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Specific Synergy of Multiple Substrate–Receptor Interactions in Platelet Thrombus Formation under Flow

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Summary

We have used confocal videomicroscopy in real time to delineate the adhesive interactions supporting platelet thrombus formation on biologically relevant surfaces. Type I collagen fibrils exposed to flowing blood adsorb von Willebrand factor (vWF), to which platelets become initially tethered with continuous surface translocation mediated by the membrane glycoprotein Ibα. This step is essential at high wall shear rates to allow subsequent irreversible adhesion and thrombus growth mediated by the integrins $\alpha_2\beta_1$ and $\alpha_{IIb}\beta_3$. On subendothelial matrix, endogenous vWF and adsorbed plasma vWF synergistically initiate platelet recruitment, and $\alpha_2\beta_1$ remains key along with $\alpha_{IIb}\beta_3$ for normal thrombus development at all but low shear rates. Thus, hemodynamic forces and substrate characteristics define the platelet adhesion pathways leading to thrombogenesis.

Introduction

Platelets adhere and aggregate at sites of vascular injury contributing to the arrest of bleeding but also to the pathologic occlusion of diseased vessels. At shear rates equivalent to those generated by blood flow in normal arterioles (Tangelder et al., 1988) or in stenotic arteries (Back et al., 1977), adhesion requires von Willebrand factor (vWF) (Sakariassen et al., 1979) present as endogenous subendothelial matrix component (Sporn et al., 1989) or adsorbed onto tissues exposed to plasma (Pareti et al., 1987). The membrane glycoprotein (GP) Ibα can tether platelets to immobilized vWF A1 domain opposing elevated shear forces, but alone cannot support permanent attachment, owing to a high dissociation rate. This interaction, therefore, results in platelet translocation on reactive surfaces, but at greatly reduced velocity relative to free flow, a step instrumental in the formation of additional bonds that eventually mediate stable adhesion and aggregation (Savage et al., 1996). Collagen, particularly types I, III, and VI, present in different layers of the vessel wall is likely to play a key role in the latter process (Baumgartner et al., 1977) by binding to different platelet membrane glycoproteins. These include $\alpha_2\beta_1$ (GP Ia-IIa) (Nieuwenhuis et al., 1985), GP VI (Moroi et al., 1996), GP IV (CD-36) (Diaz-Ricart et al., 1993), and a fourth receptor recently cloned (Chiang et al., 1997). The functional integration of the distinct adhesion pathways involved in the initiation of platelet thrombus formation has not yet been defined. To address this issue, we have studied thrombogenesis on two relevant model substrates, fibrillar type I collagen, and subendothelial matrix, obtaining three-dimensional measurements of surface coverage in real time. Our results demonstrate that the initial GP Ibα–mediated function of vWF leads to irreversible platelet adhesion and aggregation in concert with specific activities of $\alpha_2\beta_1$ and $\alpha_{IIb}\beta_3$. These synergistic interactions support thrombus development responding to variations in substrate composition and hemodynamic conditions.

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Results

Role of Plasma vWF and Platelet GP Ibα in Concert with Integrins $\alpha_{IIb}\beta_3$ and $\alpha_2\beta_1$ in Thrombus Formation on Type I Collagen Fibrils

Platelets adhered firmly to type I collagen fibrils at wall shear rates between 100 and 1500 s^{-1}, forming thrombi whose number and growth rate depended on the volume of blood perfused per unit of time (Figure 1). Inhibition of $\alpha_{IIb}\beta_3$ permitted stable single platelet adhesion but abolished thrombus growth at all shear rates. In contrast, inhibition of plasma vWF A3 domain binding to collagen obliterated all initial platelet surface interactions at 1500 s^{-1}, but had no effect on thrombus formation at 500 s^{-1} or lower shear rates (Figure 1). Blockade of GP Ibα abolished platelet deposition at 1500 s^{-1} and caused a partial but significant ($p < 0.01$) reduction of thrombus volume at 500 s^{-1}, but had no effect ($p > 0.1$) at 100 s^{-1} (Figure 1). The specificity of inhibition by monoclonal antibodies was demonstrated with four distinct isotype-matched IgGs against different epitopes in vWF, GP Ibα, and the integrin β_3 subunit. All control antibodies allowed thrombus formation with mean volume within ±17% of that obtained with untreated blood.

The total volume of thrombi formed after a 3 min perfusion at 1500 s^{-1} was lower on surfaces prepared with collagen coating concentration of 0.01, as compared to 0.1 or 2.5 mg/ml, but thrombus height remained constant (Figure 2A). Thus, differences in total volume appear to result from changes in the number rather than size of individual thrombi, reflecting fibril density more than the degree of platelet activation. Blockade of $\alpha_2\beta_1$ with a monoclonal antibody caused greater than 50% inhibition of total thrombus volume, as well as decreased height, albeit only on surfaces with low collagen concentration (Figure 2A), suggesting an influence on both size and number of individually growing thrombi. No effect was seen with a control antibody against a different epitope in $\alpha_2\beta_1$. We then evaluated whether the receptor participates in platelet adhesion preceding thrombus growth by measuring surface coverage at 10 s intervals from the onset of flow at the wall shear rate of 1500 s^{-1}. Blocking $\alpha_2\beta_1$ was inconsequential when the collagen

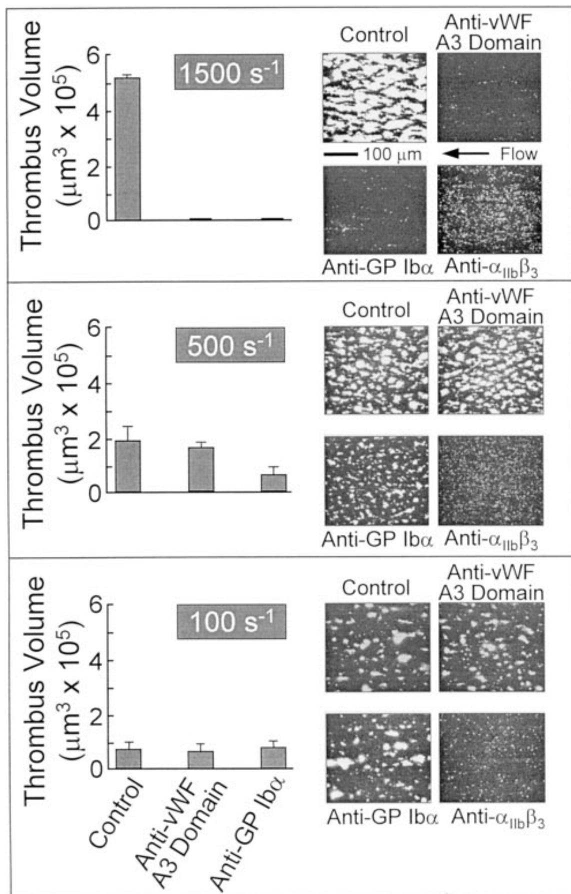

Figure 1. Plasma vWF, GP Ibα, and $\alpha_{IIb}\beta_3$ Participate in Platelet Thrombus Formation on Type I Collagen Fibrils

