Two likely mechanisms for the initiation of arterial platelet thrombus formation under conditions of elevated fluid shear stresses are: (1) excessive adhesion and aggregation of platelets from rapidly flowing blood onto the exposed subendothelium of injured, atherosclerotic arteries; or (2) direct, fluid shear stress-induced aggregation of platelets in constricted arteries with intact endothelial cells. Mechanism (1) was simulated using a parallel plate flow chamber, fibrillar collagen type I-coated slides, and mepacrine-labeled (fluorescent) platelets in whole blood anticoagulated with citrate, hirudin, unfractionated porcine heparin, or low molecular weight heparin flowing for 1 to 2 minutes at wall shear rates of 100 to 3,000 seconds\(^{-1}\) (4 to 120 dynes/cm\(^2\)). The precise sequence of interactions among von Willebrand factor (vWF), glycoprotein (GP)Ib, and GPllb-IIIa during platelet adhesion and subsequent aggregation were resolved by direct real-time observation using a computerized epifluorescence video microscopy system. Adhesion at high shear rates was the result of the adsorption of large vWF multimers onto collagen and the binding of platelet GPIb to the insolubilized vWF. Aggregation occurred subsequently and required the binding of ligands, including vWF

I

N SMALL ARTERIES partially obstructed by atherosclerosis or vasospasm, fluid shear stresses may increase many-fold above the normal time-average level of about 20 dynes/cm\(^2\) and may lead to platelet thrombosis. Among the most likely possibilities for the acute initiation of arterial platelet thrombi are: (1) excessive adhesion of blood platelets onto the exposed subendothelium of injured arteries and arterioles containing collagen and von Willebrand factor (vWF), with subsequent platelet aggregation and accumulation; or (2) direct, excessive aggregation of platelets from the blood in constricted areas of the arterial circulation that are lined with intact endothelial cells. Presently, there is controversy regarding the precise sequence of involvement of GPIb and GPIIb-IIIa in platelet adhesion and aggregation onto reactive surfaces. In this report we describe the use of video microscopy coupled with digital imaging techniques to resolve the sequence of platelet adhesion and aggregation events in real-time on fibrillar collagen type I, the collagen type that predominate in atherosclerotic arterial subendothelium. Using this model of thrombotic mechanism 1 (simulated by the perfusion of blood over collagen type I at different shear rates\(^*\)), as well as a model of thrombosis mechanism 2 (simulated by shear stress*-induced platelet aggregation in a rotational cone and plate viscometer), we have investigated the effects of new potentially useful anti-arterial thrombotic agents.

It has previously been demonstrated that vWF becomes rapidly insolubilized on the exposed subendothelium of human arteries,\(^2\) as well as onto collagenous components of the vessel wall,\(^3,4\) and that this precedes and augments platelet adhesion.\(^5\) It has also been shown\(^6\) that platelet adhesion to human arterial subendothelium can be inhibited by a monoclonal antibody (MoAb) against glycoprotein (GPIb) whereas an MoAb against GPIIb-IIIa eliminates platelet aggregate formation. At higher wall shear rates, the GPIIb-IIIa antibody also suppressed the spreading of adherent platelets. Fressinaud et al\(^7\) used MoAbs against vWF that prevent its binding to

\(\text{Real-Time Analysis of Shear-Dependent Thrombus Formation and Its Blockade by Inhibitors of von Willebrand Factor Binding to Platelets}

By B. Rita Alevriadou, Joel L. Moake, Nancy A. Turner, Zaverio M. Ruggeri, Bernard J. Folie, Martin D. Phillips, Alain B. Schreiber, Michael E. Hrinda, and Larry V. McIntire

* \(\text{Shear}^*\) refers to the relative parallel motion between adjacent fluid planes during flow. The difference in the velocity between adjacent layers of blood at various distances from the vessel wall determines the local shear rate, expressed in \(\text{cm/sec per cm}\), or inverse seconds (s\(^{-1}\)). Fluid shear stress in dynes/cm\(^2\) is a measure of the force required to produce a certain rate of flow of a viscous liquid, and is proportional to the product of shear rate and blood viscosity. Fluid shear stresses in the circulation are imposed on the surfaces of blood cells, where they may produce alterations in the structure, exposure or clustering of membrane-associated molecules.

\(\text{From the Cox Laboratory for Biomedical Engineering, Rice University; the Department of Hematology, Baylor College of Medicine, Houston, TX; Roon Research Laboratory for Arteriosclerosis and Thrombosis, the Division of Experimental Hemostasis and Thrombosis, the Department of Molecular and Experimental Medicine and Committee on Vascular Biology, Scripps Research Institute, La Jolla, CA; Exxon Chemical, Antwerp, Belgium; and Rhone-Poulenc Rorer Central Research, King of Prussia, PA.}

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\(\text{Address reprint requests to Joel L. Moake, MD, Cox Laboratory for Biomedical Engineering, Rice University, PO Box 1892, Houston, TX 77251.}

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GPIb, GPIIb-IIIa, or collagen, and found that all three antibodies inhibited platelet adhesion to equine and human collagen type III by ~30% at 2,600 s⁻¹; however, a mixture of all three was required to inhibit almost completely platelet accumulation on collagen.⁸,⁹ In a later report,¹⁰ they also concluded that Arg-Gly-Asp-Ser (RGDS)-containing peptides that block vWF binding to platelet GPIb-IIIa inhibit vWF-mediated platelet adhesion to collagen over 5-minute periods of blood flow at 2,600 s⁻¹. Recently, Sheppeck et al¹¹ reported that platelet adhesion to a polyethylene surface at 300 s⁻¹ required GPIIb-IIIa, and that GPIb was necessary for subsequent platelet aggregation.

In experiments using purified human ¹² ⁵-vWF multimers insolubilized onto glass capillary tubes, Olson et al¹² found that the adherence of ¹³¹I-labeled platelets (suspended with red blood cells [RBCs]) to the insolubilized vWF increased progressively as shear rates increased to 2,500 s⁻¹. Both platelet GPIb and GPIIb-IIIa receptors were involved in platelet adhesion onto the glass surfaces coated with the vWF alone.

The various studies cited above used morphometric or radioactive techniques to evaluate the accumulation of platelets on the exposed surfaces after the occurrence of adhesion/aggregation. In contrast, we have combined a parallel-plate perfusion chamber with a recently developed computerized epifluorescence video microscopy system to observe and sequence in vWF that is an essential part of the GPIIb-IIIa receptor complex with GPIb in the platelet membrane,¹* in shear effects (including shear-induced signal transduction) has not yet been evaluated.

