The leech product saratin is a potent inhibitor of platelet integrin $\alpha_2\beta_1$ and von Willebrand factor binding to collagen

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Subendothelial collagen plays an important role, via both direct and indirect mechanisms, in the initiation of thrombus formation at sites of vascular injury. Collagen binds plasma von Willebrand factor, which mediates platelet recruitment to collagen under high shear. Subsequently, the direct binding of the platelet receptors glycoprotein VI and $\alpha_2\beta_1$ to collagen is critical for platelet activation and stable adhesion. Leeches, have evolved a number of inhibitors directed towards platelet–collagen interactions so as to prevent hemostasis in the host during hematophagy. In this article, we describe the molecular mechanisms underlying the ability of the leech product saratin to inhibit platelet binding to collagen. In the presence of inhibitors of ADP and thromboxane A\textsubscript{2}, both saratin and 6F1, a blocking $\alpha_2\beta_1$ mAb, abrogated platelet adhesion to fibrillar and soluble collagen. Additionally, saratin eliminated $\alpha_2\beta_1$-dependent platelet adhesion to soluble collagen in the presence of an Src kinase inhibitor. Moreover, saratin prevented platelet-rich plasma adhesion to fibrillar collagen, a process dependent upon both $\alpha_2\beta_1$ and von Willebrand factor binding to collagen. Furthermore, saratin specifically inhibited the binding of the $\alpha_2$ integrin subunit I domain to collagen, and prevented platelet adhesion to collagen under flow to the same extent as observed in the presence of a combination of mAbs to glycoprotein Ib and $\alpha_2\beta_1$. These results demonstrate that saratin interferes with integrin $\alpha_2\beta_1$ binding to collagen in addition to inhibiting von Willebrand factor–collagen binding, presumably by binding to an overlapping epitope on collagen. This has significant implications for the use of saratin as a tool to inhibit platelet–collagen interactions.

Collagen plays a critical role in mediating the platelet response to vessel injury in the dynamic environment of the vasculature. Exposed collagen at sites of vascular injury initiates two platelet functions fundamental to the process of primary hemostasis: initial recruitment of circulating platelets, and triggering of the platelet activation cascade required to stimulate thrombus growth [1,2]. The first step in platelet recruitment to collagen occurs indirectly, via binding of platelet glycoprotein (GP)Ib to collagen-bound von Willebrand factor (VWF) [3]. VWF plays a critical role in the tethering of platelets at high shear levels, due to the rapid on-rate of binding between GPIb and VWF. The rapid off-rate of GPIb–VWF interactions results in platelet

Abbreviations

$\alpha_2$-bio, biotinylated $\alpha_2$ integrin subunit I domain; DIC, differential interference contrast; FITC, fluorescein isothiocyanate; GP, glycoprotein; PRP, platelet-rich plasma; TxA\textsubscript{2}, thromboxane A\textsubscript{2}; VWF, von Willebrand factor.

translocation at the site of injury, allowing adhesive interactions with slower binding kinetics (such as the platelet collagen receptors GPVI and \( \alpha_2 \beta_1 \) and \( \alpha_{Ih} \beta_3 \) integrins) to mediate platelet adhesion and activation [4]. Two routes have been proposed for this second step of platelet adhesion, namely GPVI-mediated platelet activation either preceding or following \( \alpha_2 \beta_1 \) integrin-mediated platelet adhesion [5,6]. It is noteworthy that under static or low-shear conditions, the roles of VWF and GPIb are dispensable, as the collagen receptors GPVI and \( \alpha_2 \beta_1 \) can mediate platelet adhesion independently of VWF.

