Examination of the Roles of Glycoprotein Ib and Glycoprotein IIb/IIIa in Platelet Deposition on an Artificial Surface Using Clinical Antiplatelet Agents and Monoclonal Antibody Blockade

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The mechanism of platelet thrombus growth on an artificial surface is incompletely understood. While glycoprotein (GP)Iib and GPIIb/IIIa are required for normal attachment and thrombus formation on subendothelium, their roles in platelet deposition to artificial surfaces remain unclear. Using selected platelet inhibitors (aspirin [ASA], low molecular weight dextran, monoclonal antibodies 10E5 [v GP IIb/IIIa], and 6D1 [GP IIb]) we examined the mechanism of platelet deposition to polyethylene (PE) surfaces under steady laminar and oscillatory flow conditions. Polyethylene-100 (PE-100) tubes (0.86 mm internal diameter) were perfused under steady laminar flow with citrated human whole blood reconstituted with 111-indium-labeled platelets at 312 seconds⁻¹ shear rate in the presence and absence of platelet inhibitors. The effect of oscillatory flow on platelet deposition was examined in a microcuvette system using 3/16-inch diameter discs of National Heart, Lung, and Blood Institute primary reference PE as the test surface. ASA and dextran did not significantly (P > 0.05) inhibit platelet deposition in laminar flow (not tested in oscillatory). Antibody 10E5 was a potent inhibitor (laminar <1%, P < 0.0001, oscillatory <1.6%, P < 0.01) of platelet deposition in both systems, and in this case, true adhesion (first attached layer) was blocked. Antibody 6D1 unexpectedly inhibited 70% of platelet deposition (P < 0.01) in steady laminar flow and 56.5% in oscillatory flow (P < 0.01). Scanning electron microscopy demonstrated platelets atop platelets in the controls, rare platelets in the 10E5 group, and a patchy monolayer of platelets in the 6D1 group. Transmission electron microscopy of cross-sections confirmed these observations. We conclude that the adhesion of the first platelet layer to an artificial surface requires GP IIb/IIIa. The data also suggest that GP IIb is required for the development of the second layer in vertical platelet thrombus growth.

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flow conditions. The effect of specific GP blockade was compared with that seen after treatment of blood with two clinically used antiplatelet drugs (aspirin [ASA] and low molecular weight dextran [LMWD]), which do not act via GP receptor blockade.

Our results demonstrate that identical mechanisms are used for both adhesion and paradhesion under each type of flow condition. Currently used antiplatelet drugs, tested only under laminar flow, do not significantly decrease platelet deposition, but blockade of GPIIb/IIIa can completely block platelet deposition in both systems through inhibition of the adhesion step. Blockade of the GPIb receptor significantly reduces platelet deposition, limiting the process to formation of a patchy monolayer. This result suggests that GPIb is required for paradhesion to occur. The mechanism(s) through which supradhesion occurs has not been determined, but interplatelet attachment at this level is presumably similar to aggregation in the fluid phase, and would therefore hypothetically require primarily fibrogen and GPIIb/IIIa.

The artificial surface represents a unique activation signal to the platelet that results in the utilization of GPIIb/IIIa and GPIb in a novel manner to accomplish platelet deposition. The ability to completely block platelet adhesion to an artificial surface and/or the ability to restrict this growth to a monolayer may have clinical importance.

**MATERIALS AND METHODS**

**Materials.** Adenosine diphosphate (ADP), collagen, ristocetin, aspirin, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), bovine serum albumin (BSA), Na-citrate, citric acid, Apyrase, NaOH, Sepharose 2B, adenosine triphosphate (ATP) assay (luciferin/luciferase) were purchased from Sigma Chemical Co (St Louis, MO). 8-Hydroxyquinoline (oxine) was from Aldrich Chemical Co (Milwaukee, WI). 111Indium oxyquinoline solution was purchased from Amersham Corp (Arlington Heights, IL).

Mouse MoAbs versus GPIIb/IIIa (10E5), and GPIb (6D1) were kindly provided as F(ab')2 fragments in 0.1 mol/L Tris, 0.05% NaOH, Sepharose 2B, and equilibrated with the same buffer. The platelet suspension was layered on a 1.0-cm column packed with Sepharose 2B and equilibrated with the same H/T buffer.

