Platelet Control of Fibrin Distribution and Microelasticity in Thrombus Formation Under Flow

Frauke Swieringa, Constance C.F.M.J. Baaten,* Remco Verdoold,* Tom G. Mastenbroek, Niek Rijnveld, Koen O. van der Laan, Ernst J. Brelé, Peter W. Collins, Marcus D. Lancé, Yvonne M.C. Henskens, Judith M.E.M. Cosemans, Johan W.M. Heemskerk, Paola E.J. van der Meijden

Objective—Platelet- and fibrin-dependent thrombus formation is regulated by blood flow and exposure of collagen and tissue factor. However, interactions between these blood-borne and vascular components are not well understood.

Approach and Results—Here, we developed a method to assess whole-blood thrombus formation on microspots with defined amounts of collagen and tissue factor, allowing determination of the mechanical properties and intrathrombus flow conditions. Confining the collagen content resulted in diminished platelet deposition and fibrin formation at high shear flow conditions, but this effect was compensated by a larger thrombus size and increased accumulation of fibrin in the luminal regions of the thrombi at the expense of the base regions. These thrombi were more dependent on tissue factor–triggered thrombin generation. Microforce nanoindentation analysis revealed a significantly increased microelasticity of thrombi with luminal-oriented fibrin. At a low shear rate, fibrin fibers tended to luminally cover the thrombi, again resulting in a higher microelasticity. Studies with blood from patients with distinct hemostatic insufficiencies indicated an impairment in the formation of a platelet–fibrin thrombus in the cases of dilutional coagulopathy, thrombocytopenia, Scott syndrome, and hemophilia B.

Conclusions—Taken together, our data indicate that (1) thrombin increases the platelet thrombus volume; (2) tissue factor drives the formation of fibrin outside of the platelet thrombus; (3) limitation of platelet adhesion redirects fibrin from bottom to top of the thrombus; (4) a lower shear rate promotes thrombus coverage with fibrin; (5) the fibrin distribution pattern determines thrombus microelasticity; and (6) the thrombus-forming process is reduced in patients with diverse hemostatic defects. (Arterioscler Thromb Vasc Biol. 2016;36:00-00. DOI: 10.1161/ATVBAHA.115.306537.)

Key Words: blood platelet ■ elasticity ■ fibrin ■ thrombin ■ thrombosis

During hemostasis, blood flow and platelet activation in combined action with the coagulation system determine the formation of platelet–fibrin thrombi at the injured vessel wall. Multiple components of the damaged endothelial and subendothelial layers are known to influence the dynamics of thrombus growth and fibrin polymerization. Key vascular constituents triggering this process are collagen fibers and tissue factor (TF), serving as potent platelet- and coagulation-stimulating agents, respectively. Thrombin that is formed on the surface of activated platelets and on injured vascular cells is a central player in the hemostatic process by enhancing platelet activation, producing activated coagulation factors and converting fibrinogen into fibrin. Yet, the complex role of thrombin in the formation of a platelet–fibrin thrombus under flow is at best incomplete studied.

Marine in vivo models of arterial thrombus formation have shown that the type and severity of vascular injury are determinative for the precise triggering of this process. Exposure of the vasculature to FeCl₃, resulting in endothelial denudation, leads to collagen-dependent platelet activation with an additional role of TF, particularly on milder FeCl₃ injury. Thrombin and fibrin generations are elicited on procoagulant platelets exposing phosphatidylserine. Platelets, furthermore, control the coagulation process by secreting procoagulant and anticoagulant proteins, whereas there is ample evidence for a role of platelet-exposed TF. On the other hand, in most laser-induced injury models, thrombus formation is primarily triggered by thrombin that is generated via vascular TF. Severe laser injury, however, also leads to collagen exposure and collagen-dependent platelet activation.
Together, these in vivo findings indicate that the relative availability of platelet-adhesive (like collagen) and coagulation-triggering (like TF) substances determines the buildup of a thrombus and, by implication, the roles of thrombin and fibrin herein. In support of this idea, different patterns of fibrin deposition have been detected in injury models, such as fibrin structures around a thrombus,10 and thrombin/fibrin-rich areas in the thrombus core region.17,18 How the platelet and coagulant activities of the thrombogenic surface, for example, the relative abundance of collagen and TF, influence the buildup and composition of a platelet–fibrin thrombus has not been examined so far.

