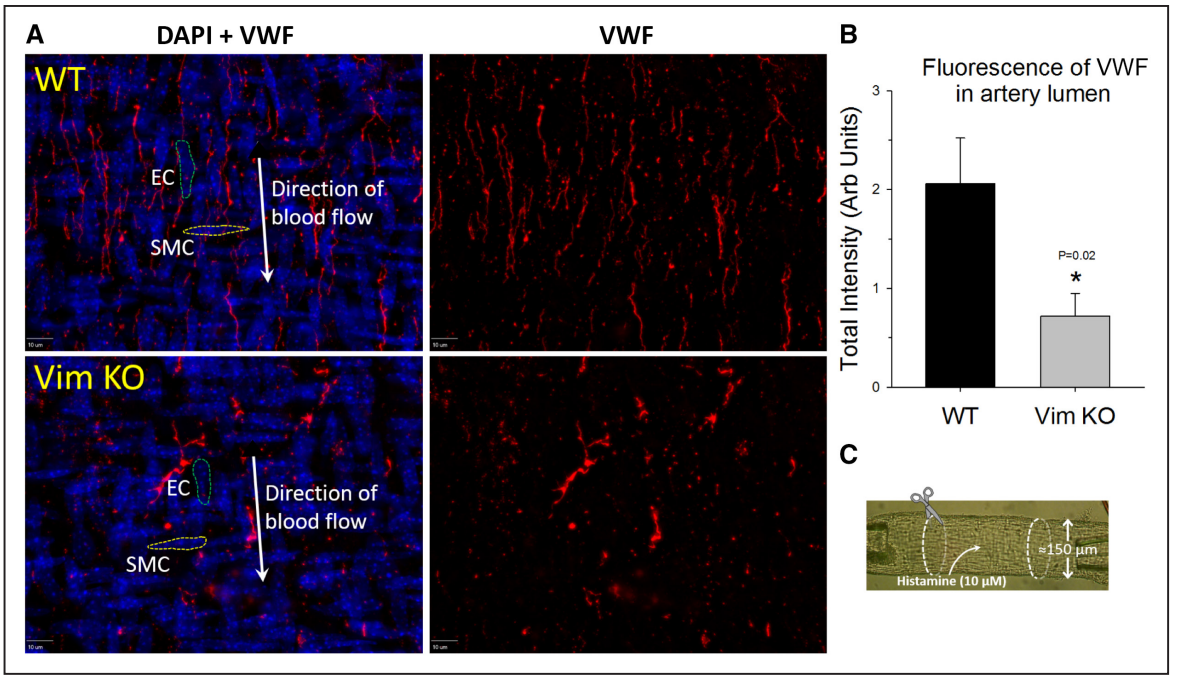

Figure 1. Demonstration of reduced VWF (von Willebrand factor) string formation in pressurized cerebral artery of Vim KO (vimentin knockout) mice compared with WT (wild type). **A**, VWF strings are detected by anti-VWF antibody (red). Endothelial and smooth muscle cell (SMC) nuclei are stained with 4',6-diamidine-2'-phenylindole dihydrochloride (DAPI; blue). The direction of blood flow is indicated by arrows parallel to long axis of endothelial cell (EC) nuclei. **B**, Summary data demonstrate a significant reduction in luminal VWF string formation in arteries of Vim KO mice. **C**, Representative isolated cerebral artery. **P*<0.05.

(*P*=0.031; 2-way RM-ANOVA). More regional analysis of the reperfusion response demonstrated significantly improved rCBF with A2 protein treatment within the core injury region (*P*=0.023) and the watershed region between the MCA and anterior cerebral artery territories (*P*=0.020; Figure 3). A2 protein treatment did not improve reperfusion within the ipsilateral anterior cerebral artery territory (*P*=0.147). Taken as a whole, these studies provide evidence for VWF string formation in the cerebrovasculature after endothelial activation and further demonstrate the potential to pharmacologically disrupt the adverse effect on blood flow after stroke through A2 protein administration in vivo.

