Immunoneutralization of Glycoprotein Ibα Attenuates Endotoxin-Induced Interactions of Platelets and Leukocytes With Rat Venular Endothelium In Vivo

Tomihiro Katayama, Yasuo Ikeda, Makoto Handa, Takuya Tamatani, Shinji Sakamoto, Masaharu Ito, Yuzuru Ishimura, Makoto Suematsu

Abstract—This study aimed to examine molecular mechanisms for endotoxin-induced adhesive changes in platelets in vivo. Platelets labeled with carboxyfluorescein diacetate succinimidyl ester were visualized in rat mesenteric venules through intravital microscopy assisted by a high-speed fluorescence video imager at 1000 frames per second or by a normal-speed intensifier under monitoring of erythrocyte velocity. Leukocyte rolling was examined by normal-speed transmission video images. The velocity of platelets traveling along the centerline of venules followed that of erythrocytes, whereas that measured at the periendothelial space was significantly smaller than the erythrocyte velocity; a majority of these cells exhibited transient but notable rolling with endothelium. Administration of endotoxin increased the density of periendothelial platelets and reduced the rolling velocities of platelets and leukocytes in venules: All events were attenuated by anti–rat P-selectin monoclonal antibody s789G or by anti–human glycoprotein (GP) Ibα monoclonal antibody GUR83/35, which blocks ristocetin-induced aggregation of rat platelets. Isolated rat platelets injected into endotoxin-pretreated rats were able to roll on the venules. This event was attenuated by pretreatment of platelets in vitro with GUR83/35 but not with s789G, suggesting involvement of endothelial P-selectin and platelet GP Ibα in the endotoxin-induced responses. Furthermore, isolated human platelets showed similar rolling interactions with endotoxin-preexposed rat venules, and pretreatment of the platelets with GUR83/35, but not with s789G, significantly reduced such interactions. Our results provide the first evidence for involvement of GP Ibα in endotoxin-induced microvascular rolling of platelets and leukocytes, and this system serves as a potentially useful tool to examine GP Ibα–associated function of human platelets in vivo. (Circ Res. 2000;86:1031-1037.)

Key Words: platelets ■ endothelial cells ■ P-selectin ■ shear stress ■ endotoxin

Under physiological conditions, platelets are thought to circulate in close contact with microvascular endothelial cells without adhering to their surface. The cells have been thought to neither adhere to the site of injury nor undergo activation until these cells are exposed to platelet-activating subendothelial substances such as collagen.1 On the initial adhesive process, platelets can release varied mediators and activate other platelets.2 Once the first layer of adhered platelets forms the stable thrombogenic surface, growth of the hemostatic plug occurs dependently on the mediator-elicited platelet-to-platelet interactions.2 These 2 events represent adhesion and aggregation of platelets, respectively.

Detailed knowledge of the density or velocity distribution of platelets flowing along the periendothelial space in vivo could help us understand mechanisms for the initial process of thrombogenesis and subsequent inflammatory responses involving leukocyte adhesion for several aspects: First, determination of the density of platelets in the local area allows us to estimate the rate of local platelet delivery. Second, analyses of velocity distribution of the periendothelial platelets make it possible to estimate alterations in adhesive force between platelets and endothelial cells under disease conditions and thus provide important information on changes in their adhesivity under flow conditions. Third, considering the presence of varied adhesion molecules on platelets that might bind to ligand molecules on the surface of leukocytes, the density of platelets in the periendothelial space could determine venular leukocyte recruitment in acute inflammatory processes.3,4 However, little knowledge of the behavior of individual platelets circulating close to the periendothelial space has been available because of technical difficulties in visualizing these cells in a reliable manner. We applied intravital ultrahigh-speed intensified microscopy assisted by carboxyfluorescein diacetate succinimidyl ester, a fluoro-
chrome that can stain platelets intravitaly. The present results provide evidence that under disease conditions such as endotoxemia, circulating platelets display the periendothelial rolling in postcapillary venules through mechanisms involving endothelial P-selectin and glycoprotein (GP) Ibβ on platelets and help leukocyte rolling and adhesion in vivo.