Viscoelasticity is considered to be an important hemostatic property of a fibrin-containing thrombus. For instance, in patients with severe factor XI deficiency, a low-density fibrin network is associated with impaired hemostasis.19,20 However, the conditions that favor high elasticity of a platelet–fibrin thrombus or clot are largely unknown. The limited evidence available suggests that a local, high concentration of thrombin availability of platelet-adhesive (like collagen) and coagulation pathway resulted in a delayed formation of fibrin only for microspots not containing TF (data not shown). In the absence of collagen (only TF), neither platelets nor fibrin deposited on the surface.

Reconstruction of stacks of confocal images in 3-dimension indicated that the platelet–fibrin thrombi formed on high collagen microspots were relatively small and dense when compared with the more dispersed thrombi formed on low collagen microspots (Figure 1A and 1D). Persistent thrombin activity on each type of microspot was confirmed by the addition of a fluorogenic-thrombin substrate, which was continuously cleaved (data not shown). Markedly, with hirudin added to the blood, platelet deposition decreased on high collagen microspots but unaltered on low collagen, whereas fibrin formation was greatly delayed in either case (Table I in the online-only Data Supplement). Pretreatment of the blood with a corn trypsin inhibitor to block the intrinsic coagulation pathway resulted in a delayed formation of fibrin only for microspots not containing TF (data not shown). In the absence of collagen (only TF), neither platelets nor fibrin deposited on the surface.

In this present article, we investigated how the type of thrombogenic surface—with defined relative amounts of collagen and TF—affects the formation and composition of platelet–fibrin thrombi that are formed under high and low shear flow conditions. We developed standardized protocols, in which citrate-anticoagulated blood was flowed over defined collagen/TF microspots, and the deposition of platelets and fibrin(ogen) was assessed simultaneously. We then determined the distribution of fibrin within and outside the thrombus by confocal microscopy, as well as the microelasticity of the formed thrombi by a novel method of nanoindentation. The data reveal a surface- and flow-dependent thrombus buildup with a different location of fibrin.

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

Results

Collagen–TF Surface Determines the Buildup of Platelet–Fibrin Thrombus and Extent of Fibrin Formation Under Flow

To study the roles of platelets and coagulation in whole-blood thrombus formation under flow, we applied a previously developed microspot method of thrombus formation in parallel-plate flow chambers,23 which was adapted to operate under strictly controlled coagulant conditions. The changes included (1) preparation of microspots with defined amounts of collagen and TF, (2) confusion of citrate-anticoagulated blood with Mg\(^{2+}/Ca^{2+}\) recalcification medium to obtain physiological concentrations of divalent cations, (3) adequate mixing of blood with medium by pushing through a tube-slit converter in the flow chamber, and (4) real-time confocal recording of both platelet deposition and fibrin generated on the microspots using 3,3′-dihexyloxacarbonylamine iodide and Alexa Fluor 647 fibrinogen as probes that were preadded to the blood samples (Figure I in the online-only Data Supplement).

Pilot data indicated that cocooating of 10 pg TF per microspot resulted in optimal shortening of time to fibrin formation, as checked with bright-field image recording, such in agreement with published findings.26 Perfusion of blood samples at a shear rate of 1000 s\(^{-1}\) over microspots enriched in collagen (100 ng per microspot) resulted in a rapid formation of platelet thrombi that were rich in fibrin (Figure 1A–1C). Lowering the collagen content (20 ng per microspot) significantly decreased platelet deposition after 7 minutes of perfusion (area covered, 12.3%±1.4% versus 33.8%±3.0%; \(P=0.005\)) and delayed the onset of fibrin formation (3 versus 5 minutes) when compared with the high collagen microspots (Figure 1A–1C). In the absence of TF, when coagulation is triggered via the intrinsic pathway,27 platelet deposition was decreased on high collagen but unaltered on low collagen, whereas fibrin formation was greatly delayed in either case (Table I in the online-only Data Supplement). Pretreatment of the blood with a corn trypsin inhibitor to block the intrinsic coagulation pathway resulted in a delayed formation of fibrin only for microspots not containing TF (data not shown). In the absence of collagen (only TF), neither platelets nor fibrin deposited on the surface.