The evolution of a panoply of molecules to interfere with the process of hemostasis has allowed the leech to continue its alimentary habit of hemato-phagy. The presence of anticoagulants in the salivary glands of the leech, *Hirudo medicinalis*, was originally discovered by Haycraft in 1884 and led to the isolation of hirudin, a potent antithrombin anticoagulant [7]. In addition to molecules that target the coagulation cascade, a number of leech-derived substances have been discovered that inhibit platelet adhesion and activation. Three such molecules, LAPP (an approximately 13 kDa leech antplatelet protein isolated from *Haementeria officinalis*) and calin and saratin (approximately 65 kDa and 12 kDa proteins, respectively, both isolated from *H. medicinalis*), have been shown to specifically block platelet–collagen interactions by inhibiting VWF binding to collagen [8–11]. Depraetere *et al.* [12] then went on to demonstrate that both LAPP and calin block the binding site on collagen for the platelet integrin \( \alpha_2 \beta_1 \). The saratin-binding site on collagen responsible for the inhibition of VWF binding is presently unknown.

Saratin, which consists of 103 amino acids and contains three disulfide bridges [13], has been cloned and produced in recombinant form in *Hansenula polymorpha*. Barnes *et al.* were the first to demonstrate that saratin specifically blocks purified VWF binding to collagen, as well as potently inhibiting platelet aggregate formation on immobilized collagen under shear flow [8], therefore leading to the extensive use in the literature of saratin as a VWF–collagen inhibitor [14–18]. Furthermore, saratin has been shown to inhibit lumen stenosis in carotid endarterectomized rats [19] and to reduce platelet adhesion and intimal hyperplasia in both a nondiseased environment [20] and in the state of hyperhomocystinemia [21]. Moreover, Vilahur *et al.* demonstrated that local administration of saratin inhibited atherosclerotic plaque thrombogenicity under shear conditions [22].

The main collagen-binding site on VWF resides within the A3 domain (residues 923–1109) of VWF [23–25]. Structural studies on the VWF A3 domain showed that it assumes the same fold as the binding site for collagen on the \( \alpha_2 \beta_1 \) integrin, namely the homologous integrin \( \alpha_2 \) I domain [26]. The present study demonstrates that saratin interferes with integrin \( \alpha_2 \beta_1 \) binding to collagen, in addition to inhibiting VWF–collagen binding, presumably by binding to an overlapping epitope on collagen. This has significant implications for the use of saratin as a tool to inhibit platelet–collagen interactions, and may provide the basis for the therapeutic use of saratin as a potent antithrombotic agent.

### Results

#### Delayed collagen-induced aggregation of platelets in the presence of saratin

We initially investigated the effects of the leech product saratin on the ability of platelets to aggregate in response to fibrillar collagen. Consistent with previous findings [8], dose–response and maximal aggregation of platelets did not differ in the presence of saratin (data not shown). However, onset of aggregation was significantly delayed in the presence of saratin (Fig. 1A), and this lag time was particularly evident at low fibrillar collagen concentrations (Fig. 1B). Moreover, a similar delay in collagen-induced aggregation was observed in the presence of the \( \alpha_2 \beta_1 \)-blocking antibody 6F1 (data not shown), consistent with previous reports demonstrating an \( \alpha_2 \beta_1 \)-dependent lag phase for collagen-induced aggregation [29]. Together, these findings led us to question whether saratin blocks platelet \( \alpha_2 \beta_1 \) binding to collagen in addition to functioning as an inhibitor of VWF–collagen binding, as had been previously described by Barnes *et al.* [8].

#### Dissection of the molecular actions of saratin on fibrillar collagen

Experiments were designed to evaluate the ability of saratin to inhibit platelet adhesion to collagen. We gently pipetted purified human platelets onto surface-immobilized fibrillar collagen, and recorded the degree of adhesion and spreading using Normarski differential interference contrast (DIC) microscopy. In agreement with previous reports, human platelets undergo complete spreading on fibrillar collagen in the absence of external stimulation (Fig. 2A). The degree of platelet adhesion to fibrillar collagen was only slightly reduced by the presence of either an \( \alpha_2 \beta_1 \)-blocking antibody or saratin; however, these effects were statistically insigni-
significant (Fig. 2B). In comparison, a 40% reduction in the degree of platelet adhesion to fibrillar collagen was observed in the presence of apyrase/indomethacin (Table 1). In contrast, 6F1 needed to be present in the suspension to achieve blockade (Table 1). However, it is noteworthy that the inhibition of platelet adhesion observed in the presence of 6F1 or saratin in the absence of secondary mediators could be overcome by treatment of platelet suspensions with the G protein-coupled receptor agonist thrombin (Fig. 2C,D).

