ORIGINAL ARTICLE

Fibronectin binding to von Willebrand factor occurs via the A1 domain

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Abstract

Background: Collagen interactions with von Willebrand factor (VWF) perform an important role in initiation of hemostasis.

Objectives: We hypothesized that in addition to collagen, other extracellular matrix (ECM) proteins such as fibronectin can bind VWF.
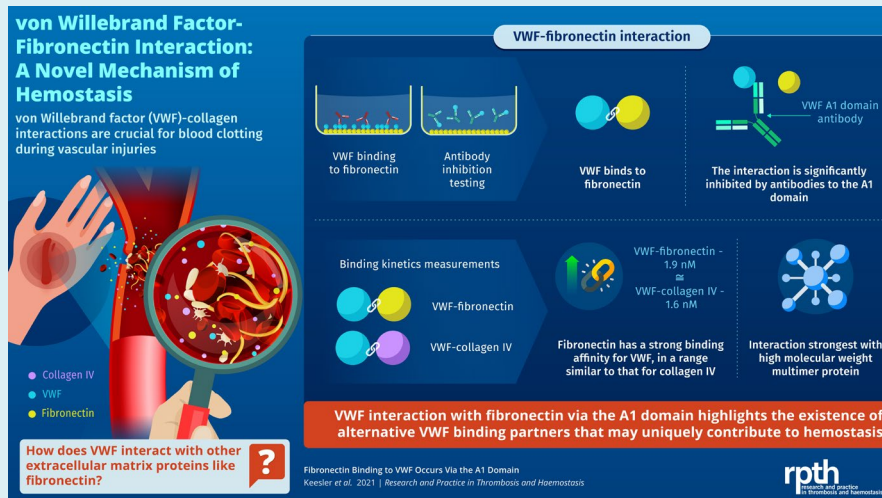
Methods: Fibronectin-VWF interactions were measured by ELISA using both plasma-derived and recombinant VWF–containing variants in specific domains. Inhibition was measured by antibody competition using antibodies directed against both VWF and fibronectin. Binding affinities were measured by the Octet Biosensor for fibronectin and collagen IV.

Results: Fibronectin was able to bind both plasma-derived and recombinant wild-type VWF. This interaction was inhibited by both anti-VWF antibodies and collagen types III and IV. Several VWF A1 domain variants in the region of the collagen IV binding site also demonstrated absent fibronectin binding, as did variants with defects in high-molecular-weight multimers. Binding affinity testing showed fibronectin has a strong affinity for VWF, in a range similar to that of collagen IV. Fibronectin binds VWF via a restricted region of the A1 domain. This interaction requires high-molecular-weight multimers and is similar to that seen with vascular collagens.

Conclusions: Therefore, VWF would appear to be the common factor linking platelet adhesion to various ECM proteins and facilitating hemostasis under conditions of ECM exposure.

von Willebrand Factor-Fibronectin Interaction: A Novel Mechanism of Hemostasis

von Willebrand factor (VWF)-collagen interactions are crucial for blood clotting during vascular injuries

KEYWORDS

collagen, extracellular matrix, fibronectins, hemostasis, von Willebrand factor

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Essentials

- Von Willebrand factor (VWF) binds to extracellular matrix protein fibronectin through the VWF A1 domain.
- This process is inhibited by antibodies to the VWF A1 domain.
- VWF binds to fibronectin with a similar affinity as its binding to collagen IV.
- The importance of this interaction in hemostasis remains unclear.

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1 | INTRODUCTION

Vascular endothelial injury exposes blood to the subendothelial extracellular matrix (ECM), which is primarily composed of proteoglycans and fibrous proteins. The major fibrous proteins that contribute to the ECM include collagens, elastins, fibronectins, and laminins.[1] Other contributors include thrombospondin and vitronectin.[2] ECM adhesive proteins play a crucial role in primary hemostasis during vascular injury by binding platelets and coagulation factors to the site of injury.[3,4] In addition, the ECM is critical for megakaryocyte function and platelet formation.[5,6]

Von Willebrand factor (VWF) is a multimeric coagulation factor that binds platelets to injured vascular endothelium. Several collagen-VWF interactions have been well categorized. Collagens I and III bind the VWF A1 and A3 domains of VWF,[7] and collagens IV and VI bind the VWF A1 domain.[8,9] Defects in the ability of VWF to bind vascular collagens have been implicated in von Willebrand disease (VWD).[9-12] Bleeding symptoms also occur with defects in collagen. Patients with Ehlers-Danlos syndrome have abnormal vascular collagen, which can lead to fragile capillaries, resulting in easy bruising and bleeding.[13]

