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ORIGINAL ARTICLE

Dual roles of fucoidan-GPIbα interaction in thrombosis and hemostasis: implications for drug development targeting GPIbα

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Abstract

Background: Platelet GPIbα–von Willebrand factor (VWF) interaction initiates platelet adhesion, activation, and thrombus growth, especially under high shear conditions. Therefore, the GPIb–VWF axis has been suggested as a promising target against arterial thrombosis. The polysaccharide fucoidan has been reported to have opposing prothrombotic and antithrombotic effects; however, its binding mechanism with platelets has not been adequately studied.

Objective: The objective of this study was to explore the mechanism of fucoidan and its hydrolyzed products in thrombosis and hemostasis.

Methods: Natural fucoidan was hydrolyzed by using hydrochloric acid and was characterized by using size-exclusion chromatography, UV-visible spectroscopy, and fluorometry techniques. The effects of natural and hydrolyzed fucoidan on platelet aggregation were examined by using platelets from wild-type, VWF and fibrinogen-deficient, GPIbα-deficient, and IL4Rα/GPIbα-transgenic and αIIb-deficient mice and from human beings. Platelet activation markers (P-selectin expression, PAC-1, and fibrinogen binding) and platelet-VWF A1 interaction were measured by using flow cytometry. GPIbα–VWF A1 interaction was evaluated by using enzyme-linked immunosorbent assay. GPIb-IX–induced signal transduction was detected by using western blot. Heparinized whole blood from healthy donors was used to test thrombus formation and growth in a perfusion chamber.

Results: We found that GPIbα is critical for fucoidan-induced platelet activation. Fucoidan interacted with the extracellular domain of GPIbα and blocked its interaction with VWF but itself could lead to GPIbα-mediated signal transduction and,

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subsequently, αIIbβ3 activation and platelet aggregation. Conversely, low-molecular weight fucoidan inhibited GPIb-VWF–mediated platelet aggregation, spreading, and thrombus growth at high shear.

Conclusion: Fucoidan-GPIbα interaction may have unique therapeutic potential against bleeding disorders in its high-molecular weight state and protection against arterial thrombosis by blocking GPIb–VWF interaction after fucoidan is hydrolyzed.

KEYWORDS
fucoidan, glycoprotein Ib, platelet, thrombosis, von Willebrand factor

1 | INTRODUCTION

Platelet adhesion, activation, and aggregation at the sites of vascular injury are essential for hemostasis. However, this mechanism can cause thrombosis when triggered pathologically and lead to life-threatening cardiovascular diseases such as heart attack and stroke [1–3]. Although current antiplatelet agents reduce the risk of thrombosis [4], they can also compromise hemostasis and lead to bleeding disorders.

Shear rate plays an important role in the mechanism that determines platelet response to vascular injury. Platelets experience a broad range of hemodynamic shear stresses *in vivo*, ranging from low shear in venules and large veins (typical wall shear rates < 500 s^{-1}), high in small arterioles (shear rates up to 5000 s^{-1}), or extremely high in stenosed or partially occluded arteries where shear rates can reach to more than 10 000 s^{-1} [5]. The interaction of the platelet mechanoreceptor glycoprotein (GP) Ib-IX to von Willebrand factor (VWF) is essential for initial platelet adhesion and activation under high shear stress. We and other groups previously also demonstrated that the GPIb–VWF interaction is required for the late stage of thrombosis that leads to vessel occlusion, which is the leading cause of elevated morbidity and mortality rates in patients with ischemic heart disease and stroke [6–8]. Therefore, GPIb-IX has been considered a promising target to treat arterial thrombosis without significantly compromising hemostasis at moderate to low shear conditions [9,10].

The GPIb-IX complex is a transmembrane platelet surface receptor consisting of 3 membrane bound subunits, GPIbα, GPIbβ, and GPIX. The GPIbα subunit is integral in the initiation of platelet adhesion and aggregation through interactions with VWF under high shear forces [2,11,12]. Under high shear conditions, VWF undergoes a conformational change that exposes its A1 domain and binds the GPIbα N-terminal ligand-binding domain (LBD). This GPIb–VWF interaction can tether platelets to the subendothelium for platelet adhesion or bridge platelet–platelet at high shear for platelet aggregation that may lead to vessel occlusion [6]. In both cases, VWF binding delivers force and changes the conformation of the GPIb mechanosensory domain (MSD), which induces subsequent GPIb-mediated outside-in signaling into platelets [13–15]. GPIb-mediated signaling further causes an 'inside-out' signaling cascade that leads to the activation of αIIbβ3 integrin [16,17] and αIIbβ3-mediated platelet aggregation. Although several αIIbβ3 inhibitors have been already developed for antithrombotic therapies [4,18], currently, no anti-GPIb-IX drug has been successfully developed for clinical use. However, preclinical studies in murine [7,19] and nonhuman primates [10,20,21], along with recent clinical studies have proven GPIb as an effective and may be a safe target for antithrombotic drug development [9,22].

Fucoidans are naturally produced polysaccharides that contain L-fucose subunits with sulfate ester groups. Fucoidans are mainly derived from brown seaweeds and echinoderms. They have broad therapeutic potential because of their anticancer, anti-infection, immunoregulation, and cardioprotective effects [23,24]. It has been reported that fucoidans also possess both anticoagulant and procoagulant activities that are dose and structure dependent. In general, anticoagulant activity is only

Essentials

- GPIbα is essential for thrombosis at high shear and is a drug target against arterial thrombosis.
- Fucoidans have prothrombotic and antithrombotic effects; however, its platelet interaction is not clear.
- High-molecular weight fucoidan-GPIbα interaction delivers signals that activate platelets.
- Low-molecular weight fucoidan-GPIbα interaction blocks von Willebrand factor-bridged shear-induced thrombus growth.

observed at high concentrations; therefore, fucoidans have been referred to as non-anticoagulant sulfated polysaccharides (NASPs) [24–26]. Fucoidans exert procoagulant activity by inhibiting tissue factor pathway inhibitor (TFPI) and improves plasma clotting in patients and animals deficient in coagulation factor VIII or IX (referred to as hemophilia A or B). Therefore, fucoidans have been proposed as a novel therapeutic for hemophilia [25,27,28].

Growing evidence shows that fucoidans of either moderate-molecular weight (27-32 kDa) or low-molecular weight (<8 kDa) significantly inhibited arterial thrombosis and that was more effective than the anticoagulant heparin in different animal models, suggesting that fucoidans can inhibit platelet involvement in thrombosis [29–31]. However, fucoidans have been reported to induce human and mouse platelets activation through C-type lectin-like receptor 2 (CLEC-2) [32] and platelet endothelial aggregation receptor 1 (PEAR1), respectively, in a way facilitated by GPIbα [33]. Therefore, the anti-platelet and proplatelet activity of fucoidan has not been well elucidated, and the effect of fucoidan on platelet mechanosensor-mediated thrombus formation has never been explored.

In this study, we found fucoidan from the seaweed *Fucus vesiculosus* (FFV) bound to the ectodomain of GPIbα and triggered GPIb-IX-mediated outside-out signaling, resulting in the activation of integrin αIIbβ3 (inside-out signaling) and platelet aggregation. Interestingly, the low-molecular weight fucoidan (LMF) specifically inhibited GPIb-IX-induced platelet adhesion, aggregation, and thrombus growth at high shear. We determined molecular weight (MW) characterization of fucoidan and found the size-dependent dual role of fucoidan in regulating platelet activation using GPIbα. Our results highlight the potential therapeutic benefits of fucoidan in thrombosis and hemostasis.

2 | MATERIALS AND METHODS

2.1 | Platelet preparation and aggregation

Human blood samples were drawn from antecubital veins of healthy volunteers after they provided informed consent [34,35]. Human platelet-rich plasma was obtained by spinning at 250 × g for 8 minutes. Gel-filtered platelets were purified from platelet-rich plasma by using a Sepharose 2B chromatography column (CL2B300, Sigma) with 1,4-Piperazinediethanesulfonic acid (PIPES) buffer (5 mM PIPES, 137 mM NaCl, 4 mM KCl, and 0.1% glucose, pH 7.0) as we previously described [34,36–38]. After a 5-minute incubation at 37 °C, platelet aggregation was initiated by the addition of fucoidan, ristocetin, botrocetin, adenosine 5′-diphosphate (ADP), collagen, or proteinase-activated receptor (PAR) agonist in separate experiments and stirred with 1000 rpm at 37°C in a Chrono-log aggregometer (Chrono-Log). Platelets from wild-type (WT), GPIbα-deficient (GPIbα$^{-/-}$), IL4Rα/GPIbα-transgenic (GPIbα/IL4R-tg), VWF and fibrinogen-deficient (VWF$^{-/-}$Fg$^{-/-}$), and αIIb- or β3 integrin-deficient mice were collected as we previously described [34,38–40].

