



Targeting shear gradient activated von Willebrand factor by the novel single-chain antibody A1 reduces occlusive thrombus formation *in vitro*

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ABSTRACT

Intraluminal thrombus formation precipitates conditions such as acute myocardial infarction and disturbs local blood flow resulting in areas of rapidly changing blood flow velocities and steep gradients of blood shear rate. Shear rate gradients are known to be pro-thrombotic with an important role for the shear-sensitive plasma protein von Willebrand factor (VWF). Here, we developed a single-chain antibody (scFv) that targets a shear gradient specific conformation of VWF to specifically inhibit platelet adhesion at sites of shear rate gradients (SRG) but not in areas of constant shear. Microfluidic flow channels with stenotic segments were used to create SRG during blood perfusion. VWF-GPIIb/IIIa interactions were increased at sites of SRG compared to constant shear rate of matched magnitude. The scFv-A1 specifically reduced VWF-GPIIb/IIIa binding and thrombus formation at sites of SRG but did not block platelet deposition and aggregation under constant shear rate in upstream sections of the channels. Significantly, the scFv A1 attenuated platelet aggregation only in the later stages of thrombus formation. In the absence of shear, direct binding of scFv-A1 to VWF could not be detected and scFv-A1 did not inhibit ristocetin induced platelet agglutination. We have exploited the pro-aggregatory effects of SRG on VWF dependent platelet aggregation and developed the shear gradient-sensitive scFv-A1 antibody that inhibits platelet aggregation exclusively at sites of SRG. The lack of VWF inhibition in non-stenosed vessel segments places scFv-A1 in an entirely new class of anti-platelet therapy for selective blockade of pathological thrombus formation while maintaining normal hemostasis.

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Introduction

Atherothrombotic events precipitating myocardial infarction and ischemic stroke are the most common causes of death worldwide.¹ These events are triggered by intraluminal plaque rupture causing an inappropriate platelet response which leads to occlusion of the vessel lumen.² As a preventative measure, prescribed anti-platelet drugs such as aspirin and clopidogrel target major platelet activation pathways such as the TXA₂- and ADP-P2Y₁₂-pathways, thereby reducing platelet reactivity.³ While this is a clinically proven strategy to reduce cardiovascular events, this approach can also cause serious side effects, most notably bleeding, as the targeted pathways also play a critical role in normal hemostasis thereby partly offsetting the benefits gained from these drugs.^{4,6} Thus, a strategy to uncouple thrombosis from hemostasis to target one without the other is an urgent unmet clinical need.

Platelet aggregates formed during a hemostatic response typically do not grow

beyond 50% of the vessel lumen^{7,8} a restriction that is insufficient to markedly change the shear rates at the site of injury.⁹ However, studies into the hemodynamic regulation of thrombus formation have led to the concept that rapidly changing shear conditions at the site of a cardiovascular insult contribute significantly to the pathological thrombotic response.^{10,11} Plaque rupture with its release of highly thrombogenic content may cause the formation of a large thrombus that reduces the vessel lumen which in turn causes local steep increases in blood shear rates, also termed shear rate gradients (SRG). In recent years, the effects of SRG flow patterns on platelet reactivity and von Willebrand factor (VWF) unfolding and activation have been increasingly studied.¹¹⁻¹³ VWF is a large blood-borne protein critical to platelet adhesion in areas of high shear such as in arterioles or larger arteries with luminal constriction. In normal circulation, VWF has a globular conformation, which unfolds under high shear stress thereby exposing binding epitopes, most importantly the platelet GPIIb α binding domain A1. This unfolding of VWF is referred hereafter as VWF activation.

It is thought that SRG are one to two orders of magnitude more efficacious in unfolding VWF, compared to constant shear.^{14,15} Indeed, we and others have previously demonstrated that SRG strongly unfold and subsequently activate VWF and that shear gradient dependent platelet aggregation mechanisms promote arterial thrombosis in a VWF-dependent fashion.^{15,16}

Given the central role of VWF in platelet adhesion at arterial shear rates, and even more so under pathological flow conditions (i.e., SRG), we hypothesized that activated (i.e., unfolded) VWF is a potential target for a new class of anti-thrombotic therapy.

Here we investigated the effect of SRG on VWF-mediated platelet adhesion and thrombus formation. Moreover, we report on the generation of the single-chain antibody (scFv) A1, designed to target shear activated VWF. Here, we developed a scFv based on the sequence of parent immunoglobulin (Ig) G antibodies raised against the monomeric 39/34-Kd fragment of VWF which encompasses the VWF-A1 domain and a proximal N-terminal sequence to VWF-A1. Our scFv targets a shear gradient specific conformation of VWF to inhibit VWF-platelet binding specifically at sites of SRG but not in areas of constant shear.

This antibody specifically inhibits VWF-platelet interactions at pathological shear but not under physiological shear regimens and may therefore form the basis of a new and safer class of anti-thrombotic therapy.

Methods

A more detailed description of the methods is published in the *Online Supplementary Appendix*.

Single-chain antibody generation

A single-chain antibody was designed by combining the variable regions of the light and heavy chains of two selected antibodies from a panel of parent IgG antibodies that we raised against a 34/39 kDa fragment of VWF.¹⁷

Blood sampling

Blood samples were collected in trisodium citrate (0.32% weight/volume [w/v] final) and immediately processed for further

use. The study was approved by the Alfred Hospital's (Melbourne, Australia) ethics committee no 67/15.

Microfluidic chips and *in vitro* flow perfusions

In-house designed polydimethylsiloxane (PDMS) microfluidic chips (Figure 1A) contained channels of 52 μm height with a semi-circular stenotic section, creating 80% lumen reduction. Chips were manufactured as previously reported.¹⁶ Citrated whole blood was incubated with DiOC₆ (0.5 $\mu\text{g mL}^{-1}$) (Sigma-Aldrich) and drawn through the microfluidic channels by a programmable syringe pump (Legato 130, KD Scientific) over a coated surface of 100 $\mu\text{g/mL}$ collagen type III or type I.

Computational fluid dynamics and calculations

Fluid dynamics were simulated with a COMSOL Multiphysics 4.2 laminar flow module applied to three-dimensional meshes. For all simulations, no-slip boundary conditions and Newtonian fluids were assumed. Both the computational fluid dynamical simulation and the calculations of fluid dynamical and geometrical parameters were carried out with the assumption that the channels and vessels were filled with Newtonian fluids. This assumption is valid because the shear rates are in a regime in which the non-Newtonian behavior of blood is limited.¹⁸

Platelet agglutination

Platelet agglutination in response to 0.75 mg mL^{-1} ristocetin (Helena Biosciences, Gateshead, UK) was performed for 10 minutes (min) on an AggRam system from Helena Laboratories, USA. Isolated platelets (150 or 300 μL at $3 \times 10^8/\text{mL}$) and platelet poor plasma (50 μL) were incubated with 2, 2.5, 4, 5, 40 and 80 $\mu\text{g mL}^{-1}$ scFv A1.

Western blot

Denatured full-length VWF (Mybiosource, USA) was electrophoresed at 0.1, 10, 100 ng and 1 μg using 5% SDS-polyacrylamide gel at 125 V for 90 min. The protein was transferred onto PVDF membrane at 90 V for 90 min followed by incubation with 1 $\mu\text{g mL}^{-1}$ scFv A1 overnight and the membrane was probed with horse-radish peroxidase (HRP)-conjugated detection antibody (1:2,500, Sigma). Later, the membrane was stripped and reprobed with 1 $\mu\text{g mL}^{-1}$ polyclonal sheep anti-VWF antibody (Abcam), followed by donkey anti-sheep HRP-conjugated secondary antibody (1:2,500, R&D Systems, USA).