We studied the effects of polymeric aurin tricarboxylic acid (ATA) and a recombinant fragment of vWF (rvWF²⁴⁵-⁷³³) that contains amino acids 445 to 733 (including binding domains for platelet GPIb, unfractionated porcine heparin, and collagen¹⁵) of the mature vWF monomer in both the viscometer and perfusion models of arterial thrombosis. Polymeric ATA binds to large vWF multimers and prevents their interaction with platelet GPIb under conditions of elevated levels of fluid shear stress,²⁰,²² whereas rvWF²⁴⁵-⁷³³ (after reduction and alkylation to prevent polymerization) binds to GPIb in the absence of any modulator.¹⁹ Both ATA and rvWF²⁴⁵-⁷³³ inhibit platelet aggregation in vivo in dog²³ and monkey²². Foils models of constrictive coronary thrombosis, and it was our additional goal to determine their effects in our two model systems in the presence of citrate, hirudin, unfractionated porcine heparin, and low molecular weight heparin.

**MATERIALS AND METHODS**

**Blood Collection and Preparation**

The final concentration of anticoagulant used was either 0.38% wt/vol sodium citrate, 0.3 to 10 U/mL (1.8 to 59 μg/mL) unfractionated porcine heparin (heparin sodium; Elkins-Sinn Inc, Cherry Hill, NJ), 1 U/mL (15 μg/mL, anticoagulant activity = 63 U/mg) low molecular weight heparin (Enoxaparin; Rhone Polenc Rorer, King of Prussia, PA), or 200 U/mL recombinant hirudin, a specific thrombin inhibitor (P H. Johnson, SRI International, Menlo Park, CA). No observable fibrin formation occurred using the various anticoagulants (and combinations) in these concentrations. Blood was collected from nonsmoking and medication-free donors into polypropylene syringes containing anticoagulant with or without 10 μmol/L of the fluorescent dye mepacrine (quinacrine dihydrochloride; Sigma, St Louis, MO). Mepacrine is concentrated in the dense granules of platelets and the granules of leukocytes and has no effect on normal platelet function at this concentration.²³ These two cell types are clearly distinguished by epifluorescence microscopy. Any fluorescence from within the erythrocytes is quenched by hemoglobin. Leukocytes do not adhere to the collagen-coated surface at the relatively high shear rates used in our studies. Platelet secretion of mepacrine from dense granules after adhesion is sufficiently limited in rate and extent that platelet fluorescence does not decrease detectably under our experimental conditions during adhesion and subsequent aggregation.

**Viscometer Experiments**

PRP was prepared by centrifugation of citrated, hirudin-treated, or heparinized whole blood at 150g for 15 minutes. After the PRP samples (0.6 mL) were incubated for 5 minutes with either buffer or test sample (MoAb, rvWF²⁴⁵-⁷³³, or polymeric ATA), two 10-μL aliquots were dispensed into 10 mL of Coulter Isoton II (Coulter Electronics, Inc, Hialeah, FL) containing 0.5% glutaraldehyde for a pre-shear platelet count. The PRP was subjected to controlled shear stress levels ranging from 90 to 180 dynes/cm² in a stainless steel cone and plate viscometer (Model 781; Ferranti Electric, Inc, Commack, NY) for 30 seconds at room temperature. After shearing, 10-μL duplicates of each sample were dispensed into 10 mL of the Isoton/glutaraldehyde. Platelet counts and size distributions were determined with an electronic particle counter (Coulter Multisizer; Coulter Electronics, Inc) using 100-μL sample volumes and a 50-μm diameter aperture. The percent decrease in platelets, compared with unsheared controls, was directly related to the percent of platelet aggregation in each sample.¹⁴,¹⁵ Each experiment was repeated at least three times (in duplicate), and the study used more than 20 different normal blood donors.

**Preparation of Collagen Solution and Collagen-Coated Coverslips**

Suspensions of 2.1 mg/mL type I acid-insoluble collagen fibrils from bovine achilles tendon (Sigma) in 0.5 mol/L acetic acid, pH
Unlike native vWF, rvWF445-733 binds to GPIb without the domains of vWF that interact with GPIb, heparin, and collagen. Amino acid residues 445 to 733 of the mature vWF subunit with the trocetin, rvWF445-733 inhibits the binding of vWF multimers to GPIb without affecting thrombin-induced vWF binding to GPIIb-IIIa.

The other whole MoAbs used in this study caused no detectable platelet aggregation under our experimental conditions. LC-CP8 is a murine monoclonal IgG, that reacts with platelet GPIb-IIIa and completely blocks the binding of vWF, fibrinogen, vitronectin, and fibronectin. It is a murine monoclonal IgG, obtained by immunization with the Arg-Gly-Asp (RGD) sequence and flanking amino acid residues of vWF and reacts specifically with the GPIb-IIIa binding site of vWF, but has no cross-reactivity with the RGD-containing domains of fibrinogen, fibronectin, or vitronectin. JI-L155B/16 is a murine monoclonal IgG, that binds to the Aα chain of fibrinogen, but not to vWF. It does not alter the function of fibrinogen and was used as control, along with vWF fragment buffer (5% [wt/vol] mannitol, 10 mmol/L citric acid, 1.0 mmol/L lysis mono-hydrochloride, 1.5 mmol/L NaCl, pH 3.5) or HEPES buffer (10 mmol/L HEPES, 145 mmol/L NaCl, 5.5 mmol/L KCl, 0.5 mmol/L Na2HPO4, 1 mmol/L MgSO4, pH 7.4). All IgG antibodies were purified on protein-A Sepharose (Sigma), dialyzed against HEPES buffer, and stored at -40°C until use.

Polymeric ATA

The trisodium salt of ATA (Aldrich Chemical, Milwaukee, WI) was dissolved in phosphate-buffered saline (PBS) (10 mmol/L Na2HPO4, 140 mmol/L NaCl, pH 7.4). ATA interacts with vWF and inhibits the binding of large vWF multimers to platelet GPIb. It inhibits ristocetin-induced, vWF-mediated platelet agglutination, and shear-induced, vWF-mediated platelet aggregation in the viscometer in the absence of ristocetin. Higher molecular weight ATA polymers were separated from lower molecular weight forms at 4°C by a 50-Kd cut-off dialysis membrane (Spectra/Por; Spectrum Medical Industries, Inc, Los Angeles, CA). A filter-sterilized stock solution of 8.5 mg/mL polymeric ATA with a mass greater than 2.5 Kd was prepared and stored at room temperature.