Materials and Methods
Our study protocol was approved by the Animal Care and Utilization Committee of Keio University School of Medicine. Male Wistar rats were anesthetized for intravitreal microscopy of mesenteric microvessels in vivo5–8 and treated with an intravenous injection of carboxyfluorescein diacetate succinimidyl ester, a nonfluorescent precursor taken up into platelets and partly into leukocytes, forming a stable fluorochrome, carboxyfluorescein succinimidyl ester (CFSE), in the cells.5,6 CFSE-labeled platelets were visualized through multifunctional intravitreal fluorescence microscopy as described previously.5,7 Advantages of the present approach compared with previous methods for platelet visualization are described in the online-only Materials and Methods (see http://www.circresaha.org). Six main protocols were used: Rats in group 1 were intravenously perfused with physiological saline as a vehicle for 30 minutes. In group 2, rats were injected with lipopolysaccharide (LPS, O111B4; 1.0 mg · kg⁻¹ · h⁻¹ IV for 30 minutes) according to the previous experimental model of endotoxemia.9 Animals in groups 3 and 4 were pretreated with new monoclonal antibodies (mAbs) against rat P-selectin s789G and s84F. Rats in groups 5 and 6 were pretreated with anti–human GP Ibα mAbs, GUR83/35 and WGA3. GUR83/35 is reported to recognize a conformation-specific epitope between residues 1 and 302 of GP Ibα and to block the binding of von Willebrand factor to human platelets in the presence of ristocetin, whereas WGA3 binds to the same region but does not block the ristocetin-induced aggregation of platelets.10,11 These mAbs were injected at 1.5 mg/kg IV 5 minutes before the start of the LPS infusion. Individual erythrocytes and platelets were visualized through an ultrahigh-speed video microscope. This system allowed us to visualize platelets and erythrocytes at 1000 frames per second for 1 second under epifluorescence illumination and transillumination, respectively. Velocities of individual platelets were normalized by dividing the values by the regional erythrocyte velocity in the centerline or the periendothelial region, with the result designated as the relative platelet velocity (Vp/Vr) in each region. This value serves as a semiquantitative index of the adhesion energy between platelets and endothelial cells. We examined differences in circulating platelet counts and the density of platelets between the periendothelial and centerline regions of the microvessels as shown in previous studies using acridine red-assisted intravital microscopy.12 The normalized densities of CFSE-positive platelets in the centerline (Dc) and periendothelial (Dp) regions were defined as described in the online-only Materials and Methods (see http://www.circresaha.org). The relative rolling velocity of leukocytes (Vp/Vr) and the density of adherent cells were determined separately with normal-speed transmission video images as described elsewhere.5,7 In other experiments, isolated rat and human platelets were injected intravenously into rats undergoing 30-minute exposure to the LPS infusion, and their behavior in venules was examined with a silicon-intensified target (SIT) camera.8 Function of isolated platelets was examined with a laser-scattering platelet aggregometer. Anti–P-selectin mAbs were generated, and their ability to block P-selectin–mediated cell adhesion in vitro was characterized. Differences among groups were examined by 1-way ANOVA with Fisher’s multiple comparison test.

Results
Individual Platelets Labeled With CFSE
Figure 1 illustrates the effects of intravital fluorescence labeling with CFSE on platelet aggregation. As seen in Figures 1A and

Figure 1. Representative pictures showing individual platelets labeled with CFSE in vivo. A and B, Representative phase-contrast transillumination image and the corresponding fluorograph of the platelet-rich suspension. Bar: 25 μm. C, Effects of labeling with CFSE on stimulus-dependent formation of microaggregates. Data collected from the CFSE-untreated [CFSE(−)] and CFSE-treated [CFSE(+)] platelets, respectively. Arrows in these panels show time of the application of ristocetin or ADP. s and l indicate time history of small and large microaggregate formation, respectively.