While collagen is the most prominent ECM protein, there are several other critical proteins that contribute to vascular structure and function. Fibronectin is a key ECM component, with a plasma form synthesized in the liver and a cellular variant synthesized in specific cell types.[14] Platelet adhesion to collagen is impaired in the absence of fibronectin, leading to the conclusion that both fibronectin and VWF are required for optimal platelet adhesion to collagen.[15] In fact, some data suggest that fibronectin can actually substitute for VWF in collagen-induced platelet aggregation.[16] However, previous studies did not find evidence for a direct association of VWF and fibronectin.[17,18] It is important to note that these experiments examining the role of fibronectin under flow conditions also take into consideration the role of shear in thrombus formation.

Based on these data, we hypothesized that fibronectin could contribute specifically to hemostasis by binding VWF.

2 | METHODS

2.1 | VWF binding to fibronectin

VWF binding to plasma fibronectin was measured by ELISA. Amine binding maleic anhydride plates (Thermo Fisher Scientific, Waltham, MA, USA) were coated at 1 μg/mL with human purified plasma-derived fibronectin (Haematologic Technologies, Inc., Essex Junction, VT, USA) diluted in phosphate-buffered saline (PBS; pH 7.4) and incubated at 2 to 4°C overnight. The fibronectin-coated plates were treated with human VWF diluted in blocking buffer (PBS with 1% bovine serum albumin, pH 7.4) and incubated at room

temperature for 1 hour. At least two dilutions of VWF were tested for each sample. Plasma samples were loaded at 1:100 and 1:200, with concentrations of 50 to 100 IU/dL VWF. Recombinant samples were loaded at 1:20 and 1:40 with concentrations of 5 to 12 IU/dL. A combination of two monoclonal biotinylated anti-VWF antibodies (AVW-1 and AVW-15, mouse anti-human VWF, Versiti Blood Research Institute, Milwaukee, WI, USA) was used for detection of VWF fibronectin-binding complexes. Detection used streptavidin binding to alkaline phosphatase. With no international standard for fibronectin binding to VWF, an arbitrary value of 100 U/dL was used for comparison to a control plasma standard. Results were expressed as a ratio to VWF antigen (VWF:Ag) to compensate for any difference in VWF expression for the recombinant VWF preparations.

2.2 | VWF preparations

Plasma VWF was tested using platelet-poor plasma from healthy human donors. VWF-deficient plasma was obtained from patients with type 3 VWD and no detectable VWF:Ag. All plasma samples were collected into sodium citrate tubes and platelet-poor plasma generated by centrifugation. Samples were then frozen until use. Recombinant VWF was generated as previously described.[19] VWF variants were expressed in the pCIneo vector in HEK293T cells and supernatants collected for use in experiments. No additional treatment of the supernatants was performed. The recombinant VWF preparations were diluted in PBS for use in experiments. Preparations of purified VWF multimers of varying sizes including ultra-high-, high-, medium-, and low-molecular-weight multimers were the kind gift of Baxter Biosciences (Deerfield, IL, USA) and have been previously studied in our laboratory.[20,21] VWF concentrations for the multimer samples were 415, 815, 471, and 249 μg/mL. Fibronectin-binding results were normalized to the VWF:Ag for each multimer preparation. Recombinant human fibronectin (R&D Systems, Minneapolis, MN, USA) was also tested with healthy donor plasma, type 3 VWD plasma, and human recombinant 1399H constructs to confirm the results since the plasma fibronectin could potentially contain other contaminants.

2.3 | Antibody inhibitory testing

Antibodies were tested for their ability to inhibit VWF-fibronectin interactions. Inhibitory testing was done by ELISA, as described above, with variation in the VWF treatment step. The antibodies were diluted to 50 μg/mL in blocking buffer and added to the VWF solution. The fibronectin-coated plates were then treated with the VWF-antibody mixture at room temperature for 1 hour. Antibody testing included monoclonal anti-VWF antibodies (AVW-3 and AVW-5, mouse anti-human VWF, Versiti Blood Research Institute), monoclonal anti-fibronectin (mouse anti-human fibronectin, Thermo Fisher Scientific), and a polyclonal anti-VWF antibody (rabbit anti-human VWF; Dako, Glostrup, Denmark). ECM proteins were tested

to examine if they could inhibit binding of fibronectin to VWF. Human fibronectin, laminin (EMD Millipore, Billerica, MA, USA), thrombospondin (Haematologic Technologies, Inc.), and vitronectin (Haematologic Technologies, Inc.) were individually diluted to 10 μg/mL and incubated with VWF before plating. Inhibitory testing was also performed with human collagen type III (Southern Biotech, Birmingham, AL, USA) and human collagen type IV (Southern Biotech) at 25 μg/mL.