Enzyme-linked immunosorbent assay

Purified full-length VWF or isolated A1 domain was coated at 0.1, 1 and 10 $\mu\text{g mL}^{-1}$ in the presence of ristocetin (0.75 mg mL^{-1}) and blocked with 2% bovine serum albumin followed by incubation with 5 $\mu\text{g mL}^{-1}$ of scFv A1 or sheep anti-VWF antibody and detection by HRP-conjugated secondary antibody

BLItz

Bio-layer interferometry was performed on a BLItz System (Pall Forte Bio, USA) using protein-A biosensors tagged with VWF-A1 domain (130 $\mu\text{g mL}^{-1}$). Remaining binding sites were blocked by exposure to a polyclonal mouse IgG for 5 min. Sequential adhesion of scFv-A1 at 0, 20, 40 and 100 $\mu\text{g mL}^{-1}$ was monitored for 5 min per concentration.

Results

Shear rate gradients activate von Willebrand factor (VWF) and reduce platelet rolling velocity on VWF/collagen type III

As previously reported¹⁶ exacerbated thrombus forma-

tion at atherosclerotic geometries is dependent on the degree of intraluminal sidewall protrusion which creates SRG. Thus, to elucidate the underlying mechanism of exacerbated thrombus formation under SRG, platelet adhesion and VWF-activation were studied at sites of SRG utilizing a microfluidic platform. These channels exhibit a stenotic feature which resembled rheological parameters in larger carotid arteries in humans (i.e., slope of stenosis, wall shear rates at the inlet versus stenotic region, and maximal flow elongational rate) while keeping required blood volume low.¹⁶ Channels incorporated a semi-circular stenotic geometry of 600 μm in length to reduce the chan-

nel width by 80 % from 300 μm to 60 μm (Figure 1A). Computational fluid dynamic analysis indicated that the wall shear rate was symmetrically distributed around the stenosis, with an 8-fold higher shear rate in the apex region compared with the pre-stenotic segment. At an input wall shear rate of 300 s^{-1} , platelets traveling through the stenosis are therefore exposed to an increase in shear rate to 2,400 s^{-1} and a shear gradient (Δs^{-1}) of approximately 7.1 $\text{s}^{-1}\mu\text{m}^{-1}$ followed by an identical shear decrease in the outlet of the stenosis (Figure 1B). Platelet rolling velocities, a measure of VWF-GPIIb bond strength, were reduced at SRG (assessed in a 140 μm zone around the

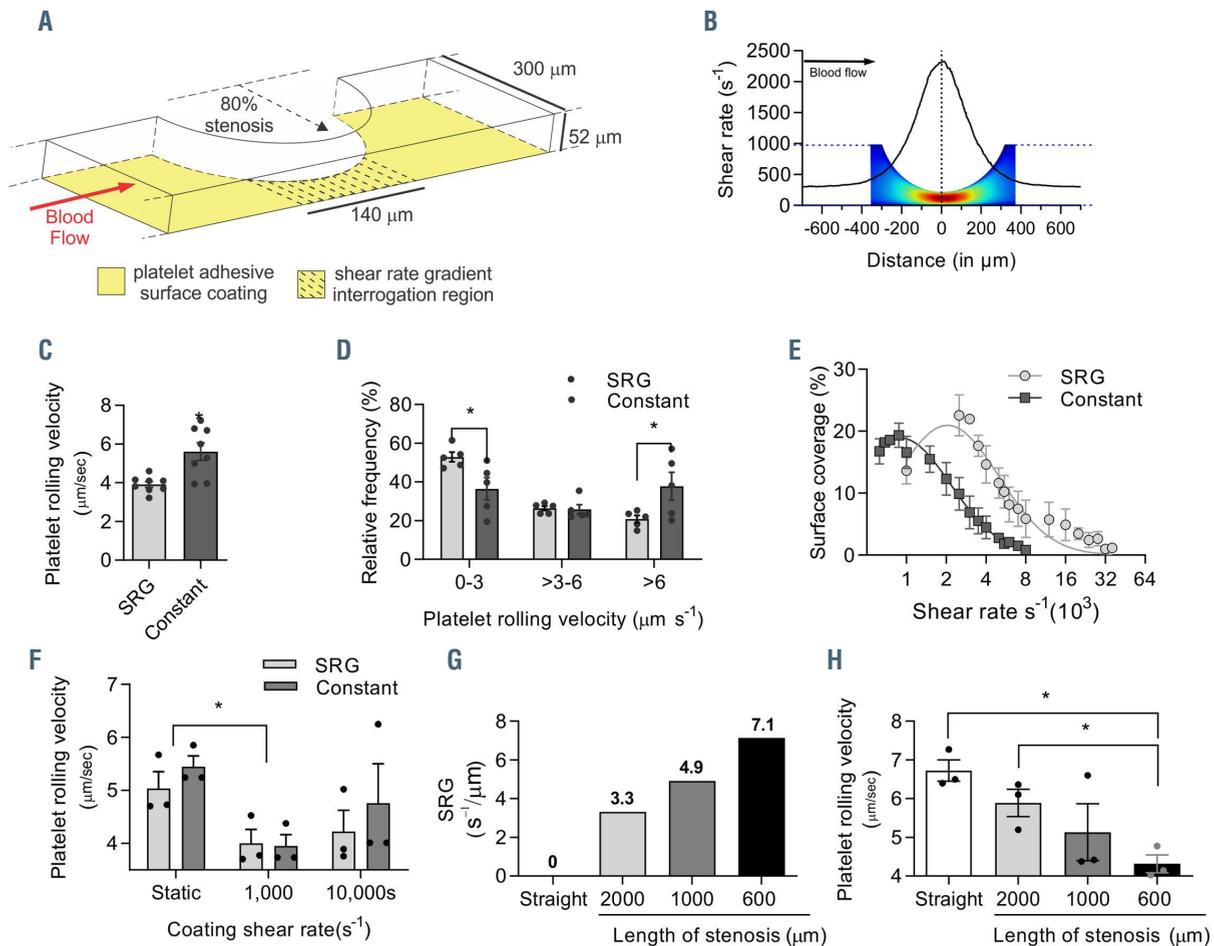


Figure 1. Shear rate gradients increase von Willebrand factor-GPIIb bond strength and reduce platelet rolling velocity. (A) Complex flow dynamics were studied in microfluidics exhibiting a semi-circular stenotic site with a diameter of 600 μm which reduced the channel width from 300 μm to 60 μm . (B) Computational fluid dynamics identified the stenosis apex as a zone with an 8-fold increase in shear compared to the inlet, followed by a negative but equal drop in shear rate in the stenosis outlet. (C) Abciximab-treated (20 $\mu\text{g mL}^{-1}$), citrated whole blood, labelled with 0.5 $\mu\text{g mL}^{-1}$ DiOC₆ was perfused over a collagen III-coated surface to promote binding of blood borne von Willebrand factor (VWF) under flow and subsequent platelet adhesion. Input shear rate was set to 300 s^{-1} which then increased to 2,400 s^{-1} in the apex of the stenosis. Control channels with straight walls were used to generate a constant shear rate of 2,400 s^{-1} . Data represented as mean \pm standard error of them mean; n=8; * P <0.05 (unpaired t-test). (D) Platelet rolling velocity on VWF-collagen III was assessed at shear rate gradients (SRG) (platelets rolling through the apex of the stenotic site) or constant shear (upstream of the stenotic site) using particle tracking software. Platelet rolling velocities were divided into three bins (0-3 $\mu\text{m s}^{-1}$; >3-6 $\mu\text{m s}^{-1}$; >6 $\mu\text{m s}^{-1}$) and presented as relative frequencies. Data represented as mean \pm standard error of the mean (SEM); n=5; * P <0.05 (multiple unpaired t-tests). (E) Surface coverage of abciximab-treated, citrated whole blood, labeled with DiOC₆ perfused over a collagen III-coated surface. Input shear rates ranged from 125 s^{-1} up to 8,000 s^{-1} for stenotic channels, resulting in peak shear rates of 1,000 s^{-1} to 36,000 s^{-1} in the apex of the stenosis; or matched shear rates in a parallel control channel exhibiting a constant shear rate throughout. (F) Collagen type III-coated channels were incubated either statically or perfused at 1,000 s^{-1} or 10,000 s^{-1} with platelet poor plasma in the absence of SRG to allow VWF adhesion to collagen. Abciximab-treated, citrated whole blood, labeled with DiOC₆ was perfused over the differentially coated collagen III/VWF matrix as described in (B). Platelet rolling velocity on VWF/collagen III was assessed at SRG (platelets rolling through the apex of the stenotic site) or constant shear (upstream of the stenotic site) for all three coating regimens using particle tracking software. Data represented as mean \pm standard error of the mean (SEM); n=3; * P <0.05 (multiple unpaired t-tests) (G) Abciximab-treated, citrated whole blood, labeled with DiOC₆ was perfused over a collagen III matrix, through 300 μm wide channels incorporating semi-circular stenotic segments which reduced the channel width to 60 μm . The diameters of semi-circular stenotic elements ranged from 600 μm and 1,000 μm to 2,000 μm . Whereas Δ shear of all three stenotic segments was equal, levels of elongational flow differed. (H) Platelet rolling velocity on VWF-collagen III was assessed the same conditions as in (C) under SRG (platelets rolling through the apex of the stenotic site) or constant shear (upstream of the stenotic site) using particle tracking software. Data represented as mean \pm SEM; n=3; * P <0.05 (Dunnett's multiple comparison one-way ANOVA).