The platelets were eluted using the same H/T buffer, into a volume of 2 mL. To this volume were added 10 μL 1 mmol/L PGE2, 12.5 μL 1 mg/mL oxine and 40 μCi 111indium-oxine. The reaction mixture was incubated at 37°C for 1 minute. A 50-μL sample was removed and immediately centrifuged at 14,000 rpm × 2 minutes in a table-top microcentrifuge (Eppendorf no. 5415; Brinkman Instruments Inc, Westbury, NY). Pellet and supernatant were separated and gamma counts of each were measured (Beckman Gamma 5500; Beckman, Fullerton, CA). Labeling efficiency ranged from 90% to 95%.

The remaining platelets were again pelleted by centrifugation at 1,100g for 6 minutes. During this time the pH of the PPP was returned to 7.4 by titration with 0.15 mol/L NaOH. On completion of the centrifugation the unbound 111indium in the supernatant was removed by decanting. The sides of the container were gently washed two times with H/T buffer, pH 7.4, without added Apyrase or PGE2. The 111indium-labeled platelets were then gently resuspended in PPP and returned to their original plasma volume. Resuspended platelets were counted with an electronic particle counter (Coulter model ZBI; Coulter, Hialeah, FL). Their concentration was 334,816±4,171 in PRP. The 111indium-labeled platelets were incubated at 37°C for 1 hour before testing.

Aggregation and secretion were tested using 0.45-μL samples of PRP using a Lumi-aggregometer (model 660; Chrono-Log Corp, Havertown, PA). ATP secretion from dense granules in response to 20 μmol/L ADP was measured in the presence of 0.04 mL of luciferin/luciferase, added to the PRP before aggregation. Aggregation with ADP, collagen, thrombin, and ristocetin were different from that measured in control platelets after 111indium labeling.

**Preparation of reconstituted whole blood.** 111Indium-labeled PRP was incubated at 37°C. Packed red blood cells (PRBC) in autologous plasma (hematocrit 60) were maintained in capped tubes at 22°C. Before perfusion, 4 mL 111indium-labeled PRP and 6 mL PRBC were gently mixed in a 10-mL syringe and incubated at 37°C for 5 minutes (final hematocrit 40 ± 3, platelets 1.5 × 1011/mL ± 0.19 × 1011). Blood for the miniewell experiments was prepared in the same manner, but only 1-mL volumes were required. Test samples of 200 μL of reconstituted whole blood were drawn out of this 1-mL volume. In experiments where MoAbs or LMWD were used, these reagents were added to the reconstituted whole blood before incubation.

Addition of MoAb 10E5 or 6D1 resulted in a maximal final azide concentration of 0.035 mg/mL. Experiments performed with 0.035 mg/mL azide resulted in inhibition of secondary aggregation with ADP, but no significant difference in platelet deposition under either flow condition, confirming the observation of McPherson et al.18 Experiments performed in the presence of a nonspecific mouse MoAb IgG, F(ab'), 10 μg/mL also demonstrated no difference in platelet deposition from control experiments.

**Laminar flow experiments.** The perfusion apparatus is shown in Fig 1. Straight 30-cm long sections of medical grade polyethylene tubing (PE-100), ID 0.086 cm (Intramedic nonradiopaque polyethylene tubing [Clay Adams division of Becton Dickinson, Parsip-
performed at room temperature. Similar observations have been measured by the time the perfusion was complete. Comparison of counter with a 1-minute counting time.

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Fig 1. Schematic of microconduit perfusion system. Perfusion system consists of a 30-cm long straight segment of PE-100 tubing (ID 0.86 mm). Citrate anticoagulated human venous blood (hematocrit 40 ± 3) containing $^{111}$indium-labeled platelets (1.5 x 10⁷/cm² ± 0.186 x 10⁷) was perfused at a constant flow rate of 1.17 cm³/min (312 seconds⁻¹) against an 80-cm H₂O vented pressure head for 7 minutes, 45 seconds. By turning the proximal stopcock, a flush with H/T buffer (no added Ca²⁺, PGE₁, or Apyrase, pH 7.4) at the same flow rate was performed for an additional 5 minutes. Tubing was prepared for electron microscopic examination by filling with fixative after the buffer flush.