2.2 | Fluorescence labeling of fucoidan

The covalent conjugation of 0.2 mg/mL fucoidan to fluorescein isothiocyanate (FITC, Sigma-Aldrich) was individually performed through nucleophilic addition reactions in 6.2 N NaOH at 4 °C. Fucoidan-FITC conjugation reactions were quenched by adding 6.5 N HCl. Fractions of product precipitations were collected, exchanged to phosphate-buffered saline (PBS) buffer pH 7.4, and purified by using the size-exclusion chromatography (SEC) method as described in section 2.9.

2.3 | Flow cytometry

The binding of FITC-labeled PAC-1 (MA5-28564, ThermoFisher), Alexa Fluor 647-labeled fibrinogen (F35200, ThermoFisher) or PE antihuman P-selectin antibody (304906, BioLegend) to platelets was measured by using a Sony SP6800 spectral cytometer as we previously described [41–44]. The binding of FITC-labeled fucoidan to platelets was performed by using WT, GPIbα-deficient (GPIbα$^{-/-}$), and GPIbα/IL4R-tg mouse samples. Platelets were prepared in PBS plus 1 mM EDTA and gently mixed with 1.6 nM fucoidan (LMF), incubated at 25 °C for 5 minutes, and washed (1000 g, 5 minutes) to remove unbound labeled fucoidan. Spectral cell flow data were acquired by using a BD LSR Fortessa X-20 cytometer and analyzed by using a supplied FlowJo 8.1 software package.

2.4 | Preparation of native and partially hydrolyzed fucoidan solution

Analytical grade of fucoidan compound FFV was obtained from Sigma (F8190) and prepared as previously described [32,33]. The hydrolyzed LMF was obtained by dissolving 10 mg of natural fucoidan polysaccharide compound in 1 mL of 0.01 N HCl and heating at 95 °C for 20 minutes, followed by neutralization with 1 mL of 0.01 N NaOH [45].

2.5 | O-Sialoglycoprotein endopeptidase treatment of platelets

O-Sialoglycoprotein endopeptidase (OSGE) treatment of platelets was performed as previously described [42,46]. Briefly, the gel-filtered platelets (1 × 10^9/mL) were resuspended in Tyrode's buffer containing 1 mM $CaCl_2$ and incubated with 100 μg/mL OSGE (CLE100, Cedarlane) at 37 °C for 30 minutes. Aliquots of the platelet suspensions were analyzed using flow cytometry or subjected to aggregation assay in the presence of plasma.

2.6 | *Ex vivo* perfusion chamber

To study the effect of fucoidan on thrombus formation under shear force, we used an *ex vivo* perfusion chamber system as we previously described [7,35,37,38,47]. Briefly, μ-Slide (μ-Slide VI 0.1, ibidi) were coated with 100 μg/mL collagen (NC9533954, Fisher Scientific) for 2 hours at room temperature. Heparinized whole blood samples were

collected from healthy human volunteers as described above. Blood samples were pretreated with the LMF for 5 minutes at room temperature (25 °C) and fluorescently labeled by 1 μM DiOC6 (3,3′-dihexyloxacarbocyanine iodide, Sigma). Then, the blood was perfused over the collagen-coated surface by using a syringe pump (Harvard Apparatus) under a shear rate of 300 s^{-1}, 1800 s^{-1}, or 5000 s^{-1} for 3 minutes. Platelet aggregation and thrombus formation were recorded in real time with the Zeiss Axiovert 135-inverted fluorescence microscope (60X-W objective). Quantitative dynamics of platelet fluorescence intensity were acquired by SlideBook software (Intelligent Imaging Innovations Inc).

2.7 | Platelet spreading and confocal microscopy

Platelet spreading assay was performed as we previously described [39,41,47]. Briefly, washed platelets suspended in Tyrode's buffer B were added to 100 μg/mL recombinant VWF A1 domain or fibrinogen (F3879, Sigma) coated 10 mm coverslips and incubated at 37 °C with the fucoidan. Platelets were fixed in 4% paraformaldehyde at room temperature for 15 minutes, permeabilized in 0.3% Triton X-100 (T8787, Sigma-Aldrich, Sigma) for 10 minutes, washed and blocked with 2% bovine serum albumin (800-095-EG, Wisent) in PBS for 1 hour. Then, the slips were stained with FITC-labeled phalloidin (P5282, Sigma) and washed and viewed with a confocal microscope Zeiss LSM 700. Platelet numbers and areas were measured by using ImageJ 1.35h software.

2.8 | Enzyme-linked immunosorbent assay

A 384-well plate (464718, Thermofisher) was coated overnight at 4 °C with 10 μg/mL BSA or recombinant GPIb extracellular domain diluted in 0.1 M sodium bicarbonate buffer (pH 9.6). Between each step, wells were washed 3 to 5 times with Phosphate Buffered Saline with 0.05% Tween-20, pH 7.4. Wells were blocked with 3% skim milk for 1 hour, then treated with His-tagged dimeric VWF A1 domain with or without native or hydrolyzed fucoidan for 2 hours. Wells were then treated with a goat anti-His HRP-conjugated antibody (1:5000 dilution) for 1 hour. Enzyme-linked immunosorbent assay substrate solution (34028, Thermofisher) was then added and quenched with 2 M sulfuric acid. Absorbances at 450 nm were quantified on a BioTek Epoch 2 Microplate Reader (Agilent).

2.9 | MW characterization of fucoidan

SEC experiments were performed by using an ÄKTA fast purification liquid chromatography instrument equipped with a HiPrep 16/60 Sephacryl S-300HR column with the geometric column volume (CV) of 120.64 mL (GE Healthcare) at 25 °C. The column was equilibrated with 2 CV of running buffer (10 mM sodium phosphate, pH 7.4, 137 mM NaCl, and 4 mM KCl) before each sample injection. All solutions were filter sterilized by using 0.2 μm microfilters. The columns were washed thoroughly with 2 CV 0.02% w/v sodium azide solution after each experiment to avoid the growth of microorganisms inside the column. A sample of 100 μL 10 mg/mL fucoidan in running buffer was injected onto the column, and experiments were run at the volumetric flow rate of 0.5 mL/min for 2 CV. Elutions were monitored by the eluate UV light absorbance at 254 nm and analyzed as a function of retention time (t_R) and retention volume (V_R) by using the provided Unicorn 4.11 software package (GE Healthcare). The column exclusion and permeation limits were calibrated by measuring t_R values of molecular weight standards as a function of log MW in Daltons by using the OriginPro software package (OriginLab, 2016) to quantify the molecular weight of eluate samples. The obtained standard curve was analyzed by applying a linear logarithmic transform function:

$$y = a - b \cdot \ln(x + c) \quad \text{Eq. (1)}$$

where a and b are the transform coefficients, and c is the vertical asymptote [48].

2.10 | UV-visible spectroscopy

UV-visible is the light absorptivity of fucoidan samples before and after hydrolyzes were measured by using a Varian Cary 100 spectrophotometer and 10-mm fused quartz cells in a concentration range of 0.10 to 0.33 mg/mL in running buffer. Temperature was kept constant at 25 °C by using a Peltier thermal controller. The obtained absorbance values at (280 ± 1) nm were analyzed as a function of the concentration. The extinction coefficient of both natural and hydrolyzed fucoidan samples was quantified by using the slope of the linear regression plot [49].

2.11 | Fluorescence spectroscopy

The intrinsic fluorescence of both natural and hydrolyzed fucoidan samples was, individually, measured in an excitation wavelength of 300 to 450 nm and an emission wavelength range of 350 to 600 nm exploiting a Varian Eclipse spectrofluorometer and 10-mm fused quartz cells in a concentration range of 0.10 to 0.33 mg/mL in running buffer. Temperature was kept constant at 25 °C by using a Peltier thermal controller. The acquired fluorescence spectra were analyzed for the inner-filter effect of labeled fucoidan experiments [50].

