Adhesion of normal erythrocytes at depressed venous shear rates to activated neutrophils, activated platelets, and fibrin polymerized from plasma

Mukul S. Goel and Scott L. Diamond

Deep vein thrombosis (DVT) is a low flow pathology often prevented by vascular compression to increase blood movement. We report new heterotypic adhesive interactions of normal erythrocytes operative at low wall shear rates (γ_w) below 100 s^{-1}. Adhesion at $\gamma_w = 50$ s^{-1} of washed red blood cells (RBCs) to fibrinogen-adherent platelets was 4-fold less ($P < .005$) than to collagen-adherent platelets (279 ± 105 RBC/mm^2). This glycoprotein VI (GPVI)–triggered adhesion was antagonized (> 80% reduction) by soluble fibrinogen (3 mg/mL) and ethylenediaminetetraacetic acid (EDTA). RBC-platelet adhesion was reduced in half by antibodies against CD36 or GPIb, but not by antibodies against GPIIb/IIIa, von Willebrand factor (VWF), thrombospondin (TSP), P-selectin, β_1, α_v, or CD47. Adhesion of washed RBCs to fibrinogen-adherent neutrophils was increased 6-fold in the presence of 20 μM N-formyl-Met-Leu-Phe to a level of 67 RBCs per 100 neutrophils after 5 minutes at 50 s^{-1}. RBC-neutrophil adhesion was diminished by anti-CD11b (76%), anti-RBC Landsteiner-Wiener (LW) (ICAM4; 40%), or by EDTA (> 80%), but not by soluble fibrinogen or antibodies against CD11a, CD11c, CD36, TSP, β_1, α_v, or CD47. RBC adhesion to activated platelets and activated neutrophils was prevented by wall shear stress above 1 dyne/cm^2 (at 100 s^{-1}). Whereas washed RBCs did not adhere to fibrin formed from purified fibrinogen, adhesion was marked when pure fibrin was precoated with TSP or when RBCs were perfused over fibrin formed from recalcified plasma. Endothelial activation and unusually low flow may be a setting prone to receptor-mediated RBC adhesion to adherent neutrophils (or platelets/fibrin), all of which may contribute to DVT. (Blood. 2002;100:3797-3803)

Introduction

Adhesion of erythrocytes to the blood vessel wall is of considerable significance in pathologies involving vasoocclusive events such as sickle cell anemia. Most previous studies on adhesive interactions of erythrocytes have been targeted toward sickle red blood cells or plasmodium falciparum-infected erythrocytes. Some of the molecules mediating sickle red cell adhesion to the endothelium are P-selectin,[1] thrombospondin (TSP),[2,3] von Willebrand factor (VWF),[4] CD36,[3,5] sulfated glycolipid,[6] $\alpha_4\beta_1$ integrin,[7] $\alpha_V\beta_3$,[8] CD47,[9] and possibly GPIb.[1] Possible mediators of plasmodium falciparum–infected erythrocytes to the endothelium include P-selectin[10] and CD36.[11,12] Although it is known that sickle erythrocytes can directly adhere to the endothelium, a common assumption is that normal red blood cells are relatively nonadhesive during coagulation events and become passively entrapped in fibrin.

Platelets adhere to the blood vessel wall through interactions mediated by glycoproteins, GP Ia/IIa, GP IIb/IIIa, and GP Ib-IX, which bind primarily to collagen,[13] fibrinogen,[14] and VWF,[15] respectively, on the vessel wall surface. Similarly, the capture of neutrophils to the vessel wall involves neutrophil P-selectin glycoprotein ligand 1 (PSGL-1)–mediated rolling[16] and membrane tethering[17] on P-selectin presented by activated endothelium or spread platelets,[18] followed by β_2-integrin–mediated firm arrest.[19] Recent studies have shown that leukocyte Mac-1 and leukocyte function–associated antigen-1 (LFA-1) can also bind the red cell intercellular adhesion molecule 4 (ICAM-4; Landsteiner-Wiener [LW] blood group glycoprotein),[20] which is a receptor associated mainly with erythroid cells[21] and is expressed almost concurrently with the Rh group during erythropoiesis.[22]

TSP, a complex trimeric glycoprotein, can interact with platelets through multiple adhesive molecules and receptors such as collagen, laminin, fibronectin, fibrinogen,[23] sulfated glycolipids,[24] VWF,[25] CD36, and possibly GPIa/IIa.[26] TSP may promote platelet aggregation by crossbridging platelet-bound fibrinogen.[27] TSP, abundant in clots[28] and uncrosslinked fibrin,[29] may accelerate fiber growth during fibrin polymerization.[28] Additionally, a role for TSP in adhesion of neutrophils to the vessel wall has also been suggested.[30,31]

The objective of this study was to examine capture and adhesion of normal erythrocytes to surface-adherent neutrophils and platelets under low flow conditions as a mechanism of red blood cell (RBC) accumulation distinct from passive entrapment within fibrin fibers. We hypothesized that red blood cells could adhere to activated neutrophils, activated platelets, and surface-deposited fibrin under venous flow conditions through receptor-mediated adhesion. This hypothesis is motivated by the observation that deep vein thrombosis (DVT) is a pathology associated with depressed shear rates and is significantly prevented in high-risk postsurgical

From the Department of Chemical Engineering, Institute for Medicine and Engineering, University of Pennsylvania, Philadelphia.

Submitted March 6, 2002; accepted June 26, 2002. Prepublished online as *Blood* First Edition Paper, July 5, 2002; DOI 10.1182/blood-2002-03-0712.

Supported by a grant from the National Institutes of Health (NIH) RO1 HL 56621. S.L.D. is an Established Investigator of the National American Heart Association. M.S.G. is an NIH predoctoral fellow (T32 HL 07954-02).

Reprints: Scott L. Diamond, Institute for Medicine and Engineering, Department of Chemical Engineering, University of Pennsylvania, 1010 Vagelos Research Laboratories, 3340 Smith Walk, Philadelphia, PA 19104; e-mail: sld@seas.upenn.edu.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 U.S.C. section 1734.

patients by vascular compression to prevent stasis. However, it is unclear how depressed flow serves as a causative agent or predisposing factor during DVT. Our studies support a role for inflammation causing neutrophil or platelet arrest and activation on activated endothelium with subsequent receptor-mediated capture of red blood cells (or rouloux) under low flow conditions to help precipitate DVT.