pany, NJ) were cannulated with 0.5-cm long blunt tipped needles made from intravenous needles (18 gauge, no. 5196; Becton Dickinson, Rutherford, NJ). A specially modified syringe pump (model no. 907, modified by increasing the gear ratio to 3:1; Harvard Apparatus, South Natick, MA) was used to perfuse the tubing under steady, laminar flow (Reynolds no. 7.6). Perfusions were conducted against a constant 80-cm H₂O pressure head administered by an H/T buffer-filled vented tube attached to the distal end of the tubing. A three-way stopcock between the syringe and the proximal tubing cannula allowed flow to be switched at the end of the perfusion from the blood syringe pump to a second syringe pump, which then perfused the tubing with 5.5 mL of H/T buffer, pH 7.4. The flush was performed at the same flow and shear rate as the preceding blood perfusion.

On completion of the buffer flush, the tubing was severed at the cannula and sectioned at the first centimeter and then at subsequent 4-cm intervals, discarding the distal 1-cm segment. $^{111}$Indium activity in these segments was then determined using a gamma counter with a 1-minute counting time.

Initial experiments were performed with blood perfusate warmed to 37°C, after which the perfusion was immediately conducted on the bench top at room temperature. A 7°C temperature drop was measured by the time the perfusion was complete. Comparison of experiments performed in a custom-made incubator in which the complete apparatus was enclosed at 37°C showed no statistically significant difference in platelet deposition versus experiments performed at room temperature. Similar observations have been made by Goodman.²⁹

Blood was sampled after reconstitution and also after perfusion for lactate dehydrogenase (LDH) activity (Technicon RA 500; Technicon Instruments Corp, Tarrytown, NY) to test for the possibility of RBC lysis. Plasma LDH activity was within normal limits for the assay after reconstitution, and showed no elevation in LDH activity after perfusion.

Disturbed flow experiments. Microwells (3/8-in high, 3/8-in ID) were constructed by cutting 3/16-in diameter discs out of the NHLBI 1° reference polyethylene with a punch. These discs were cut without removing the PE from its protective PE sheathing, so that each disc was essentially a sandwich of nontesting PE sheathing on top and bottom, with the 1° reference PE still protected in between these coverings. The bottom covering was removed and an identification number gently scratched on this surface to mark the disc and also assist in later identification of the top and bottom surfaces. This disc was pressed onto the surface of a 1/8-in diameter disc of dental wax, whose surface was warmed with a heat gun (Master-Mite; Master Appliance Corp, Racine, WI) to assure a good adhesion and seal to the bottom of the PE test disc. The top PE covering was then removed, exposing the 1° reference PE for the first time. The side walls of the well were added by placing the segment of Tygon tubing over the wax disc. The bottom of the well was sealed by passing the well through a pan of melted wax. The wells were filled with H/T buffer to check for leaks and placed in a clean enclosure until used.

For an individual experiment 10 to 20 wells were constructed. The H/T buffer was aspirated from each well and a 200-µL sample of test blood added. The wells were placed in a plexiglass chamber in which a 4 x 4 gauze sponge soaked in saline was added for humidity. The chamber lid was closed and the chamber placed on a specially constructed pedestal attached to the shaft of a rotational oscillating hematology shaker (Shaker-in-the-Round, Kraft Apparatus, Model S500, Mineola, NY; Fig 2). The shaker was set at its maximum setting of 5 and activated. The wells were agitated for 30 minutes. On removal from the shaker, each well was quickly emptied and washed using six 500-µL aliquots of H/T buffer directed against the side wall of the well. The PE discs were removed and placed in counting vials containing 200 µL of fixative.

Retained $^{111}$indium activity was determined using a gamma counter with a 1-minute counting time. The number of deposited platelets was determined using the platelet-specific activity for $^{111}$indium.

Scanning electron microscopy (SEM). Immediately after the flush with buffer the PE-100 tubing was perfused for an additional 30 seconds with modified Karnovsky’s fixative.²⁵ The tubing was sectioned as described previously and placed in vials containing 1 mL of the same fixative for overnight fixation. One-centimeter long tubing sections were then bisected lengthwise before alcohol dehydration. PE discs were fixed overnight. Samples were dehydrated in graded ETOH to absolute alcohol and critical point dried (Samdri-Regulated; Tousimis Research Corp, Rockville, MD).