3 | RESULTS

3.1 | Fucoidan induced the activation of platelet

Fucoidan from brown seaweed Fucus vesiculosus (FFV) (Figure 1A) induced significant human platelet aggregation at a concentration of 40 μg/mL. FFV-induced platelet aggregation was abrogated by αIIbβ3 blocker eptifibatide and EDTA (Figure 1B, C), demonstrating that this platelet aggregation was dependent on αIIbβ3. Consistent with this finding, FFV significantly increased PAC-1 binding to platelets, which specifically recognizes the allosterically activated αIIbβ3 (Figure 1D),

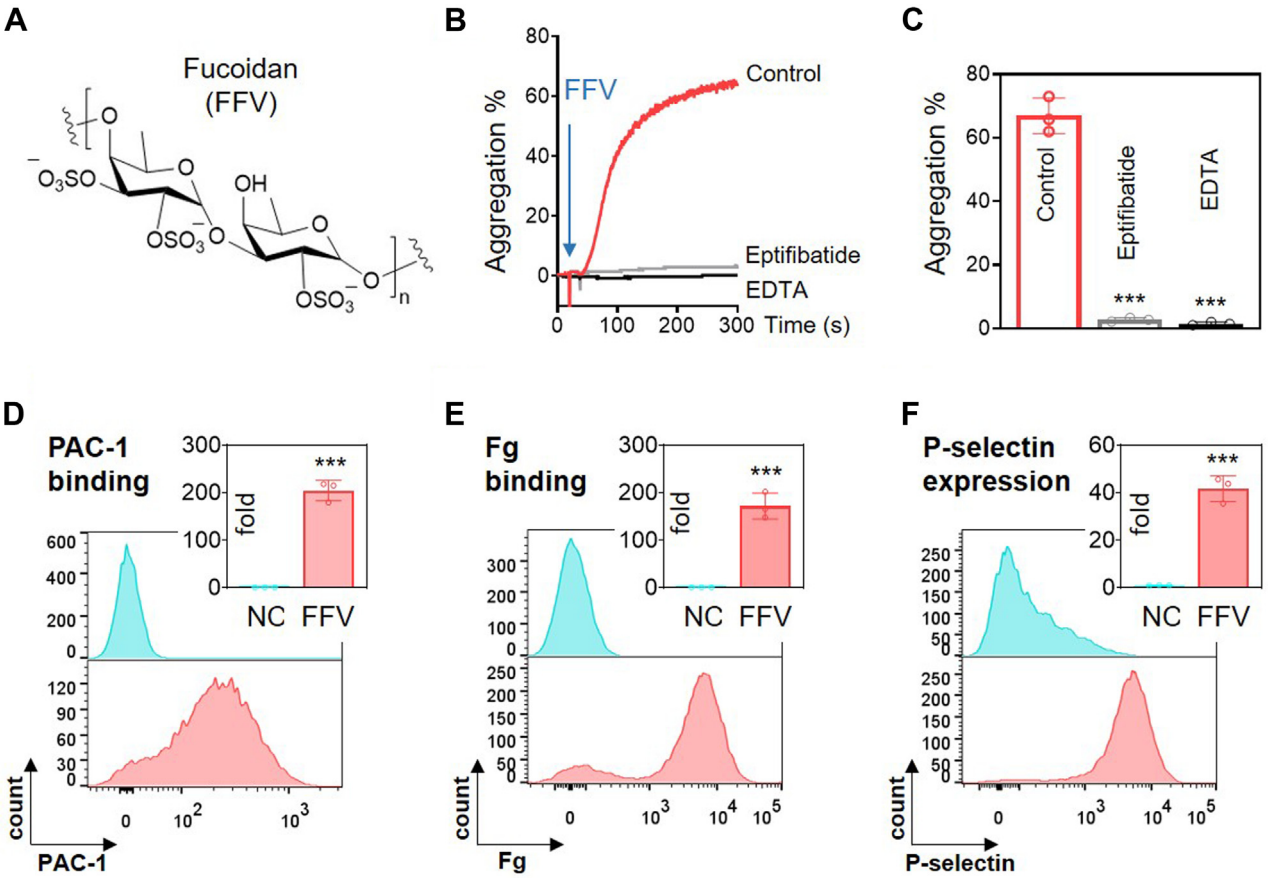

FIGURE 1 Polysaccharide fucoidan induced activation of platelet αIIbβ3. (A) Structure of fucoidan from *Fucus vesiculosus*. (B and C) Eptifibatide (0.5 mM) and EDTA (5 mM) inhibited fucoidan (FFV, 40 μg/mL)-induced platelet aggregation in gel-filtered human platelets. (D) Fucoidan increased the binding of PAC-1 to platelets. (E) Fucoidan increased the binding of fibrinogen (Fg) to platelets. (F) Fucoidan increased the expression of P-selectin on platelet membrane. $N = 3$

and therefore increased fibrinogen binding to platelets (Figure 1E). FFV also markedly induced platelet degranulation as P-selectin expression on platelet surface was significantly increased (Figure 1F). These results suggest that fucoidan induces the activation of αIIbβ3, with subsequent aggregation by ligand binding to αIIbβ3.

3.2 | GPIbα is critical in polysaccharide fucoidan-induced activation of platelet

To investigate whether GPIbα plays a role in fucoidan-induced platelet activation, we treated human platelets with OSGE (Figure 2A), which cleaves the N-terminal extracellular domain of GPIbα (Supplementary Figure S1) [42,51,52]. Interestingly, we found that OSGE treatment almost abolished FFV-induced human platelet aggregation (Figure 2A). This suggests a critical role of GPIb-IX in fucoidan-induced platelet aggregation, which is consistent with a recent study that highlights the importance of GPIb in platelet aggregation induced by a fucoidan-like sulfated polysaccharide [33].

To further elucidate the mechanism of fucoidan-induced platelet activation, we evaluated the effect of fucoidan on platelets from WT mice, VWF and fibrinogen (Fg) double-knockout mice (DKO) [6,36,37], IL4Rα/GPIbα-transgenic (IL4R/GPIbα-tg), GPIbα knockout mice [40,42,53], and integrin αIIb knockout mice [54]. FFV-induced similar aggregation levels of platelets from WT and VWF and fibrinogen double-deficient mice ($VWF^{-/-}Fg^{-/-}$), suggesting that VWF and Fg may be not required (Figure 2B), as we observed in Fg/VWF DKO and Fg/VWF/pFn triple knockout mice [6,36,37]. However, platelets deficient in either GPIbα or its ectodomain markedly decreased FFV-induced platelet aggregation (Figure 2B). Furthermore, polyclonal antibodies against GPIbα significantly inhibited fucoidan-induced platelet aggregation (Figure 2C). To further validate the role of GPIbα in FFV-induced platelet activation, we compared αIIbβ3 activation and P-selectin expression by using platelets from WT, GPIbα-deficient, and αIIb-deficient mice. FFV significantly induced αIIbβ3 activation and P-selectin expression on WT platelets but was markedly attenuated in GPIbα-deficient platelets, suggesting that GPIbα is required for FFV-induced complete activation of αIIbβ3 and