apex of the stenosis with a maximal shear rate of $2,400 \text{ s}^{-1}$ compared to matched constant wall shear rate of $2,400 \text{ s}^{-1}$ ($3.9 \pm 0.2 \text{ s}^{-1}$ vs. $5.4 \pm 0.7 \text{ s}^{-1}$) (Figure 1C), with $52.9 \pm 2.5\%$ of slow-rolling platelets at SRG compared to $36 \pm 5.7\%$ at constant shear (Figure 1D). This finding indicates that shear rate gradients reduce platelet translocation, presumably through the unfolding of the VWF-A1 domain leading to stronger VWF-GPIb interactions. In order to confirm this finding, platelet surface coverage, a measure of VWF-platelet interaction was measured in stenotic channels over a wide range of input shear rates ranging from 125 s^{-1} to $8,000 \text{ s}^{-1}$, resulting in shear rates of $1,000 \text{ s}^{-1}$ to $36,000 \text{ s}^{-1}$ in the stenosis apex and SRG of approximately $3 \mu\text{m s}^{-1}$ to $1,000 \mu\text{m s}^{-1}$. SRG facilitated higher levels of transient platelet adhesion, with maximal platelet adhesion of $20.1 \pm 3.4\%$ at $2,500 \text{ s}^{-1}$ and sustained platelet adhesion up to $7,000 \text{ s}^{-1}$, ($7.89 \pm 1.86\%$; $P=0.0462$) while platelet adhesion rapidly decreased under constant shear from a maximum of $19.3 \pm 1.9\%$ at 875 s^{-1} and was abolished above $2,000 \text{ s}^{-1}$ ($13.6 \pm 2.0\%$; $P=0.0341$) (Figure 1E). In order to elucidate the parameters critical for platelet-VWF interaction under SRG, VWF deposition onto collagen type III was tested under either static conditions or under flow in the absence of SRG. Following VWF deposition onto collagen, the coated strips were overlaid with micro-channels featuring straight or stenotic sections. Platelet rolling velocities at SRG or constant shear

rate (CSR) were assessed at an effective wall shear rate of $2,400 \text{ s}^{-1}$. The rolling velocity of platelets on collagen strips that were statically incubated with VWF was higher (SRG: $5.03 \pm 0.31 \mu\text{m s}^{-1}$; CSR: $5.44 \pm 0.2 \mu\text{m s}^{-1}$) than those in channels incubated with VWF at a shear rate of $1,000 \text{ s}^{-1}$ (SRG: $4 \pm 0.26 \mu\text{m s}^{-1}$; CSR: $3.95 \pm 0.21 \mu\text{m s}^{-1}$, $P<0.05$) (Figure 1F) indicating a role for flow in VWF deposition to collagen. However, when VWF was bound to collagen before platelet perfusion, the SRG specific increase of the GPIb α -VWF bond strength was lost (Figure 1F), suggesting that VWF deposition needs to occur under real-time SRG to have subsequently increased engagement of GPIb α .

In order to test the effect of the steepness of the stenosis gradient on platelet rolling velocity, channels containing different stenotic segment lengths were manufactured. These ranged from 600 to $2,000 \mu\text{m}$ in length, resulting in the same peak shear rate in the apex, but different SRG, ranging from $7.1 \text{ s}^{-1} \mu\text{m}^{-1}$ to $3.2 \text{ s}^{-1} \mu\text{m}^{-1}$ (Figure 1G). Interestingly, platelet rolling velocities correlated with the magnitude of SRG. Rolling velocities were negatively correlated with the stenosis gradient, producing the slowest rolling velocity ($4.32 \pm 0.23 \mu\text{m s}^{-1}$) when traveling through a $600 \mu\text{m}$ stenosis and the highest ($5.89 \pm 0.35 \mu\text{m s}^{-1}$) when traveling through a $2,000 \mu\text{m}$ stenosis. However, the rolling velocity in the absence of SRG (i.e., under constant shear) was still higher ($6.72 \pm 0.28 \mu\text{m s}^{-1}$) (Figure 1H).