The samples were mounted on aluminum stubs with double-sided tape and sputter coated with gold/palladium for 2 minutes (Hummer VI D/C Sputter Coating System; Technics West, Dublin, CA). They were examined using a JEOL series 300 scanning electron microscope.
electron microscope (Tokyo, Japan) at 20 kV, magnifications of 1,000 to 5,000×. Photographs of representative areas were obtained using Polaroid type 55 black and white film (Polaroid Corp, Cambridge, MA).

Transmission electron microscopy (TEM). Samples for TEM examination were selected from the same experiments and treatment groups as samples examined by SEM. These samples were postfixed in 1% osmium for 1 hour, then dehydrated through graded alcohols to propylene oxide. Samples were then embedded in epon. After 24 hours at 60°C, the section of PE tubing or disc was cracked out of the epon block, leaving the embedded platelets still on the epon block. Blocks representing control and 6D1-treated PE-100 tubing perfusions were sent to J.W. in a blinded fashion for sectioning and TEM examination. Blocks of control and 6D1-treated PE discs were also sectioned, stained with uranyl acetate/lead citrate, and examined at the University of Pittsburgh, using a Phillips 300 transmission electron microscope (Phillips Electronic Instruments Corp, Mahwah, NJ).

Statistical analysis. Differences in platelet deposition between laminar flow experiments performed with inhibitors added to the blood (ASA, LMWD, 6D1, and 10E5) and their respective control experiments (same donor blood without addition of any inhibitor) were investigated using univariate repeated measures analysis of variance. All statistical assumptions, including sphericity, were met. When appropriate, posthoc comparisons were made using Scheffe’s procedure.

Analysis of oscillatory flow experiments was performed using Dunnett’s test.28,29

RESULTS

Platelet deposition on control tubing. A total of 26 perfusions of tubing without addition of antibody or other inhibitors were performed during the course of this study (Fig 3). The pattern of platelet deposition along the length of the tube (depicted by the bars in Fig 3) is very consistent with that predicted for RBC-enhanced platelet transport across a diffusion boundary layer (solid line). A similar curvilinear pattern of platelet deposition has been described by other investigators using tubular perfusion systems.30,31 A similar curvilinear pattern of platelet deposition has been described by other investigators using tubular perfusion systems.30,31

Fig 3. Control perfusions. Perfusion of PE-100 tubing (ID 0.86 mm) with untreated whole blood containing 111In-labeled platelets (flow rate 1.17 mL/min, shear rate 312 seconds−1, perfusion time 7 minutes, 45 seconds). These experimental results (bar) agree with those calculated (solid line) for RBC-enhanced platelet transport across a diffusion boundary layer (solid line). A similar curvilinear pattern of platelet deposition has been described by other investigators using tubular perfusion systems.30,31

Effect of ASA on platelet deposition. 111In-labeled PRP was incubated with ASA at 7.3 mmol/L final concentration for 30 minutes before reconstitution with PRBC and subsequent perfusion. Comparison of individual segments perfused with ASA-treated blood versus their respective control tubing segments indicated that ASA treatment did not significantly inhibit platelet deposition in this model (P = .92, Fig 4A).

Effect of LMWD. 111In-labeled PRP was incubated with LMWD at a final concentration of 4 mg/mL. The choice of this concentration was based on the dosage schedule applied to humans undergoing microvascular surgical procedures. Although there appears to be a slight trend toward decreased deposition in the LMWD-treated perfusions, the interaction was not statistically significant (P = .0686, Fig 4B).

Effect of (MoAb) 6D1 (v GpIb) on platelet deposition. Ten micrograms per milliliter of 6D1 completely inhibited ristocetin-induced platelet agglutination in PRP, as previously reported. Platelet deposition in the presence of 6D1 was reduced to approximately 800,000 platelets/cm² (Fig 5A). Inhibition of deposition was highly significant (P ≤ .005) in comparison with control. SEM evaluation of these segments showed a patchy monolayer of spread
GLYCOPROTEINS Ia AND IIb/IIIa

Fig 5. Effect of MoAbs against platelet adhesion receptors: (A) 6D1 (v GP Ib, n = 7, P \leq .005), (B) 10E5 (v GPIIb/IIIa, n = 6, P \leq .0005).