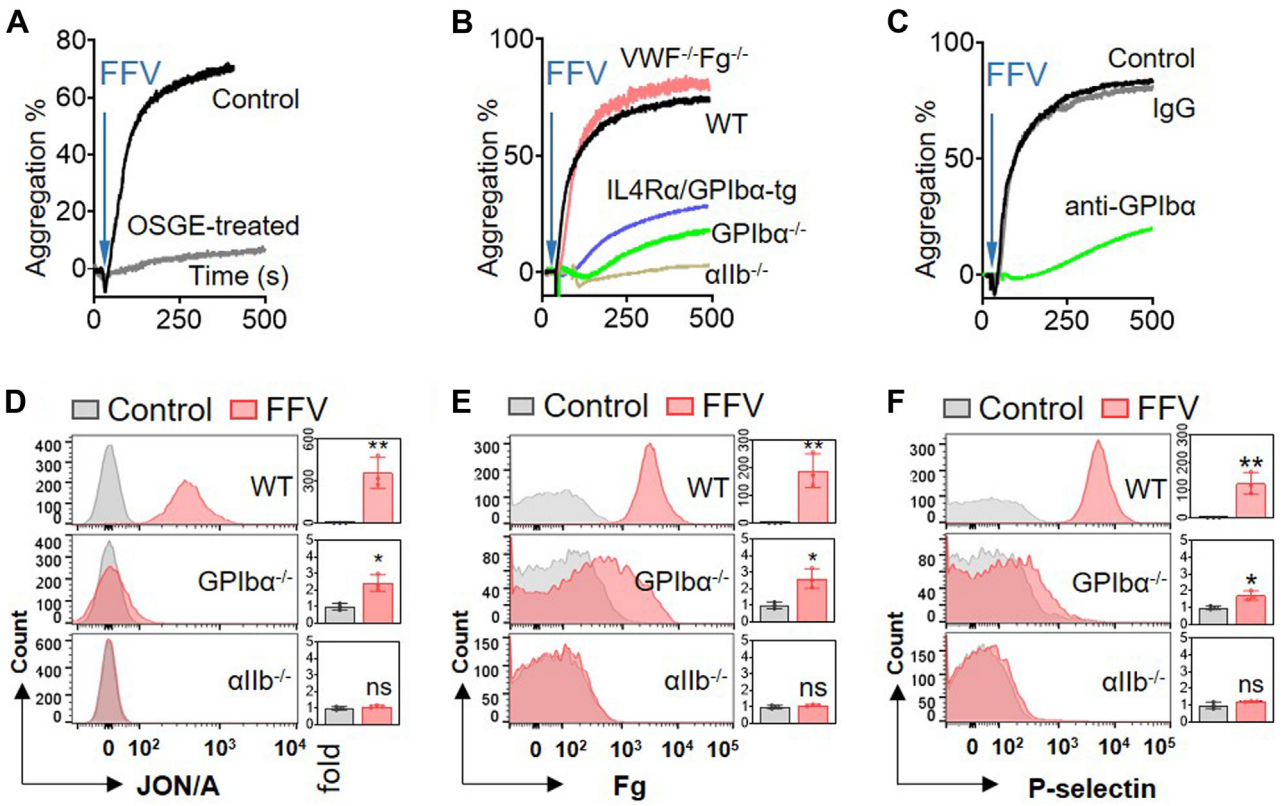

FIGURE 2 GPIb-IX is involved in fucoidan-induced activation of platelet αIIbβ3. (A) Fucoidan (FFV, 40 μg/mL) induced aggregation of human platelets with or without OSGE treatment. (B) Mouse platelet aggregation induced by fucoidan (40 μg/mL). Platelets were from wild-type (WT), VWF, and fibrinogen double knockout ($VWF^{-/-}Fg^{-/-}$), IL4Rα/GPIbα-transgenic (IL4R/GPIbα-tg), GPIbα knockout ($GPIbα^{-/-}$), and integrin αIIb knockout ($αIIb^{-/-}$) mice. (C) GPIbα antibody (40 μg/mL, emfret R300) inhibited fucoidan induced platelet aggregation. (D) The effect of fucoidan on the binding of JON/A to WT, $GPIbα^{-/-}$, or $αIIb^{-/-}$ platelets. (E) The effect of fucoidan on the binding of fibrinogen to WT, $GPIbα^{-/-}$, or $αIIb^{-/-}$ platelets. (F) The effect of fucoidan on the expression of P-selectin on WT, $GPIbα^{-/-}$, or $αIIb^{-/-}$ platelets. *N* = 3

α-granule secretion (Figure 2D-F). However, αIIbβ3 deficiency almost abolished FFV-induced P-selectin expression (Figure 2F), suggesting that FFV may have less effect on αIIbβ3-independent release of α-granules [55]. We cannot exclude that the reduced P-selectin expression may also be due to the fact that P-selectin expression in platelet is significantly less in αIIbβ3 deficient platelets [56].

3.3 | Fucoidan interacts with the extracellular domain of GPIbα and induced GPIb-mediated outside-in signaling

To further investigate whether GPIbα is directly involved in fucoidan-induced platelet aggregation, we incubated fucoidan with the recombinant human GPIbα (rhGPIbα). The rhGPIbα significantly inhibited FFV-induced platelet aggregation (Figure 3A), suggesting that FFV binds to GPIbα. Flow cytometry analysis showed that FFV was able to increase rhGPIbα binding to the platelet (Figure 3B), suggesting the possibility that FFV can crosslink multiple GPIbα on the same platelet. Previous studies reported that extracellular signal-regulated kinase (Erk) and AKT signal pathway is involved in GPIb-induced platelet activation and aggregation [17,57,58]. We found that FFV significantly induced Erk and AKT pathway activation in the presence or absence of αIIbβ3 blocker eptifibatide (Figure 3C, D). Furthermore, the FFV-induced Erk and AKT phosphorylation was significantly inhibited by GPIbα deficiency (Supplementary Figure S4), suggesting that the interaction of fucoidan with GPIbα ectodomain delivers intracellular signals that activate platelets and αIIbβ3 and therefore can induce αIIbβ3-dependent platelet aggregation.

3.4 | Fucoidan inhibited VWF-bridged platelet aggregation in the absence of αIIbβ3

Platelet aggregation described in this study includes the following: (1) Fg- or other ligands-bridged αIIbβ3 integrins on the activated adjacent platelets [5,36] and (2) VWF-bridged GPIbα on the adjacent platelets, which is also called "agglutination" by some of the researchers in this field [59,60]. Here, fucoidan-induced, αIIbβ3-mediated platelet aggregation was completely blocked by using EDTA and eptifibatide (Figure 1B). However, ristocetin-induced, VWF-bridged platelet aggregation was not significantly affected by either EDTA or eptifibatide (Figure 4A, B). Surprisingly, FFV completely blocked ristocetin-induced platelet aggregation in the presence of EDTA (Figure 4A)

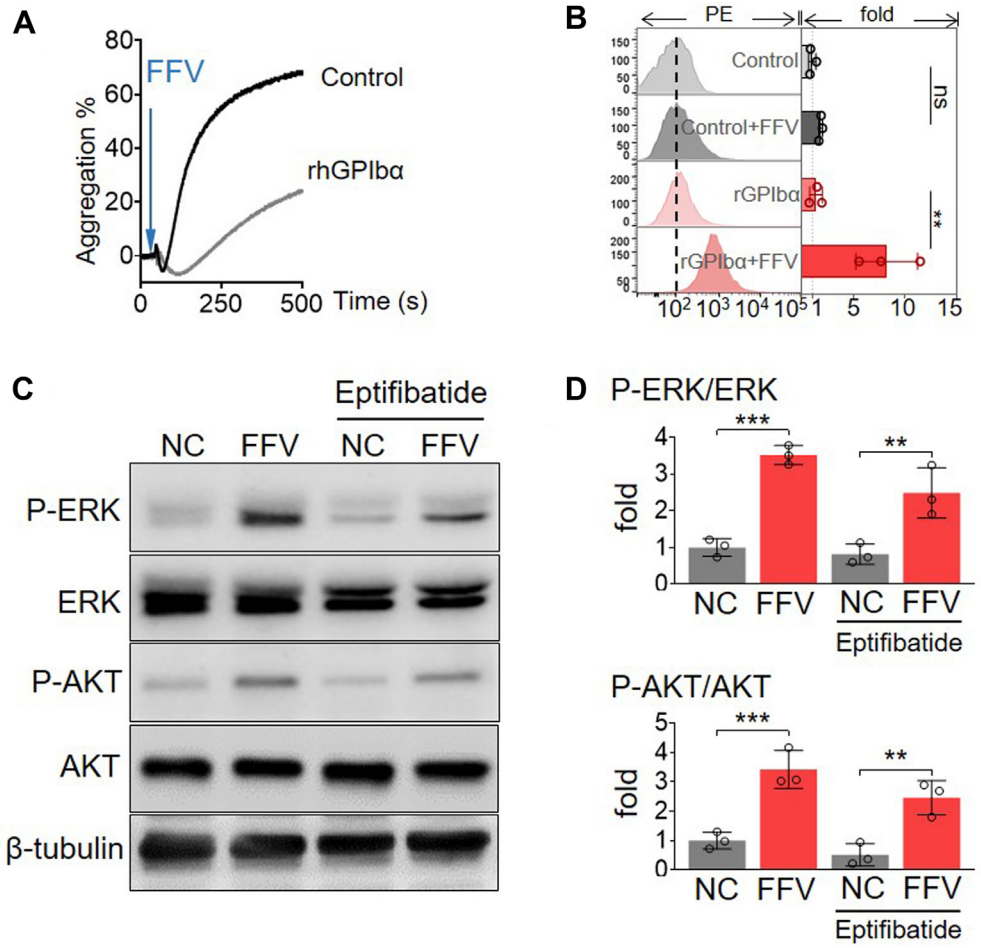

FIGURE 3 Fucoidan interact with GPIbα extracellular domain and induced GPIb-mediated signal transduction. (A) Recombinant human GPIbα (rhGPIbα, 80 μg/mL) inhibited fucoidan (FFV, 40 μg/mL) induced human platelet aggregation. (B) Fucoidan increased recombinant GPIbα binding to platelets. (C, D) Fucoidan induced GPIb-IX mediated signaling transduction in platelets in the presence or absence of eptifibatide. *N* = 3

and eptifibatide (Figure 4B), suggesting that fucoidan inhibits VWF-bridged platelet aggregation when integrin αIIbβ3 was abrogated. Further experiments with mouse platelets showed that FFV also inhibited botrocetin-induced aggregation of αIIb-deficient (Figure 4C) or EDTA pretreated platelets (Figure 4D). Therefore, despite the fact that FFV may crosslink multiple GPIbα on the same platelet and deliver GPIb-mediated outside-in signaling (Figure 3), we did not observe FFV-bridged adjacent platelets through GPIbα (Figure 4). These results suggest that although fucoidan induces GPIb-mediated integrin αIIbβ3 activation, it blocks VWF-bridged, GPIb-mediated platelet aggregation.