Recombinant vWF Fragment

A cDNA for vWF was cloned from a human cDNA library and a fragment of this cDNA was modified to allow its expression in Esherichia coli, as previously described. rvWF445-733 is composed of amino acid residues 445 to 733 of the mature vWF subunit with the seven cysteine residues reduced and alkylated. As originally demonstrated with proteolytic fragments derived from native vWF, synthetic peptides, and MoAbs, rvWF445-733 contains three functional domains of vWF that interact with GPIb, heparin, and collagen. Unlike native vWF, rvWF445-733 binds to GPIb without the requirement for exogenous modulators. In the presence of ristocetin or bo-trocetin, rvWF445-733 inhibits the binding of vWF multimers to GPIb and platelet aggregation. The lyophilized rvWF445-733 was dissolved in distilled water to a stock solution of 12.2 mg/mL. This was aliquoted and stored at -40°C.

Flow Chamber and Perfusion Studies

Before the flow chamber experiments, blood was incubated at 37°C for 15 minutes, and then an additional 5 minutes with either buffer or test sample. Control runs at the beginning and end of each experiment demonstrated a similar extent of platelet accumulation on collagen-coated slides. One side of a parallel-plate flow chamber, described in detail elsewhere, was formed by a collagen-coated glass coverslip with a flow path height of 205 μm (determined by a silicon gasket). The flow chamber was assembled and filled with isotonic saline. A syringe pump (Model 935; Harvard Apparatus, South Natick, MA) was used to aspirate blood through the flow chamber, displacing the saline, at a constant flow rate for 1 to 2 minutes. Flow rates of 0.53, 4.24, 8.0, and 16.0 mL/min produced 100, 800, 1,500, and 3,000 s-1 wall shear rates, respectively. These include the relevant range of shear rates in veins and in partially constricted arteries, and correspond to wall shear stresses of approximately 4, 32, 60, and 120 dynes/cm², respectively. Blood was considered a Newtonian fluid with a constant viscosity of 4 cp in laminar flow. The entire system was maintained at 37°C by a thermostatic air bath (Model 279; Laboratory Products, Boston, MA). The flow chamber was mounted on an inverted-stage microscope (DIAPHOT-TMD; Nikon, Garden City, NY) equipped with an epifluorescence illumination attachment (TMD-EF; Nikon), a 60× FLUOR objective, a 5× projection lens (Nikon), and a silicon-intensified target (SIT) video camera (Model C1000; Hamamatsu, Waltham, MA) suitable for very low light levels. Epifluorescence illumination was used to visualize directly platelet adhesion, and subsequent platelet aggregation and accumulation, throughout the perfusion period. The experiments were recorded in real time on a 0.5-in video cassette recorder (JVC model BR-3100U).

Measurement of Platelet Accumulation

The number of platelets deposited at the end of the perfusion period was calculated by scanning the slide and continuously recording the locally averaged fluorescence intensity over the entire field of view, using a motorized microscope stage and a computerized microphotometric measurement system. This system consists of a 40× FLUOR objective and a 1× projection lens (Nikon), a photodiode (Model PIN-10DP/5B; United Detector Technology, Hawthorne, CA) connected to the microscope, an amplifier with variable transimpedance gain (Model 101C; United Detector Technology) that also converts the signal from current to voltage, a 12-bit analog to digital converter board (ACE2-12, Strawberry Tree Computers, Inc, Sunnyvale, CA), and a microcomputer (Macintosh SE, Cupertino, CA). More detailed description of the equipment and its capabilities has been published elsewhere.

After direct observation of platelet adhesion and subsequent aggregation in real-time, the collagen-coated slide was dipped in a hemolyzing solution (ZAP-Oglobin II in Coulter Isoton II) to lyse any RBCs sticking to the surface, and crushed into 1 mL of 1% Triton X-100 (Rohm and Haas, Philadelphia, PA) to lyse the deposited platelets. The sample was sonicated for 5 seconds and centrifuged at 240g for 10 minutes to remove glass fragments. The supernatant was assayed for lactate dehydrogenase (LD-14 PL Kit; Gilford Systems, Oberlin, OH) and compared with lysates of known platelet concentrations to determine the total number of platelets on each slide. By integrating the intensity distribution along the collagen-coated surface, as recorded by the microphotometric measurement system, an intensity/platelet ratio was determined. The ratio was used to convert fluorescence intensity to platelet density, in millions/cm², at any axial position from the glass-collagen interface to the end of the collagen-coated surface. By integrating the curve of platelet...
density versus distance along the length of the slide, the total number of platelets that accumulated up to any point could be calculated. We used the first 10 mm from the glass-collagen interface to compute the platelet deposition. The average platelet deposition in millions/cm² was obtained by dividing the number of platelets deposited along this 10-mm length by the surface area.

**Evaluation of Platelet Adhesion and Subsequent Platelet Aggregation**

Videotape images, focused 0.38 mm downstream from the glass-collagen interface, were digitized and computer analyzed after 2 seconds of flow and at 15-second intervals up to 1 minute²⁴ (IC-300 Modular Image Processing Workstation; Inovision Corp, Durham, NC). The number of platelet thrombi per 3.7 X 10⁵ μm² in the microscope field were determined, along with the number of platelets in each individual thrombus. Three-dimensional representations of the platelet thrombi present at 2, 15, 30, 45, and 60 seconds during the perfusion period were constructed and photographed from the computer screen. Platelet adhesion was expressed as the number of individual thrombi/3.7 X 10⁵ μm² by using background-subtracted digitized images. The platelet aggregation that occurred subsequent to platelet adhesion was expressed as the number of platelets per thrombus and was equal to the total intensity of each thrombus (area x average intensity) multiplied by a value determined by dividing the number of single platelets in the 2-second images by the sum of the total intensities of these single platelets.