Blood containing 80 μm PPACK as anticoagulant and treated with the fluorescent dye mepacrine for platelet visualization was perfused in a parallel plate chamber at 37°C over glass coverslips coated with 2.5 mg/ml of fibrillar type I collagen. Flow was set to produce wall shear rates of 100, 500, or 1500 s^{-1}, and each experiment was recorded on tape using a videomicroscopy system. The figure shows single frame images of the surface, each corresponding to an area of 65,536 μm^2, after 2 min of perfusion at each indicated wall shear rate. Blood was either untreated or treated with monoclonal antibodies selectively inhibiting the binding of plasma vWF to collagen or the ligand-binding function of the platelet receptors GP Ibα and $\alpha_{IIb}\beta_3$. Note that inhibiting GP Ibα or plasma vWF binding to collagen essentially abolished all platelet–surface interactions at 1500 s^{-1} (only brief contact events occurred with no surface translocation) but had limited or no effects at the lower wall shear rates. These images are representative of the results obtained in six separate experiments with blood from different donors. After 2 min perfusion, the total volume of platelet thrombi present on a surface of 102,236 μm^2 was measured by confocal sectioning at 1.0 μm intervals, as described in Experimental Procedures. Volume measurements represent the mean ± SEM of four separate experiments with different blood donors. The size of single platelets can be appreciated in the panels showing the results obtained in the presence of the anti-$\alpha_{IIb}\beta_3$ antibody.

coating concentration was 2.5 mg/ml but caused a reduction in surface coverage before the appearance of aggregates at the coating concentration of 0.1 mg/ml (Figure 2A), indicating a role in initial platelet attachment. In contrast, blocking $\alpha_2\beta_1$ had modest effects on total thrombus volume when the wall shear rate was 500 s^{-1}, with significant reduction ($p < 0.05$) seen only at the coating concentration of 0.01 mg/ml (Figure 2B); inhibition was even less at 100 s^{-1} (Figure 2C). The function of $\alpha_2\beta_1$, therefore, acquires relevance for adhesion and thrombus formation when platelets are subjected to increasing hemodynamic drag on surfaces with relatively sparse collagen fibrils.

Integrins $\alpha_2\beta_1$ and $\alpha_{IIb}\beta_3$ Mediate Platelet Adhesion to Type I Collagen Fibrils with Bound vWF

Stable platelet attachment to a reactive surface is the essential first step in thrombus development. The mechanism responsible for this process was analyzed at the wall shear rate of 1500 s^{-1} in order to model the hemodynamic challenge to adhesion that may exist in arterioles (Tangelder et al., 1988) where platelets are essential for hemostasis. Chelation of divalent cations in blood by EDTA allowed tethering and continuous translocation of platelets but prevented irreversible adhesion to collagen type I fibrils, indicating the involvement of integrin–ligand interactions in the latter event (Figure 3A). Platelet displacement exhibited similar characteristics on collagen as on glass coated with purified vWF or collagen saturated with vWF before perfusion (Figure 3A), suggesting that adsorption of plasma vWF by type I collagen fibrils, like GP Ibα–mediated platelet tethering, is independent of divalent cations. As shown in Figure 1, translocation on collagen was prevented by blocking GP Ibα (Savage et al., 1996) as well as the plasma vWF A3 domain, confirming the indispensable role of immobilized vWF in establishing platelet surface contacts.

The nature of the bonds involved in the transition from initial tethering to irreversible platelet adhesion was probed using function-blocking monoclonal antibodies against $\alpha_{IIb}\beta_3$ and $\alpha_2\beta_1$. Aggregate formation and thrombus growth were abolished by inhibiting $\alpha_{IIb}\beta_3$, thus allowing direct evaluation of individual platelet interactions with the substrate. Under these conditions, the collagen surface became rapidly covered with firmly adherent platelets, and approximately 60% of first contact events resulted in prolonged adhesion with no translocation from the initial point of tethering (Figure 3B). However, when $\alpha_{IIb}\beta_3$ and $\alpha_2\beta_1$ were blocked concurrently, approximately 90% of the platelets becoming tethered to the surface moved to a position different from the point of first contact within 4 s (Figure 3B). This behavior is akin to that previously reported for platelets interacting with vWF immobilized onto glass (Savage et al., 1996). Similar results were obtained when platelet activation was inhibited with prostaglandin E_1 and $\alpha_2\beta_1$ function was simultaneously blocked (data not shown). The average velocity of platelets translocating on the collagen–vWF surface under these conditions was 5.5 μm/s (Figure 3B), similar to that measured at the same shear rate of 1500 s^{-1} on purified vWF bound to glass (Savage et al., 1996).

The time course required to establish bonds supporting stable platelet adhesion was evaluated from the onset of flow. After 8 s of perfusion, greater than 95% of platelets interacting with the surface were displaced