**Dissection of the molecular actions of saratin on soluble collagen**

We next aimed to examine platelet attachment to soluble collagen, a process that has been reported to be predominately mediated via $\alpha_2\beta_1$ integrins [31,32]. Indeed, the presence of the $\alpha_2\beta_1$ mAb 6F1 reduced platelet adhesion on soluble collagen by over 60% (Fig. 3A,B). Along these lines, a similar degree of inhibition was observed in the presence of saratin (Fig. 3B). We extended our studies to examine the effects of secondary mediators on platelet adhesion to soluble collagen. In parallel with our observations on fibrillar collagen, a 50% reduction in platelet adhesion on soluble collagen was observed in the presence of the ADP scavenger apyrase and the cyclooxygenase inhibitor indomethacin (Fig. 3B). Moreover, ADP/TxA2-independent platelet adhesion to soluble collagen was eliminated through the blockade of $\alpha_2\beta_1$ with 6F1 or treatment of collagen with saratin (Fig. 3A). As was observed with fibrillar collagen, saratin did not need to be present in suspension to have an inhibitory effect on platelet adhesion (Table 1). However, in distinct contrast to what was observed with fibrillar collagen, both the $\alpha_2\beta_1$ mAb 6F1 and saratin blocked thrombin-stimulated platelet adhesion to soluble collagen in the presence, but not the absence, of inhibitors of secondary mediators (Fig. 3C,D).

It is noteworthy that the presence of saratin did not have any effect on platelet adhesion to immobilized fibrinogen (Table 1), indicating that saratin does not inhibit platelet integrin $\alpha_{IIb}\beta_3$ binding to fibrinogen.

**Saratin blocks Src kinase-independent platelet adhesion to soluble collagen**

A set of experiments was designed to investigate the role of Src family kinases in supporting platelet adhesion and spreading on soluble collagen. As shown in Fig. 4B, a 40% reduction in the degree of adhesion was observed in the presence of the Src kinase inhib-
itor PP2, whereas platelets that bound to soluble collagen in an Src kinase-independent manner were unable to form lamellipodia. Furthermore, the presence of the $\alpha_2\beta_1$ mAb 6F1 in combination with the Src kinase inhibitor PP2 eliminated platelet adhesion to soluble collagen altogether (Fig. 4A). Importantly, saratin was capable of blocking this Src kinase-independent adhesion to soluble collagen (Fig. 4A), consistent with the ability of saratin to block $\alpha_2\beta_1$-mediated platelet binding. It is noteworthy that this series of experiments was performed in the absence of inhibitors of ADP and TxA2.

**Saratin blocks platelet-rich plasma adhesion to collagen**

Thus far, this study has utilized washed platelets in order to examine the molecular mechanisms of saratin. Physiologically, however, platelets are exposed to collagen in the presence of plasma proteins. In order to investigate the ability of saratin to inhibit receptor-mediated interactions under physiologic conditions, we layered platelet-rich plasma (PRP) over immobilized collagen. Our studies demonstrated that individual platelets in citrated PRP bound to immobilized soluble collagen; however, interestingly, these platelets were unable to form lamellipodia (Fig. 5A). Moreover, the presence of either the $\alpha_2\beta_1$ mAb 6F1 or saratin abrogated this adhesion (Fig. 5A), further demonstrating the ability of saratin to block $\alpha_2\beta_1$-mediated platelet binding. Equivalent results were observed in PPACK/heparin-anticoagulated PRP, which preserves the physiologic levels of divalent cations (36.2 ± 3.2 versus $0.74 \pm 0.25 \times 10^{-2}$ platelets/mm$^2$) on soluble collagen in the presence or absence of 10 $\mu$g/mL saratin, respectively; mean ± SEM; $n = 3$).

