Integrin $\alpha_6\beta_1$ Is the Main Receptor for Vascular Laminins and Plays a Role in Platelet Adhesion, Activation, and Arterial Thrombosis

Mathieu Schaff, PhD; ChaoJun Tang, PhD; Eric Maurer, MPhil; Catherine Bourdon, BSc; Nicolas Receiveur, BSc; Anita Eckly, PhD; Béatrice Hechler, PhD; Christiane Arnold, BSc; Adèle de Arcangeli, PhD; Bernhard Nieswandt, PhD; Cécile V. Denis, PhD; Olivier Lefebvre, PhD; Elisabeth Georges-Labouesse, PhD; Christian Gachet, MD, PhD; François Lanza, PhD; Pierre H. Mangin, PhD

Background—Laminins are major components of basement membranes, well located to interact with platelets upon vascular injury. Laminin-111 ($\alpha_5\beta_1\gamma_1$) is known to support platelet adhesion but is absent from most blood vessels, which contain isoforms with the $\alpha_1$, $\alpha_2$, or $\alpha_5$ chain. Whether vascular laminins support platelet adhesion and activation and the significance of these interactions in hemostasis and thrombosis remain unknown.

Methods and Results—Using an in vitro flow assay, we show that laminin-411 ($\alpha_5\beta_1\gamma_1$), laminin-511 ($\alpha_5\beta_1\gamma_1$), and laminin-521 ($\alpha_5\beta_1\gamma_1$), but not laminin-211 ($\alpha_5\beta_1\gamma_1$), allow efficient platelet adhesion and activation across a wide range of arterial wall shear rates. Adhesion was critically dependent on integrin $\alpha_6\beta_1$ and the glycoprotein Ib-IX complex, which binds to plasmatic von Willebrand factor adsorbed on laminins. Glycoprotein VI did not participate in the adhesive process but mediated platelet activation induced by $\alpha_5$-containing laminins. To address the significance of platelet/laminin interactions in vivo, we developed a platelet-specific knockout of integrin $\alpha_6$. Platelets from these mice failed to adhere to laminin-411, laminin-511, and laminin-521 but responded normally to a series of agonists. $\alpha_6$-Deficient mice presented a marked decrease in arterial thrombosis in 3 models of injury of the carotid, aorta, and mesenteric arterioles. The tail bleeding time and blood loss remained unaltered, indicating normal hemostasis.

Conclusions—This study reveals an unsuspected important contribution of laminins to thrombus formation in vivo and suggests that targeting their main receptor, integrin $\alpha_6\beta_1$, could represent an alternative antithrombotic strategy with a potentially low bleeding risk. (Circulation. 2013;128:541-552.)

Key Words: blood platelets • integrin $\alpha_6\beta_1$ • laminin • thrombosis

Platelet adhesion and activation are essential to limit blood loss at sites of vascular injury but can also lead to arterial thrombosis upon erosion or rupture of atherosclerotic plaques. Current antithrombotic drugs such as P2Y12 receptor antagonists and integrin $\alpha_6\beta_1$ blockers target platelet activation, thereby considerably reducing the morbidity and mortality associated with ischemic events. However, this strategy results in an increased risk of hemorrhagic complications. Experimental and clinical evidence has suggested that the inhibition of platelet adhesion to subendothelial proteins could pave the way to safer therapies causing minimal perturbation of hemostasis. Indeed, targeting the binding of collagen to glycoprotein (GP) VF or integrin $\alpha_6\beta_1$ or the GPIb/von Willebrand factor (VWF) axis efficiently reduced thrombus formation in various animal models without affecting hemostasis. In addition, it has been shown recently in phase I clinical trials that treatment with agents blocking GPVI/collagen$^2$ or GPIb/VWF$^6$ interactions resulted in no or only a modest defect in hemostasis. Whether preventing the adhesion of platelets to other extracellular matrix proteins such as laminins presents analogous advantages is unknown.

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Laminins form a family of large multidomain disulfide-linked heterotrimeric glycoproteins consisting of $\alpha$, $\beta$, and $\gamma$ chains
that give rise to >15 isoforms. They are widely expressed in various cell types of both developing and adult tissues, including vascular endothelial and smooth muscle cells. Laminins are the most abundant components of the basement membranes surrounding endothelial and medial smooth muscle cells and play a critical role in angiogenesis and the maintenance of vessel architecture through regulation of cell adhesion, proliferation, differentiation, migration, and apoptosis. Blood vessels contain 4 major isoforms, namely, laminin-411 (α1β1γ1, LM411), -421 (α1β2γ1, LM421), -511 (α2β1γ1, LM511), and -521 (α2β1γ1, LM521). Small arteries and veins, particularly in the central nervous system, also express laminin-211 (α2β1γ1) and -221 (α2β1γ1). Laminins are well located to interact with platelets upon vascular damage. Pioneer studies reported adhesion of platelets under both static and flow conditions to laminin from human placenta and laminin-111 (α1β1γ1, LM111) derived from mouse Engelbreth-Holm-Swarm sarcoma cells. Because LM111 exhibits a highly restricted distribution in adults, being absent from most blood vessels, whereas pla- central preparations contain several isoforms that are fragmented and often contaminated with other matrix proteins, the relevance of these previous findings for hemostasis and thrombosis is questionable. More recently, adhesion of platelets to recombinant human LM411 and LM511 was demonstrated, with LM521 being more reactive, but the adhesiveness under flow was not characterized. The adhesive properties of other vascular isoforms remain unexplored.

Platelets express a number of receptors with the potential to bind laminins, including several integrins (α5β1, α5β2, and α6β1), GPVI, α- dystroglycan, and a protein with a molecular mass of 67 kDa (67 LR). Only α5β1 has been found to be critical for platelet adhesion to laminins. Upon laminin binding, α5β1 activates platelets by initiating signals promoting cytoskeletal reorganization and filopodia emission. In addition, α5β1 has been postulated to bring laminin in close contact with GPVI, which in turns mediates lamellipodia formation and platelet spreading. Previous studies indicated either involvement or no participation of 67 LR in platelet adhesion to LM111, whereas the importance of α5-dystroglycan has yet to be established.

Although platelet adhesion to laminins has been established and partially characterized, whether this interaction plays a role in vivo remains completely unknown. In this study, we investigated (1) the ability of a series of vascular laminin isoforms to support platelet adhesion and activation under flow conditions in vitro and (2) the significance of platelet/laminin interactions in hemostasis and experimental thrombosis. In regard to the second objective, because laminin α5-deficient mice die embryonically while deletion of laminin α5 impairs vessel maturation resulting in weakening of the vascular wall, we generated a knockout of integrin α6β1 restricted to the megakaryocyte lineage.

**Methods**

Detailed Methods are described in the online-only Data Supplement.

**Mice**

To generate mice lacking integrin α6 in platelets, mixed-background C57BL/6×129/Sv mice containing the ITGA6 gene flanked by loxP sites (α6α6) were crossed with pure C57BL/6 transgenic animals selectively expressing Cre recombinase in the megakaryocyte lineage under control of the platelet factor 4 (PF4) promoter (PF4-Cre+). Mice expressing Cre and heterozygous for the ITGA6 recombination (PF4-Cre+/α6α6) were intercrossed to produce littermates homozygous for the floxed allele (PF4-Cre+/α6α6, hereafter named α6α6) and homozygous for the wild-type (WT) ITGA6 allele (PF4-Cre+/α6α6), serving as controls. Mice deficient in VWF (VWF−/−) or GPVI (GPVI−/−) and their WT control were of pure C57BL/6 background. All mice were anesthetized intraperitoneally with xylazine (20 mg/kg) and ketamine (100 mg/kg).