Millisecond Interactions of Platelets With Microvascular Endothelium
As shown in Figure 2A, video-recording at 1000 frames per second under transillumination allowed us to visualize indi-
individual erythrocytes packed in microvessels and recognized as granular patterns of images. Regional velocities of single erythrocytes in venules were analyzed by tracing movement of each cell per unit frame with a frame-by-frame analysis, as shown in Figure 3. At this recording rate, CFSE-labeled platelets can be visualized, but their exact localization inside the vessels could hardly be identified because of their rapidity of movement, as seen in Figure 2B. Conversely, their frame-by-frame reproduction by replaying at 30 frames per second allowed us to visualize individual platelets as pinpoint dots and to trace movement of these cells along vessel walls. As seen in serial fluorographs with 20- or 40-ms intervals in Figures 2C through 2H, a single platelet moving in the periendothelial space of the venule exhibited quite heterogeneous changes in its velocity. The distance of movement per unit time in 1 cell (arrows) differed greatly from that in another cell (asterisks), indicating a heterogeneity in velocities among different platelets.

### Spatial and Temporal Alterations in Erythrocyte Velocity in Microvessels

On the basis of the frame-by-frame analysis of transillumination images, we examined temporal and spatial profiles of the erythrocyte velocity in postcapillary venules. As seen in Figure 3A, showing the velocity of single erythrocytes (z axis) as a function of time (x axis) and position (y axis) of measurements, the velocity measured in the venular centerline region did not fluctuate greatly. The velocity of erythrocytes flowing in venules was greater in the centerline region than in the periendothelial regions.

### Table 1. Effects of In Vivo CFSE Labeling on ADP-Induced Platelet Aggregation In Vitro

<table>
<thead>
<tr>
<th></th>
<th>%T</th>
<th>Small Aggregates</th>
<th>Large Aggregates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Peak, %</td>
<td>T&lt;sub&gt;max&lt;/sub&gt;, min</td>
<td>Peak, 10&lt;sup&gt;4&lt;/sup&gt; mV</td>
</tr>
<tr>
<td>CFSE (+)</td>
<td>58.0±9.9</td>
<td>2.5±0.2</td>
<td>10.4±2.5</td>
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<tr>
<td>CFSE (−)</td>
<td>49.5±3.7</td>
<td>2.6±0.3</td>
<td>12.4±4.7</td>
</tr>
<tr>
<td>CFSE (+)</td>
<td>60.0±6.9</td>
<td>2.5±0.2</td>
<td>12.0±5.1</td>
</tr>
<tr>
<td>CFSE (−)</td>
<td>49.5±3.7</td>
<td>2.6±0.3</td>
<td>13.1±5.2</td>
</tr>
</tbody>
</table>

Data represent mean±SEM of 4 separate experiments.

### Figure 2

Sequential microfluorographs showing intravital behavior of platelets labeled with CFSE captured by ultrahigh-speed video microscopy. A, Representative transillumination image captured at 1000 frames per second. Bar=30 μm. a indicates arteriole; v, venule. B, Microfluorograph captured at 30 frames per second in the same vessels. C through H, Series of microfluorographs captured every 20 to 40 ms in the same region. Asterisks and vertical arrows indicate serial changes in positions of 2 platelets interacting with the venular wall. Horizontal arrows denote direction of blood flow in each vessel.

### Figure 3

Spatial and temporal profiles of velocities of erythrocytes and platelets in the rat mesenteric venules. A, Representative data of erythrocyte velocities determined at a venule of the LPS-untreated control. Mean values of 5 measurements in the same site were plotted as a function of time and domain in the single vessel observed. The value 100 indicates the maximum erythrocyte velocity measured at the centerline region. P<sub>c</sub> and P<sub>e</sub> denote centerline and periendothelial regions of the microvessels, respectively. B, Representative behavior of individual platelets running through centerline and periendothelial regions of the LPS-untreated control venule. C, Histogram analyses of V<sub>p</sub>/V<sub>e</sub> in the vehicle- and LPS-treated venules. Data were collected from ~100 different platelets in individual experiments using >6 rats in each group.
than in the periendothelial region. This observation led us to hypothesize that the heterogeneity of movement of platelets in the periendothelial region illustrated in Figure 2 could result from transient adhesive interactions of the cells with the endothelial surface rather than from local changes in erythrocyte velocities. To test this hypothesis, we examined alterations in the relative velocity of individual platelets versus erythrocyte velocity (Vp/Vr) in venules of the control rats. As shown in 3B, the Vp/Vr values of a single platelet flowing at the centerline of venules was constant, at ~100%, in venules. Conversely, the velocity of platelets flowing in the periendothelial regions exhibited a fluctuating pattern: The Vp/Vr values of these platelets occasionally became as low as 50% of the local erythrocyte velocity, suggesting the presence of adhesive interactions between the periendothelial platelets and microvascular endothelium.