In contrast to studies using washed platelets, where we found individual platelets to be adherent to fibrillar collagen, platelets in citrated PRP were incorporated into a fibrous mesh along the collagen fibres (Fig. 5B). The degree of platelet/fibrin deposition onto collagen fibres was unaffected by the presence of 6F1 (Fig. 5B). However, the presence of saratin eliminated the ability of PRP to form a fibrous mesh, and significantly reduced the degree of platelet adhesion to fibrillar collagen. Importantly, we observed a similar level of reduction in platelet/fibrin deposition and platelet adhesion when the $\alpha_2\beta_1$ mAb 6F1 was used in combination with...
Inhibition of 

Previous studies have shown that the T. C. White recombinant leech product saratin, we utilized a biotinylated a

In an attempt to determine whether the binding site on collagen with or without saratin. Subsequently, a con-

In selected experiments, immobilized collagen or fibrinogen was treated with the a2b1-blocking mAb 6F1 (10 µg·ml−1) or saratin (10 µg·ml−1) for 10 min, followed by washing with NaCl/Pi, prior to exposure to platelets (surface treatment). In selected experiments, 6F1 (10 µg·ml−1) or saratin (10 µg·ml−1) was added to and maintained in the suspension with the platelets throughout the adhesion assay (suspension treatment). Values are reported as follows: adherent platelets, mean ± SEM of three to six experiments; platelet surface area, mean ± SEM of 50–300 cells.

<table>
<thead>
<tr>
<th>Surface</th>
<th>Surface treatment</th>
<th>Suspension treatment</th>
<th>Platelet adhesion (cells/mm² × 10−4)</th>
</tr>
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<tr>
<td>BSA</td>
<td>–</td>
<td>–</td>
<td>2.2 ± 0.62***</td>
</tr>
<tr>
<td>Fibrillar collagen</td>
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<td>–</td>
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</tr>
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<td>6F1</td>
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</tr>
<tr>
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<td>Saratin</td>
<td>7.9 ± 1.45*</td>
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<tr>
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<td>–</td>
<td>8.2 ± 0.80*</td>
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<tr>
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<tr>
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<tr>
<td>Fibrinogen</td>
<td>–</td>
<td>–</td>
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<tr>
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<td>Saratin</td>
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<td>64.4 ± 1.60</td>
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</table>

***P < 0.01 with respect to platelet adhesion/surface area on untreated fibrillar or soluble collagen, respectively.

antagonists to VWF receptors on platelets, namely the mAb to GPIb, 6D1, and the mAb to a2b1b3, 6I-CP8. Taken together, these data are reflective of the ability of saratin to both block VWF–collagen binding and to inhibit a2b1–collagen interactions.

Inhibition of a2 integrin subunit I domain binding to collagen by saratin

In an attempt to determine whether the binding site on collagen for the platelet receptor a2b1 is blocked by the leech product saratin, we utilized a biotinylated recombinant a2 integrin subunit I domain construct. Previous studies have shown that the a2 integrin subunit I domain binds to collagen type I in a dose-dependent and saturable manner [12]. To investigate the ability of saratin to inhibit a2b1 binding, coverslips were coated with fibrillar collagen type I and preincubated with or without saratin. Subsequently, a constant amount of biotinylated a2 integrin subunit I domain (a2I-bio) was added, and the amount of a2I-bio was detected by adding streptavidin–fluorescein isothiocyanate (FITC) and visualized using fluorescence microscopy. Our results demonstrated that saratin was able to abrogate a2I-bio binding to collagen (Fig. 6). In addition, saratin was able to completely block VWF binding to immobilized collagen (data not shown), consistent with previous reports [8,14]. Taken together, our results definitively demonstrate that saratin potently inhibits a2b1 binding to collagen in addition to blocking VWF–collagen interactions.