Reconstruction of stacks of confocal images in 3-dimension indicated that the platelet–fibrin thrombi formed on high collagen microspots were relatively small and dense when compared with the more dispersed thrombi formed on low collagen microspots (Figure 1A and 1D). Persistent thrombin activity on each type of microspot was confirmed by the addition of a fluorogenic-thrombin substrate, which was continuously cleaved (data not shown). Markedly, with hirudin added to the blood, platelet deposition decreased on high collagen microspots (surface area coverage from 33.8% to 19.1%; \(P=0.001\)), whereas on low collagen, hardly any platelets were left (from 12.3% to 2.9%; \(P<0.0001\)). As expected, with hirudin present, fibrin formation was completely suppressed on either surface (Figure 1E). Quantification also learned that hirudin significantly reduced the size of individual thrombi on the high-collagen surface (from 228±76 to 121±16 \(\mu m^2\); \(P=0.007\)), a reduction that was even more pronounced on the high-collagen surface (from 228±76 to 121±16 \(\mu m^2\); \(P=0.007\)) in the presence of...
Collagen–TF Surface and Shear Rate Define Distribution of Fibrin in a Thrombus

To assess the amount and localization of thrombus-associated fibrin on different microspots, z stacks of confocal images of the fluorescent-labeled platelets and fibrin were captured at the end of high-shear flow experiments (Figure 2A). On microspots with high collagen, the majority of platelet and fibrin fluorescence was located near the base (0–10 μm) and center regions (10–20 μm) of the thrombi. On microspots with low collagen, overall thrombus volume was reduced from 1.10±0.20 to 0.46±0.10×10⁶ μm³ (P<0.001; Table I in the online-only Data Supplement). In this case, fluorescence from platelets and fibrin was more evenly distributed among the base, center, and top regions of the thrombus (Figure 2B and 2C). Markedly, the top regions of thrombi formed on low collagen had a lower fibrin/platelet ratio (P=0.013) compared with the high collagen microspots.

The images were also analyzed for colocalization of platelets and fibrin to assess the appearance of fibrin outside the aggregated platelets. On high collagen microspots, more fibrin fibers extended outside the platelet area at the base of the thrombi (28.8% ± 1.9% of fibrin-positive pixels) than at the center and top regions of the thrombus (8.9% ± 2.1% and 5.3% ± 1.8% of...
fibrin-positive pixels, respectively; \( P<0.001 \). Fibrin outgrowth was further restricted on low collagen microspots, where only 9.7%±2.8%, 2.3%±0.6%, and 5.2%±1.1% of the fibrin fluorescence were detected outside the platelet aggregates at the base, center, or top regions of thrombi, respectively.

Interestingly, when compared with a high shear rate, at a low shear rate of 150 s\(^{-1}\), overall thrombus volume reduced from 1.10±0.20 to 0.78±0.02×10\(^6\) \( \mu \)m\(^3\) (\( P=0.032 \)) on high collagen and from 0.46±0.10 to 0.29±0.04×10\(^6\) \( \mu \)m\(^3\) (\( P=0.026 \)) on low collagen. At a low shear rate, the majority of the fibrin was located outside of the platelet regions, which were low in height, such as apparent from analysis of z stacks of confocal images (Table I in the online-only Data Supplement).

Collagen–TF Surface and Shear Rate Influence Microelasticity of a Thrombus

A novel method of ferrule-top nanoindentation, related to atomic force microscopy, was used to determine how the quantity and localization of fibrin fibers affected the mechanical and microelastic properties of the thrombi formed on microspots. This method is schematized in Figure 3A and described in more detail in the online-only Data Supplement. By gradually indenting and retracting a nanoindentation tip at multiple spots per coverslip, loading and unloading curves could be obtained, wherein slopes of the unloading curves informed on the reduced Young modulus (RedYM), as an inverse measure for the microelasticity of the sample (Figure III in the online-only Data Supplement). By applying multiple nanoindentations per microspot surface in a grid-like pattern, high-resolution information on the microelasticity of the thrombi on the surface could be obtained (Figure 3B). Unspotted (not shown) or collagen-coated coverslips without thrombi gave a high RedYM of >1000 kPa (Figure 3C). In contrast, coverslips covered with coagulated, fibrin-containing plasma produced a low RedYM of 1.22±0.1 kPa, thus pointing to high microelasticity of the fibrin network. Nanoindentation measurements of thrombi formed on high collagen (78±5.9 kPa) and low collagen (20.0±8.1 kPa) microspots resulted in different RedYM values (\( P=0.019 \)), indicating that the latter had a higher microelasticity (Figure 3D). Similarly, for thrombi formed on high collagen, lowering of the shear rate from 1000 s\(^{-1}\) to 150 s\(^{-1}\) resulted in a lower RedYM (<0.0001) and hence a higher microelasticity (Figure 3D). This suggested that intrathrombus fibrin increased the microelasticity of the sample. This was confirmed by nanoindentation analysis of fibrin-poor thrombi, formed on collagen without TF, giving high RedYM values of 133.1±21.6 kPa. In the presence of hirudin to block thrombin activity, and hence fibrin formation, the RedYM even further increased to 182.9±29.3 kPa (Figure 3D). Together, this indicates that the thrombi with fibrin in the top or with outside coverage of fibrin, being formed on low-density collagen microspots or at a low shear rate, display a higher microelasticity when compared with thrombi in which fibrin is accumulated at the base.