3.5 | Fucoidan and the hydrolyzed LMF inhibited GPIb–VWF A1 interaction and VWF-bridged platelet aggregation

A previous study discovered that high-MW but not LMF from *F. vesiculosus* induced robust platelet aggregation and activation [61]; however, the reason leading to this difference has never been explored. To investigate the effect of LMF on platelet aggregation and activation, we used hydrochloric acid to hydrolyze FFV [45,62]. To characterize the MW of fucoidan, the natural fucoidan (FFV) and hydrolyzed LMF samples were individually analyzed by using SEC. Our obtained chromatograms from SEC analysis of fucoidan showed a broad elution peak in a range of 13 to 21 mL for native fucoidan and a broad elution peak in a range of 100 to 120 mL for hydrolyzed fucoidan solutions (Figure 5A). To quantify the molecular weights of FFV and LMF samples, we performed a linear logarithmic regression analysis by using known MW standards and quantified the molecular weight of FFV in an average of $(9 \pm 1) \times 10^5$ kDa (~4 million units) with no evidence of LMF mixed in the FFV sample. We further quantified MW of LFM in an average range of 0.22 to 2.0 kDa (1-10 units) (Figure 5B). To confirm that fucoidan hydrolysis was performed properly, we measured the extinction coefficient of FFV as (2.08 ± 0.01) L/g•cm and (3.62 ± 0.04) L/g•cm for LMF at 280 nm in the experimental buffer at pH 7.4 at 25 °C exploiting a UV-Vis spectroscopy method (Supplementary Figure S2A). We characterized the intrinsic fluorescence of fucoidan by using a fluorometry technique. Our obtained fluorescence spectra indicated weak fluorescence at maximum excitation and emission wavelengths of 350 nm and

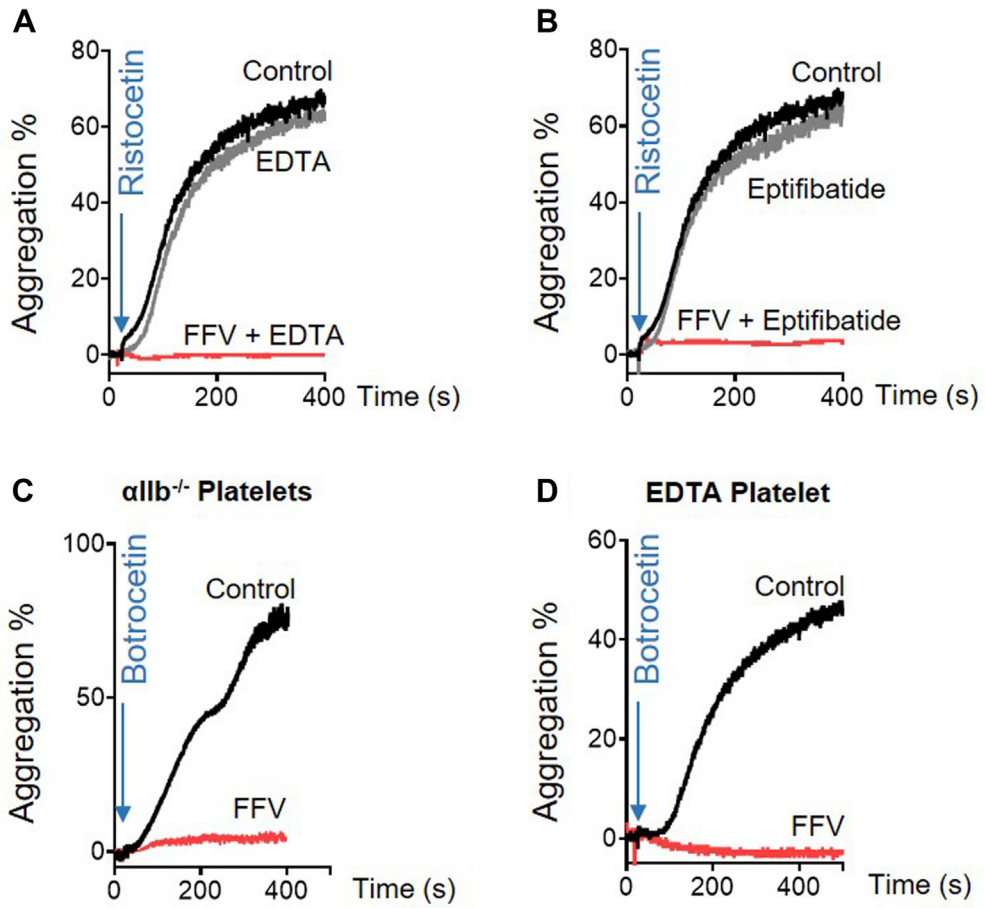

FIGURE 4 Fucoidan inhibited GPIb-mediated platelet aggregation in the absence of functional integrin αIIbβ3. Aggregation assay was performed using gel-filtered platelets with recombinant VWF (10 μg/mL). (A) Effect of fucoidan (FFV, 40 μg/mL) on ristocetin-induced human platelet aggregation in the presence of EDTA (5 mM). (B) Effect of fucoidan on ristocetin-induced human platelet aggregation in the presence of eptifibatide (0.5 mM). (C) Effect of fucoidan on botrocetin-induced platelet aggregation of $\alpha IIb^{-/-}$ mice. (D) Effect of fucoidan on botrocetin-induced mouse platelet aggregation in the presence of EDTA (5 mM). *N* = 3

450 nm, respectively, for both LMF and FFV samples (Supplementary Figure S2B).

We found that both FFV and LMF dose-dependently inhibited VWF A1 domain binding to recombinant GPIbα (Figure 5C) and human platelets (Figure 5D), despite FFV showing more potency than LMF in blocking the GPIb–VWF A1 domain interaction. Furthermore, FFV drastically dissolved the preformed, GPIb–VWF A1 domain-mediated platelet aggregates, whereas LMF also did but to a lesser extent (Figure 5E, F). Contrary to FFV, LMF did not induce significant platelet activation (Figure 5G) or aggregation, but rather blocked FFV-induced platelet aggregation (Figure 5H). We found that the deficiency of GPIbα (or GPIbα/IL4R-tg) almost abolished the FITC-labeled LMF binding to platelet. However, the binding signal was similar between GPIbα-deficient and GPIbα/IL4R-tg platelets (Supplementary Figure S3). This suggests that the ectodomain of GPIbα is the key binding site for fucoidan to platelet and GPIb complex; therefore, LMF retains the activity of blocking GPIb–VWF interaction, although it does not activate platelets.

3.6 | LMF specifically inhibited VWF-bridged, GPIb-mediated platelet aggregation

Because FFV, but not LMF, can induce platelet activation and aggregation (Figure 5G, H), we therefore investigated whether LMF can inhibit VWF-bridged, GPIb-mediated platelet aggregation. Interestingly, we found that LMF inhibited ristocetin-induced platelet aggregation dose dependently (Figure 6A, B) but had no significant effect on ADP (Figure 6C), PAR activator (PAR1AP) (Figure 6D), and collagen-induced platelet aggregation (Figure 6E). Despite previous reports showing that CLEC-2 and PEAR-1 are significantly involved in fucoidan-induced platelet aggregation and activation [32,33], we found that the CLEC-2 activator mAb AYP1 [63] and hemin [64] induced (Figure 6F, G) and PEAR1 antibody-induced (Figure 6H) human platelet aggregation was not significantly affected by LMF, suggesting that LMF specifically inhibits VWF-bridged, GPIb-mediated platelet aggregation. Collectively, these data show that polysaccharide fucoidan (FFV) induced αIIbβ3