Discussion

We present 3 major findings. First, we provide the first direct evidence for VWF strings in the cerebral circulation. Second,

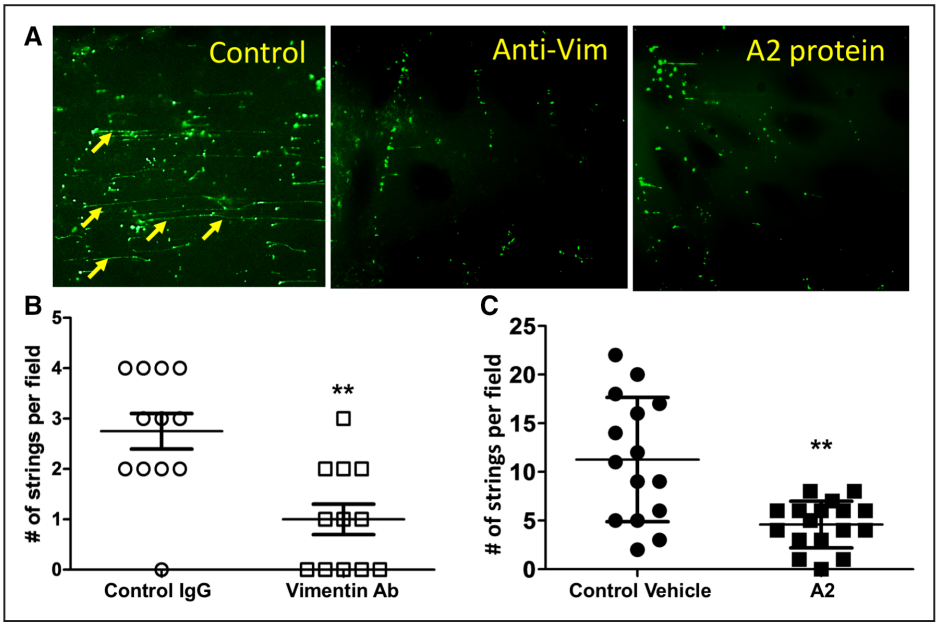

Figure 2. Histamine-stimulated VWF (von Willebrand factor) string formation is decreased in the presence of vimentin (Vim) antibodies or recombinant A2 protein. **A**, VWF strings are detected by fluorescein isothiocyanate-tagged VWF antibody. Images were analyzed for the number of VWF strings per field. Scatter plots demonstrate significantly reduced string formation in the presence of anti-Vim antibodies (20 µg/mL; **B**) or soluble recombinant A2 protein (50 µg/mL; **C**). Data represent the mean±SE of 12 or 15 different fields obtained during 2 perfusions using different batches of HUVECs. ***P*<0.01.