A

B

EFFECT OF AB-6D1

EFFECT OF AB-10E5

PLATELETS/CM² (MILLIONS)

CM FROM ORIGIN

5 9 13 17 21 25 29

CONTROL AB-6D1

CONTROL AB-10E5

PLATELETS/CM² (MILLIONS)

CM FROM ORIGIN

5 9 13 17 21 25 29

platelets, with a striking absence of vertical platelet interactions (Fig 6B). The presence of a single layer of platelets was confirmed by examining cross-sections by TEM (Fig 6D). Thus, 6D1 acted to inhibit at least paradhesion, and possibly also mildly inhibited adhesion.

Experiments using 6D1 in the oscillatory flow system yielded similar results. Platelet deposition averaged 1.47 ± 0.65 \times 10^{0}, which represented a 56.5% decrease from control (P \leq .01). Again, SEM examination of the PE surface showed a monolayer of spread platelets (Fig 7B), which was confirmed on cross-section by TEM (Fig 7D).

Effect of MoAb 10E5 (v GP IIb/IIIa). ADP (20 \mu mol/L) stimulation of platelets in PRP containing 10 \mu g/mL 10E5 resulted in normal shape change but complete inhibition of platelet aggregation, as previously reported. As shown in Fig 5B, virtually no platelet deposition occurred from blood containing 10 \mu g/cm² 10E5. These results were highly significant (P \leq .0005) in comparison with control, and the absence of platelet deposition was corroborated by SEM.

Results using 10E5 in the oscillatory flow system again paralleled those found under tubular flow. Average platelet deposition was reduced to 52,778 platelets/cm², a 98.5% decrease from control (P \leq .01). These data suggest that 10E5 acted by near-complete blockade at the adhesion step.

DISCUSSION

The interaction of blood with artificial materials is a complex, incompletely understood event. Within seconds of blood contact, the artificial surface becomes coated with a layer of plasma proteins whose configuration is dictated by the properties of the surface itself. This protein layer then serves as a new surface to which platelets can adhere, become activated, and greatly accelerate the formation of a thrombus. Currently available anticoagulants and antiplatelet agents do not significantly alter these early events in thrombosis. This hampers the clinical use of artificial materials in the circulatory system, especially at the microvascular level where the vessel surface area to blood volume ratio is high. Because platelet membrane GPIb and GPIIb/IIIa are known to mediate platelet adhesion to subendothelium, we examined the role of these receptors in the process of platelet deposition on a model artificial surface under both laminar and oscillatory flow.

A critical aspect of the investigation of blood-surface interaction is the selection of a model system. Platelets adhere to artificial surfaces in vivo under laminar and disturbed flow conditions. To examine laminar flow, we chose to use a microconduit system, similar to that described by Poot et al. because its flow characteristics are clinically relevant. Artificial microconduits, due to their high surface area to volume ratio, are rapidly occluded by thrombi almost exclusively composed of platelets after in vivo implantation. Platelet deposition under disturbed flow was examined using a gently shaken microwell system. This model readily allows for the examination of any biomaterial. The wax bottom assures blood contact with only one side of the test disc but allows the disc to be easily retrieved at the conclusion of the test period.

The direct measurement of radiolabeled platelets attached to surfaces in each model system provides great sensitivity, allowing detection of the deposition of as few as 0.1% of the total number of tested platelets. When combined with SEM and TEM, both quantitative and morphologic information can be obtained regarding platelet-surface and platelet-platelet interactions.

In the laminar flow model, we evaluated two clinically available antiplatelet agents, ASA and LMWD. Neither of these agents at clinical concentrations resulted in a significant reduction of platelet deposition to PE-100 tubing in this model, although our laboratory has seen a modest reduction when testing these agents using PTFE (polytetrafluorethylene). These results are in accord with the results of other trials using ASA and dextran, where their use has not improved microraft patency sufficiently to allow clinical use. Our results with these agents are in sharp contrast to those we obtained after selective membrane GP blockade with MoAbs (Figs 3 through 5).