Such a baseline level of the platelet-endothelium interactions was enhanced by treatment of rats with LPS. Figure 3C illustrated histograms showing distribution of the Vp/Vr values in the periendothelial space of venules. In the controls, the periendothelial platelet velocity varied greatly among different cells, whereas the mean Vp value was ~92% of the mean Vr value, and ~50% of the cells exhibited a velocity <90% of the regional erythrocyte velocity. At 30 minutes after the start of the LPS administration, venular wall shear rates did not change significantly, but the Vp/Vr histogram exhibited a marked shift to the left side. Under the present experimental conditions, venular wall shear rates in the LPS-untreated and -treated rats were 486±39 s⁻¹ and 476±52 s⁻¹ (mean±SD of 7 experiments), respectively, showing no statistical difference. These results suggest that LPS increases the adhesion energy between periendothelial platelets and venular endothelium in vivo.

**P-Selectin–Mediated Microvascular Rolling of Platelets in LPS-Treated Rats**

We determined the effects of mAbs against P-selectin and GP Ibα and then applied them to the in vivo systems. Figure 4A illustrates the effects of the anti–rat P-selectin mAbs on the intercellular adhesion between the P-selectin–expressing transfectant and HL-60 cells. The mAb s789G, but not s84F, dose-dependently attenuated the cell adhesion, indicating that the former mAb serves as a reagent to block leukocyte adhesion. Figure 4B depicted effects of anti–human GP Ibα mAbs on ristocetin-induced aggregation of rat platelets. As seen, GUR83/35, but not WGA3, significantly suppressed ristocetin-induced responses of rat cells, indicating that the former mAb can be used to block the rat GP Ibα–mediated events.

Figure 4C shows LPS-induced alterations in circulating platelet counts and density (Dp/Dc) and velocity (Vp/Vr) of periendothelial platelets in venules and depicts the effects of the aforementioned mAbs against P-selectin or GP Ibα on these parameters. Administration of LPS significantly reduced the number of platelets in peripheral blood samples. Conversely, the Dp/Dc values exhibited an ~20% elevation on the administration of LPS. The LPS-induced elevation of the periendothelial platelet density coincided with a marked reduction of the mean Vp/Vr values, indicating an increasing adhesivity between the platelets and endothelial cells. Collectively, the LPS-induced thrombocytopenic changes appeared to occur concurrently with margination of circulating platelets to the periendothelial space. Pretreatment with s789G but not with s84F significantly attenuated the LPS-induced changes in these platelet parameters, suggesting that P-selectin mediates the LPS-induced microvascular margination of platelets. Most importantly, pretreatment with the mAb GUR83/35 but not with WGA3 elicited similar inhibitory effects on the LPS-induced changes, suggesting that functional blockade of GP Ibα on circulating platelets cancels platelet-endothelium interactions in LPS-exposed venules. Conversely, the administration of 1 of the 2 mAbs to the LPS-untreated rats did not evoke alterations in these parameters (data not shown).