**Studies of Severe von Willebrand Disease (vWD) Patient Plasma and Platelets**

Perfusion experiments were performed with blood anticoagulated with citrate or porcine heparin from a severe vWD patient whose plasma contains vWF antigen levels that have been less than 1.5 U/dL on 12 different occasions by immunoradiometric and enzyme-linked immunosorbent assays (ELISAs), and vWF multimeric patterns that have been either undetectable or barely visible on prolonged exposure by sodium dodecyl sulfate (SDS)-electrophoresis/autoradiography using patient plasma or platelets.²⁴ The effect of exogenous vWF on the adhesion of vWD platelets to collagen was examined by the addition of a final concentration of 100 U/dL (100% of large vWF multimers to vWD blood samples before perfusion. vWF multimers, consisting predominately of the largest plasma-type forms, were purified from normal human cryoprecipitate using glycine- and NaCl-precipitation followed by agarose gel exclusion chromatography.¹³ The multimeric patterns were analyzed by SDS-1% agarose gel electrophoresis followed by overlay with polyclonal ¹²⁵I-anti-human vWF IgG and autoradiography. In some experiments, PGI₁-treated vWD and normal platelets were washed in HEPES buffer, as previously described,¹³ and reconstituted as either vWD platelets re-suspended in normal platelet-poor plasma (PPP) or normal platelets in vWD PPP at 400,000 platelets/μL. Normal RBCs were added to produce a 40% hematocrit.

**Statistical Analysis**

Mean ± SEM was calculated for experiments based on three or more determinations. Statistical significance of differences between means was determined for two entries (one control and one test manipulation) by two-tailed paired Student's t-test, and for more than two entries (one control and multiple test manipulations) by repeated measures analysis of variance (single factor factorial-one repeated measure ANOVA).⁴⁴⁶ In the latter case, if means were shown to be significantly different, multiple comparisons by pairs of each test manipulation with the control were performed by the two-tailed Dunnett's t-test.⁵⁶,⁶⁴ Probability values less than .05 were considered significant, and less than .01 highly significant.

**RESULTS**

**Shear-Aggregation Experiments**

**Effects of MoAbs.** The interaction of large vWF multimers with both platelet GPIb and the GPIb-IIIa complex is required for the shear-induced aggregation of platelets from normal PRP in a viscometer.¹⁴⁻¹⁶ Experiments were performed to establish the concentrations of MoAbs to be tested subsequently in the perfusion studies. Shear-induced, vWF-mediated aggregation in citrated PRP was minimal in the presence of 50 or 100 μg/mL of the anti-GPIb F(ab')₂, or 100 or 200 μg/mL of the anti–GPIb-IIIa (data not shown). Shear-aggregation was inhibited in a dose-dependent manner by 12.5 to 200 μg/mL of the antibody against the RGD sequence in vWF that mediates binding to GPIb-IIIa. The latter antibody at 200 μg/mL completely inhibited shear-aggregation at 120 dynes/cm² and reduced it by about 90% at 180 dynes/cm² (data not shown).

**Effects of rvWF⁴⁴⁵-⁷³³ and polymeric ATA.** Figure 1A compares the inhibitory effect of rvWF⁴⁴⁵-⁷³³ and polymeric ATA on shear-induced, vWF-mediated platelet aggregation in normal citrated PRP at 180 dynes/cm². rvWF⁴⁴⁵-⁷³³ inhibited shear-aggregation in a dose-dependent manner, presumably by competing with large vWF multimers for binding to platelet GPIb. Shear-aggregation was totally absent in the presence of rvWF⁴⁴⁵-⁷³³ concentrations above 50 μg/mL (Fig 1A). Polymeric ATA also produced dose-dependent inhibition of shear-aggregation (Fig 1A). The effect of different fluid shear stresses on the ability of 40 μg/mL rvWF⁴⁴⁵-⁷³³ or 80 μg/mL polymeric ATA to inhibit shear-aggregation in normal citrated PRP samples by these two different, “mirror-image” mechanisms is shown in Fig 1B.

Figure 2 demonstrates that, in the viscometer using citrated PRP, the inhibitory effectiveness of a low concentration of rvWF⁴⁴⁵-⁷³³ (30 μg/mL) was counteracted by low concentrations of polymeric ATA (10 to 20 μg/mL) and ATA and rvWF⁴⁴⁵-⁷³³ apparently interact with each other at these concentrations. At concentrations of ATA above 40 μg/mL, the inhibitory effect of ATA on shear-aggregation is neither compromised nor potentiated by the presence of 30 μg/mL rvWF⁴⁴⁵-⁷³³.

**Effect of different anticoagulants.** Shear-aggregation was compared in normal PRP samples anticoagulated with sodium citrate, recombinant hirudin, unfractionated porcine heparin, or low molecular weight heparin (Fig 3). Platelets in the PRP in each anticoagulant aggregated extensively (<20% remaining singlet platelets). These results indicate that neither the reduced calcium levels in the citrated PRP nor any interaction that may occur between plasma vWF multimers and unfractionated or low molecular weight heparin affects the extent of vWF-mediated platelet aggregation induced by shear stress (compared with hirudin-PRP). In the presence of rvWF⁴⁴⁵-⁷³³ (60 μg/mL), shear-aggregation in the PRP samples anticoagulated in citrate, hirudin, or low molecular weight heparin was almost completely reversed. In contrast, PRP anticoagulated with unfractionated porcine heparin and incubated with rvWF⁴⁴⁵-⁷³³ fully aggregated under these shear conditions. This was studied further by varying the amount of unfractionated porcine heparin added to nor-
vWF AND PLATELET THROMBI UNDER FLOW

**Fig 1.** Effect of rvWF<sup>446-733</sup> and polymeric ATA on the fluid shear stress-induced, vWF-mediated aggregation of platelets from citrated normal PRP in a cone and plate viscometer. (A) Effect of different rvWF<sup>446-733</sup> and ATA concentrations at a shear stress of 180 dynes/cm<sup>2</sup> applied at room temperature for 30 seconds. The PRP was incubated for 5 minutes at room temperature with ATA or rvWF<sup>446-733</sup> before application of the shear stress. (B) Effect of different fluid shear stresses applied for 30 seconds at room temperature on normal citrated PRP preincubated for 5 minutes with either 40 pg/mL of rvWF<sup>446-733</sup>, 80 pg/mL polymeric ATA, or buffer. Platelet counts in the PRP samples before shear were 233,000 to 340,000/µL.