Materials and methods

Materials

Anti–CD36 monoclonal antibody (mAB) SMO, anti–CD18 mAB IB4, anti–CD41a (GPIIb) mAB 96.2C1, anti–CD29 (β_1) mAB 4B7R, anti–CD51 (α_v) mAB P2W7R, anti–CD11a mAB 38, anti–CD11b mAB ICRF44, and anti–CD11c mAB 3.9 (Ancell, Bayport, MN), anti–CD62P (P-selectin) mAb AK-4, anti–CD42b (GPIb) mAB HIP1, and anti–CD47 mAB B6H12 (BD PharMingen, San Diego, CA), polyclonal rabbit anti–human VWF and polyclonal rabbit anti–human thrombospondin (Accurate Antibodies, Westbury, NY), anti–TSP-1 mAB A4.1 and anti–TSP mAB C6.7 (Lab Vision, Fremont, CA), human serum albumin (HSA; Golden West Biologicals, Temecula, CA), N-formyl-Met-Leu-Phe (fMLP; Sigma, St Louis, MO), corn trypsin inhibitor (CTI), and human fibrinogen (Enzyme Research Labs, South Bend, IN) were stored following manufacturers' recommendations. Calf skin collagen and human thrombin were obtained from Calbiochem (San Diego, CA). Convulxin was a kind gift from Dr Mark Kahn (Department of Medicine, University of Pennsylvania, Philadelphia). Anti-LW (whole serum containing polyclonal antibody against LW), a potent RBC agglutinating agent, was a kind gift from Dr Joann Moulds (Department of Microbiology and Immunology, MCP Hahnemann University, Philadelphia).

Cell isolation

Human blood was collected from healthy adult donors by venipuncture and anticoagulated with Na-citrate (9 parts blood to 1 part Na-citrate). Neutrophils were isolated by centrifugation over neutrophil isolation medium (Cardinal Associates, Santa Fe, NM) as previously described.[17,32] Platelet-rich plasma (PRP) was obtained by centrifugation of anticoagulated whole blood at 130*g* for 15 minutes. Platelet singlets were prepared by gel filtration.[32] Following isolation, neutrophils or platelets were diluted to final concentrations of 10^6 cells/mL or 10^8 cells/mL, respectively. Erythrocytes were isolated by centrifugation at 600*g* for 10 minutes, washed with Hanks balanced salt solution (HBSS) and resuspended in HBSS containing 2% HSA at 5×10^8 RBC/mL. For each experiment, a normal, healthy blood donor was chosen randomly from a pool of 10 individuals. The venepuncture protocol was approved by the University of Pennsylvania institutional review board, and informed consent was provided by all donors in accordance with the Declaration of Helsinki.

Microcapillary flow chambers

Rectangular glass capillaries (Vitrocom, Mountain Lakes, NJ) with a cross section of 0.2×2.0 mm, a length of 7 cm, and a wall thickness of 0.15 mm were used as flow chambers as previously described.[17,32] To enable adhesion of neutrophils or platelets, microcapillary flow chambers were incubated with human fibrinogen solution (100 μg/mL) for 120 minutes at room temperature or with calf skin collagen (100 μg/mL) for 4 hours at 4°C. The chambers were rinsed and cells were allowed to adhere under no-flow conditions as described previously.[32] In some experiments, adherent platelets were treated with anti-GPIIb (25 μg/mL), anti-GPIb (25 μg/mL), anti–P-selectin (25 μg/mL), anti-CD36 (25 μg/mL), anti-VWF (1:40 dilution), polyclonal anti-TSP (1:40 dilution), anti-α_v (25 μg/mL), anti-β_1 (25 μg/mL), anti-CD47 (25 μg/mL), anti–TSP A4.1 (25 μg/mL), or anti–TSP C6.7 (25 μg/mL) for 20 minutes. While anti–TSP A4.1 was used to inhibit the binding of TSP to CD36, anti–TSP C6.7 was used to block binding of the cell/platelet binding domain (CBD) through which TSP interacts with CD47/IAP.[33] In some experiments, adherent neutrophils were treated with anti-CD36 or anti-TSP. In selected experiments, a purified fibrin surface was formed by incubation of the chamber with thrombin (10 U/mL) for 2 hours at room temperature, followed by perfusion of fibrinogen solution (3 mg/mL) through the chamber for 10 minutes at 100 s^{-1} to form an observable fibrin layer. In some experiments, human TSP (25 μg/mL) was perfused over a purified fibrin surface for 20 minutes at 50 s^{-1}.

Perfusion of erythrocytes and digital imaging

Erythrocytes were perfused into the flow chambers containing defined surface compositions at a controlled flow rate using a syringe pump (Harvard Apparatus, Holliston, MA). The wall shear stress (τ_w) imposed on the surface was calculated from the solution of the Navier-Stokes equation for laminar flow of a Newtonian fluid: $\tau_w = (6Q\mu)/(B^2W)$, where Q represents the flow rate (cm^3/s), μ represents the viscosity (0.01 Poise at room temperature), B represents the total plate separation (0.02 cm), and W represents the width (0.2 cm). Consequently, the wall shear rate, γ_w (s^{-1}), can be calculated as $\gamma_w = 6Q/B^2W$. Flow rates of 20, 40, 60, and 80 μL/min corresponded to shear stresses of 0.25, 0.50, 0.75, and 1.00 dyne/cm^2 and wall shear rates of 25 s^{-1}, 50 s^{-1}, 75 s^{-1}, and 100 s^{-1}. In some experiments, the red cells were preincubated with anti-GPIIb (25 μg/mL), anti-GPIb (25 μg/mL), anti–P-selectin (25 μg/mL), anti-CD36 (25 μg/mL), anti-CD18 (25 μg/mL), anti-VWF (1:40 dilution), polyclonal anti-TSP (1:40 dilution), anti–TSP A4.1 (25 μg/mL), or anti–TSP C6.7 (25 μg/mL) for 20 minutes prior to perfusion. In other experiments, red cells were incubated with anti-CD11a (60 μg/mL), anti-CD11b (60 μg/mL), anti-CD11c (60 μg/mL), anti-α_v (25 μg/mL), anti-β_1 (25 μg/mL), anti-CD47 (25 μg/mL), or anti-LW (1:5 dilution) for the same duration. To activate surface-adherent neutrophils in the flow chamber, 20 μM fMLP was added to the erythrocytes before perfusion. To activate fibrinogen-adherent platelets, either convulxin (10 nM) or thrombin (1 U/mL) was added to the red cells prior to their perfusion over platelets. During flow experiments, the microcapillary flow chambers were mounted on a Zeiss Axiovert 135 microscope (Thornwood, NY), and a 63X (NA 1.40) oil immersion objective lens (Plan Apochromat) was used to conduct differential interference contrast (DIC) microscopy. An Argus 20 image processor (Hamamatsu, Bridgewater, NJ) was used for contrast enhancement and real-time frame averaging. Images were acquired using a closed-circuit digital (CCD) camera (Hamamatsu) or a high-speed digital camera (MotionCorder Analyzer; Eastman Kodak, New York, NY) and were recorded on videotape. Following image acquisition, adherent cells were counted for multiple fields of view ($n > 15$) to calculate the cell adherence per unit area or the cell adherence per 100 neutrophils.