Saratin reduces platelet adhesion to collagen under flow conditions

We next aimed to examine the effects of saratin on platelet adhesion in a more physiologically relevant setting. We therefore investigated platelet recruitment and aggregation as a result of the perfusion of whole blood at 1000 s−1 over immobilized fibrillar collagen. As shown in Fig. 7, substantial platelet aggregates form on collagen under flow, producing 39.6 ± 1.9 thrombi per field of view, resulting in 34.7 ± 6.2% surface coverage (Table 2). Platelet adhesion was severely reduced in the presence of the GPIb mAb 6D1, as evidenced by a dramatic reduction in surface coverage (Fig. 7, Table 2). It is noteworthy that a number of thrombi consisting of one to three platelets were observed in the presence of 6D1 (Fig. 7), whereas the number of these small thrombi was significantly reduced in the presence of the a2b1 mAb 6F1 in combination with the GPIb mAb 6D1 (Table 2). Importantly, the presence of saratin reduced both the percentage of surface coverage and the amount of thrombi formed to a similar level as observed in the presence of the GPIb and a2b1 antagonists. Similar results were observed in reconstituted blood (data not shown). Altogether, our results demonstrate that saratin, through blockade of both VWF and a2b1 binding to collagen, acts as a potent inhibitor of platelet aggregation on collagen under shear flow conditions.

Discussion

Previous studies have demonstrated that the leech product saratin functions as a potent inhibitor of VWF binding to collagen [8,14]. In this study, we extend these findings to demonstrate that saratin additionally functions as an inhibitor of platelet integrin a2b1 binding to collagen. This has important implications for the interpretation of results obtained when saratin is used as an inhibitor of platelet–collagen interactions, both in vitro [14–18] and in vivo [19–22].

The current study, in accordance with others [5,31,32], demonstrates that a2b1 integrins are not...
essential for platelet, whether purified or in plasma, adhesion to fibrillar collagen, as GPVI is capable of triggering platelet activation and release of secondary mediators (ADP and TxA2), which leads to platelet adhesion independently of $\alpha_{2}\beta_{1}$. However, in the absence of the actions of secondary mediators, GPVI-mediated activation alone is insufficient to induce platelet adhesion to fibrillar collagen in the absence of $\alpha_{2}\beta_{1}$, consistent with the current paradigm [5,33,34].

A different picture emerges for platelet adhesion to soluble collagen. This form of collagen results from the cleavage of collagen in the nontriple helical region, where covalent cross-links are found that are required for the assembly of collagen molecules into the typical banded structure found in fibrillar collagen. Soluble collagen therefore lacks the highly repetitive GPVI recognition sites characteristic of fibrillar collagen, therefore providing a means of reducing but not ablating GPVI signaling [1,35]. Consistent with previous reports [32–34,36,37], our data demonstrate that $\alpha_{2}\beta_{1}$ integrins play an important role in mediating platelet adhesion to soluble collagen in the absence of inhibitors of secondary mediators, whereas $\alpha_{2}\beta_{1}$ is essential for ADP/TxA2-independent platelet adhesion. Additionally, we demonstrate that PRP binding to immobilized soluble collagen is $\alpha_{2}\beta_{1}$-dependent. Moreover, the $\alpha_{2}\beta_{1}$ dependency of Src kinase-independent adhesion on soluble collagen further indicates the essential role of $\alpha_{2}\beta_{1}$ in the absence of platelet activation. Interestingly, we found that thrombin stimulation, which predominantly acts via the $G_{q}$ family of proteins [38], potentiated $\alpha_{2}\beta_{1}$-independent platelet adhesion to soluble collagen only in the presence of the actions of the $G_{i}$ protein-coupled agonist ADP. This supports the notion that GPVI-mediated platelet activation and adhesion on the low-GPVI-affinity soluble collagen is dependent upon a cosignal from $G_{i}$-coupled receptors in the absence of $\alpha_{2}\beta_{1}$ [37,39,40].

The ability of saratin to precisely mirror the effects of 6F1 in the aforementioned experiments, in combination with the fact that saratin abrogates the binding of the $\alpha_{2}$ integrin subunit I domain to collagen, provides unequivocal evidence that this leech product is a potent $\alpha_{2}\beta_{1}$ blocker. As the binding sites for VWF and $\alpha_{2}\beta_{1}$ on collagen are within close spatial proximity [26], saratin presumably binds to an overlapping epitope on collagen to achieve dual blockade of these interactions. Therefore,
in light of the role that \( \alpha_2\beta_1 \) plays in stabilizing collagen-bound platelets under shear [5,6], the combined ability of saratin to block both VWF-dependent and VWF-independent (via \( \alpha_2\beta_1 \)) pathways of platelet deposition on collagen makes this leech product a powerful anti-thrombotic agent.