**Statistical Analyses**

All values are reported as mean±SEM. Data were compared by 1-way or 2-way repeated-measures ANOVA with Bonferroni correction or by 2-tailed Mann-Whitney tests. Differences were considered significant at P<0.05, and all tests were performed with the use of Prism software (GraphPad, La Jolla, CA).

**Results**

**Expression of Laminin Isoforms in Mouse Carotid Arteries and Evaluation of Their Ability to Support Human Platelet Adhesion Under Flow**

Use of isoform-specific antibodies allowed us to confirm previous observations that in mice, large arteries like the common carotid show strong expression of α5 and α6 laminins along the luminal edge, outlining the endothelial basement membrane (Figure 1A and 1B). In contrast, the α6 chain exhibited a weak signal, and α5 was undetectable (Figure 1C and 1D). We next explored the ability of a series of vascular laminin isoforms containing an α5, α6, or α5 chain to support platelet adhesion under hemodynamic conditions compared with LM111, VWF, and fibrinogen. Time-lapse differential interference contrast microscopy showed that, at 1000 s−1, human platelets rapidly adhered to form a monolayer on recombinant human LM411, LM511, and LM521, whereas LM211 was not adhesive (Figure 2A and 2B). After 6 minutes of perfusion, the number of adherent platelets was highest on LM521, followed by LM511 and VWF (Figure 2A and 2B). Adhesion to LM411 was intermediate between adhesion to LM111 and fibrinogen (Figure 2A and 2B). These findings highlighted that the platelet-binding properties of laminins are largely determined by their α5 and β chains. In regard to the adhesive behavior of the cells, human platelets rolled over VWF (70±5%) and rapidly detached from fibrinogen (74±6%), whereas most of them remained stationary on LM511 (83±9%) and LM521 (82±9%; n=3; Figure 2C). LM411 and LM111 presented intermediate phenotypes with 52±6% and 39±4% (n=3) stationary adherent platelets, respectively (Figure 2C). This different behavior probably explains the enhanced platelet accumulation observed on α5-containing laminins (Figure 2A).

Similar to the findings with VWF, human platelet adhesion to LM521 and LM511 increased as a function of wall shear rate, exhibiting a bell-shaped curve with maximum at 3000 s−1 for LM521 and 2000 s−1 for LM511 (Figure 2D). Adhesion to LM411 and LM111 peaked at 800 to 1000 s−1 and was inefficient at shear rates exceeding 1500 s−1, thereby resembling that on fibrinogen (Figure 2D). These findings indicated that vascular laminins containing the α5 or α6 but not the α5 chain support efficient platelet adhesion across a wide range of arterial shear rates.
Human Platelet Adhesion to LM411, LM511, and LM521 Relies on Integrin \( \alpha_6 \beta_1 \) and the GPIb-IX/VWF Axis

Integrin \( \alpha_6 \beta_1 \) has been reported to be the main receptor for LM111,10,11,17 and accordingly we found that an anti-\( \alpha_6 \) monoclonal antibody abolished human platelet adhesion to LM411, LM511, and LM521 at 1500 s\(^{-1} \) (Figure 3A through 3C). When the \( \beta_1 \) subunit was targeted with 2 different function-blocking antibodies (4B4 and P5D2), adhesion to LM411 and LM511 was likewise inhibited (Figure 3A and 3B). Surprisingly, only partial inhibition was observed for LM521 (44±13% for 4B4, \( n=5 \) and 45±8% for P5D2, \( n=4 \); \( P<0.0001 \)), pointing to a different ability of this isoform to bind \( \alpha_6 \beta_1 \) compared with LM411 and LM511 (Figure 3C). In contrast, a \( \beta_3 \) integrin antagonist had no inhibitory effect, indicating no requirement for \( \alpha_{IIb} \beta_3 \) and \( \alpha_V \beta_3 \) (Figure 3A through 3C). We next showed that the behavior of platelet adhesion in mouse was identical to that in human and confirmed that platelets from thrombasthenic mice lacking \( \alpha_{IIb} \) adhered normally to all laminins at 1500 s\(^{-1} \) (M. Schaff, PhD, unpublished data, 2012). Blocking the VWF binding site of GPIb or the VWF A1 domain markedly diminished adhesion of human platelets to LM411, LM511, and LM521, suggesting that VWF participates in platelet attachment to laminin (Figure 3A through 3C). In contrast, a \( \beta_1 \) integrin antagonist had no inhibitory effect, indicating no requirement for \( \alpha_{IIb} \beta_1 \) and \( \alpha_{V} \beta_1 \) (Figure 3A through 3C). We next showed that the behavior of platelet adhesion in mouse was identical to that in human and confirmed that platelets from thrombasthenic mice lacking \( \alpha_{IIb} \) adhered normally to all laminins at 1500 s\(^{-1} \) (M. Schaff, PhD, unpublished data, 2012). Blocking the VWF binding site of GPIb or the VWF A1 domain markedly diminished adhesion of human platelets to LM411, LM511, and LM521, suggesting that VWF participates in platelet attachment to laminin (Figure 3A through 3C). This was confirmed with blood from VWF-deficient mice, which exhibited a reduction of 63±12%, 48±4%, and 27±6% in platelet adhesion to LM411, LM511, and LM521, respectively (n=3; \( P<0.005 \); Figure 3D). Whereas the GPIb-IX/VWF axis played a pivotal role in human and mouse platelet recruitment to vascular laminins at 1500 s\(^{-1} \), this was not the case under low shear conditions (300 s\(^{-1} \); M. Schaff, PhD, unpublished data, 2012). Overall, these results suggested that integrin \( \alpha_6 \beta_1 \) and the GPIb-IX complex are important for platelet adhesion to vascular laminins.

VWF Binds Directly to LM411, LM511, and LM521

To further investigate the capacity of VWF to mediate GPIb-IX–dependent adhesion to laminins, we determined by enzyme-linked immunosorbent assay whether these molecules directly interact with each other. As shown in Figure 3E through 3H, soluble VWF bound to immobilized LM411, LM511, and LM521 but not to bovine serum albumin and vice versa. These results clearly demonstrated that VWF interacts with LM411, LM511, and LM521, thereby supporting a model in which plasmatic VWF acts as a bridge between laminins and platelet GPIb-IX. Although it did not support platelet adhesion, LM211 also interacted with VWF but only when it was immobilized, probably because of conformational changes exposing cryptic binding sites for VWF (Figure 3E and 3I).

Activation of Platelets Upon Adhesion to LM411, LM511, and LM521

Morphological change, which represents one of the earliest measurable signs of activation, has been reported to occur in platelets adhering to LM111.10,13,14 We found that, in a manner similar to that with LM111, >99% (n=3) of the human platelets attaching to LM411, LM511, and LM521 at 300 s\(^{-1} \) rapidly transitioned from the discoid and smooth resting morphology to a spherical form extending filopodia (Figure 4A and 4B). Interestingly, the number of filopodia was higher on \( \alpha_5 \) laminins, with 32±7% and 39±4% of platelets exhibiting >11 filopodia on LM511 and LM521, respectively, compared with 20±8% on LM411 and only 8±2% on LM411 (n=3; Figure 4C). To more precisely quantify the level of platelet activation induced by the different laminins, cytosolic Ca\(^{2+} \) changes were measured with the use of a dual-dye ratiometric flow-based assay. As shown
in Figure 4D, human platelets adhering to LM111 at 300 s\(^{-1}\) displayed basal Ca\(^{2+}\) levels. In contrast, the proportion of platelets exhibiting a specific signal (ie, a series of oscillatory Ca\(^{2+}\) changes with a maximal elevation of >80 nmol/L) was 30±9% on LM411, 41±8% on LM511, and 80±5% on LM521 (n=4; Figure 4D). LM521 displayed the highest responses with a mean maximal Ca\(^{2+}\) rise of 279±26 nmol/L compared with 150±23 nmol/L on LM511 and 101±20 nmol/L on LM411 (n=4; P<0.0001; Figure 4D). Altogether, these results showed that the adhesion of platelets to vascular laminins leads to their activation.