Results

Adhesion of erythrocytes to platelets

Washed erythrocytes were perfused over collagen-adherent platelets at $\gamma_w = 50$ s^{-1} for 5 minutes to investigate the interactions between erythrocytes and platelets under flow. Many events of red cell capture, transient tethering, and/or firm adhesion to platelets were observed (Figure 1A). In a typical short-lived event, following capture, tethering, and a pause for 400 ms, an adherent red cell was released by the platelet. Of the total adhesion events observed between erythrocytes and collagen-adherent platelets in 5 separate flow chambers during a perfusion period of 5 minutes for each chamber, 46% of the cells remained adherent for over 50 seconds, and about 67% remained adherent for over 10 seconds. Only about 18% were detached within one second, a time more characteristic of low bond numbers.

To examine the dependence of erythrocyte adhesion on platelet activation, erythrocyte adhesion on collagen-adherent platelets was compared to that over fibrinogen-adherent platelets. Red cells were

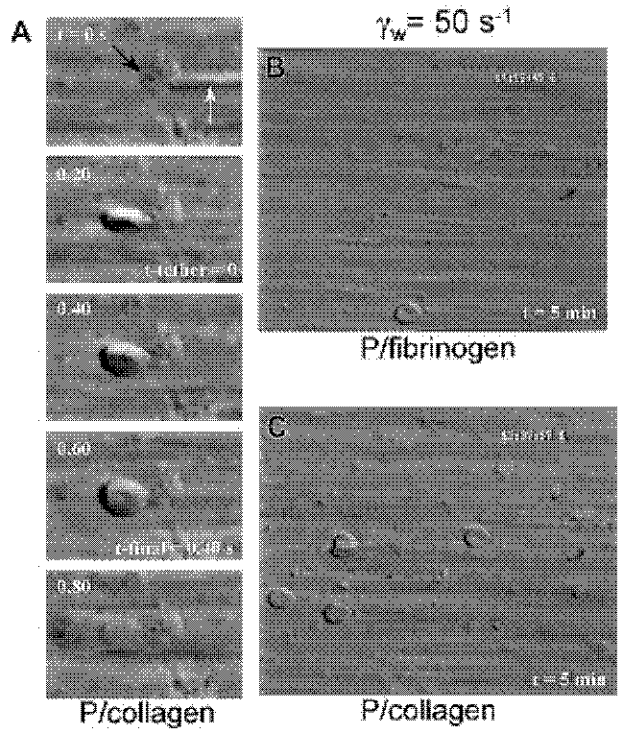

Figure 1. Erythrocyte capture by platelets and dependence on platelet activation state. (A) One of many short-lived pausing events observed as washed erythrocytes were perfused over collagen-adherent platelets at a wall shear rate of 50 s^{-1}. Following capture, membrane tethering, and a pause for 400 milliseconds, the red cell was released by the platelet. Visualization of firmly adhered erythrocytes on fibrinogen-adherent platelets (B) or collagen-adherent platelets (C) demonstrated the dependence of RBC adhesion on platelet activation. Identical results were obtained in 3 separate experiments, each conducted with an individual donor. Flow was from right to left.

perfused over fibrinogen-adherent platelets (Figure 1B) and collagen-adherent platelets (Figure 1C) in separate flow chambers at $\gamma_w = 50\ s^{-1}$ for 5 minutes. While adhesion events were observed on platelets adherent to fibrinogen or collagen, the density of red cells captured was 4-fold higher ($P < .005$, n = 3) on collagen-adherent platelets (Figure 2A). As a control, when erythrocytes were perfused at $\gamma_w = 50\ s^{-1}$ for 5 minutes over a collagen-coated surface (no platelets) incubated with albumin, no erythrocyte adhesion was observed. Because collagen-adherent platelets are more activated[32,34,35] than fibrinogen-adherent platelets, red cell adhesion to platelets at depressed venous flow conditions is regulated by the platelet activation state.

To confirm that adhesive interactions of erythrocytes with platelets are activation state–dependent, washed erythrocytes were perfused with the glycoprotein VI (GPVI)–agonist[36,37] convulxin (10 nM) over fibrinogen-adherent platelets at $\gamma_w = 50\ s^{-1}$ for 5 minutes. As a control, washed erythrocytes without convulxin were perfused over fibrinogen-adherent platelets in a separate flow chamber at the same wall shear rate. Adhesion of red cells to convulxin-stimulated platelets on fibrinogen was 2.7-fold higher ($P < .01$, n = 4) than to unstimulated fibrinogen-adherent platelets. Additionally, activation of fibrinogen-adherent platelets by thrombin (1 U/mL) resulted in a more than 5-fold increase in red cell adhesion to fibrinogen-adherent platelets (data not shown).

To investigate the role of P-selectin, CD36, GPIb, GP IIb/IIIa, TSP, or VWF in erythrocyte adhesion to collagen-adherent platelets, red cells were treated with blocking antibodies and perfused over platelets also pretreated with the same antibody. In 5 separate experiments with a polyclonal antibody against TSP and an antibody specifically directed toward blocking the interaction of TSP with CD36, no reduction in RBC adhesion to collagen-adherent platelets was found (Figure 2A). Similarly, antibodies against GPIIb, VWF, and P-selectin had no effect. Treatment with anti-CD36 or anti-GPIb showed statistically significant reduction (approximately 40%) in red cell adhesion (Figure 2A), indicating a role for CD36 and GPIb in the process. Blocking antibodies against α_v, β_1, and CD47 were used to test the possible roles of $\alpha_v\beta_3$, $\alpha_4\beta_1$ and CD47. None of these antibodies had any effect (data not shown). Additionally, the effect of an antibody targeted specifically toward the C-terminal domain of TSP (TSP Ab-3 clone C6.7), through which TSP interacts with CD47/IAP, was examined on erythrocyte-platelet interactions. The antibody did not significantly block (12%, n = 2) adhesive interactions between erythrocytes and collagen-adherent platelets. When fibrinogen (3 mg/mL) was added to the red cells prior to their perfusion over platelets, cell adhesion was significantly blocked by 80% ($P < .005$, n = 3; Figure 2A). This demonstrated that at physiologic concentrations, fibrinogen plays a protective role in preventing red cell adhesion to collagen-activated platelets.