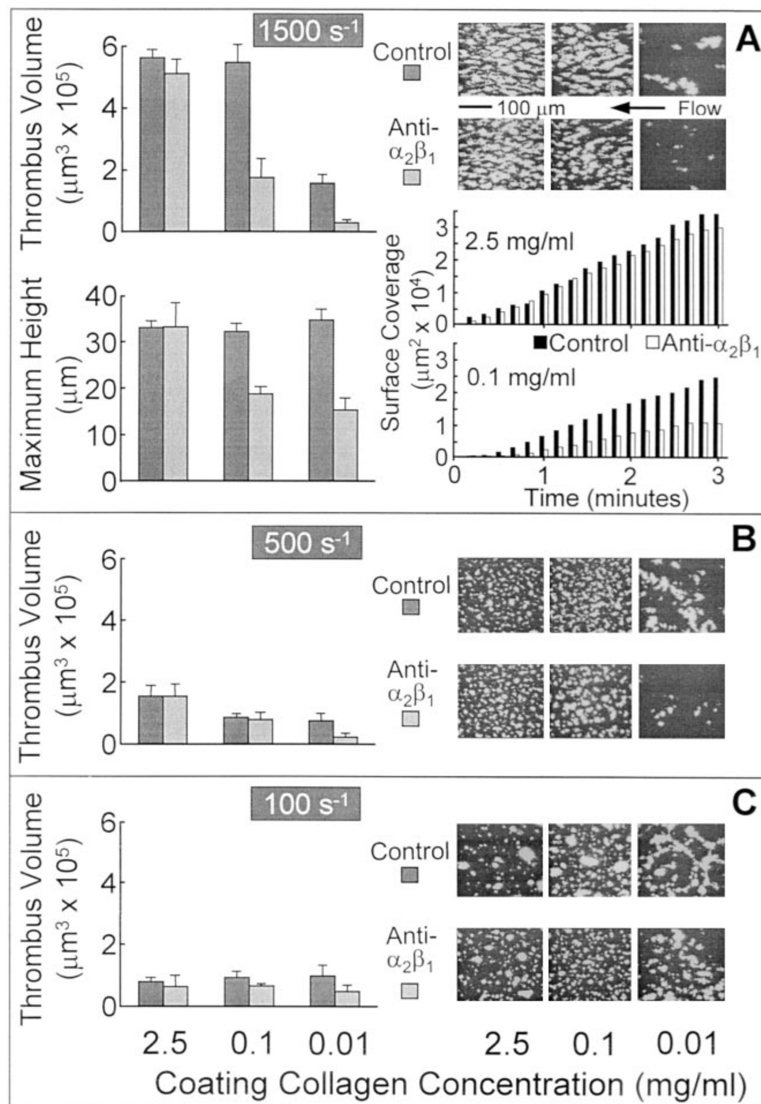

Figure 2. Role of $\alpha_2\beta_1$ in Platelet Adhesion and Thrombus Formation on Surfaces Coated with Different Densities of Type I Collagen Fibrils

The collagen concentration used for surface coating was 2.5, 0.1, or 0.01 mg/ml, as indicated. Blood, either untreated (dark bars) or after selective inhibition of $\alpha_2\beta_1$ with a monoclonal antibody (light bars), was perfused over the different collagen-coated surfaces in a parallel plate chamber as described in the legend to Figure 1. Single frame images, each corresponding to an area of 65,536 μm^2 and representative of different tested conditions after 2 min perfusion, are shown on the right. After 2 min perfusion, the total volume occupied by platelet thrombi in an area of 102,236 μm^2 was measured by confocal sectioning at 1.0 μm intervals (see Experimental Procedures).

(A) Experiments performed at wall shear rate of 1500 s^{-1}. Maximum thrombus height was estimated from series of confocal sections as the distance between the collagen surface and the tip of the highest thrombus in the area. The two bar graphs on the right show the effect of blocking $\alpha_2\beta_1$ function on the time course of platelet adhesion to surfaces coated with the two indicated concentrations of fibrillar type I collagen (open bars, $\alpha_2\beta_1$ blocked; filled bars, untreated blood). Images were captured from videotape at 10 s intervals after the onset of blood flow, and the total surface coverage by platelets in an area of 65,536 μm^2 was measured as described in Experimental Procedures.

(B) Experiments performed at wall shear rate of 500 s^{-1}.

(C) Experiments performed at wall shear rate of 100 s^{-1}.

from the point of initial contact within 5 s when $\alpha_2\beta_1$ was blocked in addition to $\alpha_{IIb}\beta_3$, as compared to approximately 60% when only $\alpha_{IIb}\beta_3$ function was inhibited. After 1 or 2 min of perfusion, approximately 90% of platelets were displaced from the point of initial tethering within 10 s when $\alpha_2\beta_1$ and $\alpha_{IIb}\beta_3$ were blocked concurrently, as opposed to less than 40% when only $\alpha_{IIb}\beta_3$ function was inhibited (Figure 4). Differences in the stability of adhesion were reflected in the total number of platelets on the surface, peaking after about 1 min, and almost doubling when $\alpha_{IIb}\beta_3$ was blocked selectively as compared to when $\alpha_2\beta_1$ and $\alpha_{IIb}\beta_3$ were blocked concurrently (Figure 4). The platelet displacement profiles and total number of platelets on the surface were essentially identical between 1 and 2 min, indicating the lack of changes in either platelet or surface reactivity with time. When only $\alpha_{IIb}\beta_3$ was inhibited, approximately 70% of platelets were firmly attached after 2 min; the remaining 30% were transiently interacting but continuously exchanged every 5 s, as reflected by the constant number of platelets on the surface (Figure 5). Under these conditions, therefore, the majority of platelets were irreversibly adherent through bonds rapidly established by $\alpha_2\beta_1$ following the initial tethering of GP Ibα to collagen-bound vWF. The stable number of this population may indicate saturation of all the collagen sites capable of interacting with $\alpha_2\beta_1$. Platelets that were translocating when $\alpha_{IIb}\beta_3$ function was inhibited represent the ones that may become irreversibly attached when the latter receptor can interact with the Arg-Gly-Asp sequence in collagen-bound vWF, a previously proven pathway of adhesion to purified vWF immobilized onto glass (Savage et al., 1996). Alternatively, or in addition, other $\alpha_{IIb}\beta_3$ ligands such as fibrinogen, fibronectin, or vitronectin (Plow et al., 1985; Thiagarajan and Kelly, 1988) may be adsorbed from plasma or released locally by activated platelets, thus contributing to stabilizing adhesion.