Another important event observed during the administration of LPS was a reduction of the rolling velocity of leukocytes in venules. As seen in Table 2, the administration of LPS induced an ~60% reduction of the Vp/Vr. Pretreatment with s789G abolished the LPS-elicted alterations, suggesting involvement of P-selectin in leukocyte rolling in the stimulated venules, which was in good agreement with previous studies. The LPS-elicted reduction of the rolling velocity was also attenuated significantly by pretreatment with GUR83/35, suggesting that functional blockade of GP Ibα attenuates the LPS-induced elevation of adhesion energy.
TABLE 2. Effects of Administration of Anti–P-selectin mAb s789G and Anti–GP Ibα mAb GUR83/35 on LPS-Induced Alternations in Rolling Velocity and Adherence of Leukocytes in Venules

<table>
<thead>
<tr>
<th>Group</th>
<th>Vp/Vn, %</th>
<th>Density of Adherent Cells, No./100-μm Venular Segment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>2.3±0.1</td>
<td>0.8±0.2</td>
</tr>
<tr>
<td>LPS</td>
<td>1.2±0.1*</td>
<td>7.6±1.3*</td>
</tr>
<tr>
<td>+s789G</td>
<td>2.1±0.2†</td>
<td>2.4±0.6‡</td>
</tr>
<tr>
<td>+GUR3/35</td>
<td>1.7±0.2†</td>
<td>5.2±1.0†</td>
</tr>
</tbody>
</table>

Data represent mean±SEM of 5 to 9 separate experiments.

*P<0.05 vs vehicle-treated controls.
†P<0.05 vs LPS-treated group.
‡P<0.05 vs group treated with LPS plus GUR83/35.

The LPS infusion also elicited a marked increase in the density of adherent leukocytes that was attenuated by pretreatment with s789G. The blockade of GP Ibα by GUR83/35 also attenuated the LPS-induced venular leukocyte adhesion significantly, although its effect was smaller than that of s789G. Collectively, these results suggest that the platelet adhesion molecule GP Ibα is a determinant involved in mechanisms for the LPS-induced microvascular leukocyte recruitment.

Role of GP Ibα in Rolling of Rat and Human Platelets in LPS-Pretreated Venules

Observations shown in Figure 4 led us to further examine the roles of P-selectin and GP Ibα in the LPS-induced interactions between platelets and venular endothelium. To that effect, differences in adhesivity of isolated rat platelets pretreated or not treated with mAbs against these adhesion molecules were examined in LPS-pretreated rats under observation with an STI camera. Once injected, the platelets displayed a transient adhesion and rolling, and some cells exhibited stationary adhesion to the venular endothelium (Figure 5). A subpopulation of the platelets flowing at the periendothelial space often attached to rolling leukocytes and made them slow down further. Formation of leukocyte-platelet complex was seen occasionally. As summarized in Figure 5, right, CFSE-labeled platelet rolling on venules was enhanced by pretreatment with LPS. When the isolated platelets were pretreated with the mAb s789G, their adhesive responses were not greatly changed. By contrast, pretreatment of the isolated platelets with GUR83/35 almost abolished their adhesion to the LPS-preexposed venules. These results showed that blockade of constitutively expressed GP Ibα on platelets is involved in mechanisms through which this mAb administered in vivo attenuated the LPS-induced platelet interactions with the endothelium.

This observation tempted us to examine whether human platelets could utilize GP Ibα-mediated mechanisms to adhere to rat venular endothelium in vivo. As seen in Figure 6A, the platelets administered into the LPS-treated rats exhibited rolling with greater frequency than those administered into the untreated controls over the whole ranges of the velocities. The population of the slow rollers with velocities <0.2 mm/s became markedly elevated in the LPS-treated group. As shown in Figure 6B, in vitro pretreatment of human platelets with the mAb GUR83/35, but not with WGA3, significantly attenuated such adhesive changes observed in vivo. Thus, the present findings demonstrated that human platelets exogenously administered into the rat vascular system are able to roll on microvascular endothelium through GP Ibα-mediated mechanisms.