Normal citrated PRP in the presence of a constant concentration of rvWF<sup>445-733</sup> (60 µg/mL) (Fig 4). The addition of unfractionated heparin alone in concentrations up to 5 U/mL had no effect on the extent of shear-aggregation, confirming the experiment in Fig 3. However, addition of 0.3 to 5 U/mL unfractionated heparin (concentrations that can be attained in vivo in humans) progressively counteracted the inhibitory effect of rvWF<sup>445-733</sup>. These results indicate that unfractionated heparin, but not low molecular weight heparin, apparently interacts with the heparin binding site of rvWF<sup>445-733</sup> molecules and prevents it from competing with large vWF multimers for attachment to platelet GPIb. However, recently reported preliminary results suggest that this effect of heparin may not be so important when rvWF<sup>445-733</sup> is administered to intact animals. In contrast, unfractionated porcine heparin had no effect on the inhibition of shear-aggregation by 80 µg/mL polymeric ATA (data not shown).

**Perfusion Experiments**

Real-time analysis of platelet adhesion and subsequent aggregation onto collagen I-coated surfaces using normal blood. Table 1 shows the results of digital image analysis of platelet accumulation from citrate-anticoagulated whole blood of four normal donors onto type I collagen. The number of individual platelet thrombi/3.7 × 10<sup>4</sup> µm<sup>2</sup>, a measurement of the platelet adhesive events that initiate thrombus formation, increased during the initial 15 seconds of flow at 1,500 s<sup>-1</sup>. As individual platelet thrombi merge into larger clumps at 30 and 60 seconds of flow, the number of individual thrombi decreased concomitantly.

The total number of platelets in millions/cm<sup>2</sup> that were deposited from normal blood onto type I collagen-coated slides using different anticoagulants (three experiments for each) were: 10.9 ± 2.0 (citrate); 14.4 ± 4.4 (porcine heparin, 4 U/mL); and 14.9 ± 5.0 (low molecular weight heparin, 1 U/mL). In one similar type of experiment using 200 U/mL of recombinant hirudin as anticoagulant, a total of 8.3 million platelets/cm<sup>2</sup> were deposited on collagen I slides. The differences among the values are not statistically significant.

Effect of exogenous vWF on platelet adhesion and subsequent aggregation onto collagen I-coated surfaces using severe vWD blood. Whole blood from a severe vWD patient (vWF...
Porcine citrate, 200 U/mL recombinant hirudin, 10 U/mL unfractionated platelet aggregation from PRP anticoagulated by 0.38% sodium citrate and plate viscometer. The shear stress was 180 dynes/cm².

Platelet counts in the PRP samples were 246,000 to 360,000/µL.

Inhibition by rvWF<sub>545-723</sub> on fluid shear-induced, vWF-mediated platelet aggregation from PRP anticoagulated by 0.38% sodium citrate. 200 U/mL recombinant hirudin, 10 U/mL unfractionated porcine heparin, or 1 U/mL low molecular weight heparin in a cone and plate viscometer. The shear stress was 180 dynes/cm² for 30 seconds at room temperature. Platelet counts in the PRP samples were 246,000 to 360,000/µL.

After the washing and resuspension of severe vWD platelets in heparinized normal plasma, platelet accumulation on type I collagen at 1,500 s⁻¹ wall shear rate was 77% of the platelet deposition from normal blood under the same perfusion conditions. Platelet deposition was 44% if normal platelets were washed and resuspended in severe vWD plasma. The results of this single experiment suggest that both plasma vWF and vWF released from normal platelets can contribute to platelet adhesion and accumulation on collagen surfaces, and are compatible with a previous report by Fressinaud et al. Platelet vWF multimers are known to be released from the α-granules of platelets activated by abnormally elevated fluid shear stresses.

Effect of anti-GPIb on platelet adhesion on collagen I. Figure 7 is a three-dimensional representation of platelet deposition onto type I collagen-coated surfaces using citrated normal whole blood that has been incubated in the presence or absence of 30 µg/mL of monoclonal anti-GPIb F(ab')<sub>2</sub>, and then perfused for 1 minute at 1,500 s⁻¹. In the experiment

Shear-rate dependence of platelet accumulation on collagen I: Contribution of vWF in plasma or released from platelets. Whole blood from a normal donor and a severe vWD patient with less than 1.5 U/dL plasma vWF was anticoagulated with porcine heparin and perfused at 100, 800, or 1,500 s⁻¹ wall shear rates for 2 minutes. Platelet deposition onto type I collagen from normal blood was shear rate-dependent, as shown by the progressive increase in platelet accumulation as the shear rate increased from 100 s⁻¹ to 1,500 s⁻¹ (Fig 6). A promoting effect of vWF on platelet adherence to collagen I can be detected, even at 800 s⁻¹. In contrast, increasing the shear rate had little effect on the number of platelets deposited onto collagen I from severe vWD blood.

| Time (s) | Controls | No. of Thrombi/Unit Area<sup>a</sup>
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<sup>a</sup> *3.7 x 10⁸ µm² coated with bovine fibrillar collagen I in this and subsequent tables. Also in this and the following tables: flow rate, 8 mL/ml; shear rate, 1,500 s⁻¹; perfusion time, 1 min; anticoagulant, sodium citrate; temp, 37°C.
shown in Fig 7 there was 94% inhibition of platelet adhesion (measured as number of individual thrombi per unit surface area at 15 seconds) onto collagen I in the presence of anti-GPIb F(ab')2 (Table 3). There was a reduction in total platelet deposition onto collagen I-coated surfaces in the presence of anti-GPIb F(ab')2 by about 85% using citrated normal whole blood (n = 3; Table 4). In the presence of anti-GPIb F(ab')2, only a few single platelets or clumps with small numbers of platelets adhered to the collagen (Table 3). There was more platelet adhesion to collagen I under the same conditions of flow using severe vWD blood with less than 1.5 U/dL plasma vWF and undetectable (or barely detectable) platelet vWF forms than in the presence of 50 μg/mL anti-GPIb F(ab')2. These findings suggest that only a small quantity of vWF insolubilized onto bovine collagen I is sufficient to initiate platelet adhesion via GPIb to this collagen type.