When red blood cells containing K_3-EDTA (4 mM) were perfused over collagen-adherent platelets, adhesion was completely blocked (100%), indicating the Ca^{++} or Mg^{2+} dependence of this process. Moreover, when erythrocytes containing EGTA

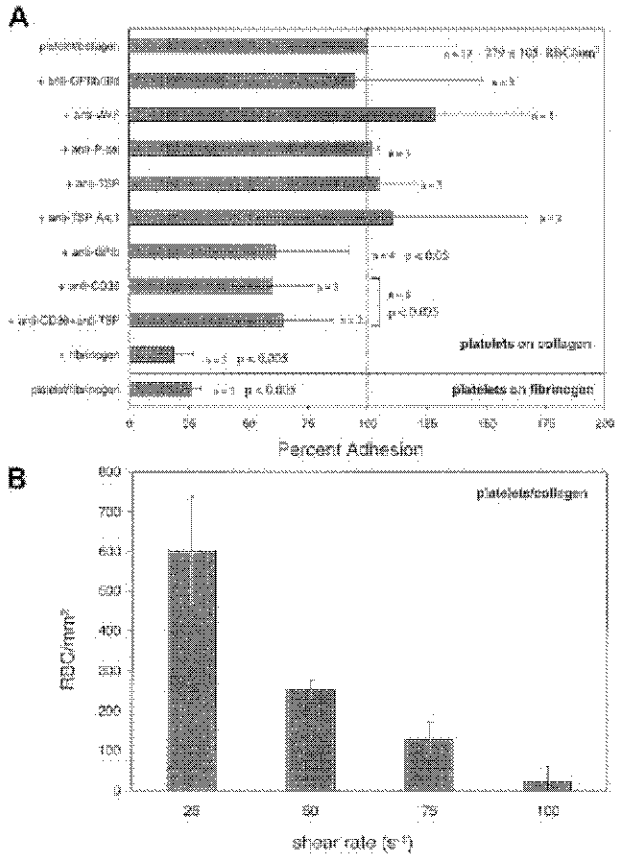

Figure 2. Effect of blocking antibodies, fibrinogen, and shear rate on erythrocyte adhesion to platelets. (A) Comparison of percent adhesion ($\gamma_w = 50\ s^{-1}$, 5 minutes) with the control demonstrated reduction in erythrocyte adhesion to collagen-adherent platelets in the presence of anti-CD36, anti-GPIb, or soluble fibrinogen. RBC adhesion on collagen-adherent platelets was 4-fold greater than on fibrinogen-adherent platelets. The average platelet density (number of platelets on surface/unit area) for both collagen-adherent platelets and fibrinogen-adherent platelets was 11.6 ($\pm$ 1.5) $\times$ 10^3 platelets/mm^2. (B) Erythrocyte adhesion at 5 minutes to collagen-adherent platelets was found to be inversely correlated to shear rate (n = 3). Data are expressed as means $\pm$ SD of n replicated experiments.

(4 mM), supplemented with Mg^{2+} (2 mM), were perfused over collagen-adherent platelets, adhesion was significantly blocked (70%), indicating the Ca^{++}-dependence of red cell adhesive interactions with platelets. In experiments at γ_w = 25, 50, 75, and 100 s^{-1} the number of adherent red cells to collagen-adherent platelets decreased as the shear rate increased (Figure 2B). These observations indicate that the RBC adhesion to platelets is significant only at depressed venous flow conditions (below 100 s^{-1}).

Adhesion of erythrocytes to neutrophils

Washed red cells were perfused over fibrinogen-adherent neutrophils at a wall shear rate of 50 s^{-1} for 5 minutes. A few transient adhesion events were observed between red cells and fibrinogen-adherent neutrophils. However, when neutrophils were activated with 20 μM fMLP (added to the erythrocytes before perfusion), the number of pausing events and firmly adherent red cells to these activated neutrophils dramatically increased. Figure 3A shows a capture/membrane tethering event lasting 800 milliseconds between an erythrocyte and an fMLP-activated neutrophil. Out of the total adhesion events observed between erythrocytes and fMLP-activated neutrophils in 4 separate flow chambers during a perfusion period of 5 minutes for each, 57% of the cells remained adherent for over 50 seconds, 66% remained adherent for over 10 seconds, and approximately 22% were detached within one second.

A comparison between Figure 3B and Figure 3C shows that the extent of red cell adhesion to activated neutrophils was considerably greater than that to normal neutrophils. fMLP caused a 6-fold increase ($P < .005$, n = 3) in RBC adhesion to neutrophils (67 RBC/100 neutrophils vs 11 RBC/100 neutrophils; Figure 4A). Also, RBC adhesion to fMLP-treated neutrophils was significantly

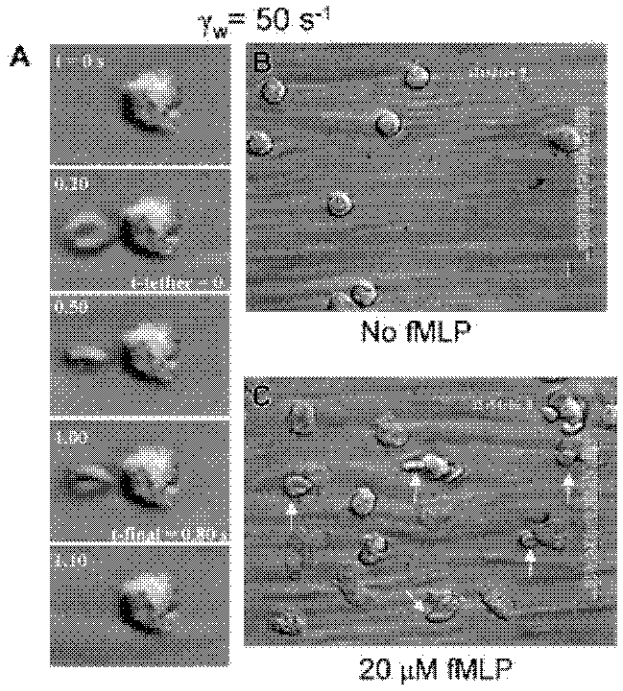

Figure 3. Erythrocyte pausing on neutrophils and dependence on neutrophil activation state. (A) One of many short-lived pausing events observed as washed erythrocytes were perfused over fibrinogen-adherent, fMLP-activated neutrophils at a wall shear rate of 50 s^{-1}. Following capture, membrane tethering, and a pause for 800 milliseconds, the red cell was released by the neutrophil. Visualization of firmly adhered erythrocytes on untreated neutrophils (B) or fMLP-stimulated neutrophils (C) demonstrated the dependence of RBC adhesion on neutrophil activation after 5 minutes at γ_w = 50 s^{-1}. Identical results were obtained in 3 separate experiments, each conducted with an individual donor. Flow was from right to left.