Discussion

High-speed microfluorography allowed us to show that platelets not only can traverse through the centerline region of microvessels in a free-flowing manner but also can move along the periendothelial region and display notable interactions with the endothelial surface at millisecond intervals even under unstimulated, ordinary conditions. Such interactions of platelets are distinct from the slow rolling behavior observed only in a subpopulation of platelets that has been detected by normal-speed videomicroscopy.13,14 Although this varied among individual cells, a majority of periendothelial platelets displayed rapid but detectable attachment to the endothelium, and some of them exhibited a velocity <50% of the erythrocyte velocity measured in situ. Such interactions of platelets were markedly enhanced by LPS treatment. The present data indicate that unstimulated platelets are able to roll on the LPS-preexposed venules and suggest a crucial role of P-selectin expressed at the side of the stimulated endothe-

Figure 5. Representative pictures showing microvascular behavior of exogenously injected CFSE-labeled rat platelets in LPS-exposed mesenteric venules and effects of immunoneutralization of GP Ibα on their density. Numbers at top left corner of each picture show time after beginning of injection of CFSE-labeled platelets. White and black asterisks denote platelets showing rolling and capturing of platelets, respectively. White arrow indicates fluorescent platelets adhered to the surface of a rolling leukocyte. Black arrows show a leukocyte undergoing collision with a free-flowing platelet, forming a complex and slowing down to become adhered to the venular wall. Bar=40 μm. Right, Effects of in vitro pretreatment with s789G or GUR83/35 on the number of CFSE-labeled platelets (PLT) rolling on the LPS-preexposed venules. *P<0.05 vs data collected from LPS-untreated controls; †P<0.05 vs data from LPS-treated rats. The data indicate mean±SEM of 4 to 5 separate experiments.
studies in vitro showing that GP Ib insight into mechanisms for platelet adhesion mediated by GP vivo appeared to be small or none. venules: Inasmuch as the s789G pretreatment did not alter the line adhesive interactions of platelets with the unstimulated were unable to address molecular determinants for the base- distribution toward the periendothelial space. However, we appears to be involved in the LPS-induced shift of platelet LPS through mechanisms involving GP Ib a

Another important event observed under GP Ib a

chance collide with leukocytes rolling directly along venular endothelium and help them slow down further for stationary adhesion. The GP Ibα-mediated elevation of the periendothelial platelet density and increase in the adhesion energy to the LPS-exposed venules could enhance both of these mechanisms for intercellular interactions and secondarily activate leukocyte adhesivity. Detailed molecular mechanisms for the GP Ibα-mediated direct interactions between platelets and leukocytes in vivo (eg, L-selectin) and involvement of plasma factors reacting with GP Ib (eg, von Willebrand factor) should be examined further.18,19

Finally, our study revealed that the rat system serves as a potentially useful tool to examine GP Ibα-associated function of human platelets in vivo, in that they can utilize GP Ibα to roll in a manner similar to that of rat platelets on the stimulated microvascular endothelial cells. GP Ib has been known to play important roles in a variety of functional alterations of platelets in vitro, such as shear-dependent aggregation19 and adhesion to immobilized von Willebrand factor under flow conditions in vitro.20 In humans, conversely, there are genetic disorders of this adhesion molecule, such as Bernard-Soulier syndrome21 or cases of heightened platelet aggregation by ristocetin.10,22 However, a pathophysiological link of such in vitro events to actual behavior of functionally impaired platelets in vivo is largely unknown because of lack of the intravital system to examine human platelet functions. Determination of impaired adhesive properties of human platelets collected from disease patients deserves further studies to understand the whole mechanisms for platelet-dependent hemostatic and thrombotic disorders from microvascular viewpoints, and such studies are under way in our laboratories.

Acknowledgments

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References


Figure 6. Rolling behavior of CFSE-labeled human platelets in vivo. A, Histogram analyses showing the number of the rolling platelets as a function of the rolling velocity (Vr) in rats pretreated with vehicle or LPS. B, Effects of in vitro pretreatment with mAbs against GP Ibα on rolling of human platelets to the LPS-preexposed venules. GUR83/35 is the function-blocking mAb, whereas WGA3 is the nonblocking mAb. *P<0.05 vs data collected from vehicle-treated rats; †P<0.05 vs data from LPS-treated rats. Data indicate mean±SEM of 5 separate experiments.

A

B


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