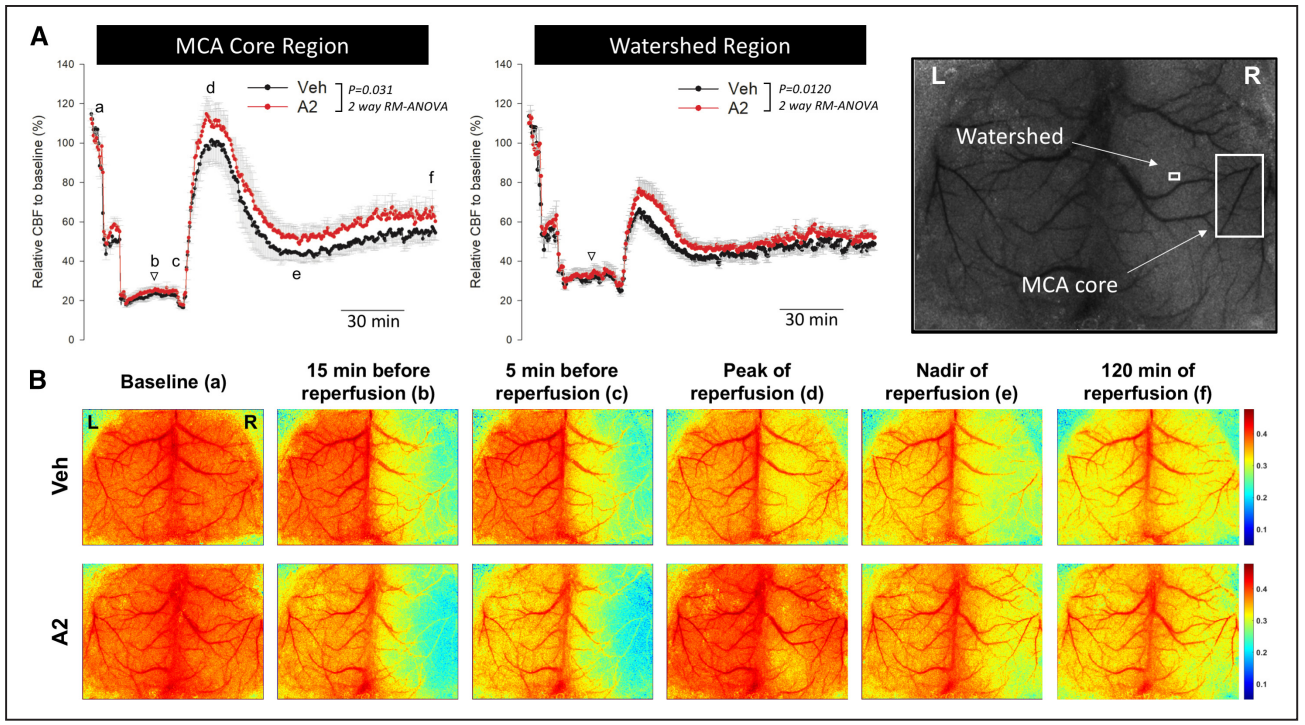

Figure 3. In vivo delivery of A2 protein improves brain reperfusion after ischemic stroke. **A**, Laser speckle contrast imaging before, during, and after Middle cerebral artery (MCA) occlusion from A2 protein and vehicle (Veh) -treated mice. Relative flow calculated using the inverse correlation time normalized to baseline. Treatment was administered 15 min before recanalization (open triangle). Summary traces reflect the MCA core region and the watershed region as indicated by the region of interest in the exemplar speckle contrast (SC) image. A2 protein treatment significantly improves reperfusion compared with Veh treatment (2-way RM-ANOVA group difference). **B**, Exemplar pseudocolored SC images representing different points of the experiment (corresponding to a–f in **A**) for both treatment groups. CBF indicates cerebral blood flow.

we demonstrate the critical role of extracellular endothelial vimentin in anchoring VWF strings through the A2 domain. Third, we provide evidence that pharmacologically disrupting the VWF/vimentin interaction can improve reperfusion after ischemic stroke.

The formation of VWF strings in pressurized cerebral arteries (Figure 1) along with the linear accumulation of discrete strings of platelets in the intact brain (Figure I in the online-only Data Supplement) demonstrates the potential and functional capacity of VWF strings to recruit platelets in the activated cerebrovasculature. Furthermore, these novel observations clearly support the possibility that VWF strings contribute to the pathology of stroke—a concept previously implied by others.[3] The rationale for this supposition is based on the known roles of VWF in platelet adhesion and thrombus formation, as well as in leukocyte adhesion and inflammatory cell recruitment. In stroke specifically, VWF KO mice (which lack capacity to produce VWF strings) demonstrated reduced injury after focal stroke.[13] In contrast, ADAMTS13 KO mice had greater stroke injury, whereas infusion of recombinant ADAMTS13 resulted in reduced stroke.[14] More recently, a study reported that low ADAMTS13 activity is associated with the risk of ischemic stroke in humans.[15] Given the potential for significant endothelial activation after stroke and accumulating evidence demonstrating impaired VWF string cleavage after oxidative modifications that are likely to occur during ischemia/reperfusion, we propose that VWF strings may play a significant role in stroke pathogenesis, particularly in the reperfusion phase.

Our current findings demonstrate a novel additional role for the exposed A2 domain[9] of newly released VWF strings, namely as a molecular binding site for vimentin at the endothelial cell surface (Figures III and IV in the online-only Data Supplement). This vimentin/A2 domain interaction seems to be critical for the adhesion of VWF strings in the vascular lumen. This role of vimentin in tethering VWF to the endothelial surface adds to our previous finding that vimentin in platelets acts as a ligand for VWF via the A2 domain.[9] Thus, delivery of A2 protein could conceivably attenuate platelet recruitment by both disrupting VWF string formation and the VWF/platelet interaction.

We explored the potential of targeting the vimentin/VWF interaction as a potential adjuvant for recanalization therapies, such as thrombolysis and thrombectomy. We used a mouse stroke model in which perfusion spontaneously declines in the reperfusion phase. This proof-of-concept experiment showed a clear beneficial effect of A2 protein treatment on the restoration of blood flow during the 2 hours of reperfusion. Although A2 protein did not fully eliminate the delayed hypoperfusion, it did produce significantly improved perfusion compared with vehicle control, suggesting that this effect is at least partially because of the effects of vimentin/VWF interactions, such as in VWF string formation. Future studies will be required to determine the resulting consequences of disrupting vimentin/VWF interaction on infarct volume and functional outcome.

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Disclosures

None.

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