In a previous study, GPVI was found to mediate platelet shape change induced by placental laminin but did not participate in the adhesive process.\(^9\) In agreement with this, we observed that adhesion of mouse platelets deficient in GPVI was unchanged on LM111, LM411, LM511, and LM521 (Figure 4E). Remarkably, although GPVI deficiency did not affect Ca\(^{2+}\) changes induced by LM411, it reduced the mean maximal Ca\(^{2+}\) elevation by 24±6% and 42±7% on LM511 and LM521, respectively (n=4; P<0.05; Figure 4F). These results revealed that GPVI contributes to platelet activation induced by \(\alpha_\text{s}\) - but not \(\alpha_\text{v}\)-containing laminins.

**Adhesive and Activatory Properties of \(\alpha_\text{v}\)-Deficient Platelets**

To study the importance of platelet/laminin interactions in vivo, we generated a mouse strain with a platelet-specific disruption of integrin \(\alpha_\text{v}\). Deletion of \(\alpha_\text{v}\) did not affect the platelet count and expression of the major surface glycoproteins, except for integrin \(\alpha_\text{v}\), which displayed an increased expression level of 24±4% (n=5; P<0.05; Table). Adhesion of mouse platelets to the laminins was critically dependent on integrin \(\alpha_\text{v}\beta_3\), because \(\alpha_\text{v}\)-deficient platelets failed to attach to any of these surfaces (Figure 5A through 5F). In contrast,
α6β1 played no important role in platelet accumulation on other subendothelial glycoproteins such as VWF, fibrillar type-I collagen, fibrinogen, or fibronectin (Figure 6A through 6D). Interestingly, there was a statistically nonsignificant trend toward reduced adhesion of α6−/− platelets to thrombospondin-1 (TSP-1; n=8; P=0.08; Figure 6E). Blockade of α6 in human platelets decreased adhesion to TSP-1 by 38±2% (n=5; P<0.0001), thereby identifying integrin α6β1 as a potentially novel platelet receptor for TSP-1 (Figure 6F).

We next examined the ability of α6−/− platelets to become activated after stimulation with a range of agonists. As shown in Figure 6G, WT and knockout platelets displayed equivalent aggregation profiles in response to ADP, thrombin, the protease-activated receptor 4 agonist peptide AYPGKF, the thromboxane A2 analogue U46619, type-I collagen, and cross-linked collagen-related peptide (CRP-XL), a GPVI-selective ligand. Soluble fibrinogen binding induced by ADP, thrombin, or AYPGKF was also normal in α6−/− null compared with control platelets.
Figure 4. Laminin-411 (LM411), LM511, and LM521 induce platelet shape change and increases in cytosolic Ca\(^{2+}\), and glycoprotein (GP) VI participates in this response on α\(_5\) laminins. A through C, Hirudinated human blood was perfused for 3 minutes at 300 s\(^{-1}\) through microcapillaries coated with 100 µg/mL LM111, LM211, LM411, LM511, or LM521. After a washing step with phosphate-buffered saline for 5 minutes at 300 s\(^{-1}\), adherent platelets were fixed and examined by scanning electron microscopy. A, Representative microscopy images are shown. Bar, 10 µm. B and C, The number of platelets extending filopodia (B) and the number of filopodia per platelet (C) were quantified in 5 random fields in 3 separate experiments and expressed as percentage of total adherent cells. D, Washed human platelets loaded with morphological and Ca\(^{2+}\) dyes were reconstituted with 50% (vol/vol) autologous packed red blood cells at a final concentration of 2.5×10\(^8\) platelets per milliliter and perfused over LM111, LM411, LM511, or LM521 at 300 s\(^{-1}\). Changes in fluorescence were monitored for 5 minutes by confocal microscopy, and cytosolic Ca\(^{2+}\) concentrations were determined. The dot plot distribution of the maximal increase relative to basal state in individual adherent platelets is shown (n=80 from 4 independent experiments). Resting refers to washed platelets allowed to settle onto phosphate-buffered saline/10 mg/mL human serum albumin–coated coverslips. Mean values are indicated by horizontal bars. E, Hirudinated blood from wild-type (WT) and glycoprotein (GP) VI\(^{-/-}\) mice was perfused over laminins at 300 s\(^{-1}\), and adherent platelets were counted in 1 random field in 3 to 6 separate experiments. F, Washed platelets from WT and GPVI\(^{-/-}\) mice loaded with morphological and Ca\(^{2+}\) dyes were reconstituted with red blood cells and perfused over LM411, LM511, or LM521 at 300 s\(^{-1}\), and cytosolic Ca\(^{2+}\) concentrations were measured, as in D. Bars represent the mean maximal increase relative to basal state (n=80 platelets from 4 independent experiments). Data were compared by 1-way (D) or 2-way repeated measures (E) ANOVA with Bonferroni correction or by 2-tailed Mann-Whitney tests (F). *P<0.05, ***P<0.0001.
P-selectin surface exposure, a marker of granule secretion, was not altered after thrombin or AYPGKF stimulation (Figure 6I). Finally, thrombin- and convulxin-induced phosphatidylserine exposure, which mediates platelet procoagulant activity, was unchanged in α₆⁻/⁻ platelets (Figure 6J). Altogether, these results indicated that integrin α₆β₁ plays no major role in the activation of platelets by a wide range of agonists.

### α₆⁻/⁻ Mice Are Protected in 3 Models of Arterial Thrombosis

Mice lacking platelet α₆β₁ were subjected to 3 models of arterial thrombosis induced by endothelial denudation without exposure of the outer vessel wall layers (Figure IA and IB in the online-only Data Supplement). In all 3 models, thrombosis was sensitive to platelet P2Y₁₂ and α₂β₃ inhibition, indicating critical requirement for platelet activation (Figure IC and ID in the online-only Data Supplement). When the common carotid artery was injured with a guidewire, α₆ deficiency led to a 69±10% (n=6; P<0.05) reduction in total thrombus surface area compared with WT mice (Figure 7A through 7C). After compression of the abdominal aorta with forceps, the thrombus area in α₆⁻/⁻ mice was likewise reduced by 56±12% (n=5; P<0.05; Figure 7D through 7F). Interestingly, mice bearing a platelet-restricted deletion of integrin β₁ exhibited similar degrees of inhibition in both models (Figure II in the online-only Data Supplement). A more pronounced decrease in thrombosis of 82±3% (n=12 vessels in 3 WT or α₆⁻/⁻ mice; P<0.0001) was found in a model of moderate laser injury of mesenteric arterioles (Figure 7G through 7I). In contrast, α₆⁻/⁻ mice were not protected from thrombosis after a deeper laser lesion exposing the media and adventitia (Figure III in the

### Platelet Count and Expression of Major Surface Glycoproteins in Wild-Type and α₆⁻/⁻ Mice

<table>
<thead>
<tr>
<th>Glycoprotein</th>
<th>Wild-Type</th>
<th>α₆⁻/⁻</th>
<th>No. of Mice per Group</th>
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<tr>
<td>GPIb/IIa (α₂β₃)</td>
<td>4.29±0.11</td>
<td>4.46±0.13</td>
<td>8</td>
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<tr>
<td>GPIa (α₂)</td>
<td>1.46±0.02</td>
<td>1.57±0.11</td>
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<tr>
<td>GPIc (α₅)</td>
<td>0.81±0.07</td>
<td>0.84±0.07</td>
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<tr>
<td>GPIc' (α₆)</td>
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</tr>
<tr>
<td>GPV</td>
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<td>0.015±0.005*</td>
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</tr>
<tr>
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<td>GPID</td>
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<tr>
<td>GPIIb</td>
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<td>8</td>
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<tr>
<td>GPIbα</td>
<td>1.96±0.04</td>
<td>2.08±0.04</td>
<td>8</td>
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<tr>
<td>Platelet count</td>
<td>1029±49×10⁶/mL</td>
<td>974±44×10⁶/mL</td>
<td>19</td>
</tr>
</tbody>
</table>

Platelet counts were determined using an animal blood cell counter and represent the mean±SEM. The surface expression of the indicated glycoproteins (GP) was determined in whole blood by staining with selective antibodies, followed by flow cytometric analysis. Results are expressed as the geometric mean±SEM of the relative fluorescence intensity, in arbitrary units. All data were compared by 2-tailed Mann-Whitney tests.