2.4 | Binding kinetics

Binding kinetics experiments were performed using the Octet Red 96 (ForteBio, Menlo Park, CA, USA), Amine Reactive Second Generation (AR2G) sensors, and reagents in the Amine Coupling Kit (ForteBio).[22] AR2G sensors were activated with the EDC+s-NHS mixture. Human type IV collagen (Southern Biotech), diluted to 5 μg/mL in pH 6 buffer, and human fibronectin, diluted to 10 μg/mL in pH 5 buffer, were immobilized and quenched to the AR2G sensors. Wild-type (WT) human recombinant VWF and recombinant VWF homozygous for the p.R1399H variant were diluted to various concentrations to measure the association and dissociation of the VWF analyte to the ECM ligands. Binding kinetics were
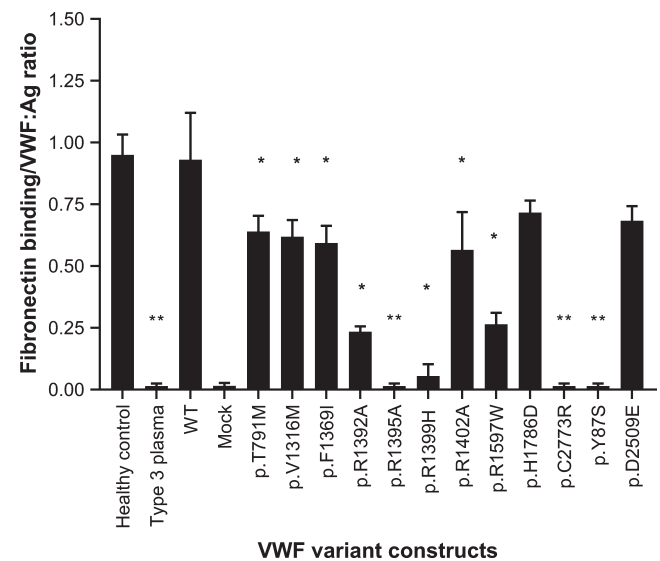
FIGURE 1 Plasma and recombinant VWF bind to fibronectin. Human fibronectin was captured on a plate and used to bind plasma VWF (healthy controls or subjects with type 3 VWD) or recombinant VWF. Decreased binding was noted for VWF constructs unable to form multimeric structures (2773R and 87S) as well as constructs with VWF A1 domain variants (1392A, 1395A, 1399H). Results are graphed as a ratio of VWF bound to fibrinogen over VWF antigen to account for minor differences in total protein. Error bars show 1 standard deviation. Results are average of ≥3 experiments. * signifies *P*<.05 and ** signifies *P*<.02 (plasma samples compared against each other, recombinant samples compared to WT recombinant VWF). VWD, von Willebrand disease; VWF, von Willebrand factor; VWF:Ag, von Willebrand factor antigen; WT, wild-type
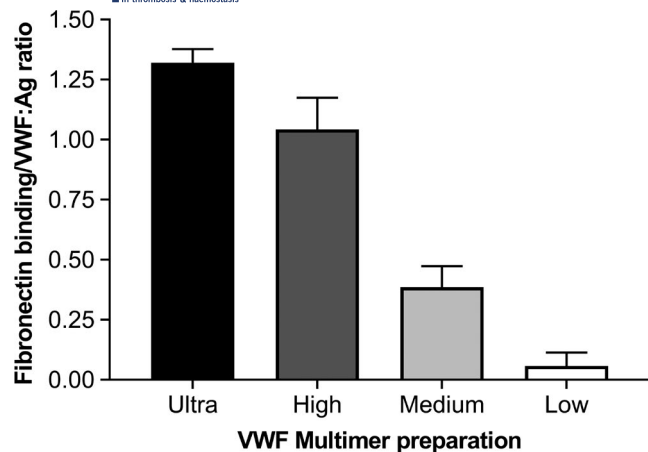
FIGURE 2 Fibronectin preferentially interacts with higher-molecular-weight VWF multimers. Binding of VWF to human fibronectin is shown using purified preparations of recombinant ultra-high-molecular-weight VWF multimers (ultra), high-molecular-weight VWF multimers (high), medium-molecular-weight VWF multimers (medium), and low-molecular-weight VWF multimers (low). Results are graphed as a fraction of WT recombinant VWF binding with all multimers present to normalize for the total amount of VWF present in each preparation. Error bars show 1 standard deviation. Results are average of ≥3 experiments. VWF, von Willebrand factor; VWF:Ag, von Willebrand factor antigen; WT, wild-type