Effects of antibodies against GPIIb-IIIa or the RGD sequence in vWF (GPIIb-IIIa binding region) on platelet adhesion and aggregation onto collagen I. Anti-GPIIb-IIIa or the antibody against the RGD sequence in vWF (both at 200
Table 2. Platelet Adhesion and Subsequent Aggregation Onto Collagen I/vWF

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<th>Time (s)</th>
<th>Severe vWD</th>
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<td>7</td>
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<tr>
<td>15</td>
<td>27 (31%)</td>
<td>87</td>
</tr>
<tr>
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Platelet thrombus size distribution

<table>
<thead>
<tr>
<th>Time (s)</th>
<th>1</th>
<th>2-5</th>
<th>6-20</th>
<th>21-100</th>
<th>301-500</th>
<th>501-2,000</th>
<th>&gt;2,000</th>
</tr>
</thead>
<tbody>
<tr>
<td>Severe vWD</td>
<td>2</td>
<td>12</td>
<td>3</td>
<td>8</td>
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<td></td>
<td></td>
</tr>
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<td>4</td>
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</tr>
<tr>
<td>Severe vWD + vWF (100 U/dL)</td>
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<td></td>
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<td>15</td>
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<td>60</td>
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Table 3. Platelet Adhesion and Subsequent Aggregation Onto Collagen I/vWF

<table>
<thead>
<tr>
<th>Time (s)</th>
<th>Control</th>
<th>Anti-GP Ib (50 μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>19</td>
<td>1</td>
</tr>
<tr>
<td>15</td>
<td>80</td>
<td>5 (6%)</td>
</tr>
<tr>
<td>30</td>
<td>44</td>
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<td>28</td>
<td>1</td>
</tr>
<tr>
<td>60</td>
<td>37</td>
<td>12</td>
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Platelet thrombus size distribution

<table>
<thead>
<tr>
<th>Time (s)</th>
<th>1</th>
<th>2-5</th>
<th>6-20</th>
<th>21-100</th>
<th>301-500</th>
<th>501-2,000</th>
<th>&gt;2,000</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
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<td>6</td>
<td>9</td>
<td>2</td>
<td>2</td>
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<td>12</td>
<td>6</td>
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<td>1</td>
<td></td>
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<tr>
<td>45</td>
<td>5</td>
<td>19</td>
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<td>28</td>
<td>6</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Anti-GP Ib (50 μg/mL)</td>
<td>2</td>
<td>1</td>
<td></td>
<td></td>
<td>4</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>30</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>45</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>2</td>
<td>5</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

μg/mL) were less inhibitory than the anti-GP Ib F(ab')2 at 50 μg/mL on total platelet accumulation onto collagen I (Table 4). (These antibody concentrations caused complete inhibition of shear-induced, vWF-mediated aggregation in the viscometer.) Preincubation of blood with 200 μg/mL of MoAbs against GPIIb-IIIa or the RGD sequence in vWF suppressed platelet deposition onto collagen by about 67% and 39%, respectively (n = 3; Table 4). In the presence of anti–GPIIb-IIIa, the number of platelet adhesive events (number of individual thrombi per unit surface area) was not decreased and thrombi consisting of one or two to five platelets were predominate (Table 5 and Fig 8). In the presence of anti-RGD in vWF there was a reduced rate of platelet aggregation, as indicated by the relatively stable number of individual thrombi over 60 seconds compared with controls (Table 5) and the three-dimensional images of platelet thrombus formation over time in Fig 9.

Effect of vWF445-733 and polymeric ATA on platelet adhesion and subsequent aggregation onto collagen I. Citrated normal whole blood was incubated for 5 minutes with polymeric ATA (28.5, 57, Or | buffer and perfused at 1,500 s⁻¹ for 1 minute (Table 6 and Fig 10A). There was concentration-dependent inhibition of total platelet accumulation onto collagen, which was about 87% at 114 μg/mL of polymeric ATA (Table 6). Inhibition was caused by ATA suppression of platelet adhesion to collagen I (Table 7 and Fig 11).

Fig 6. Platelet accumulation (in millions per cm²) after 2 minutes of perfusion at wall shear rates of 100, 800, and 1,500 s⁻¹ onto bovine collagen type I-coated glass slides using heparinized blood from a normal donor and from a patient with severe vWD. The last two columns demonstrate the effect of either plasma vWF or vWF released from normal platelets on platelet accumulation onto collagen. For these experiments: either severe vWD platelets with no detectable platelet vWF were washed and resuspended in normal PPP, or normal platelets were washed and resuspended in severe vWD-PPP containing less than 1.5 U/dL vWF antigen. Normal erythrocytes were added to produce final hematocrit values of 40%, and platelets were 400,000/μL.
Citrated normal whole blood was also incubated for 5 minutes with 30, 60, or 200 µg/mL of rvWF (2000-733) and perfused at 1,500 s⁻¹ for 1 minute. A concentration-related inhibitory effect of rvWF (2000-733) on total platelet accumulation along collagen-coated surfaces is shown in Fig 10B and Table 6. Similar results were obtained in one experiment with 30, 60, or 200 µg/mL of rvWF (2000-733) in normal blood anticoagulated with recombinant hirudin (not shown). Inhibition of total platelet inhibition was about 85% at 200 µg/mL of rvWF (2000-733) (Table 6). This inhibition was, as with polymeric ATA, to a considerable extent the result of interference with platelet adhesion to collagen I, and the subsequent formation of large platelet clumps (Table 8 and Fig 11).

Citrated normal whole blood was incubated with either buffer or 60 µg/mL rvWF (2000-733) and perfused for 1 minute at shear rates of 3,000, 1,500, 800, and 100 s⁻¹. At each shear rate, the average number of platelets per cm² deposited as a result of adherence and subsequent aggregation onto collagen I-coated surfaces was determined (Fig 12). The inhibitory effectiveness of rvWF (2000-733) was greater at higher shear rates (n = 3 for each experimental condition). In one additional experiment using 120 µg/mL of rvWF (2000-733), qualitatively similar results were obtained (with an augmented extent of inhibition) at each shear rate (data not shown).

Incompatibility between unfractionated porcine heparin and rvWF (2000-733) (containing heparin binding sites) in perfusion experiments is shown in Fig 13. These results are similar to those obtained in the shear-aggregation studies. The inhibitory effect of 60 µg/mL rvWF (2000-733) on platelet adhesion to collagen type I from normal citrated whole blood at 1,500 s⁻¹ and 1-minute perfusion time was partially reversed as porcine heparin concentrations were increased from 1 to 4 U/mL.