In order to confirm that previously observed platelet-

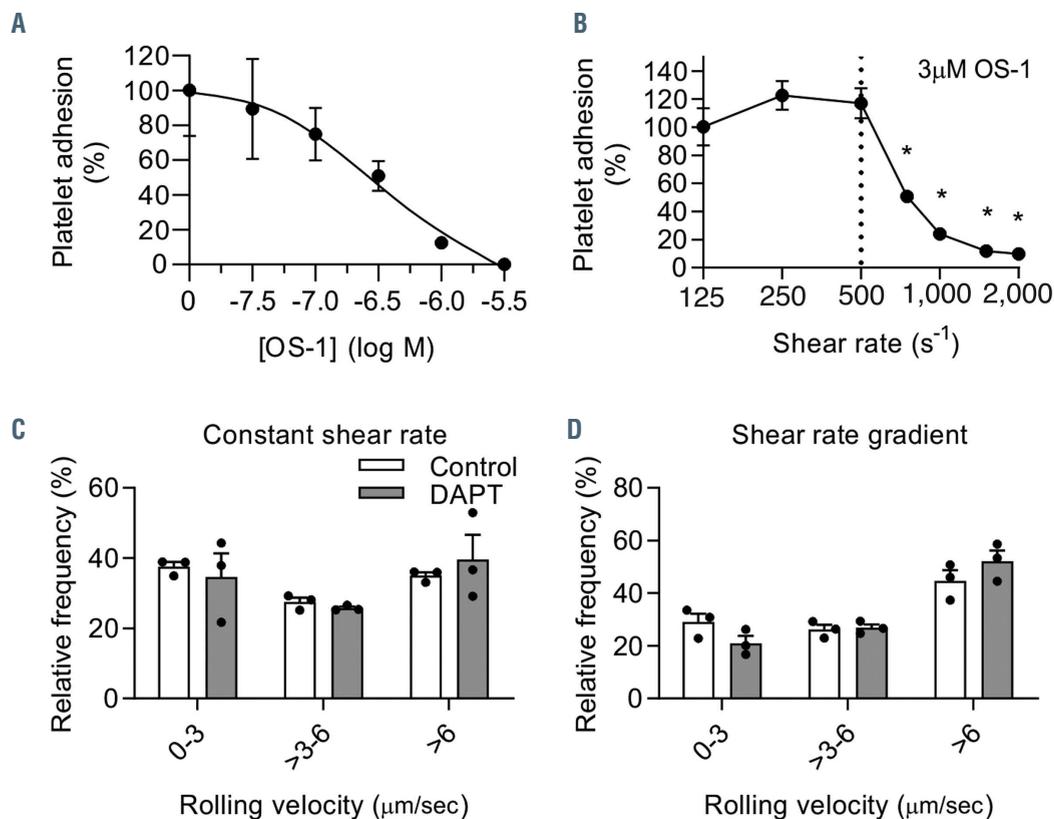


Figure 2. Platelet adhesion to collagen III is von Willebrand factor-GPIb dependent. (A) Blood, containing DiOC₆-labelled platelets, was treated with increasing concentrations of OS-1. Blood samples were drawn through collagen III coated channels at $1,500 \text{ s}^{-1}$. Fluorescence intensity was measured and expressed as relative platelet adhesion. (B) Citrated whole blood treated with $20 \mu\text{g mL}^{-1}$ Abciximab and $3 \mu\text{M OS-1}$ was perfused through collagen III-coated microfluidics at constant shear rates ranging from 125 s^{-1} to $2,000 \text{ s}^{-1}$. Data represented as mean \pm standard error of the mean (SEM); $n=4$; $*P<0.05$ (Dunnett's multiple comparison one-way ANOVA). (C) Abciximab-treated ($20 \mu\text{g mL}^{-1}$) platelets were treated with DAPT ($30 \mu\text{M}$ aspirin plus $300 \mu\text{M}$ 2-MeSAMP) or control and drawn through stenotic or straight channels. Platelet rolling velocity on collagen III bound von Willebrand factor (VWF) was assessed under constant shear (C) and at shear rate gradients (SRG) (D) using particle tracking software. Rolling velocities were divided into three bins ($0-3 \mu\text{m s}^{-1}$; $>3-6 \mu\text{m s}^{-1}$; $>6 \mu\text{m s}^{-1}$) and presented as relative frequencies (C, D). Data represented as mean \pm standard error of the mean; $n=3$; $*P<0.05$ (multiple unpaired t-tests).

surface interactions in our experimental setup were exclusively mediated through VWF-GPIb α , blood was incubated with the GPIb α inhibitor OS-1.¹⁹ Blood perfusion at a shear rate of 1,500 s⁻¹ resulted in platelet adhesion of 74.2 \pm 19.5% surface area coverage which was concentration-dependently reduced by OS-1. Platelet adhesion was completely abolished in the presence of 3 μ M OS-1 (Figure 2A). Furthermore, inhibition of platelet GPIb α by OS-1 (3 μ M) caused a sharp decrease in numbers of adherent platelets from above 500 s⁻¹ (53 \pm 4 platelets per field) to 2,000 s⁻¹ (11 \pm 4 platelets per field) (Figure 2B).

Having demonstrated that the observed interaction was GPIb α -VWF dependent with only minor if any contribution of direct collagen III interaction above 500 s⁻¹, we tested whether platelet activation played a role in the observed differential rolling velocity. Thus, platelets were treated with aspirin plus the P2Y12 inhibitor 2-MeSAMP. The drug treatment did not affect rolling velocity at sites of SRG (Figure 2D), nor at constant shear (Figure 2C), highlighting that the observed rolling velocity on collagen III is solely determined by the biophysical interaction of GPIb α with VWF independent of platelet activation.

Single-chain antibody A1 specifically inhibits shear rate gradients-dependent platelet-von Willebrand factor interaction

SRG have been shown to strongly activate VWF, which promotes platelet deposition at pathological shear rates

thereby exacerbating thrombus formation. Specifically blocking SRG-mediated VWF-activation would potentially open up a new avenue of limiting excessive thrombus growth at the latter stages of atherothrombosis, thereby keeping the blood vessels patent.

In order to investigate whether SRG-activated VWF is a potential therapeutic target, we produced several scFv, based on the sequences of the variable heavy and light chain regions derived from a panel of monoclonal antibodies raised against a 34/39 kDa VWF fragment incorporating the VWF A1 domain. The new scFv were specifically tested for their capacity to inhibit VWF-platelet interactions selectively at sites of SRG. ScFv A1 reduced platelet-VWF adhesion specifically in the stenosis inlet, where shear gradients are the greatest from 6,187 \pm 1,097 platelets/mm² to 5,158 \pm 1,032 platelets/mm²; $P=0.0013$ (Figure 3A). No differences in platelet adhesion were observed at the apex (3,934 \pm 892 platelets/mm² control; 3,805 \pm 783 platelets/mm² scFv A1, $P=0.7932$); nor in the stenosis outlet (5,042 \pm 1,167 platelets/mm² control; 4,475 \pm 1,199 platelets/mm² scFv A1; $P=0.07$). Surprisingly, scFv A1 showed an opposite effect in the straight section under constant shear, leading to increased platelet-deposition (2,047 \pm 232 platelets/mm² control; 3,096 \pm 690 platelets/mm² scFv A1; $P=0.0195$) (Figure 3A). Platelet rolling velocities were largely unaffected by the addition of scFv A1 except for the stenosis apex where a mild increase in rolling velocity was observed upon addition of

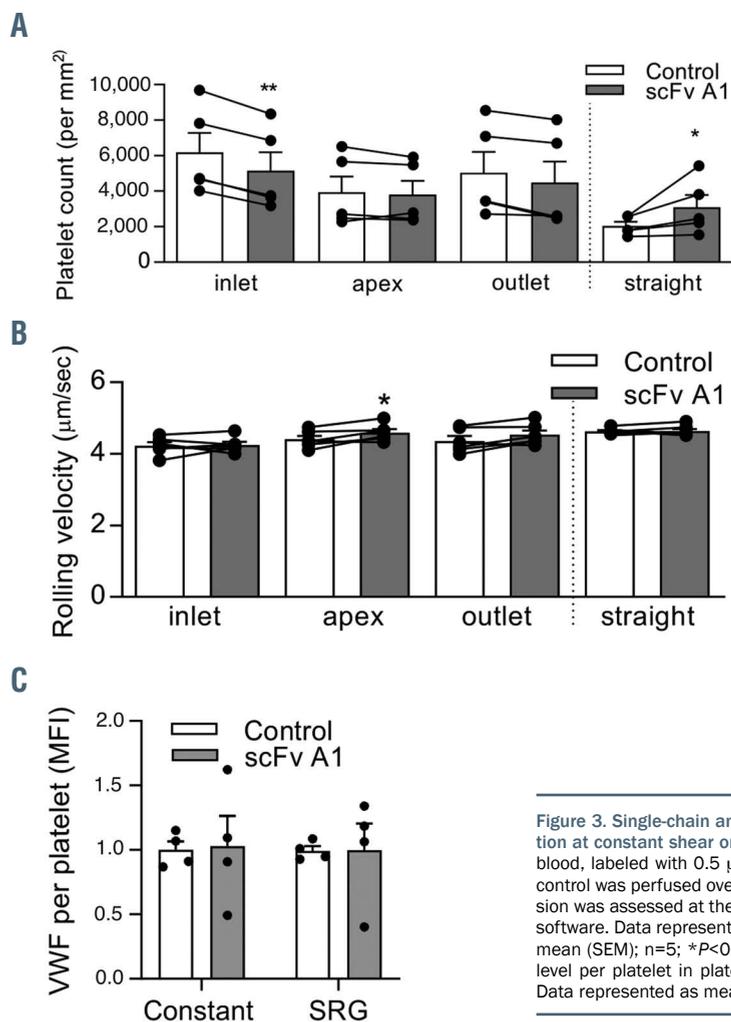


Figure 3. Single-chain antibody A1 differentially inhibits von Willebrand factor-platelet interaction at constant shear or gradients of shear. (A) Abciximab-treated (20 μ g mL⁻¹), citrated whole blood, labeled with 0.5 μ g mL⁻¹ DiOC₆ containing single-chain antibody (scFv) A1 (5 μ g mL⁻¹) or control was perfused over a collagen III-coated surface as described in Figure 1B. Platelet adhesion was assessed at the stenosis inlet, apex, outlet and upstream areas using particle tracking software. Data represented as matched individual data points and mean \pm standard error of the mean (SEM); n=5; * $P<0.05$. (C) Relative von Willebrand factor (VWF) amount corrected for the level per platelet in platelet aggregates under shear rate gradient (SRG) and constant shear. Data represented as mean \pm SEM; n=3-4; (multiple t-test).