Platelet Adhesion and Thrombus Formation on Subendothelial Matrix Require Endogenous as Well as Plasma vWF, $\alpha_2\beta_1$, and $\alpha_{IIb}\beta_3$

The mechanism supporting thrombus growth on type I collagen fibrils was confirmed by studies with subendothelial extracellular matrix. The function of GP Ibα was

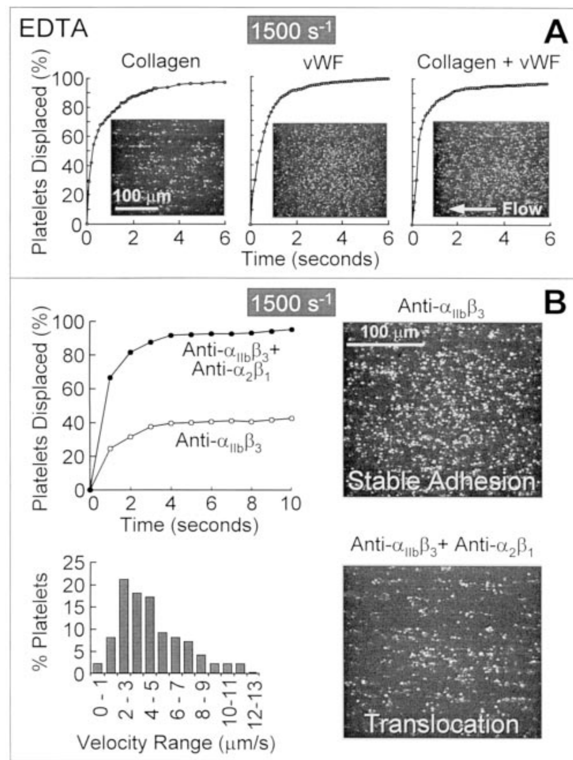

Figure 3. Integrins $\alpha_2\beta_1$ and $\alpha_{IIb}\beta_3$ Contribute to Stabilize the Adhesion of Platelets Initially Tethered to Collagen-Bound vWF

(A) Blood containing 5 mM EDTA to inhibit the function of platelet integrins was perfused at the wall shear rate of 1500 s^{-1} over a surface coated with either fibrillar type I collagen (left panel; coating concentration of 2.5 mg/ml), purified vWF (middle panel; coating concentration of 0.1 mg/ml), or collagen preincubated with purified vWF before the onset of blood flow (right panel). After 2 min perfusion, consecutive images were captured from videotapes and analyzed for platelet movement (for definition, see Experimental Procedures). A single frame image corresponding to an area of 65,536 μm^2 is shown for each condition tested. The percentage of platelets displaced from their initial position was calculated as a function of time relative to the total number of platelets attached to the surface in the first image analyzed. Note that greater than 90% of all platelets moved on the surface within 3 s of observation, indicative of predominantly transient attachment with surface translocation mediated by GP Ibα interaction with surface-bound vWF. In spite of the movement, most of the platelets remained persistently tethered to the surface.

(B) Perfusion over collagen-coated surfaces of blood containing 80 μm PPACK as anticoagulant and the function-blocking monoclonal antibodies of indicated specificity. The stability of platelet adhesion was evaluated after 2 min perfusion. Note that approximately 90% of all platelets (indicated as platelets displaced) moved on the surface within 3 s of observation when $\alpha_{IIb}\beta_3$ and $\alpha_2\beta_1$ were inhibited concurrently, as opposed to greater than 60% of the platelets remaining stationary (40% displaced) after selective inhibition of $\alpha_{IIb}\beta_3$. The frequency distribution of the average velocity of individual platelets with blocked $\alpha_{IIb}\beta_3$ and $\alpha_2\beta_1$ moving on the surface is also shown. The two single frame images on the right, each representing an area of 65,536 μm^2, depict surface coverage after 2 min perfusion of platelets with selective $\alpha_{IIb}\beta_3$ inhibition (top) or combined $\alpha_{IIb}\beta_3$ and $\alpha_2\beta_1$ inhibition.

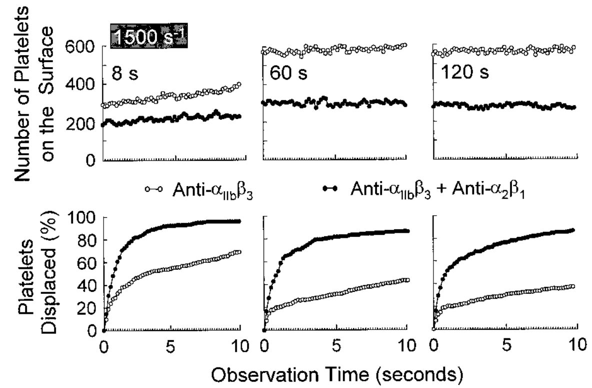

Figure 4. Time Course of the Transition from Initial Platelet Tethering with Translocation to Irreversible Adhesion Mediated by $\alpha_2\beta_1$ and $\alpha_{IIb}\beta_3$ on a Collagen-Coated Surface

Blood was perfused at a wall shear rate of 1500 s^{-1} over surfaces prepared with a collagen coating concentration of 0.1 mg/ml. Platelet $\alpha_{IIb}\beta_3$ function was blocked either selectively (open circles) or concurrently with inhibition of $\alpha_2\beta_1$ (filled circles) using specific monoclonal antibodies. Consecutive images were captured from videotapes and analyzed for the total number of platelets on the surface, as well as for platelet movement (for definition, see Experimental Procedures) at 8 s (left panels), 60 s (middle panels), and 120 s (right panels) after the onset of blood flow. Note that blocking both receptors simultaneously resulted in the displacement of greater than 80% of platelets within 10 s from the initial tethering at all time points. In contrast, up to 60% of the total platelets on the surface remained firmly attached in the same position after 2 min perfusion when only $\alpha_{IIb}\beta_3$ function was blocked.

essential for initiating platelet recruitment on this substrate when blood was perfused at the wall shear rate of 1500 s^{-1} (Figure 6), and stable attachment, but not continuous translocation, of surface-tethered platelets was dependent on the availability of divalent cations. Selective inhibition of $\alpha_{IIb}\beta_3$ allowed firm adhesion of approximately 50% of the platelets initially tethered to the matrix (Figure 6), a value similar to that seen on fibrillar type I collagen (Figure 3B). In contrast, approximately 30% of initially tethered platelets still became firmly adherent for at least 10 s when $\alpha_{IIb}\beta_3$ and $\alpha_2\beta_1$ were inhibited concurrently (Figure 6), thus more than the 10% seen on type I collagen fibrils (Figure 3B). This value was reduced by blocking the integrin subunit α_5, indicating a possible contribution of matrix or plasma fibronectins to the stabilization of platelet interaction with the subendothelium (Figure 6).

