Redundant Mechanism of Platelet Adhesion to Laminin and Collagen under Flow

INVOLVEMENT OF VON WILLEBRAND FACTOR AND GLYCOPROTEIN Ib-IX-V

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Although the role of collagen in thrombosis has been extensively investigated, the contribution of other extracellular matrices is still unclear. We have recently reported that laminin stimulates platelet spreading through integrin α5β1-dependent activation of the collagen receptor glycoprotein (GP) VI under static condition. Under physiological high and low shear conditions, platelets adhered to laminin, and this was strongly inhibited by an antibody that blocks association between GPIb-IX-V and von Willebrand factor (VWF). Moreover, platelets of type III von Willebrand disease or Bernard-Soulier syndrome adhered to laminin at a low shear condition but not at a high shear condition. The specific binding of laminin to VWF was confirmed by surface plasmin resonance spectroscopy (BIAcore). These findings suggest that laminin supports platelet adhesion depending on the interaction of VWF and GPIb-IX-V under pathophysiological high shear flow. This mechanism is similar to that of collagen. We propose that integrins, GPVI, GPIb-IX-V, and VWF represent a general paradigm for the interaction between platelets and subendothelial matrices.

Platelet adhesion to exposed subendothelial matrices at sites of damaged vessel wall is the first step in thrombosis and hemostasis. Full understanding of the role of subendothelial matrices in this process is essential toward an understanding of thrombotic diseases and new forms of therapeutic intervention. Among types of subendothelial matrices, the role of collagen for thrombosis and hemostasis has been extensively investigated. Collagen activates platelets through collagen receptors glycoprotein (GP)VI and integrin α5β1, and induces platelet plug formation and occlusion at sites of vessel damage under physiological shear condition by recruiting platelets to exposed collagen. At arterial shear rates of flow, von Willebrand factor (VWF) and its platelet surface receptor GPIb-IX-V play a critical role in initiation of thrombus formation, although the role of VWF and GPIb-IX-V is minimal in venous shear rates of flow. Under arterial shear, VWF in circulation binds to collagen via its A3 domain, and then platelets are captured or tethered to the complex of collagen and VWF through GPIb-IX-V binding to VWF (1). This is followed by platelet activation mediated by the binding of collagen to GPVI, leading to inside-out stimulation of integrin α5β1 and αIIIβ3 and platelet thrombin formation (2). Since collagen is a strong thrombogenic matrix that supports platelet adhesion and aggregation under high shear flow, collagen has been considered to play a central role in thrombosis and hemostasis.

A glycoprotein laminin, which is also a major subendothelial matrix in vasculature, is predominantly expressed in the basement membrane under endothelial cells. We have recently discovered that the laminin purified from human placenta induces platelet activation that leads to cell spreading (3). Surprisingly, human laminin stimulates platelets by binding to the collagen receptor GP VI, and this interaction is facilitated by integrin α5β1 (3). This mechanism of platelet adhesion and activation is similar to the interaction of platelets with collagen; integrin α5β1 binding to collagen facilitates the interaction between GPVI and collagen (4–7). Since both laminin and collagen are major subendothelial matrices in vasculature and both matrices activate platelets through GPVI, we hypothesized that laminin also plays a role in thrombosis and hemostasis under arterial shear condition.

To explore the physiological significance of laminin-induced platelet activation, we sought to investigate whether laminin also supports platelet adhesion under pathophysiological flow condition. The present study demonstrated that laminin also supports platelet adhesion depending on the interaction of VWF with GPIb-IX-V at a high shear condition, thereby contributing to the hemostatic process. We propose that integrins, GPVI, GPIb-IX-V, and VWF may be common molecules necessary for platelet binding to subendothelial matrices.

EXPERIMENTAL PROCEDURES

Materials—AJvW-2, an anti-VWF monoclonal antibody (8), was a gift from Ajinomoto Pharmaceutical Research Laboratories (Yokohama, Japan). Argatroban was from Mitsubishi Tanabe Pharma (Tokyo, Japan). DiOC6 was from Invitrogen. Glass capillary tube was from Camlab (Cambridge, UK). Horm type I collagen was from Nycoderm Pharma GmbH (Munich, Germany). Human laminin and collagenase were from Sigma-Aldrich. Human VWF was purified as described elsewhere (9). All other materials were from previously named sources (3).