scFv A1 ($4.41 \pm 0.1 \mu\text{m s}^{-1}$ control; $4.6 \pm 0.1 \mu\text{m s}^{-1}$ scFv A1; $P=0.038$) (Figure 3B). The relative amount of VWF per platelet deposited on aggregates in areas of SRG and constant shear was not reduced by scFv A1 (Figure 3C). Taken together these data suggest that scFv A1 interferes with a soluble fraction unfolded VWF which prevents VWF from engaging with surface-bound platelets.

Single-chain antibody A1 specifically inhibits shear rate gradient-exacerbated thrombus formation

Taking the findings of the effect of scFv A1 on platelet rolling velocity and adhesion further into a more complex scenario, we tested its capacity to specifically inhibit SRG-mediated thrombus formation. Blood containing scFv A1 ($5 \mu\text{g mL}^{-1}$) or control antibody was drawn through stenosis channels coated with collagen type I. ScFv A1 had no effect on platelet deposition at constant shear in the straight channel sections (Figure 4A, left panel). In order to highlight the differences in growth rate between constant shear and SRG, the aggregate sizes over time were nor-

malized to those measured at 1 min into the flow (set as $t=0$ in the graph) and defined as relative thrombus growth. Relative thrombus growth under constant shear ranged from 2.11 ± 0.23 versus 1.9 ± 0.36 at $2,000 \text{ s}^{-1}$ to 1.48 ± 0.16 versus 1.62 ± 0.2 at $1,500 \text{ s}^{-1}$ (Figure 4B, left panels). However, under SRG conditions, scFv A1 significantly reduced thrombus formation from 1.85 ± 0.28 to 1.17 ± 0.07 ($P < 0.0001$) at $2,000 \text{ s}^{-1}$ and from 1.58 ± 0.22 to 1.2 ± 0.09 ($P < 0.001$) at $1,500 \text{ s}^{-1}$ (Figure 4B, right panels). Inhibition of thrombus formation by scFv A1 was absent at a dose of $2.5 \mu\text{g mL}^{-1}$ whereas $5 \mu\text{g mL}^{-1}$ and $10 \mu\text{g mL}^{-1}$ showed inhibition and to a similar degree (Online Supplementary Figure S1).

Next, the inhibitory effects of ScFv A1 were compared to the platelet GPIIb/IIIa inhibitor OS-1 after 15 minutes of blood flow. Addition of $0.1 \mu\text{M}$ OS-1, a concentration that allowed residual platelet adhesion as determined in Figure 2A, inhibited platelet deposition under both constant and shear gradient conditions (Figure 4C). At $2,000 \text{ s}^{-1}$ constant shear OS-1 reduced thrombus formation from while scFv

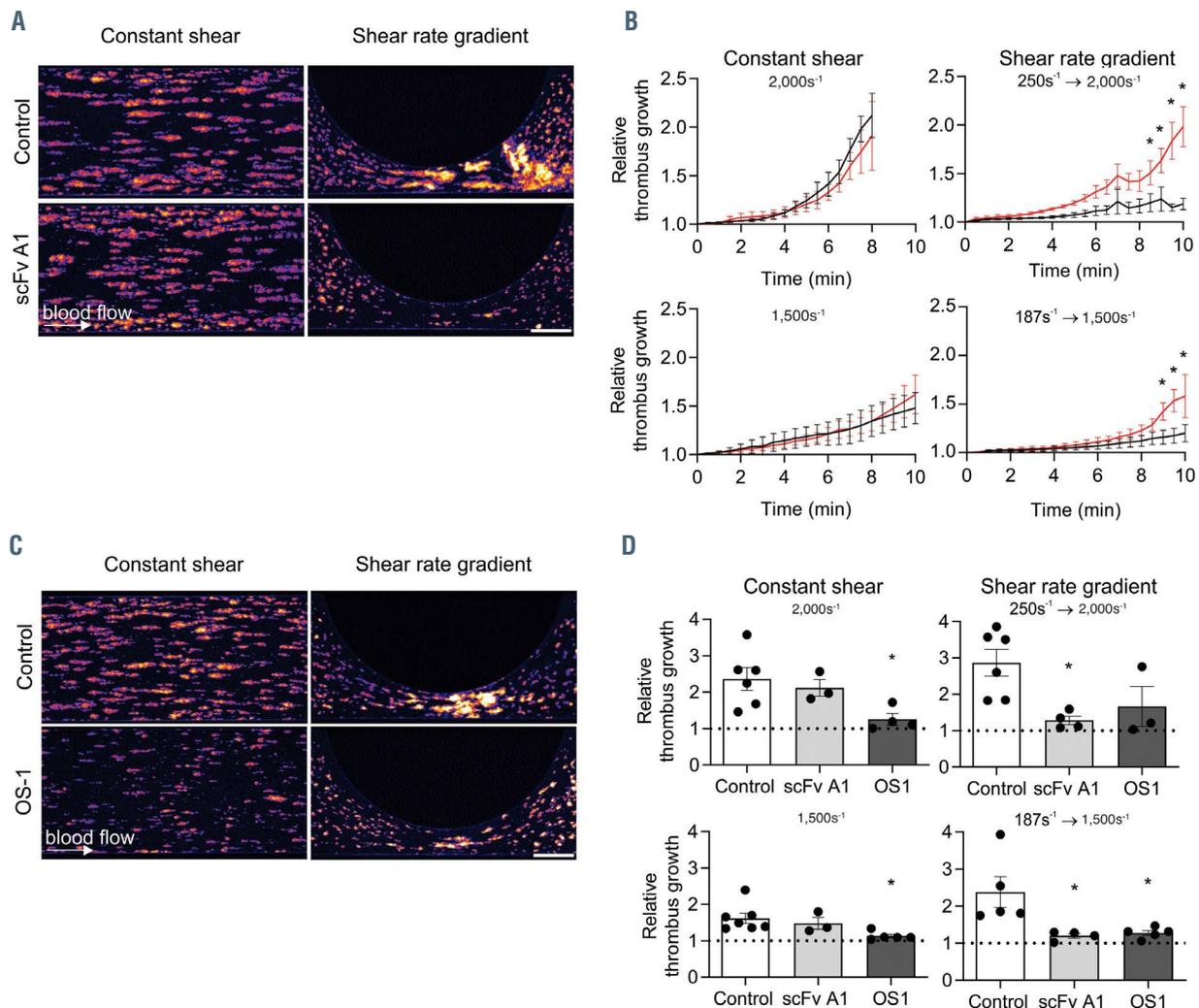


Figure 4. Single-chain antibody A1, but not OS-1 selectively inhibits thrombus formation in stenotic channel segments, a site of shear rate gradient, but not at constant shear rate. Confocal microscopy images of platelet aggregates (A) and quantification (B) of aggregate growth. Citrated whole blood, labeled with $0.5 \mu\text{g mL}^{-1}$ DiOC₆, containing $5 \mu\text{g mL}^{-1}$ single-chain antibody (scFv) A1 (black) or control (red), was perfused over a collagen type I matrix for 10 minutes at an effective shear rate of $1,500$ and $2,000 \text{ s}^{-1}$ in the stenosis apex (shear rate gradient [SRG]) or upstream (constant shear). Note the inhibitory effect of scFv A1 at SRG but not under constant shear. Data represented as mean \pm standard error of the mean (SEM); $n=3-4$; $*P < 0.05$ (two-way ANOVA). (C) Confocal images of platelet aggregate formation as in (A) in the presence of the GPIIb inhibitor OS-1 ($0.1 \mu\text{M}$) or control. (D) Quantification of platelet aggregate growth after 15 minutes at sites of SRG or at constant shear in the presence of scFv A1 ($5 \mu\text{g/mL}$), OS-1 ($0.1 \mu\text{M}$) or control. Data represented as mean \pm SEM; $n=3-6$; $*P < 0.05$ (two-way ANOVA).