### Table 4. Effect of MoAbs Against Platelet vWF Receptors or the RGD Binding Site in vWF on Platelet Accumulation Onto Collagen

<table>
<thead>
<tr>
<th>Time (s)</th>
<th>Control</th>
<th>Anti-RGD-vWF</th>
<th>Anti-GPllb-lla</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>12</td>
<td>4</td>
<td>12</td>
</tr>
<tr>
<td>15</td>
<td>90</td>
<td>70</td>
<td>81</td>
</tr>
<tr>
<td>30</td>
<td>83</td>
<td>57</td>
<td>104</td>
</tr>
<tr>
<td>45</td>
<td>64</td>
<td>78</td>
<td>94</td>
</tr>
<tr>
<td>60</td>
<td>55</td>
<td>68</td>
<td>111</td>
</tr>
</tbody>
</table>

### Table 5. Platelet Adhesion and Subsequent Aggregation Onto Collagen vWF

<table>
<thead>
<tr>
<th>Time (s)</th>
<th>Control</th>
<th>Anti-RGD-vWF</th>
<th>Anti-GPllb-lla</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>12</td>
<td>4</td>
<td>12</td>
</tr>
<tr>
<td>15</td>
<td>90</td>
<td>70</td>
<td>81</td>
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<tr>
<td>30</td>
<td>83</td>
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<td>78</td>
<td>94</td>
</tr>
<tr>
<td>60</td>
<td>55</td>
<td>68</td>
<td>111</td>
</tr>
</tbody>
</table>

Values expressed as mean ± SEM; n, number of experiments.

*P < .00035, 1P < .0005, tP < .0125, two-tailed paired Student's t-test, relative to respective controls.

### DISCUSSION

vWF multimers from normal plasma, the relatively large vWF multimeric forms purified from normal human cryoprecipitate, and vWF released from platelets apparently become insolubilized rapidly onto bovine fibrillar collagen type I under flowing conditions and are the predominant mediators of platelet-collagen adhesion at shear rates between 800 and 3,000 s⁻¹ in citrate, hirudin, and unfractionated porcine heparin. The latter does not detectably interfere with the adsorption of vWF multimers onto type I collagen, even though vWF monomers contain a binding site for unfractionated heparin. Other studies have shown that vWF deposited by endothelial cells in the extracellular matrix also supports platelet adhesion.50-52

Anti-GPlb F(ab')₂ inhibited platelet adhesion to type I collagen at 1,500 s⁻¹ by 85% to 95%. Interfering with the function of either GPllb (using rvWF (2000-733)) or the GPllb binding site of vWF (using polymeric ATA) also suppressed platelet-collagen I adhesion under the same conditions of flow. Together with our experiments using severe vWD blood (<1.5 U/dL vWF antigen in plasma) in the presence and absence of vWF, these results establish with real-time observations and analysis that platelet adhesion from rapidly flowing blood onto collagen I is predominantly mediated by the interaction between collagen-associated insolubilized vWF multimers and platelet GPllb.

Total platelet deposition onto collagen I/vWF at 1,500 s⁻¹ was inhibited by two thirds in the presence of anti-GPllb-
Fig 9. Three-dimensional representations of platelet adhesion and subsequent aggregation onto bovine collagen type I-coated surface from normal citrated blood incubated in the presence of 200 μg/mL of anti-RGD sequence in vWF (GPIIb-IIIa binding site). Blood was incubated with the MoAb for 5 minutes and perfused at 37°C at a wall shear rate of 1,500 s⁻¹ for 1 minute.

Fig 8. Three-dimensional representations of platelet adhesion and subsequent aggregation onto bovine collagen type I-coated surface from normal citrated blood incubated in the presence of 200 μg/mL of anti-GPIIb-IIIa. Blood was incubated with the MoAb for 5 minutes and perfused at 37°C at a wall shear rate of 1,500 s⁻¹ for 1 minute.

Fig 11. Three-dimensional representations of platelet adhesion and subsequent aggregation onto bovine collagen type I-coated surface from citrated normal whole blood in the presence of either 114 μg/mL polymeric ATA or 200 μg/mL of vWF 445-737. A blood sample was incubated with one or the other agent and perfused at 37°C at a wall shear rate of 1,500 s⁻¹ for 1 minute.
Table 6. Effect of rVWF<sub>446-732</sub> or Polymeric ATA on Platelet Accumulation Onto Collagen I/vWF

<table>
<thead>
<tr>
<th>n</th>
<th>Control</th>
<th>(µg/mL)</th>
<th>Total Deposited Platelets (x10&lt;sup&gt;4&lt;/sup&gt;/cm&lt;sup&gt;2&lt;/sup&gt;)</th>
<th>Total Deposited Platelets (x10&lt;sup&gt;8&lt;/sup&gt;/cm&lt;sup&gt;2&lt;/sup&gt;) (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>Buffer</td>
<td>9.6 ± 0.7</td>
<td>30 5.5 ± 0.4</td>
<td>58 ± 9&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>60 4.2 ± 0.6</td>
<td>45 ± 9&lt;sup&gt;†&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>200 1.5 ± 0.7</td>
<td>14 ± 7&lt;sup&gt;†&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values expressed as mean ± SEM; n, number of experiments. Statistical significance of differences between means of results with varying concentrations of test agents was evaluated by repeated measures ANOVA:
P < .001. Two-tailed Dunnett's t-test used for pairwise comparisons between each result with the test agent and the respective control.
* Significant at 95%.
† All others significant at 99%.

Table 7. Platelet Adhesion and Subsequent Aggregation Onto Collagen I/vWF

<table>
<thead>
<tr>
<th>Time (s)</th>
<th>2</th>
<th>5</th>
<th>10</th>
<th>20</th>
<th>30</th>
<th>60</th>
<th>120</th>
<th>240</th>
<th>360</th>
<th>600</th>
<th>1200</th>
<th>2400</th>
<th>&gt;2400</th>
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</thead>
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<td>2</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Polymeric ATA (114 µg/mL)</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
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<td>1</td>
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</table>

Platelet thrombus size distribution

<table>
<thead>
<tr>
<th>No. of Thrombi Containing These Platelet Numbers</th>
<th>101-300</th>
<th>301-500</th>
<th>501-1000</th>
<th>&gt;1000</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Polymeric ATA (114 µg/mL)</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>3</td>
</tr>
</tbody>
</table>