The volume of thrombi formed on the matrix at shear rate of 1500 s^{-1} was similar to that on type I collagen fibrils (compare Figure 7 with Figures 1 and 2) and was reduced 80% or more by blocking selectively either $\alpha_2\beta_1$ on platelets or the A1 domain of subendothelial vWF or the A3 domain of vWF in perfused blood (Figure 7). Thrombi formed on the matrix at 500 s^{-1} were larger than at 1500 s^{-1} and twice as large as on collagen, whereas at 100 s^{-1} they were about the same size on both substrates and smaller than at higher shear rates (compare Figure 7 with Figures 1 and 2). The anti-$\alpha_2\beta_1$ and anti-vWF antibodies decreased the total volume of thrombi deposited on the matrix by more than 60% at 500 s^{-1} but had no effect at 100 s^{-1} (Figure 7). As anticipated, blocking $\alpha_{IIb}\beta_3$ abolished thrombus formation at all shear rates (not shown).

Discussion

We have used real-time analysis of platelet–substrate interactions and volumetric measurements by confocal

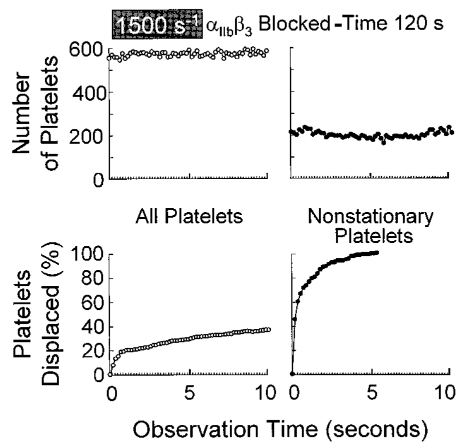

Figure 5. Stability of Platelet Attachment to Collagen after Blocking Platelet $\alpha_{IIb}\beta_3$ Function

Blood with added anti-$\alpha_{IIb}\beta_3$ monoclonal antibody was perfused at a wall shear rate of 1500 s^{-1} over surfaces prepared with a collagen coating concentration of 0.1 mg/ml. Consecutive images were captured from videotape beginning 120 s after the onset of flow and analyzed for the total number of platelets on the surface and for platelet movement (for definition, see Experimental Procedures). Left panels (open circles), total number of platelets (upper) and displaced platelets (lower) considering all those on the surface. Right panels (filled circles), total number of platelets not firmly attached (upper) and time course of their displacement (lower) after excluding stationary platelets by image processing.

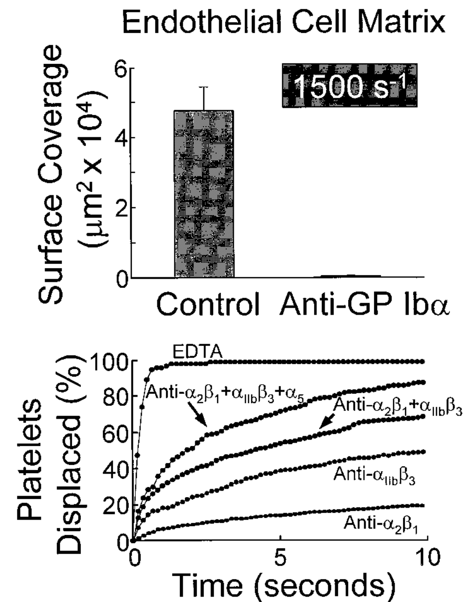

Figure 6. Mechanism of Initial Platelet Tethering and Stable Adhesion to Extracellular Matrix Deposited by Endothelial Cells

Blood for these experiments, prepared as described in the legend to Figure 1, was perfused over extracellular matrix (see Experimental Procedures) at the wall shear rate of 1500 s^{-1}.

(Top) Surface coverage over an area of 65,536 μm^2 observed during perfusion of blood without (Control) or with addition of a function-blocking anti-GP Ibα monoclonal antibody. Measurements represent the mean $\pm$ SEM of six determinations on different areas. Surface coverage was inhibited greater than 95% by blocking GP Ibα.

(Bottom) Motion analysis of platelets interacting with the surface during perfusion of blood containing EDTA (see Figure 2) or the function-blocking monoclonal antibodies of indicated specificity. Consecutive images were captured from videotapes and analyzed for platelet movement (for definition, see Experimental Procedures).

videomicroscopy to illustrate a paradigmatic mechanism of thrombogenesis. The same sequence of events appears to take place when blood flowing with arteriolar wall shear rate is exposed to type I collagen fibrils or subendothelial matrix. In either case, initial platelet tethering mediated by immobilized vWF and GP Ibα leads to integrin-dependent irreversible adhesion and thrombus growth (see Table 1). The importance of specific interactions in these processes may vary, depending on hemodynamic parameters. For example, vWF and integrin $\alpha_2\beta_1$ are not required at lower shear rates, but their key function at the higher shear rates relevant to hemostasis and thrombosis has now been identified.

In agreement with a previously proposed hypothesis, it is apparent that substrate-bound vWF, like purified vWF immobilized onto glass (Savage et al., 1996), is uniquely efficient in tethering rapidly flowing platelets to an exposed thrombogenic surface. At shear rates within the range of arteriolar flow, such as 1500 s^{-1} (Tangelder et al., 1988), binding of plasma vWF to collagen or extracellular matrix and subsequent GP Ibα binding to immobilized vWF is required for initial platelet–surface contact. As previously discussed, the latter process depends on bonds that form with fast forward rate but support slow translocation rather than arrest at the site of first contact because of an inherently short half-life (Savage et al., 1996). Nevertheless, coupling of GP Ibα to the vWF A1 domain substantially reduces the velocity of tethered platelets compared to those flowing freely in the boundary layer, enabling interactions that have relatively slow forward rates but are required for

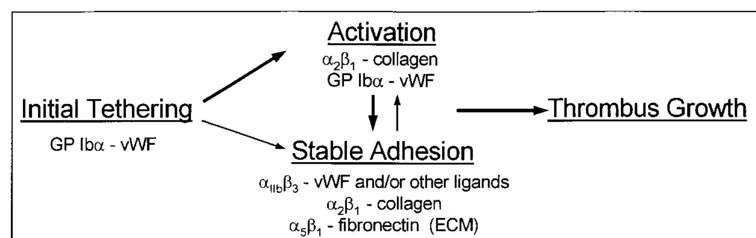

Table 1. Interactions Proposed to Participate in Mediating Platelet Adhesion and Inducing Aggregation on Subendothelial Matrix and Collagen Fibrils at High Shear Rate

The events depicted here in temporal sequence from left to right, initiating with platelet tethering to a reactive surface, are proposed to be involved in thrombogenesis at arteriolar wall shear rates (1500 s^{-1}), but some may not be relevant at lower shear rates. The scheme considers only adhesive interactions whose role has been defined in these studies and does not exclude the relevance of other ligand–receptor pairs for platelet thrombus formation, nor the role of other agonists in platelet activation. The two arrows of different weight connecting activation and stable adhesion express the hypothesis that activation may in general precede stable adhesion but the latter may contribute to enhance activation. ECM denotes an interaction that may occur on endothelial cell matrix but is not proven to occur on collagen fibrils.