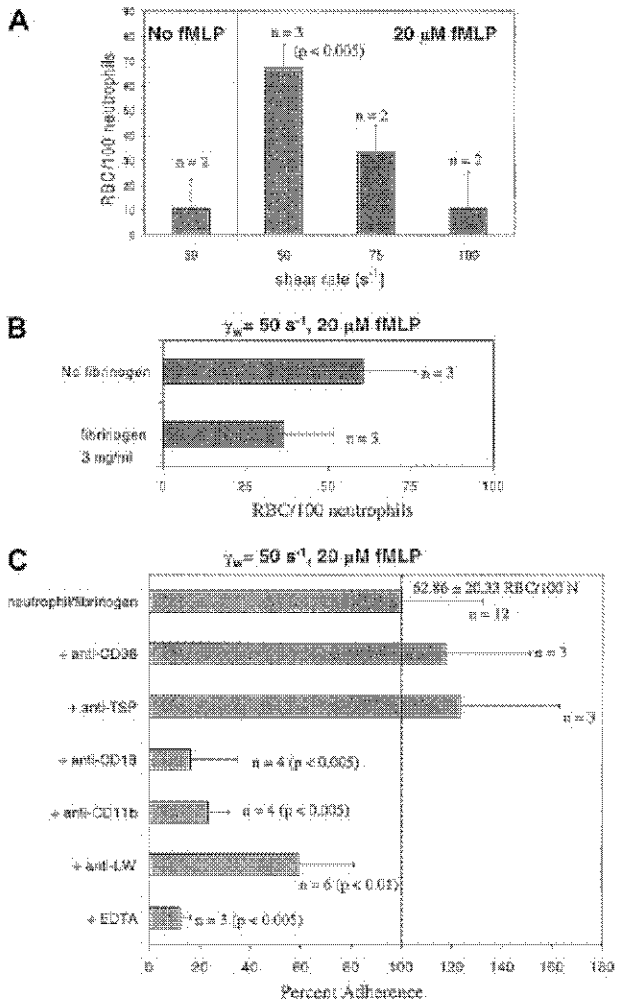

Figure 4. Effect of shear rate, soluble fibrinogen and blocking antibodies on erythrocyte adhesion to neutrophils. (A) Erythrocyte adhesion to fibrinogen-adherent, fMLP-activated neutrophils was found to be inversely correlated to shear rate. Also, RBC adhesion to fMLP-activated neutrophils was more than 6-fold greater than to untreated neutrophils. (B) RBC adhesion to activated neutrophils was insensitive to soluble fibrinogen. (C) Comparison of percent adhesion with the control demonstrated significant reduction in erythrocyte adhesion to fMLP-activated neutrophils in the presence of anti-CD11b, anti-CD18, anti-LW, or EDTA. Data are expressed as means ± SD of n replicated experiments.

reduced as γ_w increased above 100 s^{-1} (Figure 4A). When fibrinogen (3 mg/mL) was added to the erythrocytes (containing fMLP) prior to their perfusion over adherent neutrophils, the minor reduction in red cell adhesion to activated neutrophils (Figure 4B) was not statistically significant. As a control, when erythrocytes were perfused for 5 minutes over a fibrinogen-coated surface (no neutrophils) incubated with albumin, no RBC adhesion was observed. Erythrocyte adhesion to fMLP-activated neutrophils at depressed venous flows, unlike RBC adhesion to collagen-activated platelets, was not blocked by soluble fibrinogen.

The presence of anti–CD18 antibody significantly blocked adhesive interactions between red cells and fMLP-activated neutrophils by 80% ($P < .005$, n = 3; Figure 4C). Having observed the role of anti-CD18 in blocking adhesion, blocking antibodies against CD11a, CD11b, or CD11c were used to specify if the neutrophil receptor responsible for these interactions was LFA-1 (CD11a/CD18), Mac-1 (CD11b/CD18), or p150,95 (CD11c/CD18). Whereas antibodies against CD11a or CD11c had no effect on adhesion (data not shown), anti-CD11b blocked adhesion by 76% ($P < .005$, n = 4; Figure 4C) demonstrating that Mac-1 is the

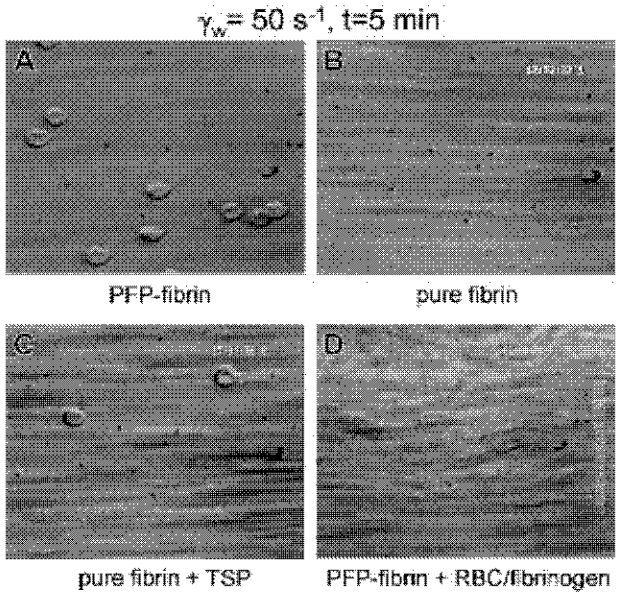

Figure 5. Erythrocyte adhesion to fibrin at low shear rate. As washed erythrocytes were perfused over fibrin polymerized from PFP at a wall shear rate of 50 s^{-1}, firm adhesion to fibrin was observed (A). When erythrocytes were perfused over purified fibrin, no firm adhesion and transient pausing to fibrin were observed (B). When this fibrin surface was coated with TSP (25 μg/mL) prior to erythrocyte perfusion, adhesion was observed (C). This adhesion was attenuated in the presence of soluble fibrinogen (D). Flow was from right to left.

major mediator of adhesion on the neutrophil. When red blood cells in anti-CD36 or anti-TSP were perfused over fMLP-treated neutrophils also pretreated with the same antibody, no significant reduction in adhesion was observed. When erythrocytes in anti-LW were perfused over fMLP-activated neutrophils, a 40% reduction ($P < .01$, n = 6; Figure 4C) in red cell adhesion to neutrophils was observed. In contrast, antibodies directed toward α_v, β_1, and CD47 had no effect on red cell adhesion to neutrophils (data not shown). Consistent with β_2-integrin function, the adhesion of erythrocytes to activated neutrophils was markedly inhibited by EDTA (Figure 4C).

Adhesion of erythrocytes to fibrin

To investigate possible adhesive interactions (not entrapment) between erythrocytes and fibrin, washed erythrocytes were perfused at 50 s^{-1} over preformed fibrin fibers polymerized from recalcified PFP. At t = 5 minutes, some RBC adhesion was observed (Figure 5A). In order to determine if this observed adhesion was a result of direct adhesive interaction between fibrin and the red cell, washed erythrocytes were perfused over fibrin fibers made from purified fibrinogen. Absence of adhesion to purified fibrin (Figure 5B) demonstrated that the interaction was not direct, but mediated by a molecule in plasma bound to fibrin. As TSP is a plasma protein that can mediate multiple interactions, we analyzed its role by perfusing red cells over a purified fibrin surface treated with TSP. The observed red cell adhesion to this surface (Figure 5C) indicated the role of TSP in mediating this interaction. However, this role of TSP could not be verified because anti–TSP A4.1 did not show any effect on RBC adhesion to fibrin from platelet-free plasma (PFP; data not shown). Finally, the dependence of adhesive interactions between erythrocytes and fibrin (from PFP) on divalent cations and their fibrinogen sensitivity were tested for comparison to interactions with platelets and neutrophils. Like adhesion to platelets, RBC adhesion to fibrin from PFP was antagonized by soluble fibrinogen (3 mg/mL; Figure 5D) and was EDTA-sensitive.