Formation of Platelet–Fibrin Thrombus Under Conditions of Impaired Hemostasis

Using several approaches, we further assessed the roles of platelets and other blood components in the formation of fibrin-containing thrombi. Considering the diminished clotting activity of patients with perioperative dilutional coagulopathy,\(^{30,31} \) we first determined the consequences of blood dilution in vitro. Blood samples were flowed at a shear rate of 1000 s\(^{-1}\) over high collagen/TF microspots. In the absence of dilution, this resulted in a high platelet deposition and fibrin formation (see above). However, dilution of the blood to 80%, 60%, and 40% resulted in a gradual decrease in platelet deposition after 7 minutes of perfusion, amounting to 14±2% (\( P=0.046 \)), 10±3% (\( P=0.004 \)), and 8±3% (\( P<0.001 \)), respectively, in comparison with the undiluted sample. In contrast, amounts of fibrin only reduced at dilution to 60% and 40% blood, which reduction was accompanied by a prolonged time to onset of fibrin formation (Figure 4A and 4B). Comparable results were obtained under conditions of a low, venous shear rate of 150 s\(^{-1}\) (Figure IV in the online-only Data Supplement). Reconstitution of the 40% diluted blood with either washed platelets or red blood cells caused partial improvement of both platelet deposition and fibrin formation (Figure 4A and 4C). Interestingly, complete recovery of the thrombus-forming process was obtained by addition of platelets in combination with red blood cells (Figure 4C). These data pointed to a limiting role of platelet adhesion (enforced by marginalization with erythrocytes) not only for platelet aggregation but also for fibrin formation under conditions of high shear flow. This was confirmed with scanning electron microscopy (Figure V in the online-only Data Supplement).
intrathrombus localization of fibrin could be analyzed in more detail. Analysis of z stacks of confocal images indicated that fibrin and platelets were similarly distributed throughout the thrombi (Figure 6A). Yet, the ratio of fibrin to platelets was higher in the top regions of thrombi (Figure 6B), with only limited fibrin extending from the platelet aggregates at base areas (12±8% of fibrin-positive pixels). This fibrin distribution pattern, concentrated in platelet regions, persisted even after prolongation of the perfusion time to 12 minutes (Figure 6C).

After 12 minutes, at high and low collagen microspots, fibrin deposition outside the platelet regions at the thrombus base gradually increased to 34±14% and 6±1%, respectively. Jointly, these results pointed to platelet control of fibrin formation and distribution in thrombi formed on collagen/TF surfaces, relying on phosphatidyserine exposure.

Discussion

This study shows that, under conditions of high shear blood flow and coagulation, platelet deposition and platelet-dependent fibrin formation are decreased when collagen as a platelet-activating substrate becomes limited. Although fewer thrombi are formed on microspots with low collagen, we see that a partly compensating effect in that thrombus size is increased and the contribution of thrombin to thrombus growth is relatively more important. Nanoindentation measuring viscoelastic properties of the sample indicates that the thrombi formed on low collagen possess enhanced microelasticity because of fibrin accumulation in the luminal region of thrombi. Interestingly, redistribution of fibrin from the base to the luminal region of thrombi is also observed under conditions of thrombocytopenia. Hence, a limitation of either the platelet-activating surface or the platelet number leads to reductions in platelet deposition and fibrin accumulation, which effects are accompanied by an altered fibrin distribution throughout the thrombus. Blood flow at low shear rate similarly enhances microelasticity by reducing platelet deposition and increasing the formation of a fibrin coat surrounding the thrombi.