A1 had no effect. A similar pattern, although weaker, was observed at $1,500\text{ s}^{-1}$. In contrast, at SRG ($300\text{--}2,000\text{ s}^{-1}$) and ($187\text{--}1,500\text{ s}^{-1}$), both OS-1 and scFv A1 inhibited thrombus formation. These data highlight the shear selective nature of ScFv A1 compared to a generic GPIIb-VWF inhibitor.

Single-chain antibody A1 does not interact with von Willebrand factor under no shear conditions

Next we investigated whether scFv A1 could also inhibit VWF-GPIIb binding under zero shear conditions. VWF dependent platelet agglutination was tested in a ristocetin-induced platelet agglutination test (RIPA). Since the exposure of VWF to ristocetin or shear reportedly exposes similar epitopes within VWF,²⁰ we hypothesized that scFv A1 may inhibit ristocetin stimulated platelet agglutination. However, scFv A1 up to $80\text{ }\mu\text{g mL}^{-1}$ (16 times higher than used in the microfluidic flow device) did not result in delayed or reduced agglutination of isolated platelets by ristocetin (0.75 mg mL^{-1}) in the presence of plasma VWF. However, when plasma VWF was preincubated with scFv A1 and ristocetin to facilitate their interaction before platelet binding, a minor inhibitory effect of scFv A1 on platelet agglutination was observed (*Online Supplementary Figure S2A*). Western blotting of denatured VWF with scFv A1 also did not show any complex formation (*Online Supplementary Figure S2B*). Similarly, scFv A1 did not bind to immobilized full-length VWF or isolated VWF A1 domain in an enzyme-linked immunosorbent assay test (*Online Supplementary Figure S2C*). Consistent with this result, we also did not observe any binding between immobilized VWF full length or VWF A1 domain with scFv using the BLItz binding assay (*Online Supplementary Figure S2D*).

Single-chain antibody A1 dampens thrombus formation in the latter stages only

During the growth of an intraluminal thrombus, the local shear environment becomes progressively more complex. Here we set out to mimic this highly dynamic process and characterize the inhibitory effects of scFv A1 within this process. Large platelet aggregates were allowed to form in straight channels and create their own local shear gradient environment during thrombus growth. Whole blood was perfused at $1,000\text{ s}^{-1}$ over discrete patches of coated collagen type I surrounded by albumin coated areas (Figure 5A). The presence of the albumin-collagen interface generates a sharp front of aggregates which grow to significant heights relative to the $52\text{ }\mu\text{m}$ high channels and as a result create a local steep shear gradient. After an initial phase of homogenous platelet accumulation across the entire collagen patch, platelet aggregation progressively increased at the front area of the patch (2.0 ± 0.2 -fold increase; front over rear) (Figure 5B). This effect could not be explained by depletion of platelets at the boundary layer as it was observed on sequential patches within a single channel. Next, we tested scFv A1 for its capacity to inhibit thrombus formation in this progressive shear gradient flow model. ScFv A1 selectively attenuated platelet aggregation at the front area of the collagen patch (Figure 5C) however, scFv A1 did not reduce platelet aggregation in the rear area where shear rates were constant and SRG were absent. Computational fluid dynamics (CFD) analysis of a representative experiment revealed that platelet aggregates at the front area of the patch experienced increased surface

shear rates compared to those in the back area of the patch (Figure 5D; upper panel), concomitant with platelet deposition patterns. As expected, scFv A1 reduced platelet deposition at the front area resulting in reduced aggregate surface shear rates (Figure 5D; lower panel). The inhibitory effect of scFv A1 was observed even though the calculated maximum SRG, expressed as $\text{s}^{-1}/\mu\text{m}$, was lower in this flow model (4.9 and $3.1\text{ s}^{-1}/\mu\text{m}$ for control and scFv A1 respectively) compared to the SRG calculated in the microfluidic stenosis channels used in Figures 1-4 ($7.1\text{ s}^{-1}/\mu\text{m}$) (Figure 5D).

Time-lapse confocal microscopic analysis of platelet aggregate formation across the collagen patch revealed a lag phase of up to 180 seconds where small aggregates formed throughout the patch, followed by a growth phase at the front area of the patch where scFv A1 was inhibitory (Figure 5E). Indeed, quantitative analysis showed that scFv A1 inhibited platelet aggregation only at the front of the patch but not at the rear (Figure 5F).

Discussion

In this study, we demonstrate that SRG exacerbate VWF-dependent platelet aggregation through increased VWF-GPIIb α bond strength and that this process can selectively be inhibited using an antibody strategy that targets a SRG-sensitive epitope within the VWF A1-domain. Thus, SRG are a potential novel drug target for the prevention of occlusive thrombus formation.

The blood flow dynamics in areas of flow restriction, stenosis or mural thrombus formation have long been known to create gradients in shear rate, or SRG. However, the activating effects of SRG on VWF-dependent platelet aggregation are still largely unknown. We have previously demonstrated the potential of micro-shear gradients in promoting thrombus formation.¹⁰ Following on from this finding, we have shown exacerbated thrombus formation in the outlet of stenotic vessel sections to be VWF dependent.¹⁶ While high constant shear in the range of approximately $5,000\text{ s}^{-1}$ to $8,000\text{ s}^{-1}$ is required to unfold and thereby activate VWF,¹⁴ SRG dramatically reduce this shear range by 10-fold. This means SRG mediated platelet aggregation can occur in virtually all vessel beds where a flow constriction occurs. Previous studies investigating the role of the VWF-GPIIb α axis in platelet adhesion and aggregation used various approaches ranging from mathematical simulations,^{21,22} single molecule force probe pulling,^{23,24} VWF multimer analysis²⁵ to functional platelet adhesive behavior under flow conditions.²⁶⁻²⁸ However, it is difficult to establish functional links between these studies and, in isolation, they provide limited insight into the overall mechanism of VWF-mediated platelet aggregation under shear conditions, particularly SRG. In our study, we used CFD analysis for the characterization of our microfluidics setup, linking the shear profile to functional readouts such as platelet aggregate formation, platelet adhesion and rolling velocity as a direct consequence of SRG induced VWF unfolding and activation.