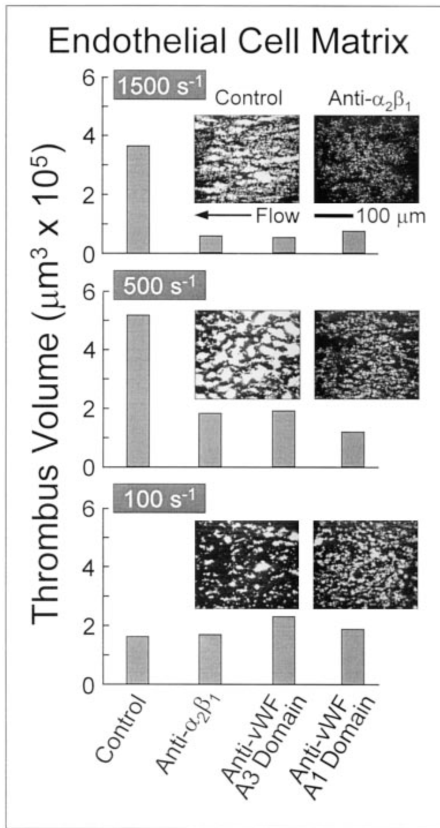

Figure 7. Mechanism of Platelet Thrombus Formation on Extracellular Matrix Deposited by Endothelial Cells

The blood used for these perfusion studies contained 80 μm PPACK as anticoagulant, without (Control) or with addition of the function-blocking monoclonal antibodies of indicated specificity (anti-$\alpha_2\beta_1$ or anti-vWF A3 domain); the experiment labeled "anti-vWF A1 Domain" was performed by perfusing control blood over subendothelial matrix previously saturated with monovalent Fab fragment of the monoclonal antibody NMC-4 (see Experimental Procedures). The total volume of platelet thrombi present on a surface of 102,236 μm^2 was measured by confocal sectioning at 1.0 μm intervals, as described in Experimental Procedures, during perfusion at the three indicated wall shear rates. The single frame images shown on the right, each corresponding to an area of 65,536 μm^2, exemplify the inhibition of platelet thrombus development on subendothelial matrix by the anti-$\alpha_2\beta_1$ monoclonal antibody.

irreversible adhesion (Savage et al., 1996). Of note, GP Ibα–mediated tethering resulted in similar velocity of translocation whether vWF was immobilized onto glass, adsorbed onto collagen under static conditions, or bound from plasma under flow conditions. This observation indicates that the affinity of vWF for GP Ibα is regulated by intrinsic properties of the immobilized A1 domain and not by conformational changes specifically induced upon binding to collagen or other matrix components, a commonly stated hypothesis never proven experimentally.

It was surprising to find that both the A3 domain of plasma vWF and the A1 domain of endogenous subendothelial vWF were needed concurrently to initiate platelet tethering and thrombus formation on matrix exposed to high shear rate (Figure 7). Possibly both the amount of endogenous vWF and the availability of vWF-binding sites are limiting, such that additional binding of plasma vWF can complement the function of the endogenous protein, but neither is sufficient independently. Alternatively, the vWF A1 and A3 domains may have yet undefined properties, in addition to promoting initial platelet tethering, that render them indispensable for thrombus development at higher shear rates. Such properties may be influenced by the substrate onto which vWF becomes immobilized, which in the case of subendothelial matrix may be collagen type VI (Rand et al., 1991) as well as a noncollagenous component (Wagner et al., 1984). Of note in this regard, thrombus formation has been shown to vary under flow conditions, depending on whether vWF is bound to collagen type I (Alevriadou et al., 1993) or VI (Ross et al., 1995).

At shear rates relevant for hemostasis in arterioles (Tangelder et al., 1988), as well as thrombosis in atherosclerotic arteries (Back et al., 1977), $\alpha_2\beta_1$ and $\alpha_{IIb}\beta_3$ are prominent in mediating stable platelet adhesion and aggregation. The role of $\alpha_{IIb}\beta_3$ as a key receptor for adhesive proteins, notably fibrinogen and vWF (Ikeda et al., 1991), that are involved in linking platelets to one another is well established (Phillips et al., 1991). Our findings now define a unique function for $\alpha_2\beta_1$ among collagen receptors, expressed by its ability to act in concert with GP Ibα–vWF and $\alpha_{IIb}\beta_3$ to promote stable platelet adhesion as well as activation. It is apparent from the experiments on subendothelial matrix that both attributes are important, since selective $\alpha_2\beta_1$ blockade caused a relatively modest inhibition of surface coverage but a pronounced decrease in thrombus volume (compare Figures 6 and 7) (Keely and Parise, 1996). Such an effect on the formation of large aggregates cannot depend on direct participation in interplatelet bonding. Rather, it may reflect the role of $\alpha_2\beta_1$ in platelet activation following adhesion, a function possibly crucial when thrombin activity is limited, as in our experimental model. Indeed, these findings provide a mechanistic explanation for the occurrence of bleeding in patients with congenital defects of $\alpha_2\beta_1$ (Nieuwenhuis et al., 1985).