Model of Thrombus Buildup, Determining Fibrin Distribution and Microelasticity

Platelet adhesion to collagen is known to result in a sustained rise in cytosolic calcium and phosphatidyserine exposure. This procoagulant surface greatly promotes the assembly of coagulation factors culminating in the formation of factor Xa and thrombin. Earlier, in flowing mouse blood, we have established that in the presence of collagen and TF, this platelet-dependent factor Xa formation is essential for the propagation of fibrin formation and for boosting platelet phosphatidyserine exposure. The present results allow to extend this coagulation–platelet activation cross talk to the human system, in that we find that human deficiency in factor IX (hemophilia B patient) or deficiency in platelet phosphatidyserine exposure (Scott syndrome patient) results in almost complete abolition of fibrin formation under flow conditions.

Although in the past years progress has been made in determining the mechanical properties of fibrin fibers formed under static conditions, little is known about the elasticity of platelet–fibrin thrombi formed under flow. Recently, a new method of nanoindentation has been used to assess the microelasticity...
of clotted murine platelet-rich plasma. In this study, we have used this technique to measure the physical characteristics of human thrombi formed at high and low shear rates and find that thrombi with a high fibrin content in the thrombus top region are the highest in microelasticity. Jointly, our results indicate that not so much the fibrin content but rather the fibrin distribution through a thrombus determines its elastic properties. We hypothesize that the high microelasticity of thrombi with luminal-oriented fibrin ameliorates the hemostatic process. In agreement with this, thromboelastometry studies have indicated that a high clot elasticity associates with less bleeding in patients with hemostatic insufficiencies.

If platelet adhesion is limited (low collagen), we see a relatively high fibrin content in the top region of an apparently loose thrombus. On the other hand, at high platelet adhesion (high collagen), fibrin seems to be primarily formed at the base region of thrombi. Summarizing these data as in Figure 7, we conclude that (1) the presence of thrombin increases platelet thrombus volume independent of the shear rate; (2) the presence of TF drives fibrin formation outside of the thrombus; (3) limitation of platelet adhesion redirects fibrin from the bottom to the top of the thrombus; (4) lowering of the shear rate results in a more fibrin-rich thrombus; and (5) the thrombus micro-elasticity is determined by the distribution pattern of fibrin.

Comparison With In Vivo Models

These findings provide an extension of the hemostatic thrombus model, based on in vivo observations, presented by Brass and Diamond, in which thrombin activity is confined to the dense thrombus core. Previous in vivo studies have shown that, on laser-induced injury of arterioles, where exposed TF drives the thrombotic process, fibrin concentrates near the vessel wall and in the vascular-oriented part of the thrombus. Our findings suggest that this vascular-oriented fibrin distribution pattern points to a relatively high abundance of platelet-adhesive substrates. These substrates can be collagens, as well as other vascular components, such as laminins and von Willebrand factor. In other words, it seems that the strength of the platelet-adhesive surface controls the platelet-packing density during thrombus buildup and thereby, the thrombin retention and fibrin distribution pattern. Confirmative evidence for this hypothesis comes from in vivo studies using PAR4-deficient mice, in which fibrin was found to redistribute through the whole thrombus as a consequence of diminished platelet activation.

A different pattern of fibrin distribution occurs under low-shear flow conditions, where relatively more (microelastic) fibrin is formed, appearing as a coat that covers the thrombi. This might be explained by a reduction in the flow-dependent
removal of thrombin and fibrin monomers. In accordance with our results, it has been shown that the thrombi formed in mesenteric venules after laser injury are surrounded by a fibrin cap.

**Effects of Hemostatic Insufficiencies**

Under conditions of (perioperative) dilution, we find that platelet deposition is more sensitive to blood dilution than fibrin formation, which is impaired already at 60% blood. This contrasts to static measurements of macroscopic clot strength by thromboelastometry, which seemed to be less sensitive to dilution effects. Single-pass flow perfusion likely makes the difference here because under flow, the deposited platelets provide a rate-limiting surface for the formation of thrombin and subsequent fibrin, whereas the same flow removes procoagulant factors and hence restricts fibrin formation. Remarkably, in reconstituted systems, the addition of both platelets and red blood cells was required for complete reversal of dilution effects. By implication, this indicates that under conditions of flow and sufficient margination of platelets by red blood cells, a coagulant activity of >60% is required for unrestricted generation of fibrin. These findings are supported by hydrodynamic simulations, indicating that platelet interactions with the vessel wall are promoted by an increasing hematocrit.