IIIa and the adherent platelets were mostly singlets or present in small clumps of 2 to 5. Using an antibody against the RGD sequence in vWF, total platelet deposition onto collagen I/vWF under these same perfusion conditions was reduced by about 40%. The inhibitory effect of the anti-RGD in vWF antibody was to slow the rate of formation of very large aggregates (>500 platelets/thrombus). These observations indicate that vWF multimers are involved in platelet-platelet cohesion (aggregation) in our perfusion model (as well as in the platelet adhesion to collagen I), even in the presence of the relatively high concentrations of fibrinogen and fibronectin in normal blood. Our finding that anti-GPIIb-IIIa was a more effective inhibitor of platelet aggregation than anti-RGD in vWF may reflect: local release of platelet vWF that was less completely inhibited by the anti-RGD in vWF; different binding affinities of the two antibodies under flowing conditions; or participation of fibrinogen, fibronectin, or thrombospondin, as well as vWF, in the platelet aggregation that occurs after platelet adhesion to collagen/vWF. There is some evidence that fibrinogen may participate relatively less than the other ligands in this type of platelet aggregation.33

![Diagram A](https://via.placeholder.com/150)

![Diagram B](https://via.placeholder.com/150)

Fig 10. Platelet accumulation along bovine collagen type I-coated surfaces from normal citrated whole blood perfused for 1 minute at 37°C at a wall shear rate of 1,500 s<sup>-1</sup> after preincubation with either: (A) 28.5, 57, or 114 µg/mL polymeric ATA or buffer; or (B) 30, 60, or 200 µg/mL of rVWF<sub>446-732</sub> or buffer.
Table 8. Platelet Adhesion and Subsequent Aggregation Onto Collagen I/vWF

<table>
<thead>
<tr>
<th>Time (s)</th>
<th>Control</th>
<th>rvWF445-733</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of Thrombi/Unit Area</td>
<td>No. of Thrombi/Unit Area</td>
</tr>
<tr>
<td>2</td>
<td>47</td>
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</tr>
<tr>
<td>15</td>
<td>100</td>
<td>46 (46%)</td>
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<tr>
<td>30</td>
<td>90</td>
<td>51</td>
</tr>
<tr>
<td>60</td>
<td>71</td>
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Platelet thrombus size distribution

<table>
<thead>
<tr>
<th>Time (s)</th>
<th>No. of Thrombi Containing These Platelet Numbers:</th>
<th>101-300</th>
<th>301-500</th>
<th>501-1,000</th>
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<td>3</td>
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<tr>
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<td>15 31 40 18 9</td>
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<tr>
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<td>30 24 32 25 3</td>
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<tr>
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<td>60 21 21 16 7</td>
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</table>

rvWF445-733 (200 µg/mL)

<table>
<thead>
<tr>
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<th>No. of Thrombi Containing These Platelet Numbers:</th>
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<th>301-500</th>
<th>501-1,000</th>
<th>&gt;1,000</th>
</tr>
</thead>
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<td>0</td>
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<tr>
<td>30</td>
<td>31 14 5</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>60</td>
<td>32 21 8</td>
<td>2</td>
<td>2</td>
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</tbody>
</table>

In contrast to our results in the real-time perfusion system, the inhibitory effects of anti-GPⅡb-Ⅲa and the antibody against the RGD sequence in vWF on shear-induced, vWF-mediated aggregation in the cone and plate viscometer were as pronounced as the inhibitory effect of anti-GPⅡb. Previous work has indicated that both vWF·GPⅡb and vWF·GPⅡb-Ⅲa interactions are required to induce direct platelet aggregation under the conditions of abnormally elevated fluid shear stresses in the viscometer.14-16

Our results lead to the conclusion that(rvWF445-733 binds to platelet GPⅡb,19 competitively interferes with the attachment of large vWF multimers insolubilized onto collagen to this binding site, and inhibits platelet adhesion in a concentration- and shear rate-dependent process. This interpretation is compatible with our finding that rvWF445-733 also interferes with GPⅡb-dependent, shear-induced, vWF-mediated aggregation (which does not involve collagen).

Unfractionated porcine heparin in concentrations that can be attained in vivo interacts with rvWF445-733 and prevents it from competing with large vWF multimers for GPⅡb binding sites, and from exerting its inhibitory effects on shear-aggregation and platelet adhesion to collagen I. These results suggest that the use of rvWF445-733 as an anti-thrombotic agent may potentially be compromised by the concurrent local or systemic administration of unfractionated heparin. However, it should be emphasized that we have performed only in vitro experiments. In vivo interactions between rvWF445-733 and unfractionated porcine heparin may not be similar for reasons that include, among others, differences in catabolism between the two compounds. Preliminary studies in pigs indicate that the concurrent use of rvWF445-733 and unfractionated heparin may be compatible in vivo.47 It remains to be determined in humans if rvWF445-733 can be given along with unfractionated heparin and retain inhibitory effectiveness with respect to arterial thrombotic processes.

Polymeric ATA interacts with, and inhibits, GPⅡb binding sites on vWF multimers.19 Therefore, polymeric ATA and rvWF445-733 both block platelet GPⅡb-vWF interactions—but by “mirror image” mechanisms. Each is capable of inhibiting platelet adhesion from citrated or hirudin-treated whole blood onto collagen I/vWF in the perfusion system, as well as the
direct aggregation of platelets from citrated or hirudin-anti-
coagulated PRP at an abnormally elevated level of fluid shear
stress (180 dynes/cm²) in the viscometer. Inhibition of these
events by more than 50% occur in vitro in both the perfusion
and viscometer model systems at relatively low concentrations
of rvWF445-733 or ATA (60 μg/mL).

In contrast to rvWF445-733, the inhibitory effect of polymeric
ATA is not counteracted by unfractionated porcine heparin.
However, negatively charged ATA molecules may attach to
rvWF445-733 because the inhibitory effect of a relatively low
concentration of rvWF445-733 is reduced in the presence of
low concentrations (10 to 20 μg/mL) of polymeric ATA. Be-
cause the shear and perfusion models may simulate condi-
tions for the two mechanisms of platelet thrombus formation
in vivo in humans, it will be prudent to consider in any ther-
apeutic trials that the anti-arterial thrombotic potential of
rvWF445-733 and polymeric ATA may be maximized if the
two agents are administered separately.

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BR Alevriadou, JL Moake, NA Turner, ZM Ruggeri, BJ Folie, MD Phillips, AB Schreiber, ME Hrinda and LV McIntire