*P<0.05.

Figure 5. Role of α₂β₁ in the adhesion of mouse platelets to LM411, LM511, and LM521 under flow. Hirudinated blood from wild-type (WT) and α₆⁻/⁻ mice was perfused at 2000 s⁻¹ through microcapillaries coated with 100 µg/mL laminin-411 (LM411) (A, B), LM511 (C, D), or LM521 (E, F). A, C, and E, Representative differential interference contrast microscopy images depicting platelets adhering after 3 minutes of perfusion. The arrow indicates the direction of blood flow. Bar, 20 µm. Data were compared by 2-way repeated measures ANOVA with Bonferroni correction (A, C, and E).***P<0.0001.
Figure 6. Role of $\alpha_6\beta_1$ in the adhesion of platelets to other subendothelial glycoproteins and in platelet activation in response to various agonists. A through E, Hirudinated blood from wild-type (WT) and $\alpha_6^{-/-}$ mice was perfused through microcapillaries coated with von Willebrand factor (VWF; 20 $\mu$g/mL; A), collagen (200 $\mu$g/mL; B), fibrinogen (FGN; 300 $\mu$g/mL; C), fibronectin (300 $\mu$g/mL; D), or thrombospondin-1 (TSP-1; 60 $\mu$g/mL; E) at a wall shear rate supporting optimal platelet adhesion: 1500 s$^{-1}$ (VWF and collagen) or 300 s$^{-1}$ (fibrinogen, fibronectin, and TSP-1). The time course of platelet adhesion was determined in 1 random field by differential interference contrast microscopy except for thrombus formation on collagen, which was monitored by confocal microscopy with the use of blood in which the platelets had been stained with an Alexa Fluor 488–coupled anti-glycoprotein Ib$\beta$ antibody. F, Hirudinated human blood was incubated for 10 minutes with irrelevant rat IgG2a (control) or a blocking monoclonal antibody against $\alpha_6\beta_1$ (GoH3, both 10 $\mu$g/mL) and perfused over TSP-1 (60 $\mu$g/mL) at 300 s$^{-1}$. After 10 minutes, the number of adherent platelets was counted in 8 random fields. A through F, Data are the mean±SEM in 3 to 8 independent experiments. G, Washed platelets from WT and $\alpha_6^{-/-}$ mice were aggregated by a range of agonists in the presence (ADP, AYPGKF, U46619, collagen, and cross-linked collagen-related peptide [CRP-XL]) or absence (thrombin) of fibrinogen (64 $\mu$g/mL). Arrows indicate the point of agonist addition, and aggregation profiles are representative of 3 separate experiments. H and I, WT and $\alpha_6^{-/-}$ washed platelets were stimulated with various agonists in the presence of Alexa Fluor 488/fibrinogen (H) or a fluorescein isothiocyanate/anti-P-selectin antibody (I). After 20 (H) or 10 (I) minutes, the samples were fixed, and the extent of fibrinogen binding or P-selectin exposure was measured by flow cytometry. Results represent the geometric mean±SEM of the relative fluorescence (fluor.) intensity in arbitrary units, in 3 separate experiments performed in duplicate (10 000 platelets analyzed per experiment). J, WT and $\alpha_6^{-/-}$ washed platelets were stimulated with the indicated agonists for 10 minutes, incubated with fluorescein isothiocyanate/annexin V for 20 minutes, and analyzed by flow cytometry. The forward light scatter and fluorescence intensity of 10 000 cells were collected with a logarithmic gain, and the percentage of annexin V–positive platelets was determined in the upper right quadrant. Data are the mean±SEM in 3 separate experiments performed in duplicate. Results were compared by 2-way repeated measures ANOVA with Bonferroni correction (A through E) or by 2-tailed Mann-Whitney tests (F and H through J). ***P<0.0001.
online-only Data Supplement), consistent with the fact that no laminin expression has been observed in these outer layers of the mesenteric arterial wall.21

\( \alpha_6^{-/-} \) Mice Display Normal Tail Bleeding

To establish whether the decreased thrombosis observed in \( \alpha_6^{-/-} \) mice is mirrored by defective hemostasis, tail-bleeding experiments were performed. The time required for first arrest of bleeding was normal in most of the \( \alpha_6 \)-null (median, 132 seconds; n=19) compared with WT (median, 123 seconds; n=19) mice (Figure 8A). In addition, the total bleeding time (Figure 8B) and volume of blood lost (Figure 8C) over 30 minutes were unchanged. Overall, these results suggested a minor contribution of \( \alpha_6 \beta_1 \) to primary hemostasis.
Discussion

Although laminins are major constituents of endothelial basement membranes, well located to interact with platelets upon vascular damage, their role in platelet functions has remained ill defined. The studies reported herein demonstrate that (1) various laminin isoforms expressed in the vascular wall efficiently support human platelet adhesion and activation under arterial shear conditions; (2) integrin α5β1, the GP Ib-IX/VWF axis, and, depending on the type of laminin, GP VI synergistically mediate these processes; and (3) integrin α5β2 deficiency provides protection against thrombosis in several experimental models.

We confirmed that platelets can adhere to recombinant human LM111 over a range of hemodynamic conditions, as reported for LM111 extracted from mouse sarcomas. However, platelets are unlikely to encounter this isoform in vivo because the α5 chain is absent from most blood vessels and in particular from large arteries such as the carotid. We obtained evidence that platelets can adhere to recombinant α1β1, the GP Ib-IX/VWF axis, and, depending on the type of laminin, GP VI synergistically mediate these processes; and (3) integrin α5β2 deficiency provides protection against thrombosis in several experimental models.

LM411 exhibited adhesive properties very similar to those of LM111, whereas LM211 did not allow platelet recruitment. In contrast, LM511 supported much higher platelet adhesion under flow, in accordance with previous results obtained under static conditions. Because LM111, LM211, LM411, and LM511 share the same β1 and γ1 chains, our results point to a crucial role of the α subunit in supporting platelet adhesion. This concurs with the fact that α chains carry in their COOH-terminal globular modules the major domains interacting with cellular receptors, including integrin α5β1. A stronger adhesiveness of αγ relative to αγ and α-containing laminins has already been reported for epithelial, endothelial, and hematopoietic stem cells and agrees with the higher affinity of the α5 chain for α5β1. Recently, laminin-3B11 (α5β1γ1, LM3B11) was identified as a novel vascular laminin predominantly expressed in capillaries and small veins. The α5 chain has the highest homology to α5 but whether LM3B11 allows platelet adhesion remains to be established.