Subendothelial matrix and collagen fibrils synthesized by other cells in the vessel wall are both likely to be involved in thrombogenesis, perhaps with greater relevance of the former in vascular disease and of the latter upon blood contact with extravascular tissues after trauma. Compared to isolated collagen fibrils, subendothelial matrix contains other components that may participate in establishing adhesive interactions, for example, fibronectin, which can bind to $\alpha_5\beta_1$ on platelets (Ginsberg et al., 1993) (Figure 6). With respect to collagen, however, $\alpha_2\beta_1$ and $\alpha_{IIb}\beta_3$ seem to provide essentially all the bonds required to mediate platelet adhesion against elevated shear stress, albeit with the necessary contribution of adsorbed plasma proteins, among which vWF is notable. The surface density of collagen may modulate these events to the extent that, when it is sufficiently high, $\alpha_{IIb}\beta_3$ alone may stabilize the attachment of enough platelets to initiate rapid thrombus formation without involving $\alpha_2\beta_1$. This function of $\alpha_{IIb}\beta_3$ has been documented to occur with purified vWF immobilized onto nonbiological surfaces when the receptor promotes platelet arrest by interacting with the Arg-Gly-Asp

sequence in the vWF C1 domain, following activation induced by initial GP Ibα tethering to the A1 domain (Savage and Ruggeri, 1991; Savage et al., 1996). A similar process may occur when plasma vWF is immobilized onto collagen fibrils. Consequently, the need for $\alpha_2\beta_1$ may be bypassed at higher collagen density because increased amounts of bound vWF may enhance signal transduction through GP Ibα, potentiating the synergism with other receptors and inducing $\alpha_{IIb}\beta_3$ function. In this regard, platelets may be activated following GP Ibα occupancy by vWF (De Marco et al., 1985; Du et al., 1994), as well as collagen interaction with GP VI (Ichinohe et al., 1997) and the 65 kDa receptor (Chiang et al., 1997), in addition to $\alpha_2\beta_1$ (Polanowska-Grabowska et al., 1993; Keely and Parise, 1996). Moreover, other adhesive proteins present in blood and known to be $\alpha_{IIb}\beta_3$ ligands, for example, fibronectin (Plow et al., 1984), may interact with collagen and provide the substrate for irreversible platelet adhesion after initial tethering to the vWF A1 domain. A composite of all these events may also take place on subendothelial matrix where, however, the function of $\alpha_2\beta_1$ may remain indispensable, perhaps reflecting a relatively limiting concentration of exposed collagen.

Our studies indicate that the adhesive potential of platelets results from the sum of distinct pathways supported by coordinated receptor–ligand interactions specifically adapted to respond to different environmental conditions. This may be a relevant paradigm to interpret the multiplicity of adhesion mechanisms in other cells. Such variables as the composition of a reactive surface and hemodynamic forces may directly modulate the process of adhesion and ensuing activation, affecting the rate of thrombus growth and determining the outcome of the hemostatic response or the occurrence of pathological thrombosis. In all these instances, functional integration of the platelet receptors GP Ibα, $\alpha_2\beta_1$, and $\alpha_{IIb}\beta_3$ with their respective ligands now appears to have direct physiopathological relevance.

Experimental Procedures

Preparation of Collagen-Coated Coverslips

Suspensions of acid-insoluble fibrillar type I collagen from bovine Achilles tendon (Sigma) in 0.5 M acetic acid (pH 2.8) were prepared as previously described (Folie et al., 1988). Two hundred microliters was applied evenly over horizontal glass coverslips (Corning, Inc.; 24 mm × 50 mm) that were then placed in a humid environment at room temperature (22°C–25°C) for 60 min. After removing excess collagen by four sequential rinses with phosphate-buffered saline (PBS) (pH 7.4), a coverslip was assembled in the flow chamber. Saturating the surface with bovine serum albumin (0.1 mg/ml) did not affect initial platelet adhesion or subsequent thrombus formation, and uncoated coverslips did not support platelet adhesion (unpublished observations).

Preparation of Coverslips Coated with Extracellular Matrix Deposited by Endothelial Cells

Human umbilical vein endothelial cells were purchased and cultured in growth medium (Clonetics Corp., San Diego, CA). After reaching confluence in T-75 tissue culture flasks, endothelial cells always before passage three were seeded and cultured on sterile 25 mm glass slides (Corning Glass, Vineland, NJ). The cells were grown on the slides for 1–3 days to reach confluence and then maintained for an additional 1–2 days in a humidified 5% CO_2 incubator at 37°C. The glass slides covered by endothelial cell monolayers were assembled as the base of rectangular parallel plate perfusion chambers used to expose cells to laminar flow, according to a previously published method (Frangos et al., 1985) with minor modifications. A shear stress of 14 dynes/cm^2 was applied for 4–6 hr, followed by 58 dynes/cm^2 for an additional 15 hr. The glass slides were then removed from the chamber, and the cells were detached by incubation for 3 min at 37°C with 20 mM NH_4OH and 0.5% Triton X-100 in phosphate-buffered saline. The matrix remaining attached to the glass slides was washed repeatedly with phosphate-buffered saline (Gospodarowicz et al., 1981) and used immediately in blood perfusion studies.

Functional Inhibition of Platelet Receptors and vWF Domains

All the murine monoclonal antibodies used in these experiments were obtained and characterized as previously described. They were purified using protein A (Sigma) chromatography according to published procedures (Ey et al., 1978). LJ-Ib1 (IgG_1) reacts with the amino-terminal 45 kDa domain of GP Ibα containing the vWF binding site (Handa et al., 1986; Vicente et al., 1988, 1990) and inhibits completely this interaction under all experimental conditions tested (Alevriadou et al., 1993). The binding of this antibody to platelets is specifically abolished by the Ala156→Val mutation described in a patient with Bernard-Soulier syndrome (Ware et al., 1993). LJ-CP8 (IgG_1) reacts with the integrin $\alpha_{IIb}\beta_3$ (GP IIb-IIIa complex) and blocks the activation-dependent binding of soluble ligands to this receptor (Niiya et al., 1987; Weiss et al., 1989) as well as platelet aggregation and thrombus formation under all experimental conditions tested (Weiss et al., 1989). This antibody also inhibits platelet adhesion to immobilized fibrinogen (Savage et al., 1992, 1996) and irreversible single platelet attachment to immobilized vWF (Savage et al., 1996). NMC-4 reacts with the A1 domain of vWF (Fujimura et al., 1991) and blocks all its functions, in particular vWF interaction with GP Ibα (Mohri et al., 1989). The epitope recognized by this antibody has been defined at the atomic level (Celikel et al., 1998). MR-5 (IgG_1) reacts with a distinct sequence (residues 948–998) in the A3 domain of vWF (Roth et al., 1986) and blocks the interaction between vWF and collagen under all experimental conditions tested (Roth et al., 1986; Pareti et al., 1987). Antibody R2–7E4 (IgG_1) was obtained from the same fusion as the previously reported antibody R2–8C8 (Diaz-Gonzalez et al., 1996); as the latter, it exhibits recognition specificity for the α2 subunit of integrin $\alpha_2\beta_1$ and inhibits the adhesion of Chinese Hamster ovary cells transfected with $\alpha_2\beta_1$ to type I collagen in the absence of shear. Antibody 12F1 (IgG_{2a}) binds to platelet $\alpha_2\beta_1$ but does not inhibit interactions with collagen (Pischel et al., 1988). Antibody MAb16 reacts with the α_5 subunit of integrin $\alpha_5\beta_1$ and inhibits interaction with fibronectin, as previously reported (Akiyama et al., 1989; Yamada et al., 1990). The murine monoclonal antibodies used as additional isotype-matched controls have been previously characterized: LJ-Ibα1 is an anti-GP Ibα IgG_1 (Ware et al., 1993); LJ-Ib10, an anti-GP Ibα IgG_{2a} (Handa et al., 1986); LJ-2.2.9, an anti-vWF IgG_1 (Schneppenheim et al., 1996); and AV-10, an anti-β3 IgG_1 (Felding-Habermann et al., 1996). Monovalent Fab fragments of antibodies NMC-4 and MR-5 were prepared according to a published method (Celikel et al., 1997) and used to block the A1 and/or A3 domain of immobilized vWF; blockade of the same domains of soluble vWF could be achieved with identical results using intact IgG. All antibodies, except NMC-4, were used at a final concentration of 100 μg/ml in blood, shown to produce their maximal specific effect. A solution of NMC-4 Fab at the concentration of 100 μg/ml was evenly distributed over the extracellular matrix for 20 min at room temperature. The substrate was then washed extensively before use in the blood flow experiments.