A limitation of this study is the absence of vascular cells, such as endothelial cells, which can also provide a procoagulant surface for the formation of thrombin. On the other hand, current overviews indicate that collagen (TF) based in vitro flow studies compare well with in vivo arterial thrombosis models, whereas the same flow removes procoagulant factors and hence restricts fibrin formation. Remarkably, in reconstituted systems, the addition of both platelets and red blood cells was required for complete reversal of dilution effects. By implication, this indicates that under conditions of flow and sufficient margination of platelets by red blood cells, a coagulant activity of >60% is required for unrestricted generation of fibrin. These findings are supported by hydrodynamic simulations, indicating that platelet interactions with the vessel wall are promoted by an increasing hematocrit.

<table>
<thead>
<tr>
<th>Collagen 100 - Thr</th>
<th>Collagen 100</th>
<th>Collagen 100 + TF</th>
<th>Collagen 20 + TF</th>
</tr>
</thead>
<tbody>
<tr>
<td>150 s⁻¹</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
</tr>
<tr>
<td>1000 s⁻¹</td>
<td><img src="image4.png" alt="Image" /></td>
<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
</tr>
</tbody>
</table>

Figure 7. Schematic representation of thrombus buildup on collagen/tissue factor (TF) surfaces. Platelet–fibrin thrombi were formed by blood perfusion over microspots with indicated amounts of collagen and TF at indicated shear rates, as described in Figure 1. Multiple parameters of thrombus formation were assessed (full data are available in Table I in the online-only Data Supplement). Cartoon images illustrate key characteristics of thrombi in terms of width (scale, 1–5), height (scale, 1–3), fibrin inside platelet thrombus (sphere size, 1–4), and fibrin outside platelet thrombus (size of shell). Presentation is for 3 thrombus zones: A, <10 μm from microspot surface; B, 10 to 20 μm; and C, >20 μm.

**Sources of Funding**

This work was supported by the Dutch Landsteiner Foundation for Blood Transfusion Research (1006) and ZonMW, Animal-free Research Techniques (114021004). P.F.J. van der Meijden was supported by the Cardiovascular Center Maastricht.

**Disclosures**

N. Rijnveld, E.J. Breel, and K.O. van der Laan are employees of Optics1, Amsterdam, The Netherlands. The other authors report no conflicts.

**References**

the fibrin distribution is determinative for the thrombus microelasticity as assessed by nanoindentation.

abundance of vascular triggers of platelet adhesion/activation (collagen) at one side and of coagulation (tissue factor) at the other side is tissue factor, the interactions between these blood-borne and vascular components are not well understood. Our data imply that the relative let- and fibrin-dependent thrombus formation is regulated by blood flow, the blood composition and vascular triggers, such as collagen and activation of blood platelets, as well as the coagulation system, is required for a normal hemostatic response. Although it is known that platelet-based control of coagulation.

Activation of blood platelets, as well as the coagulation system, is required for a normal hemostatic response. Although it is known that platelet-based control of coagulation.


Shattend SJ, Kim C, Ginsberg MH. The final steps of integrin activation: the end game. Nat Rev Mol Cell Biol. 2010;11:288–300. doi:10.1038/nrm2781.


Platelet Control of Fibrin Distribution and Microelasticity in Thrombus Formation Under Flow

Frauke Swieringa, Constance C.F.M.J. Baaten, Remco Verdoold, Tom G. Mastenbroek, Niek Rijnveld, Koen O. van der Laan, Ernst J. Breel, Peter W. Collins, Marcus D. Lancé, Yvonne M.C. Henskens, Judith M.E.M. Cosemans, Johan W.M. Heemskerk and Paola E.J. van der Meijden

Arterioscler Thromb Vasc Biol. published online February 4, 2016;
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2016 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/early/2016/02/04/ATVBAHA.115.306537

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2016/02/04/ATVBAHA.115.306537.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/
Platelet-dependent control of fibrin distribution and micro-elasticity in thrombus formation under flow