First, we investigated the effects of SRG on individual platelet-surface interactions on a collagen type III matrix because this type of collagen shows high affinity for VWF, resulting in high VWF density, while causing mild platelet activation. In order to prevent engagement of $\alpha\text{IIb}\beta_3$ with VWF during platelet adhesion/aggregation, whole blood

was preincubated with the α IIb β 3-inhibitor abciximab. Next, we investigated the effects of SRG on platelet aggregation on a collagen type I matrix because this type of collagen induces strong platelet activation and subsequent aggregate formation. Most importantly, the use of collagen I or III as a physiological VWF substrate, rather than glass-immobilised VWF, ensured correct conformation of shear-immobilised VWF.^{29,30}

Our data show that platelet rolling velocities at SRG are lower than at matched constant shear, suggesting that SRG at the inlet of the stenosis, lead to a rapid unfolding of VWF beyond the degree expected under matched constant shear. This unfolding promotes the formation of catch bonds between VWF and GPIIb α and reduces the

rolling velocity of platelets on the adhesive surface either due to a higher degree of unfolding per VWF molecule or more unfolded VWF molecules in general. In analogy to tether formation in platelets,²⁸ this feature of SRG may arrest a platelet sufficiently long to facilitate firm adhesion to the adhesive surface or a neighbouring platelet, thereby exacerbating thrombus formation under SRG.

Common antiplatelet drugs - aspirin plus a P2Y12 inhibitor - did not prevent the pro-adhesive phenotype of platelets under SRG conditions, highlighting that platelet activation does not play a key role in SRG-mediated platelet adhesion and that current antiplatelet therapy would be unable to inhibit exacerbated thrombosis which is induced by mechanical hyper-activation of VWF by SRG.

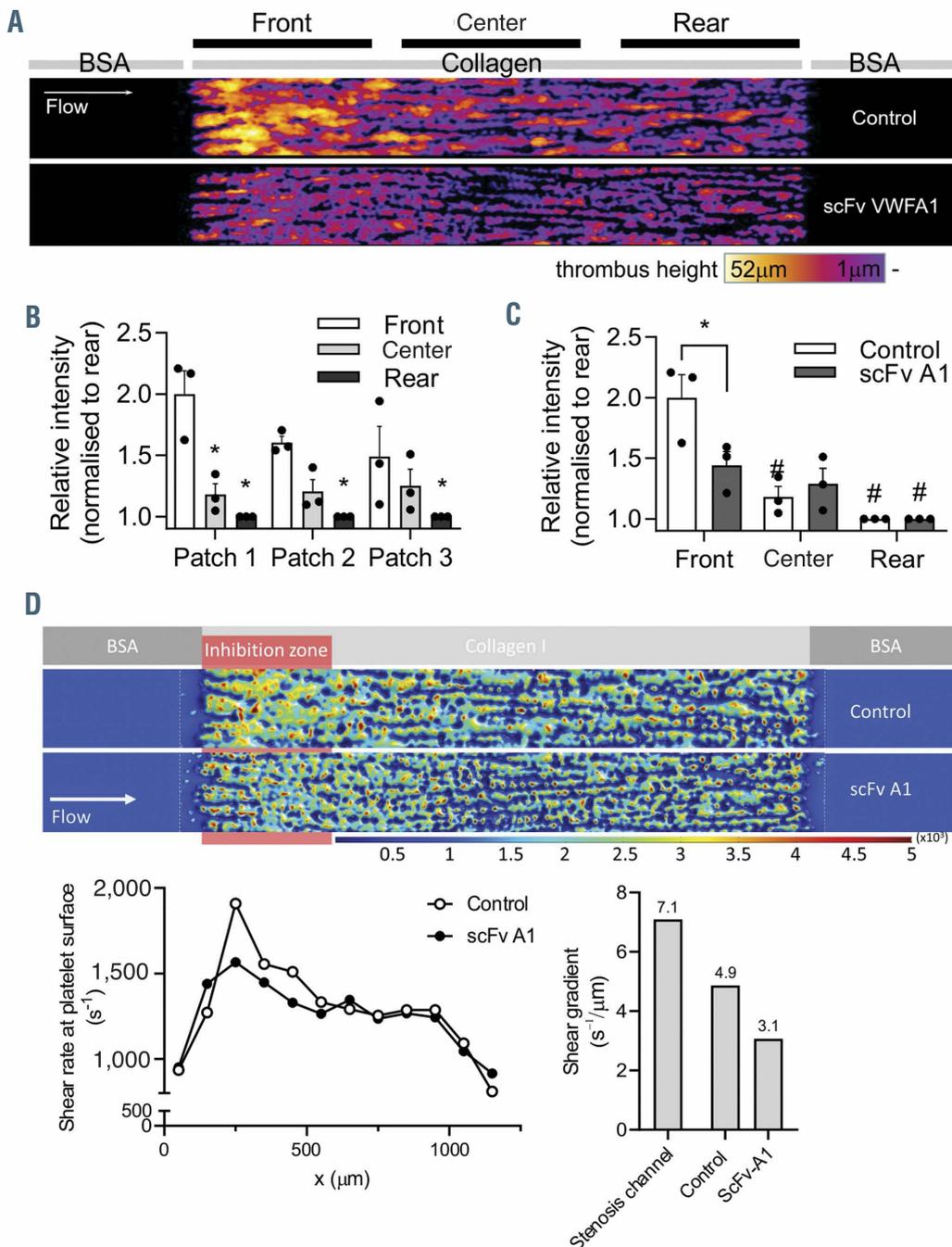


Figure 5. Figure continued on the following page.