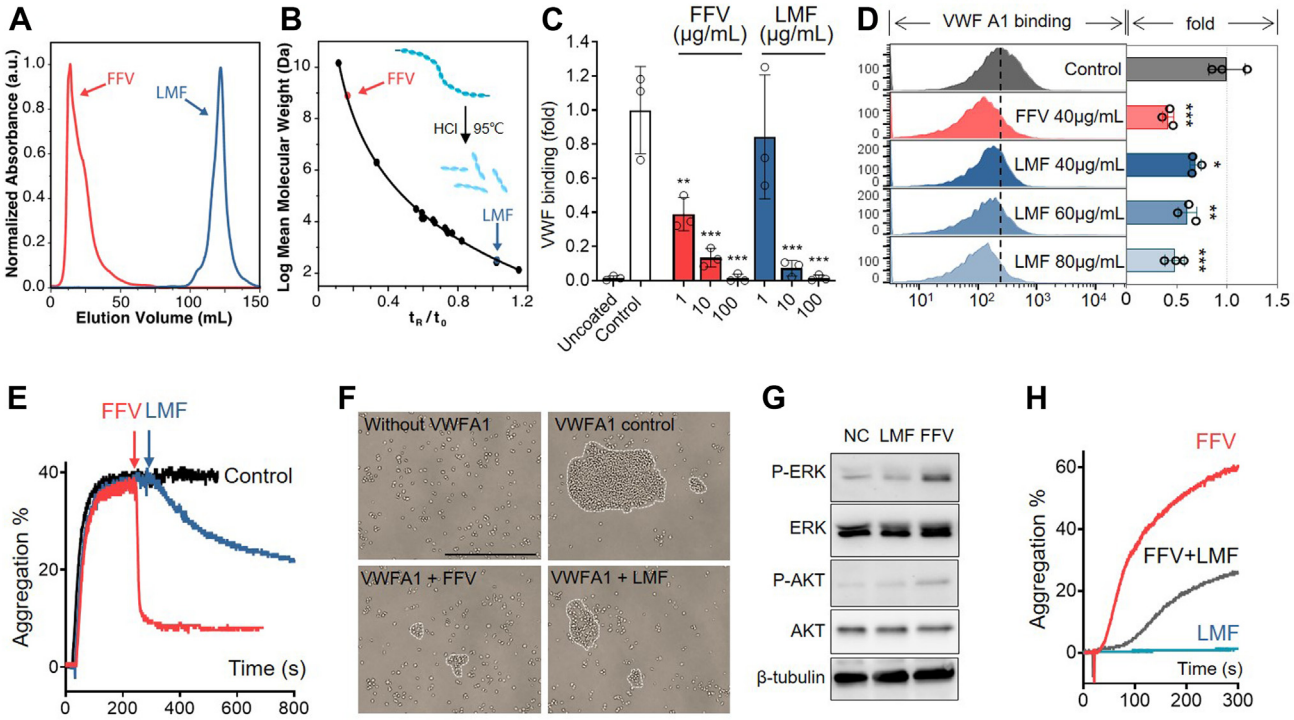

FIGURE 5 Fucoidan and the hydrolyzed low-molecular weight fucoidan inhibited GPIbα-VWF A1 interaction. (A) Superimposed SEC chromatograms of FFV (red) and hydrolyzed low-molecular weight fucoidan (LMF) (blue) in 10 mM sodium phosphate buffer pH 7.4, 137 mM NaCl, 4 mM KCl at 25 °C at 0.5 mL/min. (B) SEC calibration plot by using a linear logarithmic analysis. FFV elutes in a MW range of $(9 \pm 1) \times 10^8$ Da, and LMF elutes in a range of 220 to 2000 Da. (C) FFV and LMF dose-dependently inhibited VWF binding to recombinant GPIbα detected by enzyme-linked immunosorbent assay. (D) Effect of FFV and LMF on recombinant VWF A1 binding to platelets detected by flow cytometry. (E) Representative curves and (F) images showed FFV and LMF disassociated VWF A1-induced agglutination of the paraformaldehyde-fixed platelet. Bar = 100 μm. (G) Effect of FFV (40 μg/mL) and LMF (80 μg/mL) on GPIb-IX mediated signaling transduction in platelets. (H) Effect of FFV and LMF on platelet aggregation. *N* = 3

activation and platelet aggregation through interaction with the LBD of GPIbα. LMF retains the activity of binding to LBD of GPIbα, therefore blocking the VWF-bridged platelet aggregation but not activating platelets (Figure 6I).

3.7 | LMF inhibited GPIb-mediated platelet adhesion and spreading

GPIb-mediated platelet adhesion to VWF plays a critical role in subsequent platelet activation, spreading, and aggregation *in vivo*. Therefore, we investigated the effect of LMF on platelet adhesion and spreading on immobilized VWF and fibrinogen, respectively. LMF not only significantly decreased platelet adhesion on immobilized VWF A1 domain but also inhibited the spreading area of the attached platelets (Figure 7A-D). However, LMF has no significant effect on platelet adhesion or spreading on immobilized fibrinogen (Figure 7E-H). These results suggest that LMF specifically inhibits GPIb-IX rather than αIIbβ3-mediated platelet adhesion and spreading. We found FFV did not enhance, rather inhibited, GPIb/A1-induced platelet adhesion and spreading on immobilized VWF A1 domain (Supplementary Figure S5A-D). However, FFV increased platelet adhesion and spreading on immobilized full-length VWF (Supplementary Figure S5E-H), which is likely mediated by αIIbβ3 integrin-RGD interactions on VWF.

3.8 | LMF inhibited platelet thrombus growth under high shear forces

Collagen is a major subendothelial matrix protein that is exposed after vessel injury. Although several mechanisms mediate platelets adhesion and aggregation over exposed collagen at low shear, the GPIb–VWF interaction becomes increasingly critical at higher shear. Plasma VWF binds exposed collagen and undergoes a shear mediated structural change that exposes its A1 domain, leading to VWF-bridged, GPIb-mediated platelet adhesion and thrombus formation in arterial vessels [65]. The effect of LMF on human platelet thrombus formation and growth under different shear forces was first assessed by using an *ex vivo* perfusion chamber at the shear rate of 300 s^{-1}, 1800 s^{-1}, and 5000 s^{-1}, which can represent the hemodynamic shear stress in veins, large and small arterioles,

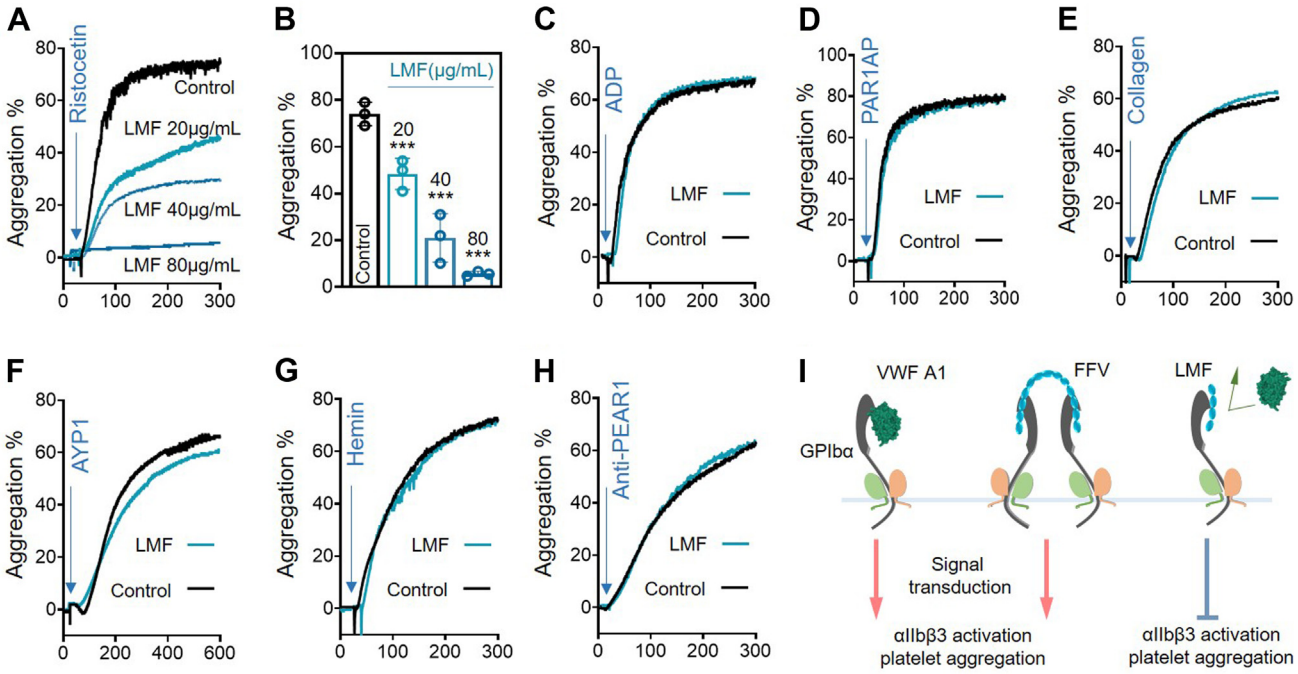

FIGURE 6 Low-molecular weight fucoidan inhibited GPIb-IX mediated human platelet aggregation. (A, B) Dose-dependent effect of low-molecular weight fucoidan (LMF) on ristocetin (1.5 mg/mL) induced platelet aggregation. Effect of LMF (80 μg/mL) on (C) ADP (10 μM), (D) PAR1AP (10 μM), (E) collagen (5 μg/mL), (F) AYP1 (10 μg/mL), (G) hemin (10 μM), and (H) anti-PEAR1 antibody-induced platelet aggregation. *N* = 3. (I) Schematic diagram shows the proposed mechanism of FFV-induced platelet activation and LMF-mediated platelet inhibition

respectively. LMF dose-dependently inhibited thrombus formation under high shear forces. The inhibitory effect was enhanced as shear stress escalates (Figure 8). These data suggest that LMF prefers inhibiting high shear stress-mediated thrombus formation, indicating a specificity for arterial thrombosis or stenosis-related thrombosis.