Interestingly, LM521 supported more platelet adhesion than LM511, indicating that the β1 chain, which has been proposed to contain integrin-binding sites, also contributes to this process. One could hypothesize that β1 laminin has a higher affinity for α5β1 than β1 laminin. Alternatively, β2 could maintain the α5 chain in a more appropriate conformation for α5β2 binding, consistent with the fact that the α5 monomers of laminins require heterotrimerization with β and γ subunits to fully exert their adhesiveness.

Besides integrin α5β1, platelet adhesion to LM411, LM511, and LM521 depended on the GP Ib-IX complex, requiring the prior binding of plasmatic VWF to laminins. This mechanism is reminiscent of that documented for platelet adhesion to collagen, fibrin, fibronec-tin, and tenascin-C to which VWF adsorbs, ensuring platelet capture through GP Ib-IX, especially under elevated flow conditions. The interaction of VWF with LM411, LM511, and LM521 could account, at least partly, for their ability to support platelet adhesion at high wall shear rates (>1000 s⁻¹). Despite the fact that LM211 also bound to VWF, it remained unable to support platelet adhesion, presumably because of the low affinity of the α5 laminin chain for integrin α5β2.

Integrin α5β2 was dispensable for adhesion to LM411, LM511, or LM521, confirming previous results obtained with LM111. Although α5β1, the second platelet β1 integrin, mediates adhesion of endothelial and mural cells to laminins through RGD sequences present in the short arm of the α5 chain, it was not critical in platelet/laminin interactions. This could be attributable to its low expression (100–500 receptors per platelet) compared with α5β2 (2000–4000 copies per platelet). Among nonintegrin laminin receptors, platelets express GP VI, α-dystroglycan, and the molecule 67 LR, which has been reported to mediate their adhesion to LM111. Adhesion of mouse platelets deficient in GP VI was unchanged on LM411, LM511, and LM521, in agreement with a previous study. Moreover, neither the antibody IIE6C4 blocking α-dystroglycan nor the synthetic peptide YIGSR against 67 LR inhibited human platelet adhesion to vascular laminins (M. Schaff, PhD, unpublished data, 2012). Overall, α5β2 and GP Ib-IX would thus appear to be the main platelet receptors for laminins. We propose a working model whereby upon endothelial denudation, exposed LM411, LM511, and LM521 adsorb plasmatic VWF, which in turns recruits circulating platelets through GP Ib-IX. Stable adhesion is then
established by direct binding to $\alpha_i\beta_1$, allowing the platelets to efficiently withstand shear stress.

Thus far, laminins were considered to be the only subendothelial glycoproteins capable of binding platelet $\alpha_i\beta_1$. Our results suggest that TSP-1 could also be a ligand for this integrin. A direct interaction between $\alpha_i\beta_1$ and TSP-1 was demonstrated previously in microvascular endothelial and fibrosarcoma cells. Blockade of $\alpha_i\beta_1$ only partially inhibited platelet adhesion to TSP-1, consistent with the presence of other receptors mediating this interaction, namely, CD36, CD47, and GPIb. Platelets adhering to LM411, LM511, and LM521 became activated, as indicated by their cytosolic Ca\textsuperscript{2+} oscillations and shape change, suggesting that the role of these proteins goes beyond their ability to recruit platelets. Interestingly, Ca\textsuperscript{2+} levels and the number of filopodia increased as a function of lamina adhesiveness and were highest on LM511 and LM521 as the result of their binding to GPVI. This agrees with a previous study demonstrating direct interaction between GPVI and placent lamina, which is enriched in $\alpha_i$-containing isoforms. Our results that GPVI deficiency did not affect Ca\textsuperscript{2+} changes downstream of integrins $\alpha_i\beta_1$ and $\alpha_i\beta_1$, also suggest that integrin $\alpha_i\beta_1$ participates in platelet activation. This is supported by the fact that Ca\textsuperscript{2+} fluxes were measured with the use of reconstituted blood without plasmatic VWF and under low shear conditions in which $\alpha_i\beta_1$ is critical in adhesion and the role of secreted VWF is unlikely. Moreover, platelet $\alpha_i\beta_1$ has been proposed to activate spleen tyrosine kinase and phospholipase $\text{Cy}2$, 2 enzymes important for both Ca\textsuperscript{2+} mobilization and cytoskeletal reorganization downstream of integrins $\alpha_i\beta_1$ and $\alpha_i\beta_1$. Until now, no indication of an in vivo role of platelet $\alpha_i\beta_1$ has been reported. This study highlights a major contribution of this integrin to arterial thrombus formation in 3 models of mechanical and laser denudation of the endothelium. Interestingly, in the 2 mechanical injury models, the reduction of thrombus formation in $\alpha_i\beta_1$ mice was very similar to that in $\beta_1$ mice, suggesting that among the $\beta_1$ integrins expressed at the platelet surface, $\alpha_i\beta_1$ might be particularly important. Our results indicate that laminins and, to a lesser extent, TSP-1 significantly contribute to platelet adhesion and thrombus formation. In contrast, after FeCl\textsubscript{3} injury of the common carotid artery, $\alpha_i\beta_1$ mice were not protected against thrombosis (C. Bourdon, BSc, unpublished data, 2012). This observation is in agreement with our previous results that deletion of integrin $\beta_1$ does not affect thrombosis in this model, probably because of the fact that ferric ion–filled bodies denature the subendothelial adhesive proteins. In addition, $\alpha_i\beta_1$ animals were not protected against thrombosis after a deep laser injury of a mesenteric arteriole resulting in exposure of the media and adventitia, which do not contain any laminin. Although small amounts of laminins located at the edge of the lesion could be exposed to the flowing blood in this model, the lack of effect suggests a minor contribution compared with other subendothelial proteins such as collagen or tissue factor. Overall, our findings point to an important role of $\alpha_i\beta_1$ in experimental thrombosis depending on the type and severity of injury.

Pharmacologically, targeting integrin $\alpha_i\beta_1$ may represent a potential antithrombotic strategy. Anti-$\alpha_i\beta_1$ agents could reduce the initial step of platelet adhesion and activation to an injured vessel and thereby prevent excessive thrombus growth and vessel occlusion. Moreover, although bleeding in patients is multifactorial, the normal tail bleeding times and volume of blood lost in $\alpha_i\beta_1$ mice suggest that such a therapy could have a lower bleeding risk than currently available drugs.

In conclusion, $\alpha_i$- and $\alpha_i$-containing laminins, which are widely expressed in the vasculature, were found to efficiently support platelet adhesion and activation across a wide range of arterial shear rates by direct binding to integrin $\alpha_i\beta_1$, or GPVI and indirect engagement of the GPIb-IX complex through plasmatic VWF bridging. Using mice with a genetic deletion of $\alpha_i\beta_1$, we obtained the first in vivo evidence that platelet/laminin interactions are of importance during thrombus formation after superficial arterial injury while having no impact on hemostasis. The profound antithrombotic protection achieved in 3 distinct thrombosis models suggests that $\alpha_i\beta_1$ could represent a potential antithrombotic target. Because platelets also participate in embryonic development, immunity, and diseases such as atherogenesis and tumor malignancy, further studies are required to evaluate the importance of platelet/laminin interactions in these processes.

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Disclosures

None.