measured using biolayer interferometry on the Octet. Association and dissociation curves were then exported to the ForteBio Data Analysis software (version 9.0) for analysis. Since collagen IV and fibronectin were the ligands and VWF was the analyte, a 1:1 binding model analysis was done on the interaction of the proteins.[22]

3 | RESULTS

3.1 | VWF binding to fibronectin

Plasma samples from healthy human controls and subjects with type 3 VWD were examined to characterize fibronectin binding in human subjects (Figure 1). Results for each sample were compared to wild-type (WT) recombinant VWF binding to fibronectin. In addition, several VWF constructs containing specific variants at different ligand-binding sites were also examined in terms of their binding to plasma-derived fibronectin. Variants were chosen to represent specific VWF functional defects. The p.T791M variant removes factor VIII binding (type 2N); p.V1316M contains a gain-of-function platelet-binding variant (type 2B); p.F1369I decreases glycoprotein Ib binding (type 2M); p.R1392A, p.R1395A, p.R1399H, and p.R1402A all affect collagen IV binding; p.R1597W results in decreased VWF binding and decreased high-molecular-weight multimers (type 2A); p.H1786D removes type III collagen binding; p.C2773R and p.Y87S remove C terminal dimerization and N-terminal multimerization, respectively; and the p.D2709E variant removes αIIbβ3 binding. These variants were included to observe
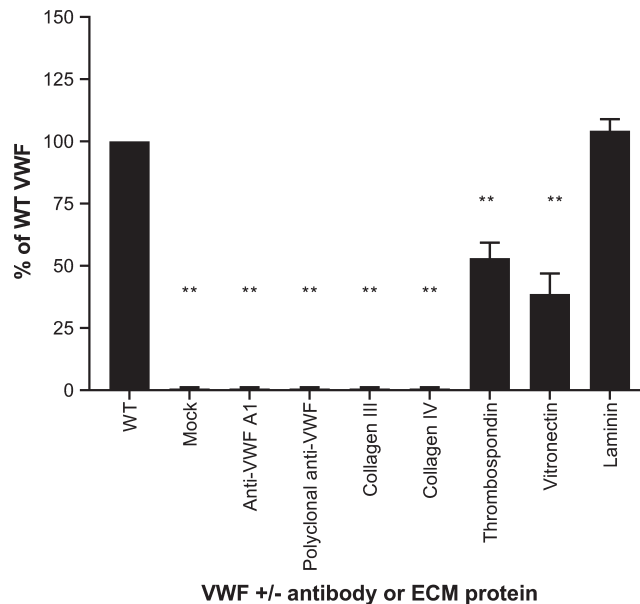
FIGURE 3 Inhibition of VWF-fibronectin interactions. VWF binding to human fibronectin was measured alone (WT), and in the presence of anti-VWF antibody AVW-3, which blocks the A1 domain (anti-VWF A1), a polyclonal VWF antibody (polyclonal anti-VWF), collagen type III and IV, and extracellular matrix proteins thrombospondin, vitronectin, and laminin. Results are graphed as a percent of WT VWF bound to fibronectin in the absence of antibody. Error bars show 1 standard deviation. Results are average of three or more experiments. ** signifies $P < .01$. ECM, extracellular matrix; VWF, von Willebrand factor; VWF:Ag, von Willebrand factor antigen; WT, wild-type

the behavior of VWF binding to fibronectin in different types of VWD. The VWF variants tested are mapped in Figure S1. Both normal plasma and WT recombinant VWF exhibited binding to fibronectin. A sample standard curve for the normal control plasma is shown in Figure S2. VWF binding to fibronectin was absent for type 3 VWD samples, although it should be noted that the type 3 samples serve here as a negative control. Since there was no detectable VWF in the samples, no binding would be expected. Recombinant fibronectin showed similar results as the plasma-derived fibronectin (data not shown). All experiments were repeated a minimum of three times.