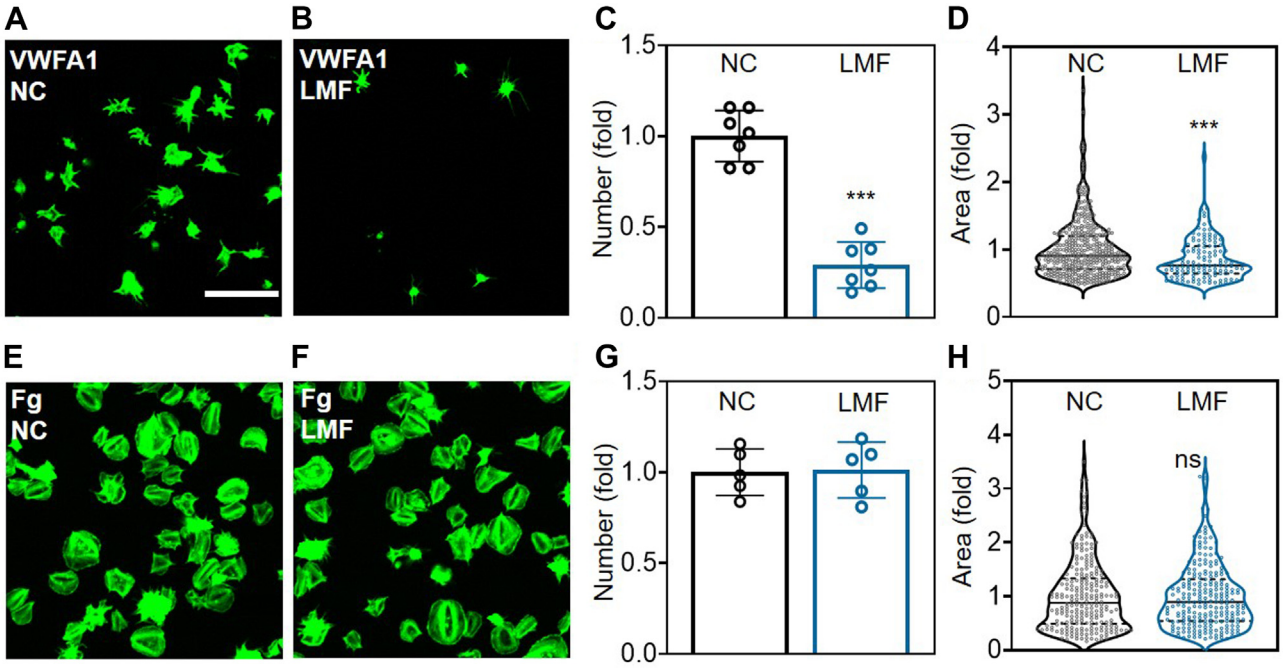

FIGURE 7 Low-molecular weight fucoidan inhibited GPIb-IX mediated platelet adhesion and spreading. (A-D) Effect of low-molecular weight fucoidan (LMF) (80 μg/mL) on platelet adhesion and spreading on immobilized VWF A1 domain. (E-H) Effect of LMF (80 μg/mL) on platelet adhesion and spreading on immobilized fibrinogen. Bar = 20 μm

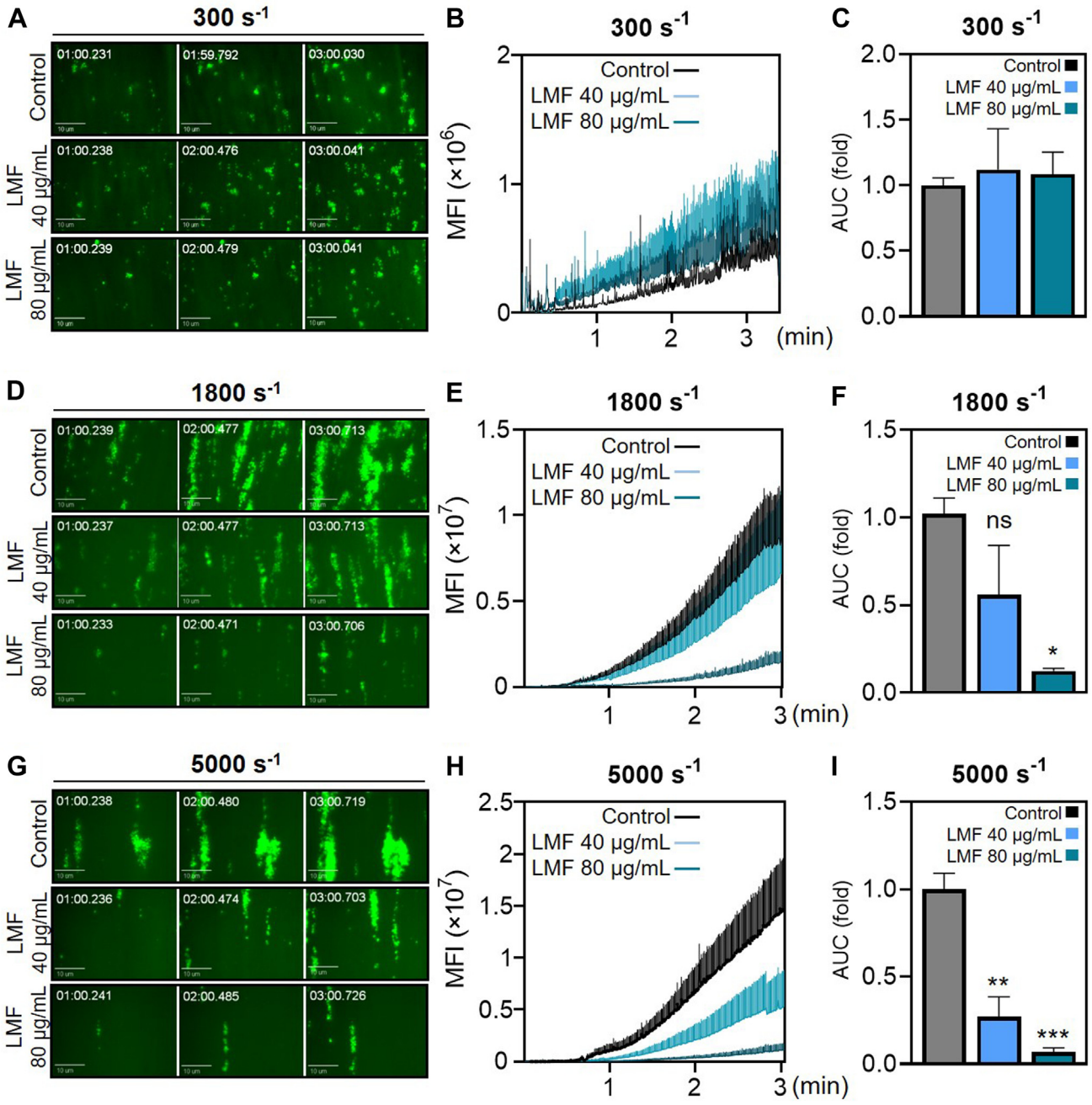

FIGURE 8 Low-molecular weight fucoidan inhibited human platelet thrombus formation and growth under high shear force. An *ex vivo* perfusion chamber model was used to investigate the effect of low-molecular weight fucoidan (LMF) on thrombus formation and growth under shear. The representative images and quantification of platelet thrombosis formation and growth with or without LMF at the shear rate of 300 s^{-1} (A-C), 1800 s^{-1} (D-F) and 5000 s^{-1} (G-I). The curve represent platelet mean fluorescence intensity (MFI) and the shaded regions represent SEM of 4 to 5 independent experiments. Significance analysis was performed by using one-way analysis of variance with the areas under the curves (AUC). Bar = 10 μm.

4 | DISCUSSION

In this study, we demonstrated that *F. vesiculosus*-derived fucoidan interacted with the N-terminal LBD of GPIbα, leading to GPIb-IX-mediated outside-in signaling and subsequent αIIbβ3 activation (inside-out signaling) and platelet aggregation. The binding of fucoidan to GPIbα resulted in the blockade of GPIbα-VWF interaction. The hydrolyzed LMF did not induce platelet activation but remains to specifically inhibit GPIbα-VWF interaction, therefore leading to the inhibition of GPIb-induced platelet adhesion, aggregation, and thrombus growth at high shear. This study highlights the therapeutic potential of LMF in the treatment of arterial thrombosis.