Flow Adhesion Assay—Whole blood from healthy donors, a patient with type III von Willebrand disease (type III VWD), or
a patient with Bernard-Soulier syndrome (BSS) was collected into a syringe and anticoagulated with 0.1 μg/ml argatroban and 5 units/ml heparin. All subjects provided written, informed consents in accordance with the Ethical Committee of University of Yamanashi and the Declaration of Helsinki. Capillary tubes (0.3 × 1.2 mm, 50 mm long) were coated with 100 μg/ml laminin or collagen overnight at 4 °C. Capillaries were washed and blocked with phosphate-buffered saline containing 2% BSA for 2 h at room temperature. Laminin-coated capillaries were treated with 0.1 mg/ml collagenase for 2 h at 35 °C before BSA blocking as described previously (3). Then they were rinsed with modified Tyrode’s buffer supplemented with 2 mM CaCl₂ and 1 units/ml heparin and connected to a syringe filled with the anticoagulated blood that had been pretreated with 5 μM DiOC₆ for 30 min. Blood was perfused into capillaries at indicated shear rates in the presence or absence of 10 μg/ml A1vW-2, and adherent platelets were visualized using a fluorescent video microscope (IX71, Olympus, Tokyo, Japan). Movie data were converted into sequential photo images, and the percentage of area covered by adherent platelets at 5 min was measured using NIH Image (National Institutes of Health, Bethesda, MD). Since platelet count is significantly low in BSS patients when compared with normal donors, we diluted blood from a normal donor by the addition of platelet-poor plasma and added washed red blood cells from the same donor to adjust blood cell counts so that comparison could be made between BSS blood and diluted normal blood.

Surface Plasmon Resonance Spectroscopy—A specific interaction between VWF and laminin was analyzed using a BIACore X (BIAcore AB, Uppsala, Sweden). VWF coupling, blocking, and regeneration of a CM5 chip were performed as described before (3). Laminin in HBS-EP buffer (10 mM HEPES, 0.15 mM NaCl, 3 mM EDTA, and 0.005% Tween 20, pH 7.4, BIAcore) at several concentrations was perfused over the control surface or an immobilized VWF surface at a flow rate of 10 μl/min at 25 °C, and the resonance changes were recorded. The response from the VWF surface was subtracted from that of the control. The dissociation constants (Kₐ) were determined using BIAEvaluation software.

Statistics—Statistical analysis was performed using the unpaired Student’s t test.

RESULTS

Laminin Supports Platelet Adhesion under High Shear Flow—Whole blood labeled with DiOC₆ was flowed over laminin or collagen surfaces under high (1500 s⁻¹) shear rate for 5 min. Fig. 1 showed that laminin as well as collagen supports platelet adhesion (Fig. 1, A and B). Under high shear rates, platelets attached to, tethered on, and stably adhered to both collagen and laminin surfaces, whereas some of them detached after transient adhesion. The majority of adherent platelets to collagen formed large aggregates in 5 min (Fig. 1A), whereas only small aggregate formation was observed on laminin under high shear flow (Fig. 1B). Under high shear flow, VWF in circulation binds to exposed collagen, and the initial contact of platelets with collagen is mediated through GPIb-IX-V and VWF bridges. In this process, VWF plays an essential role in platelet adhesion to collagen. Since laminin supports platelet adhesion under high shear flow, we hypothesized that VWF and GPIb-IX-V on platelet membranes are also involved in this process, as is the case with collagen. To investigate this hypothesis, an anti-VWF antibody against A1 domain (AjvW-2) that blocks association of GPIb-IX-V with VWF was utilized. Pretreatment of blood with AjvW-2 significantly inhibited platelet adhesion to laminin as well as platelet adhesion to collagen at 1500 s⁻¹. The percentage of area covered by adherent platelets to a laminin-coated capillary was reduced in the presence of AjvW-2 from 38.6 ± 4.40% to 0.65 ± 0.84% at 5 min (mean ± S.D., p < 0.005), and that to a collagen-coated capillary was reduced from 39.3 ± 6.53% to 1.66 ± 1.71% at 5 min (mean ± S.D., p < 0.005) (Fig. 1, A and B). In contrast, AjvW-2 did not inhibited platelet adhesion to either laminin or collagen at a shear rate of 300 s⁻¹ (data not shown). These results suggested that VWF and GPIb-IX-V play a crucial role in platelet adhesion to laminin, which is quite reminiscent of the role of VWF and GPIb-IX-V for platelet adhesion to collagen under high shear condition.

Platelet Adhesion to Laminin Requires VWF and GPIb-IX-V under High Shear Flow—The inhibitory effect of AjvW-2 on platelet adhesion to laminin under high shear prompted us to further investigate the role of VWF and GPIb-IX-V in shear-resistant platelet adhesion to laminin using blood from a patient who lacks VWF (type III VWD) or GPIb-IX-V (BSS). As shown in Fig. 1C, platelets from a normal donor adhered to laminin and collagen at high and low shear rates. On the other hand, the adhesion of platelets from a VWD patient to laminin- or collagen-coated surfaces was greatly attenuated at a shear rate of 1500 s⁻¹, although they almost normally adhered to either surface at a shear rate of 300 s⁻¹, suggesting that VWF plays a crucial role in platelet adhesion to laminin under high shear conditions (Fig. 1C). Involvement of GPIb-IX-V in platelet adhesion to laminin under flow was also investigated using whole blood from a patient of BSS, whose platelets lack GPIb-IX-V. Similar to the results with VWF-deficient blood, platelets from a BSS patient adhered to both laminin and collagen under low shear condition but failed to adhere to either surface under high shear conditions (Fig. 1D), indicating that GPIb-IX-V is also involved in platelet adhesion to laminin under this condition.