References


CLINICAL PERSPECTIVE

The central role of platelets in arterial thrombosis renders them attractive targets for antithrombotic drugs. Current antiplatelet agents such as P2Y12 receptor antagonists and integrin αIIbβ3 blockers target platelet activation and aggregation, thereby considerably reducing the morbidity and mortality associated with ischemic events, especially myocardial infarction. However, this strategy results in an increased risk of hemorrhagic complications, particularly in stroke patients and in those undergoing coronary bypass grafting, endarterectomy, or neurosurgery. Experimental and clinical evidence has suggested that the inhibition of platelet adhesion to glycoproteins of the arterial wall such as von Willebrand factor and collagen could pave the way to safer therapies causing minimal perturbation of hemostasis. In the present study, we investigated the relevance of targeting the interaction of platelets with subendothelial laminins. Using an in vitro blood perfusion assay, we showed that integrin αIIbβ3 plays a critical role in platelet adhesion to laminins with an α5 or α6 chain, which are the major isoforms expressed in arteries. Compared with wild-type mice, those with a platelet-specific deletion of integrin α5 presented a marked decrease in thrombosis in 3 models of mechanically- or laser-induced injury of the carotid artery, aorta, and mesenteric arterioles. In contrast, the tail bleeding time and volume of blood lost remained unchanged in α5-deficient mice, suggesting normal hemostasis. In conclusion, this study provides the first evidence that inhibiting platelet integrin αIIbβ3 could represent an alternative antithrombotic strategy with a potentially low bleeding risk.
Integrin $\alpha_6\beta_1$ Is the Main Receptor for Vascular Laminins and Plays a Role in Platelet Adhesion, Activation, and Arterial Thrombosis

Mathieu Schaff, ChaoJun Tang, Eric Maurer, Catherine Bourdon, Nicolas Receveur, Anita Eckly, Béatrice Hechler, Christiane Arnold, Adèle de Arcangelis, Bernhard Nieswandt, Cécile V. Denis, Olivier Lefebvre, Elisabeth Georges-Labouesse, Christian Gachet, François Lanza and Pierre H. Mangin

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Integrin α6β1 is the main receptor for vascular laminins and plays a role in platelet adhesion, activation and arterial thrombosis

Mathieu Schaff, PhD1*; ChaoJun Tang, PhD1*; Eric Maurer, MPhil1; Catherine Bourdon, BSc1; Nicolas Receveur, BSc1; Anita Eckly, PhD1; Béatrice Hechler, PhD1; Christiane Arnold, BSc2; Adèle de Arcangelis, PhD3; Bernhard Nieswandt, PhD4; Cécile V Denis, PhD5; Olivier Lefebvre, PhD2; Elisabeth Georges-Labouesse, PhD3; Christian Gachet, MD, PhD1; François Lanza, PhD1; Pierre H Mangin, PhD1

1UMR_S949, INSERM, Université de Strasbourg (UdS), Etablissement Français du Sang-Alsace, Strasbourg, F-67065, France, 2UMR_S1109, INSERM, UdS, Strasbourg, F-67200, France, 3UMR_7104, CNRS, UMR_S964, INSERM, UdS, Institut de Génétique et de Biologie Moléculaire et Cellulaire (IGBMC), Illkirch-Graffenstaden, F-67404, France, 4Rudolf Virchow Center, University of Würzburg, Würzburg, D-97080, Germany, 5UMR_S770, INSERM, Université Paris-Sud, Le Kremlin-Bicêtre, F-94270, France

* These authors contributed equally to this work.

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Materials

Alexa Fluor 488-labeled fibrinogen (FGN), DIOC₆ (3,3’-dihexyloxacarbocyanine iodide), DAPI (4’,6-diamidino-2-phenylindole dihydrochloride), Oregon Green 488 BAPTA-1/AM (1,2-bis(2-aminophenoxy)ethane-N,N,N’,N’-tetraacetic acid/tetra(acetoxyethyl) ester) and Calcein Red-Orange/AM were from Molecular Probes (Paisley, UK), fluorescein isothiocyanate (FITC)-coupled anti-P-selectin antibody (RB40.34) was from BD Pharamingen (Le Pont-De-Claix, France) and FITC-conjugated annexin V was from Roche Diagnostics (Meylan, France). Fatty acid-free human serum albumin (HSA), bovine serum albumin (BSA), human FGN, bovine thrombin, U46619 and adenosine 5’-diphosphate (ADP) were provided by Sigma-Aldrich (Lyon, France). Cross-linked collagen-related peptide (CRP-XL) was obtained from Dr. R.W. Farndale (University of Cambridge, Cambridge, UK) and convulxin from Dr. M. Jandrot-Perrus (INSERM U698, Paris, France). Horm fibrillar type-I collagen from equine Achilles tendon for aggregation assays was purchased from Nycomed (Zurich, Switzerland), while the protease-activated receptor 4-selective agonist AYPGKF peptide was synthesized by PolyPeptide Group (Strasbourg, France). Apyrase was purified from potatoes as previously described.¹ Glutaraldehyde was from Electron Microscopy Sciences (Euromedex, Souffelweyersheim, France), Fluorsave reagent was from Millipore (Molsheim, France) and optimal cutting temperature (OCT) compound (Shandon Cryomatrix) and O-phenylenediamine were from Thermo Scientific (Cergy-Pontoise, France). A number of adhesive proteins were isolated in our laboratory according to published methods: VWF from human factor VIII concentrates,² thrombospondin-1 (TSP-1) from washed human platelets,³ fibronectin from human plasma⁴ and acid-soluble fibrillar type-I collagen (ASC) from bovine Achilles tendon (Sigma-Aldrich).⁵ Recombinant human laminins were purchased
from Biolamina (Stockholm, Sweden) and checked for purity by LC-MS/MS spectrometry. Recombinant hirudin was from Transgene (Illkirch-Graffenstaden, France), clopidogrel from Sanofi (Toulouse, France), eptifibatide from Schering-Plough (Kenilworth, NJ) and acid citrate dextrose (ACD) solution from Bioluz (St-Jean-de-Luz, France).

**Antibodies**

Azide-free blocking monoclonal antibodies against human platelet receptors were as follows: anti-integrin $\beta_1$, 4B4 (Beckman Coulter, Villepinte, France) and P5D2 (Santa Cruz Biotechnology, Tebu-bio, Le Perray-en-Yvelines, France); anti-integrin $\alpha_6$, GoH3 (Santa Cruz Biotechnology); anti-integrins $\alpha_{Ib}\beta_3$ and $\alpha_{V}\beta_3$, chimeric 7E3 Fab fragment abciximab (E. Lilly, Indianapolis, IN); anti-GPlb$\alpha$, AK2 (GeneTex, Euromedex); anti-VWF A1 domain, clone 701, kindly provided by Dr. C.V. Denis. Other antibodies used were: mouse IgG$_1\kappa$ and rat IgG$_{2\alpha}\kappa$ isotype controls (Biolegend, Ozyme, St-Quentin-en-Yvelines, France), horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Beckman Coulter), HRP-conjugated anti-VWF (Dako, Glostrup, Denmark), anti-laminin $\alpha_2$, clone 5H2 (Millipore), anti-laminin $\alpha_4$, clone 3H2 (Santa Cruz Biotechnology), anti-laminin $\alpha_5$, clone 4C7 (Millipore) and Cy3-labeled goat anti-rat or -rabbit IgG (Jackson ImmunoResearch, Newmarket, UK). For immunohistofluorescence, highly-selective primary antibodies against laminins $\alpha_1$ (clone 200),$^6,7$ $\alpha_2$ (clone 401),$^8$ $\alpha_4$ (clone 377)$^8$ and $\alpha_5$ (clone 405)$^8$ were kind gifts from Dr. Lydia Sorokin (University of Münster, Münster, Germany).