Finally, to mimic pathophysiologic conditions more closely in the presence of soluble fibrinogen and limited contact pathway activation, PFP containing red blood cells, CTI (to inhibit Factor XIIa of the contact pathway of coagulation[32,38]), and fMLP was perfused at $\gamma_w = 50$ s^{-1} over a collagen-adherent cell mixture of neutrophils and platelets. Red blood cells in plasma frequently displayed events of pausing and capture to collagen-adherent platelets and neutrophils. After 25 minutes, when fibrin polymerization was first detectable as expected,[32] erythrocyte adhesion increased dramatically and included observable RBC adhesions with neutrophils and platelets as well as to the fibrin deposited on the surface (Figure 6). Direct visualization of RBC interaction with fibrin indicated that RBC adhesion to the fibrin strands was not via passive entrapment.

Discussion

We report that adhesion of normal erythrocytes to platelets at depressed venous shear rates below 100 s^{-1} is enhanced by activation mediated through platelet spreading on collagen relative to fibrinogen. Direct stimulation of fibrinogen-adherent platelets by convulxin elevates erythrocyte firm adhesion to the levels supported by collagen-adherent platelets. RBC adhesion to collagen-adherent platelets can be significantly reduced by soluble fibrinogen, EDTA, and to a lesser extent by antibodies against CD36 or GPIb. Receptor-mediated adhesion of erythrocytes to fMLP-activated neutrophils is also inversely correlated to shear rate and can be essentially blocked by antibodies against CD11b, CD18, LW, or EDTA, but not by physiologic levels of soluble fibrinogen. Adhesion of erythrocytes to fibrin formed from PFP was blocked by soluble fibrinogen and EDTA. Although RBCs do not adhere to purified fibrin at $\gamma_w = 50$ s^{-1} they do adhere to a TSP-coated purified fibrin surface. In addition to firm adherence of RBCs, short-lived adhesion events (t < 5 seconds) between red blood cells and platelets, neutrophils, or fibrin were also visualized; they

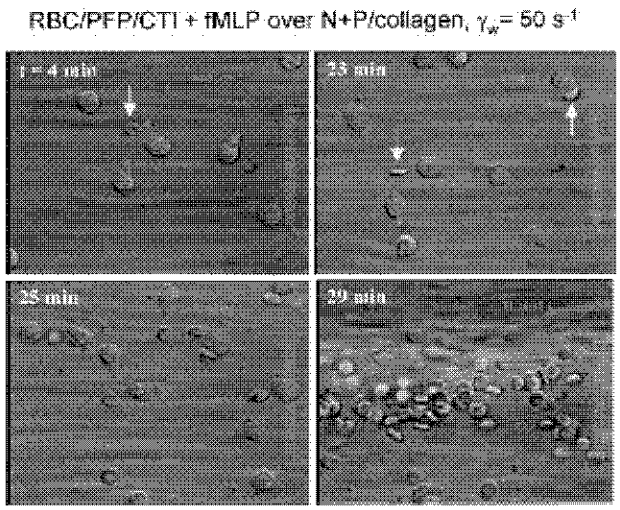

Figure 6. Erythrocyte adhesion under pathophysiologic conditions. As PFP containing resuspended RBC, CTI (50 μg/mL), and fMLP was perfused over a collagen-adherent cell mixture of platelets and neutrophils, events of RBC pausing and capture to platelets and neutrophils were observed. After 25 minutes, when fibrin polymerization first became evident, a striking increase in erythrocytes firmly adherent to platelets, neutrophils, and deposited fibrin was observed. Flow was from right to left.

were all accompanied by RBC membrane deformation and membrane tethering upon binding. Whereas physiologic concentrations of soluble fibrinogen considerably reduce erythrocyte adhesion to platelets and fibrin, the adhesion to neutrophils was fibrinogen insensitive.

Because platelet receptor GPVI mediates the adhesion and signaling responses to collagen[37,39] and convulxin is a GPVI selective agonist,[36,37,39] our results indicate that platelet GPVI is triggering signaling that leads to the adhesive interactions of erythrocytes with platelets. In addition to being EDTA-sensitive, the interaction was found to be ethyleneglycotetraacetic acid (EGTA)–sensitive (70% block), demonstrating the Ca^{++} dependence of the adhesive interactions between erythrocytes and platelets. As GPVI-mediated platelet activation occurs through Ca^{++}-dependent pathways,[39] the Ca^{++} dependence of erythrocyte-platelet adhesion events may involve GPVI signaling in mediating these interactions.

Using a Dacron graft thrombosis model, Palabrica et al[40] observed that anti–P-selectin inhibited neutrophil and fibrin accumulation as well as reduced RBC adhesion to the clot surface by an unknown mechanism. This decrease in red cells observed by Palabrica et al may be explained by adhesive mechanisms described in the present study. Although P-selectin may not have been directly involved in normal red cell adhesion, the reduction in fibrin and number of neutrophils in the clot may have led to diminished erythrocyte adhesion in the harvested grafts examined by Palabrica et al.[40]

Recent studies have suggested a role for adherent neutrophils in sickle cell vascular occlusion.[41] The initiation and propagation of vaso-occlusive processes in sickle disease has been associated with neutrophil activation.[42] We believe that adhesive interactions of normal erythrocytes with adherent activated neutrophils, like the ones we have presented in this study, can also lead to vaso-occlusion under low shear rates ($< 100\ s^{-1}$) that occur in DVT. A slight increase in flow may provide DVT prophylaxis to the patient by preventing and/or reversing erythrocyte adhesion to neutrophils and platelets. Studies by Stewart,[43] which suggest a link between inhibition of neutrophil adhesion to the vessel wall and reduction in the occurrence of DVT following hip replacement, also support our hypothesis. In a different set of studies, a drug designed as an inhibitor of neutrophil adhesion during inflammation reduced experimentally induced venous thrombosis and restored blood movement by a mechanism that is unclear.[44] Moreover, hip arthroplasty in patients is accompanied by an increased CD11b expression on neutrophils.[45] It is possible that DVT, which is more frequent in patients undergoing hip or knee arthroplasty (as a result of venous stasis), might be linked with increased CD11b-mediated red cell adhesion to neutrophils.