3.2 | Localization of the fibronectin-binding domain in VWF

To determine the domain of VWF responsible for the VWF interaction with fibronectin, several recombinant VWF constructs containing amino acid sequence variants were tested (Figure 1). Two single-nucleotide A1 domain sequence variants (p.R1395A and p.R1399H) had reduced or undetectable binding of VWF to fibronectin. A sequence variant in the VWF A3 domain known to disrupt VWF binding to collagen III (p.H1786D) but with preserved multimer formation resulted in binding of 70% that seen

TABLE 1 Binding affinities of VWF for collagen IV and fibronectin

ECM protein	VWF concentration (µg/mL)	K_D (×10^{-9})	R^2
Collagen IV	2.5	2.25	0.80
	1.25	1.78	0.85
	0.63	2.01	0.88
	0.31	0.87	0.84
	0.16	1.06	0.86
	0.08	2.11	0.90
	0.04	1.40	0.81
Fibronectin	2.75	1.63	0.67
	1.38	1.62	0.73
	0.69	2.62	0.80
	0.34	1.75	0.73
	0.17	1.36	0.71
	0.00	1.69	0.73
	0.04	2.61	0.71

Abbreviations: ECM, extracellular matrix; K_D, dissociation constant; VWF, von Willebrand factor.
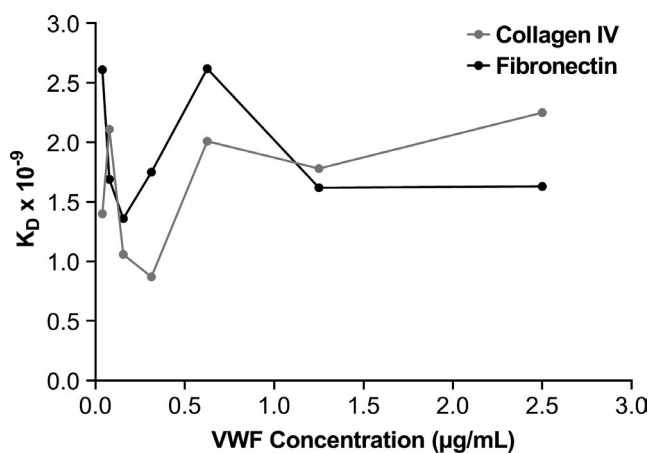
FIGURE 4 VWF binding to fibronectin and collagen IV. VWF binding to human fibronectin (black) and human collagen IV (gray) was measured using the Octet Biosensor to determine binding affinities. The x axis shows varying VWF concentrations from 0 to 3 µg/mL, and the y axis shows the K_D in nM. K_D, dissociation constant; VWF, von Willebrand factor

with normal VWF. Similar results were seen with other variants outside the VWF A1 domain, including p.T791M (64%) and p.D2509E (68%). Two single-nucleotide sequence variants (p.Y87S and p.C2773R) known to disrupt multimer formation showed no binding to fibronectin. An A2 domain variant (p.R1597W) that has a mild effect on multimer formation showed a moderate decrease in VWF binding to fibronectin.

VWF fragments of varying multimerization levels were tested to confirm that the VWF-fibronectin interaction was multimer size dependent (Figure 2). Fibronectin binding paralleled the level of multimerization, with highest binding noted for the ultra-high-molecular-weight multimers and lowest binding observed for the low-molecular-weight multimers. A multimer gel of the different

preparations is shown in Figure S3. Taken together, these results suggest that the binding of VWF to fibronectin is multimer dependent.

3.3 | Inhibition of VWF binding to fibronectin

Anti-VWF antibodies were tested for inhibition of fibronectin binding (Figure 3). Results for each sample were examined as a fraction of WT recombinant VWF binding to fibronectin without any inhibitory antibody present. Rabbit polyclonal anti-VWF (Dako) inhibited 99% of fibronectin binding. Among the monoclonal antibodies tested, AVW-3, an A1 domain–specific antibody, also resulted in significant inhibition of VWF binding to fibronectin. Collagen III and collagen IV resulted in nearly complete inhibition, while thrombospondin and vitronectin protein resulted in partial inhibition of VWF binding (Figure 3) of 40% and 44%, respectively.