**Experimental procedures**

**Reagents**

Fibrillar type I collagen (Horm) from equine tendon was purchased from Nycomed (Munich, Germany). Soluble, nonfibrillar type I collagen from rat tail was purchased from Sigma (St Louis, MO, USA). LJ-CP8 was generously provided by Z. M. Ruggeri (Scripps Research Institute, La Jolla, CA, USA). 6F1 and 6D1 were a kind gift from...
B. Coller (Rockefeller University, New York, NY, USA). The Src kinase inhibitor PP2 was purchased from Calbiochem (San Diego, CA, USA). Recombinant saratin, produced in the yeast *Han. polymorpha* as previously described [8], was supplied by BioVascular, Inc. (La Jolla, CA, USA). Other reagents were obtained from Sigma or previously named sources [27,28].

Preparation of washed platelets

Human venous blood was drawn from healthy volunteers into sodium citrate and acid/citrate/dextrose as previously described [3]. PRP was prepared by centrifugation of whole blood at 200 g for 20 min (5702 R centrifuge, Eppendorf, Hamburg, Germany, rotor F-35-30-17). The platelets were then isolated from PRP by centrifugation at 1000 g for 10 min (5702 R centrifuge, Eppendorf, Hamburg, Germany, rotor F-35-30-17) in the presence of prostacyclin (0.1 μg·mL⁻¹). The pellet was resuspended in modified Hepes/Tyrodes buffer (129 mm NaCl, 0.34 mm Na₂HPO₄, 2.9 mm KCl, 12 mm NaHCO₃, 20 mm Hepes, 5 mm glucose, 1 mm MgCl₂, pH 7.3) containing 0.1 μg·mL⁻¹ prostacyclin, washed, and resuspended (2 × 10¹¹ mL⁻¹) in Hepes/Tyrode buffer.

In selected experiments, platelet suspensions were treated with 10 μg·mL⁻¹ 6F1, 100 μg·mL⁻¹ LJ-CP8, 10 μg·mL⁻¹ 6D1, 10 μg·mL⁻¹ saratin, 1 U·mL⁻¹ thrombin, 20 μM PP2, and/or 2 U·mL⁻¹ apyrase and 10 μM indomethacin for 10 min before use in the assays. It is noteworthy that this dose of saratin is well above the IC₅₀ reported for platelet–collagen binding [8]. All experiments were performed in the absence of exogenously added Ca²⁺.

Platelet adhesion assays

Glass coverslips were incubated with a suspension of fibrillar collagen (100 μg·mL⁻¹) or soluble collagen (50 μg·mL⁻¹) overnight at 4 °C. Surfaces were then blocked with denatured BSA (5 mg·mL⁻¹) for 1 h at room temperature, and this was followed by subsequent washing with NaCl/P, before use in spreading assays. In selected experiments, collagen-coated surfaces were treated for 10 min with saratin (10 μg·mL⁻¹), and this was followed by washing with NaCl/P. Quiescent platelets failed to bind or spread on surfaces coated with denatured BSA (Table 1). For spreading experiments, washed platelets (2 × 10⁷ mL⁻¹) were incubated on collagen-coated coverslips at 37 °C for 45 min. Subsequently, coverslips were gently washed with Hepes/Tyrode buffer to remove unbound cells.
Table 2. Effects of saratin on platelet adhesion/aggregation on collagen under flow. Human whole blood was perfused over immobilized collagen at 1000 s\(^{-1}\). Blood was pretreated for 10 min with the GPIb mAb 6D1 (10 \(\mu\)g\(\cdot\)mL\(^{-1}\)) with or without the \(\alpha_2\beta_1\) mAb 6F1 (10 \(\mu\)g\(\cdot\)mL\(^{-1}\)). In separate experiments, collagen-coated coverslips were pretreated with saratin (10 \(\mu\)g\(\cdot\)mL\(^{-1}\)) for 10 min, whereas saratin (30 \(\mu\)g\(\cdot\)mL\(^{-1}\)) was maintained in whole blood during flow. Values are reported as mean ± SEM of three experiments.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Surface coverage (%)</th>
<th>Number of thrombi/field of view</th>
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<tr>
<td>–</td>
<td>34.7 ± 6.18</td>
<td>39.6 ± 1.95</td>
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<tr>
<td>6D1</td>
<td>6.9 ± 2.77*</td>
<td>36.4 ± 5.85</td>
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<tr>
<td>6D1 + 6F1</td>
<td>3.8 ± 0.96*</td>
<td>13.0 ± 4.19**</td>
</tr>
<tr>
<td>Saratin</td>
<td>5.7 ± 2.43*</td>
<td>15.4 ± 5.27**</td>
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</table>