The GPIb-IX complex is abundantly and almost exclusively expressed in platelets and megakaryocytic lineage. The interaction of

GPIb-IX with VWF, the principal ligand of GPIb-IX in plasma, is crucial for initial platelet adhesion at sites of vascular injury and subsequent thrombus growth, especially at high hemodynamic shear forces [1,6,7,11]. This interaction can induce intracellular signals that activate integrin αIIbβ3, which synergistically enhances thrombus growth [13–15]. Our previous study also demonstrated that GPIb–VWF interaction is essential for occlusive thrombus growth because shear stress escalates before vessel occlusion [6]. Therefore, theoretically, GPIb-IX is a promising target to treat arterial thrombosis and related cardiovascular ischemia. Intensive preclinical studies by using different thrombosis models (eg, stroke and thrombotic thrombocytopenic purpura) in murine [7,19], nonhuman primates [10,20,21], and recent clinical studies have suggested GPIb-IX as an efficient and may be a safe target for antithrombotic drug development [9,22]. However, no drug targeting GPIbα has been successfully developed to date.

Our study first discovered that hydrolyzed LMF from seaweed *F. vesiculosus* markedly inhibited human thrombi growth at high shear by blocking GPIb-IX. The inhibitory effect was enhanced as shear stress escalates (Figure 8), suggesting that LMF has a great potential in protecting arterial thrombosis and resultant occlusive ischemic stroke or myocardial infarction. Previous studies indeed showed that fucoidan of either middle- or low-MW inhibited arterial thrombosis and was more effective than the anticoagulant heparin in different animal models, which suggests that fucoidan-mediated platelet inhibition is significantly involved *in vivo* [29–31]. The inhibitory effect of LMF on high shear-induced thrombosis was not because of its potential anticoagulant activity because there were no significant changes in thrombelastogram parameters under the concentration that LMF markedly inhibited thrombi growth (Supplementary Figure S6). It has been reported that fucoidan inhibited microvascular thrombus formation independently from its P- and L-selectin targets [66]. Meanwhile, it is also not likely that LMF inhibited shear-induced thrombosis using the previously described targets CLEC-2 and PEAR1 [32,33] because LMF did not inhibit CLEC-2 or PEAR1 agonist-induced platelet aggregation, and CLEC-2 is not required for platelet thrombus growth at arteriolar shear [67]. In combination with the data from platelet aggregation (Figure 6), adhesion (Figure 7), and thrombus growth (Figure 8) assays, we demonstrate that LMF is highly selective toward GPIb-induced arterial thrombosis. However, the deficiency or inhibition of GPIbα did not completely inhibited FFV-induced platelet aggregation. This suggests that other receptors such as CLEC-2 [32] and PEAR1 [33] may also be involved in multivalent FFV-induced, GPIb-independent platelet aggregation.

The polymerization degree of fucoidan, as reflected by the MW, is highly relevant to the biological activities of fucoidan [26]. A minimal sugar chain length of 70 (≈15 kDa) is required for the desired procoagulant activity of fucoidan [26]. However, it is unclear whether and how the polymerization of fucoidan affects its effect on platelets [31,61,68]. A recent study using synthetic sulfated α-L-fucoside-pendant glycopolymers showed that fucoidan with an average monomeric units of 13 significantly induced platelet activation but showed less potency than that with 329 units [33]. Therefore, the proplatelet and antiplatelet activities of fucoidan may also be highly determined by its polymerization degrees. By using the previously established HCl hydrolysis treatment [45,62], we generated the partially hydrolyzed LMF. The higher polymerized natural fucoidan, rather than LMF, can induce GPIb-IX dependent signaling, therefore leading to sequential intracellular activation of αIIbβ3 and platelets. However, LMF (0.22-2.0 kDa) retained to block the ectodomain of GPIbα despite in a less potent manner than the larger fucoidan chains (Figure 5). Therefore, LMF can competitively inhibit fucoidan-induced platelet aggregation, block GPIb–VWF interaction, and block resultant platelet aggregation and thrombus growth at high shear (Figures 6–8). We cannot exclude that hydrolysis treatment may not only decrease the polymerization level but also slightly decrease the degree of sulfation from the fucoidan polymers [45,62], which may synergistically affect the binding affinity of LMF to GPIbα. Collectively, our data first indicated that the polymerization degree (or size) determines the GPIb-IX mediated proplatelet and antiplatelet activities of fucoidans.

Fucoidan has been previously proposed as promising agent to treat hemophilia A and B by improving blood coagulation through blocking TFPI. This protective role in hemostasis has been proven in studies examining both murine and canine deficient in coagulation factor VIII or IX [25,27]. VWF and Fg have long been recognized as 2 essential molecules that mediate platelet adhesion and aggregation and are also critically involved in blood coagulation [6,36]. Deficiencies in these molecules are associated with von Willebrand disease (VWD) and afibrinogenemia, inherited bleeding disorders with limited treatment strategies [6,36,69,70]. Here, we discovered that fucoidan can markedly induce aggregation of platelets from VWF- and Fg-deficient mice (Figure 2B), suggesting therapeutic potential of fucoidan in the treatment of these bleeding disorders by inducing platelet hemostatic plugs. However, we found that αIIbβ3 is indispensable for fucoidan-induced platelet aggregation (Figure 2B), suggesting other ligands that mediate αIIbβ3 activation (eg, fibronectin [38,71], vitronectin [72], Cadherin 6 [73], and multimerin 1 [74]) may be required for fucoidan-induced hemostatic plug formation. The molecular basis for fucoidan-induced, VWF- and Fg-independent platelet aggregation is not fully understood but is worthwhile to be clarified to further address the therapeutic potential of fucoidan for VWD, afibrinogenemia, and other bleeding disorders.

Generally, fucoidan is accepted as a cardioprotective agent and has been developing as a bioactive oral supplement worldwide with no reported adverse effects [23,24]. Several studies characterized that fucoidan inhibited arterial thrombosis without affecting hemostasis [29–31]; however, the mechanism has not been adequately studied. Clarifying this mechanism may further promote the medical application of fucoidans as antithrombotic drugs, especially for stroke or myocardial infarction that mainly resulted from arterial thrombosis. The primary antithrombotic drugs currently used in the treatment of cardiovascular thrombotic disorders are aspirin, clopidogrel, and αIIbβ3 antagonists [4,18]. They are effective in platelet inhibition and thrombosis prevention but may cause thrombocytopenia and bleeding disorders in some patients [9,41,75]. GPIb-IX is specifically expressed in platelet/megakaryocyte and is predominantly activated under high hemodynamic shear *in vivo* [5–8]. We found that LMF specifically

inhibited GPIb-induced platelet aggregation (Figure 6), adhesion (Figure 7), and thrombus growth at high shear (Figure 8), without significantly affecting blood coagulation (Supplementary Figure S6). These prerequisites may make LMF a unique/superior GPIbα inhibitor that is eligible for arterial thrombosis treatment and prevention, through exhibiting minimal bleeding side effects [7,9,10,21].

In conclusion, we have exemplified the GPIb-IX-mediated, size-dependent, proplatelet and antiplatelet activities of fucoidan. Interaction of the higher MW fucoidan with GPIbα delivers intracellular signals that activate platelets and therefore can induce αIIbβ3-dependent platelet aggregation. Opposingly, interaction of LMF with GPIbα blocks GPIb–VWF interaction, therefore inhibiting GPIb-induced platelet adhesion, aggregation, and thrombus growth. Our study suggests the size-dependent, dualistic therapeutic potential of fucoidans against bleeding disorders and arterial thrombosis through GPIbα interaction.

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AUTHOR CONTRIBUTIONS

C.S. designed the research studies, performed the experiments, analyzed data, generated the figures, and wrote the manuscript. D.T.M., A.A.S., R.X., W.M, and E.C. performed the experiments, analyzed the data, and made critical revision of the manuscript. P.B. generated the recombinant proteins and made critical revision of the manuscript. D.K., Z.C., and P.C. breed the mouse strains, contributed to the animal protocol, and made critical revision of the manuscript. B.H. and L.L. analyzed the data and made critical revision of the manuscript. G.Z., X.L., and P.E.J. provided critical regents and made critical revision of the manuscript. H.N. is the Principal Investigator who supervised the project, analyzed the data, and wrote and made critical revision of the manuscript. All authors read and approved the final manuscript.

DECLARATION OF COMPETING INTEREST STATEMENT

The authors declare that they have no competing interests.

ETHICS STATEMENT

All animal studies were approved by the Animal Care Committees of St. Michael's Hospital, Toronto, Canada. Procedures using human blood samples were approved by the Research Ethics Board of St. Michael's Hospital, Toronto, Canada.

DATA AVAILABILITY

All data generated or analyzed during this study are included in this article (and its Supplementary Information files).

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SUPPLEMENTARY MATERIAL

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