3.4 | Binding kinetics results

Serial dilutions of recombinant WT VWF were bound to fibronectin and collagen IV. Dissociation constant (K_D) and coefficient of determination (R^2) were measured for each concentration (Table 1). Binding of WT VWF at 2.75 µg/mL to fibronectin had a K_D of 1.63 nM with an R^2 of 0.671. Figure S4 shows a representative binding curve. Binding of fibronectin across all WT VWF concentrations had a K_D ranging between 1.36 and 2.62 nM (Figure 4) with R^2 ranging between 0.671 and 0.803. The average K_D for VWF binding to fibronectin was 1.9 nM.

Binding of WT VWF at 2.5 µg/mL to collagen IV had a K_D of 2.25 nM with an R^2 of 0.941. Binding of collagen IV across all WT VWF concentrations had a K_D ranging between 0.873 and 2.25 nM

(Figure 4), with R^2 ranging between 0.890 and 0.981. The average K_D for VWF binding to collagen IV was 1.6 nM.

4 | DISCUSSION

Collagen is the most abundant ECM protein.[23] The effects of VWF binding to collagen have been well studied, and the importance of high molecular weight multimers in VWF activity is well known.[24,25] Our data show that fibronectin plays a role in binding VWF, and is also multimer size dependent. Collagen III has been shown to have a K_D of 8 nM to the VWF A1 domain, similar to the K_D of 6 nM seen with collagen I.[26] Factor VIII binding to VWF has a much lower K_D, reported as 0.32 nM.[27] Laminin has a reported K_D of 524 nM.[28] When we examined binding of VWF to fibronectin, we found a K_D of 1.9 nM (1.36– 2.62). The affinity of VWF to collagen IV was in a similar range at 1.6 nM (0.873–2.25).

The interaction of VWF with fibronectin appears to be localized to a specific portion of the A1 domain of VWF. Collagen IV binding is abrogated with sequence variants in amino acids 1392, 1395, 1399, and 1406.[9] While there is overlap with the collagen IV binding domain, the fibronectin-binding domain appears to be a more restricted region, as binding to VWF was only significantly affected by sequence variants in amino acids 1395 and 1399. Fibronectin may provide an alternate substrate to bind VWF at sites of injury and bring platelets in close proximity to both VWF and factor VIII. There may be physiological reasons for the restricted binding site, or this may represent an area of VWF most readily exposed to other binding partners.

Fibronectin has recently been reported to be important in the pathophysiology of heparin-induced thrombocytopenia.[29] Fibronectin, and to a lesser extent vitronectin, inhibited platelet factor 4–heparin complex binding to platelets.[29] These results demonstrate the importance of cross-talk between platelets and the ECM.

Previous data have shown that VWF does not inhibit fibronectin binding to collagen I, suggesting a slight difference in binding sites.[30] However, because collagen is helical in nature, this could also reflect binding of VWF at different locations on the collagen protein. In addition, this study looked at fibronectin binding to collagen as the end point, not fibronectin binding to VWF. Our study used amine binding as a method of localization for the fibronectin, which could also contribute to the difference in results. Our data also reflect that of Houdijk and colleagues,[15,31] who showed that fibronectin was critical in thrombus formation. Additional mouse studies have shown that decreased plasma fibronectin results in a delay in thrombus formation.[32] Plasma-derived fibronectin also appears to function earlier than, and independent of, VWF.[33]

Limitations of this study include the use of plasma-derived fibronectin from a single source for most of the results. Although similar results were obtained with recombinant proteins, we cannot exclude the possibility that different fibronectins have different behavior and that fibronectin in vivo may differ from the preparations used in these experiments. In addition, cellular-derived fibronectin may have different properties to the plasma-derived fibronectin investigated here. While direct binding affinities were assessed, the results may vary under different flow conditions in vivo. Nevertheless, there was clear binding between WT VWF and fibronectin, suggesting that this interaction does play a role in hemostasis.

Our data show that VWF binds to fibronectin, with affinity just slightly lower than VWF's affinity for collagen. Since fibronectin is a crucial component of the ECM, having VWF able to interact directly with exposed fibronectin may help with cessation of bleeding upon injury. The ability of VWF to bind fibronectin directly offers a view of alternative binding partners that may represent a unique contribution to hemostasis. In addition, variants in fibronectin could also potentially contribute to bleeding and/or thrombosis and provide a new phenotype to explore.

RELATIONSHIP DISCLOSURE

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

DAK performed experiments, wrote the manuscript, and assisted in research design. TLS performed experiments, assisted in research design, and edited the manuscript. CEK performed experiments and assisted in research design. CWS and SLH performed experiments and edited the manuscript. VHF supervised experiments, designed the research, and edited the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.