Although it is known that vascular compression devices help prevent DVT, the molecular mechanisms by which they do so have been unclear.[46,47] Our observation that firm adhesion of red cells to platelets and neutrophils is efficient at shear rates below $100\ s^{-1}$ may explain these phenomena. Reduced shear rates can allow red cell adhesion to the cells adherent to inflammed venular endothelium causing occlusion and pain. Vascular compressions may lead to a slight increase in blood movement and wall shear rate and shear stress, which, as explained above, may impede erythrocyte capture and/or reverse adhesion and subsequent vaso-occlusion thus providing DVT prophylaxis to the patient.

Although the adhesion of erythrocytes to activated platelets is GPVI-triggered and partly CD36-dependent and their adhesion to activated neutrophils is mediated by Mac-1 and LW, the mediator(s) for RBC-fibrin interaction remains unclear. Even though RBCs firmly adhered to a TSP-coated fibrin surface, a TSP antibody did not block adhesion to PFP fibrin. Because TSP is a complex molecule that interacts with cells through multiple pathways including fibrinogen cross-bridging,[27] it may be difficult to antagonize its effects using an antibody.

Why have normal RBC adhesion processes not been detected or described previously? Prior studies of platelet, neutrophil, and sickle cell adhesion have generally been conducted at $\tau w \geq 1$ dyne/cm^2.[2,48,49] With the recent discovery of "hydrodynamic thresholding,"[50,51] where shear forces facilitate bonding, reduced shear rates in the range of 10 to $100\ s^{-1}$ are used to generate low forces of 0.1 to 1 dyne/cm^2. Additionally, whole blood forms rouloux at shear rates below $100\ s^{-1}$ and these structures may obscure visualization of this process. Furthermore, DIC microscopy used in this study facilitated visualization of events near the surface even in the presence of high hematocrit, while real-time digital frame averaging to average out moving red cells from captured cells also enhanced the detection of short-lived adhesion events in a visual background of flowing blood. Finally, the hypothesis that normal RBCs are entirely passive precludes the study of RBC adhesion during coagulation or inflammation. We report direct observations that normal RBCs do in fact form adhesive interactions under low flow conditions.

References

1. Matsui NM, Borsig L, Rosen SD, Yaghmai M, Varki A, Embury SH. P-selectin mediates the adhesion of sickle erythrocytes to the endothelium. Blood. 2001;98:1955-1962.
2. Manodori AB, Barabino GA, Lubin BH, Kuypers FA. Adherence of phosphatidylserine-exposing erythrocytes to endothelial matrix thrombospondin. Blood. 2000;95:1293-1300.
3. Sugihara K, Sugihara T, Mohandas N, Hebbel RP. Thrombospondin mediates adherence of CD36+ sickle reticulocytes to endothelial cells. Blood. 1992;80:2634-2642.
4. Wick TM, Moake JL, Udden MM, Eskin SG, Sears DA, McIntire LV. Unusually large von Willebrand factor multimers increase adhesion of sickle erythrocytes to human endothelial cells under controlled flow. J Clin Invest. 1987;80:905-910.
5. Browne PV, Hebbel RP. CD36-positive stress reticulocytosis in sickle cell anemia. J Lab Clin Med. 1996;127:340-347.
6. Hillery CA, Du MC, Montgomery RR, Scott JP. Increased adhesion of erythrocytes to components of the extracellular matrix: isolation and characterization of a red blood cell lipid that binds thrombospondin and laminin. Blood. 1996;87:4879-4886.
7. Swerlick RA, Eckman JR, Kumar A, Jeitler M, Wick TM. Alpha 4 beta 1-integrin expression on sickle reticulocytes: vascular cell adhesion molecule-1-dependent binding to endothelium. Blood. 1993;82:1891-1899.
8. Kaul DK, Tsai HM, Liu XD, Nakada MT, Nagel RL, Coller BS. Monoclonal antibodies to alphaVbeta3 (7E3 and LM609) inhibit sickle red blood cell-endothelium interactions induced by platelet-activating factor. Blood. 2000;95:368-374.
9. Brittain JE, Mlinar KJ, Anderson CS, Orringer EP, Parise LV. Activation of sickle red blood cell adhesion via integrin-associated protein/CD47-induced signal transduction. J Clin Invest. 2001;107:1555-1562.
10. Senczuk AM, Reeder JC, Kosmala MM, Ho M. Plasmodium falciparum erythrocyte membrane protein 1 functions as a ligand for P-selectin. Blood. 2001;98:3132-3135.
11. McCormick CJ, Newbold CI, Berendt AR. Sulfated glycoconjugates enhance CD36-dependent adhesion of plasmodium falciparum-infected erythrocytes to human microvascular endothelial cells. Blood. 2000;96:327-333.
12. Gruarin P, Primo L, Ferrandi C, et al. Cytoadherence of plasmodium falciparum-infected erythrocytes is mediated by a redox-dependent conformational fraction of CD36. J Immunol. 2001;167:6510-6517.
13. Saelman EU, Nieuwenhuis HK, Hese KM, et al. Platelet adhesion to collagen types I through VIII under conditions of stasis and flow is mediated by

GPIa/IIa (alpha 2 beta 1-integrin). Blood. 1994; 83:1244-1250.