To briefly review their actions, studies of platelet adhesion to vessel subendothelium have identified the platelet membrane GPIb as the receptor involved in initial platelet
adhesion under high shear conditions. Platelet activation and spreading then occur, followed by attachment of additional platelets, both mechanisms presumably requiring GPIIb/IIIa. These mechanisms have also been forwarded to explain the process through which platelets deposit on artificial surfaces, although this remains controversial. If these mechanisms mediated platelet interaction with PE, one would predict that blockade of GPIb by 6D1 should completely prevent platelet adhesion. Blockade of GPIIb/IIIa, on the other hand, should not completely
inhibit platelet adhesion, but should significantly reduce deposition through inhibition of the paradhesion step, leaving a monolayer. Our results with receptor blockade do not support this model. We observed the opposite.

Inhibition of the platelet Ib/IIa complex by MoAb 10E5 essentially abolished platelet adhesion to both PE tubing (laminar flow) and PE discs (oscillatory flow), indicating that GPIb/IIa is the primary mediator of platelet adhesion to the protein layer on this artificial surface under each flow condition. Although GPIIb/IIIa has been shown to bind multiple plasma proteins (due to the presence of RGD regions in these proteins), it has the greatest affinity for fibrinogen. Our results do not identify the surface-bound ligand to which GPIIb/IIIa attaches, although pilot experiments performed with afibrinogemic plasma implicate fibrinogen as the attachment ligand. Nevertheless, our results also agree with reports of platelet deposition in systems in which vWF or fibronectin have been preadsorbed on the test surface.

As opposed to initial adhesion, we unexpectedly found that availability of GPIb was essential for platelets to adhere atop other platelets, with its specific requirement between the first and second layers (ie, the paradhesion layer). When MoAb 6D1 binds to GPIb, it prevents vWF from binding to this receptor. Under laminar flow, blockade of GPIb by 6D1 decreased platelet deposition by an average of 70% versus control, and converted the deposition profile to a flat line. Mass transfer calculations would predict a curvilinear decline in platelet deposition along the perfused tube. This flat profile with 6D1 treatment indicates that the process of platelet deposition proceeds to a similar degree and then stops at the same early stage throughout the length of the tube. This interpretation was confirmed by SEM and cross-section TEM evaluation that showed a patchy monolayer of spread platelets throughout the tube, with no evidence of vertical platelet layering.

Blockade of GPIb with antibody 6D1 under disturbed flow yielded similar results. Platelet deposition was decreased 56.5% versus control. Again, SEM and TEM examination confirmed the presence of a monolayer of platelets.

We interpret these results to indicate that inhibition of GPIb prevents attachment of a second (paradherent) layer of platelets. In pilot experiments performed using vWF-deficient plasma, paradhesion was found to also depend on the presence of vWF. The mechanism(s) responsible for the attachment of third and subsequent layers of platelets are not testable under the conditions of these experiments, so no conclusions can yet be drawn regarding them.

This model of platelet deposition wherein GPIIb/IIIa (and probably fibrinogen) is required for initial adhesion and GPIb (and probably vWF) for paradhesion has also been supported by other work in our laboratory in studies of a clinically relevant biomaterial (PTFE). We have also observed the same receptor requirements in studies of human platelet deposition to stainless steel (Sheppeck et al, unpublished data, 1989).

These results support earlier findings using glass bead columns. Through the use of one- and two-stage retention tests, fibrinogen was shown to be necessary for initial platelet retention. As expected, thrombasthenic platelets were not retained. vWF was shown to be required for second-stage retention, ie, after the initial layer(s) of platelets had interacted with the surface in some way. However, interpretation of these results has been limited by undefined flow conditions in the glass bead column, the possibility of platelet aggregation in the column, and the sensitivity of this system to blood handling and column preparation.

Our results obtained in two separate model systems that encompass both laminar and oscillatory flow parallel and expand on those obtained by others using glass bead columns. Using a combination of SEM and TEM, we have made an additional observation on the effect of GPIb blockade. Based on the above results, we have drawn the following conclusions: (1) The artificial surface represents a unique activation signal to the platelet, requiring a novel mechanism of GPIIb/IIIa and GPIb participation to support platelet adhesion and paradhesion. (2) Initial platelet adhesion requires GPIIb/IIIa. (3) Attachment of a second platelet to the initially adherent platelet (paradhesion) requires GPIb. (4) The presence or absence of laminar flow conditions does not alter the basic requirement of GPIIb/IIIa for initial adhesion and GPIb for paradhesion.

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