Perfusion Chamber and Epifluorescence Videomicroscopy

Platelet interaction with immobilized collagen under various flow conditions was studied using a modification of a Hele-Shaw flow chamber described elsewhere (Usami et al., 1993; Savage et al., 1996). Collagen-coated coverslips formed the lower surface of the chamber, and a flow path height of 254 μm was determined by a silicone rubber gasket. The flow chamber was assembled and filled with PBS (pH 7.4). A syringe pump (Harvard Apparatus Inc.) was used to aspirate blood through the flow chamber. Flow rates of

1.94, 0.65, and 0.13 ml/min produced initial wall shear rates of 1500 s^{-1}, 500 s^{-1}, and 100 s^{-1}, respectively, at the inlet of the flow chamber where all measurements of platelet adhesion and thrombus formation were made. Blood was collected from the antecubital vein of healthy adult donors with normal cell count profiles through a 19-gauge needle into syringes containing as anticoagulant the thrombin inhibitor D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone dihydrochloride (PPACK; 80 μM, final concentration). None of the donors had taken aspirin or other drugs known to affect platelet function in the preceding 10 days. Platelets were labeled in whole blood by direct incubation with the fluorescent dye mepacrine (quinacrine dihydrochloride; 10 μM, final concentration). Although this dye also labels leukocytes, these cells could be readily distinguished from platelets by their relatively large size, nuclear morphology, and sparsity; moreover, permanent leukocyte attachment to collagen was negligible at wall shear rates above 500 s^{-1}. At a wall shear rate of 100 s^{-1}, the contribution of adherent leukocytes to the total thrombus volume was typically less than 10% (unpublished observations). Red cells were not visualized, due to fluorescence quenching by hemoglobin. Mepacrine concentrates in the dense granules of platelets and has no effect on normal platelet function at the concentration used (Dise et al., 1982). Platelet secretion after adhesion does not prevent their visualization. Furthermore, previous studies have shown that mepacrine does not interfere with platelet adhesion (Savage et al., 1996). The flow chamber, mounted on an epifluorescence microscope (Axiovert 135M inverted microscope, Carl Zeiss Inc.), allowed direct visualization in real time of the platelet adhesion process, which was recorded with a VCR (Magnavox).

Measurement of Platelet Stability

Platelets were considered to move on the surface when exhibiting spatial displacement greater than one platelet diameter (Savage et al., 1996). To estimate motion, a series of images from the recorded experiment was digitized at a sampling rate of six frames per second using a computer-controlled VCR (Sony 9500) and a frame grabber (Matrox Image LC). The images were binarized after application of a threshold to distinguish platelets from the background. Time was calculated by referring to the frame number. Image analysis was performed using the Metamorph software package (version 2.76, Universal Imaging Corp). The first two consecutive frames in a series were superimposed using the logical AND function, so that the resulting image represented only the overlapping areas of single platelets at two different times. The new image was then superimposed to the next frame, and the process was continued until the overlapping area was equal to zero. When this occurred, a platelet had moved by a distance greater than its diameter; if this did not occur, a platelet was considered firmly attached during the period of observation. The time for each individual platelet displacement was computed and the process repeated until all the platelets in the microscopic field had moved, or for a preselected time interval, whichever occurred first.

Measurement of Platelet Surface Velocity

To calculate the velocity of platelets interacting with the surface, images were digitized from the recorded experiment at a sampling rate of 30 frames per second, and a total of 300 frames were analyzed for each experiment. Digitized images had a standard size of 512 × 512 pixels and an optical resolution of 0.5 μm. Image processing was performed with the Metamorph software package. The position of the centroid of the platelet was calculated for every frame, and their relative coordinates (*x* and *y*) were measured. Centroid displacement was computed as a function of time. Velocity was calculated as the distance traveled by the centroid divided by the time interval.

Thrombus Volume Measurements

The total volume occupied by thrombi in an area of 102,236 μm^2 was estimated from a series of confocal sections at 1.0 μm intervals in the *z* axis (LSM confocal microscope, Carl Zeiss Inc.). These were obtained while blood was flowing using a laser wavelength of 488 nm and a scanning time of 2 s per section. The microscope settings—including contrast, brightness, magnification, and pinhole aperture—were maintained constant in order to facilitate comparisons between different experiments. Confocal sections were analyzed using the Metamorph software package. A threshold was applied to all the images in a stack to distinguish platelets from the background; the same value was then used in all subsequent analyses of confocal images for a given experiment. The area occupied by all thrombi in a given plane was calculated, and the volume corresponding to a 1.0 μm thick section was estimated by multiplying the average area covered by platelets in two consecutive planes by the height of the section (1.0 μm). The total volume occupied by all thrombi was then estimated by summation of the sectional volumes.

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