***P < 0.01 with respect to untreated and 6D1-treated blood, respectively.

Platelet spreading was imaged using Köhler illuminated Nomarski DIC optics with a Zeiss 63× oil immersion 1.40 NA plan-apochromat lens on a Zeiss Axiovert 200M microscope (Carl Zeiss, Thornwood, NY, USA), and recorded using STALLION 4.0 (Intelligent Imaging Innovations, Inc., Denver, CO, USA). To compute the degree of adhesion and surface area of spreading platelets, images were manually outlined and quantified by determining the number of pixels within each outline using a Java plug-in for IMAGE J software, as previously described [28]. Imaging a graticule under the same conditions allowed the conversion of pixel size to micrometers.

Flow adhesion studies

Glass coverslips were coated with fibrillar collagen as described above. Coverslips were assembled onto a flow chamber (Glyotech, Gaithersburg, MD, USA) and mounted on the stage of an inverted microscope (Zeiss Axiovert 200M). In selected experiments, coverslips were treated with 10 \(\mu\)g\(\cdot\)mL\(^{-1}\) saratin for 10 min prior to the flow assay. PPACK (40 \(\mu\)M) anticoagulated whole blood was perfused through the chamber for 3 min at a wall shear rate of 1000 s\(^{-1}\), and this was followed by washing for 4 min at the same shear rate with modified Tyrodes buffer and imaged using DIC microscopy.

Measurement of platelet aggregation

To prepare heparinized PRP, blood was collected from healthy human donors into syringes containing heparin sodium (10 U\(\cdot\)mL\(^{-1}\) final concentration). PRP was obtained by centrifugation of heparinized blood at 200 \(\times\) g for 15 min (5702 R centrifuge, Eppendorf, rotor F-35-30-17). Optical aggregation studies were carried out using a Born aggregometer (Chronolog, Havertown, PA, USA) with high-speed stirring (1200 r.p.m.) at 37°C. Platelet shape change and aggregation were monitored by measuring changes in light transmission as previously described [29].

Binding competition assays

The recombinant \(\alpha_2\)I domain-encoding region was generated, purified and biotinylated as previously described [30]. Purified material was characterized by SDS/PAGE, and the concentration of \(\alpha_2\)I-bio was quantified using a detergent compatible-protein assay (Biorad, Hercules, CA, USA).

Coverslips were coated overnight at 4°C with 1 mg\(\cdot\)mL\(^{-1}\) fibrillar collagen. Wells were then blocked with denatured BSA (5 mg\(\cdot\)mL\(^{-1}\)) for 1 h at room temperature, and this was followed by subsequent washing with NaCl/P, before incubation with vehicle or saratin (10 \(\mu\)g\(\cdot\)mL\(^{-1}\)) for 10 min. A constant amount of \(\alpha_2\)I-bio (0.3 \(\mu\)M) or VWF (10 \(\mu\)g\(\cdot\)mL\(^{-1}\)) was then added and allowed to bind for 90 min. Following copious washing, bound \(\alpha_2\)I-bio or VWF was detected by adding streptavidin–FITC or anti-VWF–FITC, respectively, for 1 h at RT, and visualized using fluorescence microscopy.

Analysis of data

Experiments were carried out at least three times, and images shown are representative data from one experiment. Where applicable, results are shown as mean ± SEM. The statistical significance of differences between means was determined by ANOVA. If means were shown to be significantly different, multiple comparisons were performed by the Tukey test. Probability values of P < 0.01 were considered to be statistically significant.

Acknowledgements

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References