14. Bennett JS, Shattil SJ, Power JW, Gartner TK. Interaction of fibrinogen with its platelet receptor: differential effects of alpha and gamma chain fibrinogen peptides on the glycoprotein IIb-IIIa complex. J Biol Chem. 1988;263:12948-12953.
15. Vicente V, Houghten RA, Ruggeri ZM. Identification of a site in the alpha chain of platelet glycoprotein Ib that participates in von Willebrand factor binding. J Biol Chem. 1990;265:274-280.
16. Moore KL, Patel KD, Bruehl RE, et al. P-selectin glycoprotein ligand-1 mediates rolling of human neutrophils on P-selectin. J Cell Biol. 1995;128: 661-671.
17. Schmidtke DW, Diamond SL. Direct observation of membrane tethers formed during neutrophil attachment to platelets or P-selectin under physiological flow. J Cell Biol. 2000;149:719-730.
18. Buttrum SM, Hatton R, Nash GB. Selectin-mediated rolling of neutrophils on immobilized platelets. Blood. 1993;82:1165-1174.
19. Diacovo TG, Roth SJ, Buccola JM, Bainton DF, Springer TA. Neutrophil rolling, arrest, and transmigration across activated, surface-adherent platelets via sequential action of P-selectin and the beta 2-integrin CD11b/CD18. Blood. 1996;88: 146-157.
20. Hermand P, Huet M, Callebaut I, et al. Binding sites of leukocyte beta 2 integrins (LFA-1, Mac-1) on the human ICAM-4/LW blood group protein. J Biol Chem. 2000;275:26002-26010.
21. Spring FA, Parsons SF, Ortlepp S, et al. Intercellular adhesion molecule-4 binds alpha(4)beta(1) and alpha(V)-family integrins through novel integrin-binding mechanisms. Blood. 2001;98:458-466.
22. Southcott MJ, Tanner MJ, Anstee DJ. The expression of human blood group antigens during erythropoiesis in a cell culture system. Blood. 1999;93: 4425-4435.
23. Bacon-Baguley T, Ogilvie ML, Gartner TK, Walz DA. Thrombospondin binding to specific sequences within the A alpha- and B beta-chains of fibrinogen. J Biol Chem. 1990;265:2317-2323.
24. Roberts DD, Haverstick DM, Dixit VM, Frazier WA, Santoro SA, Ginsburg V. The platelet glycoprotein thrombospondin binds specifically to sulfated glycolipids. J Biol Chem. 1985;260:9405-9411.
25. Xie L, Chesterman CN, Hogg PJ. Control of von Willebrand factor multimer size by thrombospondin-1. J Exp Med. 2001;193:1341-1349.
26. Kowalska MA, Tuszynski GP. Interaction of thrombospondin with platelet glycoproteins GPIa-IIa and GPIIb-IIIa. Biochem J. 1993;295:725-730.
27. Bonnefoy A, Hantgan R, Legrand C, Frojmovic MM. A model of platelet aggregation involving multiple interactions of thrombospondin-1, fibrinogen, and GPIIbIIIa receptor. J Biol Chem. 2001; 276:5605-5612.
28. Bale MD, Mosher DF. Effects of thrombospondin on fibrin polymerization and structure. J Biol Chem. 1986;261:862-868.
29. Tuszynski GP, Srivastava S, Switalska HI, Holt JC, Cierniewski CS, Niewiarowski S. The interaction of human platelet thrombospondin with fibrinogen: thrombospondin purification and specificity of interaction. J Biol Chem. 1985;260: 12240-12245.
30. Suchard SJ, Burton MJ, Dixit VM, Boxer LA. Human neutrophil adherence to thrombospondin occurs through a CD11/CD18-independent mechanism. J Immunol. 1991;146:3945-3952.
31. Mansfield PJ, Suchard SJ. Thrombospondin promotes both chemotaxis and haptotaxis in neutrophil-like HL-60 cells. J Immunol. 1993;150:1959-1970.
32. Goel MS, Diamond SL. Neutrophil enhancement of fibrin deposition under flow through platelet-dependent and -independent mechanisms. Arterioscler Thromb Vasc Biol. 2001;21:2093-2098.
33. Chung J, Gao AG, Frazier WA. Thrombspondin acts via integrin-associated protein to activate the platelet integrin alphaIIbbeta3. J Biol Chem. 1997;272:14740-14746.
34. Briede JJ, Heemskerk JW, van't Veer C, Hemker HC, Lindhout T. Contribution of platelet-derived factor Va to thrombin generation on immobilized collagen- and fibrinogen-adherent platelets. Thromb Haemost. 2001;85:509-513.
35. Heemskerk JW, Vuist WM, Feijge MA, Reutelingsperger CP, Lindhout T. Collagen but not fibrinogen surfaces induce bleb formation, exposure of phosphatidylserine, and procoagulant activity of adherent platelets: evidence for regulation by protein tyrosine kinase-dependent Ca2+ responses. Blood. 1997;90:2615-2625.
36. Polgar J, Clemetson JM, Kehrel BE, et al. Platelet activation and signal transduction by convulxin, a C-type lectin from Crotalus durissus terrificus (tropical rattlesnake) venom via the p62/GPVI collagen receptor. J Biol Chem. 1997;272:13576-13583.
37. Chen H, Locke D, Liu Y, Liu C, Kahn ML. The platelet receptor GPVI mediates both adhesion and signaling responses to collagen in a receptor density-dependent fashion. J Biol Chem. 2002; 277:3011-3019.
38. Holmes MB, Schneider DJ, Hayes MG, Sobel BE, Mann KG. Novel, bedside, tissue factor-dependent clotting assay permits improved assessment of combination antithrombotic and antiplatelet therapy. Circulation. 2000;102:2051-2057.
39. Quinton TM, Ozdener F, Dangelmaier C, Daniel JL, Kunapuli SP. Glycoprotein VI-mediated platelet fibrinogen receptor activation occurs through calcium-sensitive and PKC-sensitive pathways without a requirement for secreted ADP. Blood. 2002;99:3228-3234.
40. Palabrica T, Lobb R, Furie BC, et al. Leukocyte accumulation promoting fibrin deposition is mediated in vivo by P-selectin on adherent platelets. Nature. 1992;359:848-851.
41. Turhan A, Weiss LA, Mohandas N, Coller BS, Frenette PS. Primary role for adherent leukocytes in sickle cell vascular occlusion: a new paradigm. Proc Natl Acad Sci U S A. 2002;99:3047-3051.
42. Lard LR, Mul FP, de Haas M, Roos D, Duits AJ. Neutrophil activation in sickle cell disease. J Leukoc Biol. 1999;66:411-415.
43. Stewart GJ. Neutrophils and deep venous thrombosis. Haemostasis. 1993;23:127-140.
44. McEver RP. P-selectin and PSGL-1: exploiting connections between inflammation and venous thrombosis. Thromb Haemost. 2002;87:364-365.
45. Fassbender K, Kaptur S, Becker P, Groschl J, Hennerici M. Adhesion molecules in tissue injury: kinetics of expression and shedding and association with cytokine release in humans. Clin Immunol Immunopathol. 1998;89:54-60.
46. Coleridge Smith PD, Hasty JH, Scurr JH. Deep vein thrombosis: effect of graduated compression stockings on distension of the deep veins of the calf. Br J Surg. 1991;78:724-726.
47. Chen AH, Frangos SG, Kilaru S, Sumpio BE. Intermittent pneumatic compression devices: physiological mechanisms of action. Eur J Vasc Endovasc Surg. 2001;21:383-392.
48. Roest M, Sixma JJ, Wu YP, et al. Platelet adhesion to collagen in healthy volunteers is influenced by variation of both alpha(2)beta(1) density and von Willebrand factor. Blood. 2000;96:1433-1437.
49. Reinhardt PH, Kubes P. Differential leukocyte recruitment from whole blood via endothelial adhesion molecules under shear conditions. Blood. 1998;92:4691-4699.
50. Finger EB, Puri KD, Alon R, Lawrence MB, von Andrian UH, Springer TA. Adhesion through L-selectin requires a threshold hydrodynamic shear. Nature. 1996;379:266-269.
51. Lawrence MB, Kansas GS, Kunkel EJ, Ley K. Threshold levels of fluid shear promote leukocyte adhesion through selectins (CD62L, P, E). J Cell Biol. 1997;136:717-727.