**Mice**
To generate mice lacking integrin α6 in platelets, mixed background C57BL/6 x 129/Sv mice containing the ITGA6 gene flanked by loxP sites (α6^fl/fl) (Dr. E. Georges-Labouesse)^9,10 were crossed with pure C57BL/6 transgenic animals selectively expressing Cre recombinase in the megakaryocyte lineage under control of the platelet factor 4 (PF4) promoter (PF4-Cre^+^) (Pr. R. Skoda, University Hospital Basel, Basel, Switzerland).^11 Mice expressing Cre and heterozygous for the ITGA6 recombination (PF4-Cre^+/α6^+/-^) were intercrossed to produce littermate mice homozygous for the floxed allele (PF4-Cre^+^/α6^+/-^, hereafter named α6^-/-^) and homozygous for the WT ITGA6 allele (PF4-Cre^+^/α6^+/-^), serving as controls. α6^-/-^ mice were viable, fertile and had no gross abnormality. β1^fl/fl^ (Dr. R. Fässler, Max Planck Institute, Martinsried, Germany),^12 VWF^-/-^ (Dr. C.V. Denis)^13, GPVI^-/-^ (Pr. B. Nieswandt)^14 and αIIb^-/-^ (Dr. W. Vainchenker, INSERM U1009, Villejuif, France)^15 mice were backcrossed for at least 12 generations on C57BL/6 background. Subsequently, the β1^fl/fl^ mice were mated with PF4-Cre^+^ animals as described above. C57BL/6 WT mice were obtained from Charles River (L’Arbresle, France). All mice were fed standard mouse chow containing 3% fat (Safe, Augy, France) and maintained in the animal facilities of the Etablissement Français du Sang-Alsace. Unless otherwise indicated, we used 8-10-week-old mice, anesthetized intraperitoneally with a mixture of xylazine (20 mg/kg, Rompun®, Bayer, Leverkusen, Germany) and ketamine (100 mg/kg, Imalgene 1,000®, Merial, Lyon, France). Ethics approval for animal experiments was received from the French Ministry of Research in accordance with the guidelines of the European Union and the Guide for the Care and Use of Laboratory Animals.

Immunohistofluorescence

One-year-old male WT mice were anesthetized and perfused with PBS by cardiac puncture in the left ventricle using a 5 mL syringe with a 25-gauge needle. An incision in the right
atrium allowed removal of blood. The carotid arteries were then excised and immediately snap-frozen in OCT embedding compound. Transverse sections of the carotids (8 µm thick) were cut with a cryostat (Leica CM3050 S, Leica Microsystems, Wetzlar, Germany), collected onto Superfrost/plus glass slides (VWR, Fontenay-sous-Bois, France), air-dried and stored at -80°C. For immunostaining, frozen sections were left to dry at room temperature and incubated overnight at 4°C in a moist chamber with antibodies against laminin α1 (clone 200; 1:100), α2 (clone 401; 1:1,000), α4 (clone 377; 1:500) or α5 (clone 405; 1:500, all in PBS), as previously performed.6-8 The tissues were then washed three times in PBS and incubated for 1 h at room temperature with Cy3-coupled goat anti-rat or -rabbit IgG secondary antibodies (1 µg/mL in PBS). After washing, nuclei were stained with DAPI (0.2 µg/mL in PBS) and the slides were mounted in Fluorsave reagent. Control sections were processed as above with omission of the primary antibody. Observations were made using a Provis AX60 epifluorescence microscope equipped with a 40x, 0.75 numerical aperture air objective and an F-view digital camera (Olympus, Hamburg, Germany). Images were acquired and analyzed using DP-Soft software (Olympus). Minimal background was observed in control sections.

**In Vitro Flow-Based Adhesion Assay**

Flow experiments were performed as previously described.16 Briefly, glass microcapillaries were coated with purified proteins overnight at 4°C and blocked with PBS-10 mg/mL HSA for 30 min at room temperature. In preliminary experiments we determined the lowest concentrations of proteins providing maximal platelet adhesion. Hirudinated (100 U/mL) whole blood from healthy human volunteers or drawn from the abdominal aorta of mice was perfused through the coated capillaries with a syringe pump (Harvard Apparatus, Holliston, MA) at 37°C and various flow rates. Platelet adhesion was monitored in real time (15 images/s) by differential interference contrast (DIC) microscopy (Leica DMI4000B) using a
40x, 1.25 numerical aperture oil objective and a Photometrics CoolSNAP HQ Monochrome charge-coupled device (CCD) camera (Roper Scientific, Evry, France). Images were acquired and analyzed with Metamorph software (Molecular Devices, Roper Scientific). The adhesive behavior of platelets was examined frame by frame and classified as (i) rolling when the cells moved constantly over the surface, (ii) stationary adhesion when they did not move more than one half of a single cell diameter over a 10 s period, and (iii) detaching when detachment occurred within 2 s following the initial contact. Thrombus formation on ASC collagen was studied by perfusing whole blood in which the platelets had been labeled by incubation for 15 min at 37°C with an Alexa Fluor 488-conjugated monoclonal antibody against GPIbβ (RAM.1, 5 µg/mL, produced in our laboratory\textsuperscript{17}). Fluorescence emission was measured in the range 490-595 nm after excitation with a 488 nm argon-ion laser using a confocal Leica SP5 inverted microscope with a resonant scanner and a 40x oil objective. Series of optical sections in xyzt were taken from the base to the peak of the thrombi (Leica LASAF software). Images were then stacked and the volume of the thrombi was determined with ImageJ (National Institutes of Health, Bethesda, MD).

\textit{In Vitro} Binding Assays

Binding assays were performed according to a published method.\textsuperscript{16} Briefly, 96-well ELISA plates were incubated overnight at 4°C with PBS-20 mg/mL BSA or with carbonate buffer (pH 9.6) containing 2 µg/mL laminin or 5 µg/mL VWF. After blocking with PBS-20 mg/mL BSA for 1h at 37°C, increasing concentrations of VWF or laminin in blocking solution (1:1,000 Tween 20, 1 mg/mL BSA, 0.5 mmol/L MgCl\textsubscript{2} and 1 mmol/L CaCl\textsubscript{2} in PBS) were added to the coated wells for 2 h at 37°C. To detect bound VWF, an HRP-conjugated anti-VWF antibody (0.4 µg/mL in blocking solution) was added for 1 h at room temperature. Bound LM211, LM411, LM511 and LM521 were detected using antibodies against the α₂
(5H2; 1:10,000), α₄ (3H2; 2 µg/mL) or α₅ (4C7; 1:10,000) chain, followed by an HRP-coupled goat anti-mouse IgG antibody (0.4 µg/mL, all in blocking solution and incubated for 1 h at room temperature). O-phenylenediamine was then added for 5 min, after which the reaction was discontinued with 50 µL of 3 mol/L H₂SO₄ and the plates were read at 490 nm.

**Scanning Electron Microscopy**

Adherent platelets were fixed for 45 min with 25 mg/mL glutaraldehyde in 0.1 mol/L cacodylate buffer containing 20 mg/mL sucrose (305 mOsm, pH 7.3). Scanning electron microscopy was performed as described elsewhere.¹⁸

**Analysis of Cytosolic Ca²⁺ Fluxes**

Intraplatelet Ca²⁺ changes upon adhesion to laminin were measured using our published dual-dye ratiometric method.¹⁶ Briefly, washed human or mouse platelets were simultaneously loaded with morphological (Calcein Red-Orange/AM, 4 µmol/L) and Ca²⁺ (Oregon Green 488 BAPTA-1/AM, 5 µmol/L) dyes for 40 min at 37°C. After a washing step, the platelets were resuspended in Tyrode’s albumin buffer containing 0.02 U/mL apyrase, and reconstituted with 50% (v/v) autologous packed red blood cells at 2.5x10⁸ platelets/mL just prior to perfusion over the laminins. Fluorescence measurements were performed under a confocal Leica SP5 inverted microscope equipped with a resonant scanner and a 63x, 1.4 numerical aperture oil objective. The fluorescence emission of Calcein Red-Orange/AM was measured in the range 572-700 nm after excitation with a 543 nm helium/neon laser, while Ca²⁺-dependent fluorescence was measured in the range 495-535 nm after excitation with a 488 nm argon-ion laser. The ratio of signal intensity between the two dye channels was then converted to cytosolic Ca²⁺ concentrations using Leica LASAF software. A specific Ca²⁺
response was defined as any increase of more than 80 nmol/L, which corresponds to twice the mean maximal elevation in platelets allowed to settle onto non-reactive PBS-10 mg/mL HSA-coated coverslips under static conditions (n=80 in 4 independent experiments).