The Direct Binding of Laminin to VWF Was Observed by a Surface Plasmon Resonance Spectroscopy—Our findings hitherto strongly suggested that VWF binds to laminin under high shear flow, and then platelets bind to laminin through the interaction between GPIb-IX-V and VWF. To verify the binding of laminin with VWF, we next measured Kₐ for the interaction of laminin with VWF using BIACore. As shown in Fig. 2, a direct binding between laminin and VWF was observed. The Kₐ for the interaction of laminin with VWF was calculated as 524 ± 63.9 nM (mean ± S.D.), and that of Horm collagen with VWF was calculated as 5.90 ± 1.03 nM (mean ± S.D.). This suggests that the affinity between laminin and VWF is about 100-fold lower than that between Horm collagen and VWF.

DISCUSSION

Although a previous study reported that laminin supports platelet adhesion under high shear (11), its mechanism has not
been elucidated to date. We have recently reported that human laminin supports platelet adhesion through integrin $\alpha_6\beta_1$ and activates platelets that induce spreading through a collagen receptor GPVI under static condition (3). The similarity of the mechanism of platelet adhesion and activation between laminin and collagen under static condition prompted us to hypothesize that laminin supports platelet adhesion under high shear flow through a mechanism similar to collagen.

First, we compared platelet adhesion to laminin with that of collagen at a high shear rate. When blood was flowed over collagen, platelets rapidly adhered to the monolayer and formed large aggregates in 5 min, indicating that major signaling receptors GPVI strongly activates platelets upon adhesion to collagen. Although platelets adhered to laminin, the majority of adherent platelets formed only small aggregates in 5 min. These differences may be accounted for by the lower affinity of GPVI for laminin relative to collagen; the $K_d$ for the interaction of GPVI with laminin ($6.36 \pm 1.44 \mu M$) is 10 times larger than that of GPVI with collagen ($0.57 \pm 0.73 \mu M$) (3).

We next demonstrated that the shear-resistant adhesion of platelets to laminin is greatly dependent on the interaction between GPIb-IX-V and VWF using an anti-VWF antibody or blood donated by patients of VWD and BSS. It is of note that VWF and GPIb-IX-V are involved in shear-resistant adhesion to laminin under flow. Our results may account for the previously unexplained finding by Wagner et al. (12), which showed that VWF binds to extracellular matrices independently of collagen. We also confirmed the direct interaction of laminin with VWF by using BIAcore in this study, although a previous report failed to detect the direct binding of VWF to laminin by enzyme-linked immunosorbent assay (13). We assume that the direct binding of VWF with laminin may be difficult to be detected by enzyme-linked immunosorbent assay because of the weak binding affinity between VWF and laminin.

We have recently reported that laminin activates platelets through a collagen receptor GPVI and that the interaction between GPVI and laminin is facilitated by another laminin receptor integrin $\alpha_6\beta_1$ (3). In the present study, we demonstrated that shear-resistant adhesion of platelets to laminin requires an interaction among laminin, VWF, and GPIb-IX-V. This is
quite reminiscent of collagen-induced platelet activation, for which GPVI, integrin $\alpha_\text{IIb}\beta_3$, VWF, and GPIb-IX-V are required. We propose that GPVI, integrins, VWF, and GPIb-IX-V may represent a general paradigm for the interaction between platelets and subendothelial matrices. Since both laminin and collagen are major subendothelial matrices in the vessel wall, this redundant mechanism of platelet adhesion to laminin and collagen under high shear flow may be suitable for cessation of bleeding at sites of damage to the vasculature and may also lead to pathological thrombus formation.

FIGURE 2. The specific interaction between VWF and laminin was analyzed using BIACore X. Laminin or collagen (inset) at several concentrations was perfused over a control and a VWF surface of a sensor tip at a flow rate of 10 $\mu$L/min at 25 °C, and the resonance changes were recorded. The results are shown from one experiment that is representative of four others. A, 240 nM; B, 120 nM; C, 60 nM; D, 30 nM; E, 22 nM; F, 334 nM; G, 250 nM; H, 167 nM; I, 66 nM. RU, resonance units.

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