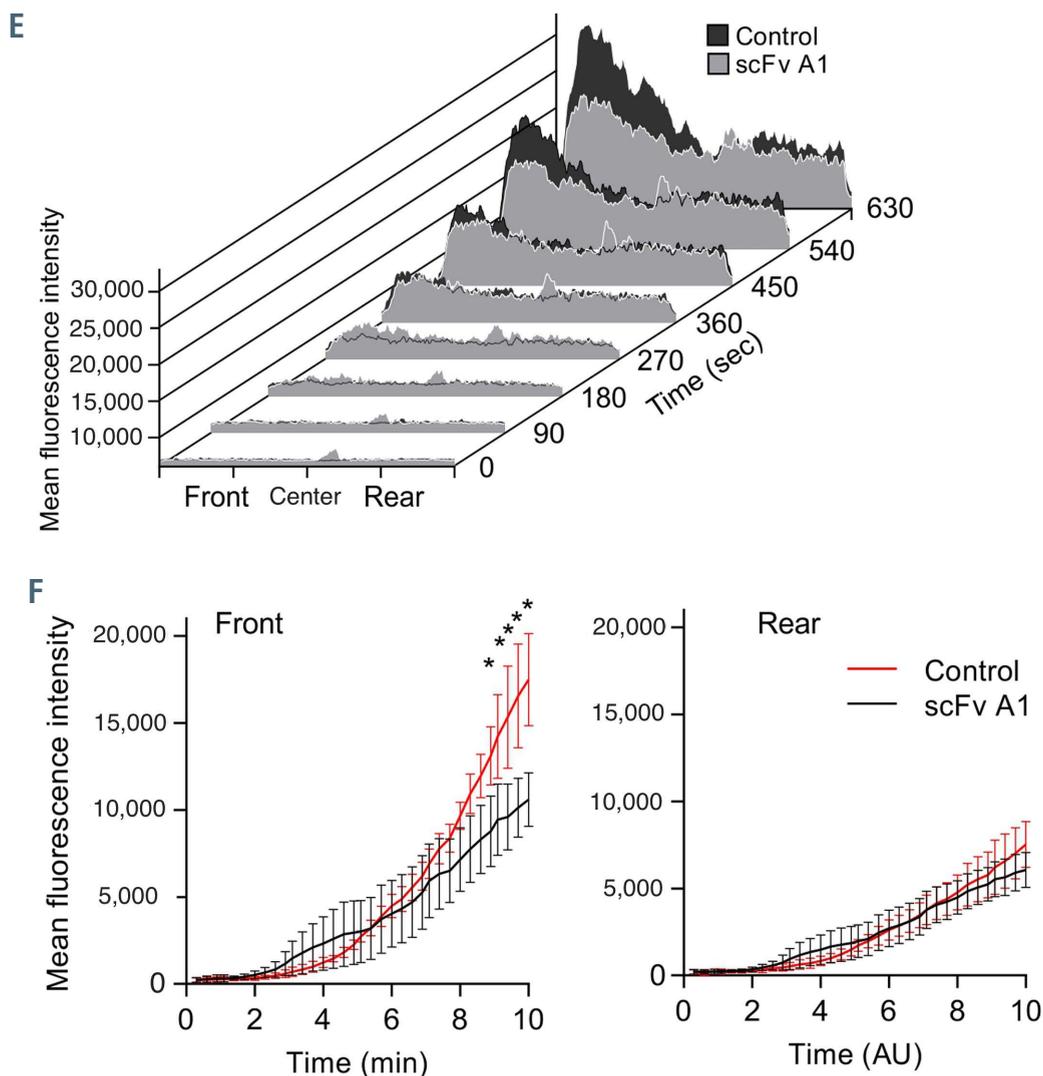


Figure 5. Single-chain antibody A1 specifically inhibits thrombus formation at sites of shear rate gradients but not at constant shear rate. Citrated whole blood, labelled with $0.5 \mu\text{g mL}^{-1}$ DiOC₆, containing $5 \mu\text{g mL}^{-1}$ single-chain antibody (scFv) A1 or control, was drawn at $1,000 \text{ s}^{-1}$ through flow channels exhibiting a patch of perpendicularly-coated collagen type I ($100 \mu\text{g mL}^{-1}$) on the bottom surface, creating a BSA-collagen interface. (A) Z-slices of confocal microscopy images were binarized and compiled to z-projections showing thrombus height for control (top panel) and scFv A1-loaded blood (bottom panel). (B) Relative fluorescence intensities of platelet aggregates on sequential collagen patches in the same flow channel. Data represented as mean \pm standard error of the mean (SEM); $n=3$; $*P<0.05$ (multiple t-tests). (C) Relative fluorescence intensities of platelet aggregates in the front, center and rear areas of the collagen patch after 10 minutes of flow. Data represented as mean \pm SEM; $n=3$; $*P<0.05$ (multiple t-tests). (D) Computational fluid dynamics (CFD) analysis of shear rates present at the surface of platelet aggregates in control blood and scFv A1-treated blood, expressed as a heat map and numerical. Maximal shear rate gradients are shown for the microfluidic stenosis channels used in Figures 1 to 4 and at the surface of the aggregates shown in the heat map. (E) Graphical presentation of the platelet fluorescence distribution over the entire collagen patch at various timepoints. Data is mean of $n=3$ flows. (F) Quantification of time-lapse confocal microscopy images showing reduced platelet deposition in the presence of scFv A1 at the front area but not the rear area of the patch. Data represented as mean \pm SEM; $n=3$; $*P<0.05$ (two-way ANOVA).

Others have identified the GPIIb α -VWF axis as a potential target and various drug candidates are currently under investigation.³¹ In contrast to our SRG-specific approach, these drugs take a systemic approach, thereby potentially affecting hemostasis. We sought an approach that would allow inhibition of VWF specifically at sites of SRG, a feature of atherothrombosis, without affecting VWF activation under constant shear, which prevails during normal hemostasis.

Our blocking strategy contained the novel scFv A1, which was based on the sequences from a previously developed human monoclonal antibody panel raised in mice against a 39/34-kDa VWF fragment (Leu-480/Val-481-Gly-718) which encompasses the A1 domain and a sequence N-terminal to that.¹⁷ The basis for the generation

of our scFv A1 involved combining the variable heavy chain of an antibody which recognizes a linear epitope in VWF-A1 with the variable light chain of another antibody which recognises a conformation-specific epitope in the VWF A1 domain.¹⁷

Assessing single platelet binding to the adhesive surface, our scFv A1 showed selective platelet-VWF inhibition at stenotic sites where SRG are prevalent compared to areas of constant shear suggesting that the scFv A1 did not systematically block the VWF-GPIIb α interaction but rather specifically at the stenosis and in a transient manner. Similarly, under constant shear conditions platelet aggregation on collagen-bound VWF in the presence of scFv A1 was not inhibited. Taken together, these results suggest that scFv A1 targets the transiently present hyperactive

form of VWF at the site of a stenosis. In agreement with this, the presence of hyperactive VWF at sites of SRG has previously been reported.¹⁶

The activation state of VWF during the flow immobilization to collagen or immobilized platelets appeared to modulate the subsequent recruitment of additional platelets. When VWF was deposited onto collagen before the flow experiment, SRG did not exert this pro-adhesive effect on platelets, indicating that the activation state of VWF in the fluid phase at the moment of deposition onto collagen or immobilized platelets is a key determinant in the pro-thrombotic phenotype of SRG. Similar observations have recently been reported by Receveur *et al.*³² who used a flow model which introduced SRG of the same magnitude as those used in this study. VWF unfolding in an accelerating flow field is likely to be very transient, with apparent rapid refolding in areas downstream of SRG where shear rates resume a constant level again. Therefore, the binding of scFv A1 to VWF is an elusive event that could only be achieved under shear gradient forces in the microfluidic flow assays. Indeed, we were unable to establish evidence of scFv A1 binding to VWF in other assays with no shear being present, likely due to the epitope for scFv A1 being shielded or not in a correct conformation. Since SRG are thought to be mechanistically similar to very high constant shear, we aimed to mimic the shear unfolding of VWF with the biochemical modulator ristocetin. This antibiotic is known to modulate the VWF-GPIb interaction, binding similar epitopes to those exposed by elevated shear.²⁰ After optimizing experimental conditions for platelet agglutination, scFv A1 inhibited ristocetin-induced platelet agglutination in a minor way suggesting that ristocetin may expose epitopes in VWF that are recognized by scFv A1. Although ristocetin-treated VWF exposes similar epitopes as shear stress, we cannot claim that the topology of the epitopes is the same under both conditions, particularly because we create SRG conditions whereas the literature is based on constant shear rates. This likely explains why the inhibitory effect of our scFv-A1 in the ristocetin induced aggregation assays was only modest and requires further investigations.

One limitation of this study is its exclusive *in vitro* approach to address these questions. The scFv A1 antibody recognizes human VWF and thus would require a fully humanized mouse model of the VWF-GPIb axis to study the efficacy of the antibody in an *in vivo* setting. These *in vivo* approaches will be addressed in subsequent studies. Therapeutic development would include full humanization of the scFv replacing structural elements

that are murine based. Current humanization technologies are based on decades of experience with antibody scaffolds and in the majority of cases the antibodies keep their properties. However, there is always a small risk that the properties will change and the antibody might become less affine or specific. In case this is observed further affinity maturation might be required which ultimately could make the antibody more potent. A scFv typically clears the circulation in 1/2 hour compared to days of a full IgG antibody. The ultimate clinical application (acute treatment of thrombosis vs. prophylaxis) will determine which format is optimal. There might be scope to a fast and slow clearing antibody alone or in combination as required based on the desired pharmacokinetics.

In conclusion, we have shown that pro-thrombotic effects of SRG, which are known to lead to “hyperactivation” of VWF³²⁻³⁵ can be site-specifically inhibited with our scFv A1 antibody without interfering with the VWF-GPIb α interaction under normal flow. We speculate that our targeting strategy could provide an important cornerstone in future antithrombotic therapy which mechanically decouples thrombosis from hemostasis and therefore does not contribute to an increased bleeding tendency, one of the main culprits in current antiplatelet drug development.

Disclosures

The molecular design and sequence of said single-chain antibody scFv A1 is subject to a patent application (provisional patent no. 2020902148) and is therefore not disclosed in this manuscript.

Contributions

TH, AR, HA and EW performed research; TH, AR, BN, SJ, HA, AM, EW performed data analysis; EG, RA provided critical reagents and supervision; TH, AR and EW wrote the manuscript; CH and EW supervised the study.

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