**Preparation of Washed Platelets and Measurement of Platelet Aggregation, Soluble FGN Binding and Exposure of P-selectin and Phosphatidylserine**

Blood drawn into ACD anticoagulant from the abdominal aorta of 2 to 4 mice was pooled and platelets were washed by sequential centrifugations and adjusted to 3x10^8 cells/mL in Tyrode’s albumin buffer containing 0.02 U/mL apyrase. Washed platelet aggregation was measured turbidimetrically in a 4-channel CARAT TX4 aggregometer (Entec, Ilmenau, Germany). Agonist-induced binding of soluble FGN and exposure of P-selectin were determined as previously published. Phosphatidylserine exposure was quantified by FITC-annexin V binding.

**Injury of the Carotid Artery with a Guidewire**

The carotid arteries of anesthetized mice aged 50-60 days were exposed. The right common and internal carotids were clamped and the external carotid was ligated with a 9-0 silk suture. An arteriotomy was then made in the external carotid proximal to the silk suture and an angioplasty guidewire (HI-TORQUE CROSS-IT 100XT®, Abbott Vascular, Santa Clara, CA) was introduced and advanced to 3 mm proximal to the carotid bifurcation. The common carotid was injured by moving the wire back and forth 20 times. After removal of the wire, the external carotid was ligated proximal to the arteriotomy. The clamps were released to re-establish blood flow from the common to the internal carotid and thrombus formation was monitored.
Injury of the Aorta with Forceps

The abdominal aorta of anesthetized male mice aged 50-60 days was exposed and mechanically injured by pinching it with forceps (type 11063-07, F.S.T., Heidelberg, Germany) for 15 s. After removal of the forceps, thrombus formation was monitored.

Recording of Thrombus Formation after Mechanical Injury

In both the above models, platelets were labeled prior to injury by injection of DIOC₆, a membrane fluorescent dye, into the exposed left jugular vein (5 µL of a 100 µmol/L solution per g of body weight). Thrombus formation was monitored in real time (1 image/s) under a fluorescent macroscope (MacroFluo, Leica Microsystems) equipped with a 5x, 0.5 numerical aperture objective and a CCD camera (CoolSNAP HQ2, Photometrics, Roper Scientific). Image acquisition and analysis were performed with Metamorph.

Transmission Electron Microscopy (TEM) Analysis of Injured Arteries

Intact and mechanically-injured arteries of C57BL/6 WT mice were fixed by transcardial perfusion with 25 mg/mL glutaraldehyde in 0.1 mol/L sodium cacodylate buffer containing 20 mg/mL sucrose (305 mOsm, pH 7.3). The arteries were then excised, post-fixed overnight at 4°C and processed for TEM as described elsewhere. TEM indicated that both types of lesion caused uniform and complete denudation of the endothelial cell layer, leading to platelet adhesion to the exposed subendothelium, while the internal elastic lamina and media kept intact appearance (Supplemental Figure IA and IB).
Laser-Induced Thrombosis in a Mesenteric Arteriole

Thrombosis was performed in 27-28-day-old mice as previously described. Briefly, platelets were labeled by injection of DIOC₆ and a localized superficial or deep injury of a mesenteric arteriole was induced with a high intensity 440-nm-pulsed nitrogen dye laser applied with a Micropoint system (Photonic Instruments, Andor Technology, Belfast, UK). Superficial injury has been shown to remove the endothelium without damaging the underlying structures, while exposure of all vascular layers occurs upon deep injury. Thrombus formation was monitored in real time (1 image/s) by bright field and fluorescence microscopy (Leica DM IRB) using a 40x oil objective and a Sensicam CCD camera (The Cooke Corporation, Romulus, MI). Images were acquired with Metamorph and analyzed with SlideBook software (Intelligent Imaging Innovations, Göttingen, Germany).

Bleeding Time

The bleeding times and volume of blood lost were determined by transversally severing a 3-mm segment from the distal tail of 50-60-day-old mice, as published. Briefly, the amputated tail was immersed in 9 mg/mL isotonic saline at 37°C during 30 min and the time required for arrest of bleeding was recorded. The tube containing saline with blood was then homogenized, centrifuged and a lysis buffer was added to the pellet. After homogenization, the optical density was read at 540 nm and compared to a standard curve to determine the volume of blood loss.
Supplemental Figure I

A. Control

Guidewire-injured

B. Control

Forceps-injured

C. Guidewire-injured

Thrombus area (μm²)

Time (s)

Control
Hirudin
Clopidogrel
Eptifibatide

D. Forceps-injured

Thrombus area (μm²)

Time (s)

Control
Hirudin
Clopidogrel
Eptifibatide
Supplemental Figures Legends

Supplemental Figure I. TEM analysis and pharmacological characterization of thrombosis induced by guidewire injury of the carotid artery or forceps compression of the aorta. A and B, The right common carotid (A) or abdominal aorta (B) of C57BL/6 WT mice was dissected free, injured or not with a guidewire (A) or forceps (B) and immediately fixed by perfusion with glutaraldehyde. TEM cross-section images of intact (a) and injured (b) arteries are shown. The photographs are representative of several sections taken from at least 2 mice. Bars=2 (A) or 5 (B) µm. The inserts show a platelet adhering to exposed subendothelium. Bars=0.5 µm. P, platelet; EC, endothelial cell; IEL, internal elastic lamina; SMC, smooth muscle cell; M, media. C and D, A carotid guidewire (C) or an aortic forceps (D) injury was generated in C57BL/6 WT mice pretreated with vehicle, clopidogrel (50 mg/kg), hirudin (20 mg/kg), or eptifibatide (20 mg/kg) and thrombus formation was monitored by fluorescence microscopy. Time-course of thrombus growth, represented by its mean surface area, is shown. The shading under the curves corresponds to the SEM and the data are from 5 mice.

Supplemental Figure II. β1 integrin-deficient mice exhibit a decreased thrombotic response after guidewire injury of the carotid artery or forceps injury of the aorta. A carotid guidewire (A, B) or an aortic forceps (C, D) injury was generated in WT and β1−/− mice and thrombus formation was monitored by fluorescence microscopy, as in Figure 7A-F. A and C, Time-course of thrombus growth, represented by its mean surface area. The shading under the curves corresponds to the SEM. B and D, Dot plots depict the area under curve for individual mice. Mean ±SEM values are indicated by horizontal bars. Data are from 4 (A, B) or 4-6 (C, D) mice (*p<0.05, **p<0.01, 2-tailed Mann-Whitney tests).
Supplemental Figure III. $\alpha_6^{-/-}$ mice display a normal thrombotic response after deep laser injury of a mesenteric arteriole. A localized deep laser injury of a mesenteric arteriole was generated in WT and $\alpha_6^{-/-}$ mice and the ensuing thrombus formation was monitored by fluorescence microscopy. A, Time-course of thrombus growth, represented by its mean surface area. The shading under the curves corresponds to the SEM. B, Dot plots depict the area under curve for individual mice. Mean ±SEM values are indicated by horizontal bars. Data are from 5-6 vessels in 3 mice (p=0.25, 2-tailed Mann-Whitney tests).

Supplemental References