Frauke Swieringa¹, Constance C.F.M.J. Baaten¹*, Remco Verdoold¹*, Tom G. Mastenbroek¹, Niek Rijneveld², Koen O. van der Laan², Ernst J. Bree², Peter W. Collins³, Marcus D. Lancé⁴, Yvonne M.C. Henskens⁵,⁶, Judith M.E.M. Cosemans¹, Johan W.M. Heemskerk¹, Paola E.J. van der Meijden¹

¹Department of Biochemistry, Cardiovascular Research Institute Maastricht (CARIM), Maastricht University, Maastricht, The Netherlands; ²Optics11, Amsterdam, The Netherlands; ³Arthur Bloom Haemophilia Centre, School of Medicine, Cardiff University, Cardiff, United Kingdom; Departments of Anaesthesiology⁴ and Internal Medicine⁵ and Central Diagnostic Laboratory⁶, Maastricht University Medical Center, Maastricht, The Netherlands
*Equal contribution

Methods

Healthy volunteers and patients
Blood was taken from healthy volunteers and patients after informed consent was obtained in accordance with the Declaration of Helsinki. Studies were approved by the local medical ethics committee (METC 10-3-023). All donors were free from antiplatelet and anticoagulant medication for at least 2 weeks. Blood was taken from healthy control donors and from two patients with hemophilia B (both 5 % factor IX), a patient with Scott syndrome (deficient anoctamin 6 expression), two patients with immune-induced thrombocytopenia (platelet count 85 x 10⁹/L and 22 x 10⁹/L), or from three patients with dilutional coagulopathy due to massive fluid infusion during cardiothoracic surgery (4.5-5 L), collected in the operating theatre. At least three blood samples were analyzed for all conditions.

Blood collection and preparation
Blood was collected in 1:10 (v/v) 3.2 % trisodium citrate for whole blood perfusion experiments and the preparation of washed red blood cells, or in 1:6 (v/v) acidic citrate dextrose (ACD, 80 mM trisodium citrate, 52 mM citric acid and 180 mM glucose) for the isolation of washed platelets. Platelets and red blood cells were isolated as described. Cell count was determined using a thrombocounter (Coulter Electronics, High Wycombe, United Kingdom).

Formation of platelet-fibrin thrombi under conditions of coagulation and flow
Thrombus formation under flow conditions was determined by perfusion of citrated whole blood over a collagen/tissue factor (TF) coated glass coverslip in a transparent parallel-plate perfusion chamber (width 3 mm, depth 50 µm, length 300 mm). Coverslips were coated with microspots containing 20 or 100 ng Horm type I collagen (Nycomed Pharma, Munich, Germany) in the absence or presence of 10 pg recombinant human TF (Innovin, Dade Behring, Deerfield IL, USA). After coating, surfaces were blocked with 1 % bovine serum albumin (BSA, Sigma, St. Louis MO, USA) in Hepes buffer pH 7.45 (10 mM Hepes, 136 mM NaCl, 2.7 mM KCl, 2 mM MgCl₂), and used within 4 h. Immediately before use, blood samples were pre-labeled with the membrane probe DiOC₆ (f.c. 0.5 µg/mL, Anaspec, Reeuwijk, The Netherlands) to identify platelets (green) and with Alexa fluor (AF)647-fibrinogen (f.c. 16.5 µg/mL, Molecular Probes, Life Technologies, New York NY, USA) to
detect fibrinogen and fibrin (red). Using two pulse-free micro-pumps (Model 11 Plus, 70-2212, Harvard apparatus, Holliston MA, USA) and a Y-shaped flattened mixing tube, blood samples in 1 mL plastic syringe (Becton Dickinson, Franklin Lakes NJ, USA) were mixed with coagulation medium (Hepes buffer pH 7.45 supplemented with 32 mM MgCl₂ and 63 mM CaCl₂) also in 1 mL syringe at a volume ratio of 10:1 (push mode). This resulted in complete mixing and physiological (mM) concentrations of Ca²⁺ and Mg²⁺. Where indicated, blood samples were pre-treated with 3 µg/mL hirudin (Refludan, Schering, Berlin, Germany) to block formed thrombin. Blood perfusion over microspots in the flow chamber was at a wall-shear rate of 150 or 1000 s⁻¹ for at least 7 min.

For assessing platelet contraction in a thrombus, citrated blood samples were spiked with 3% of autologous, washed DiOC₆-labeled platelets. Where indicated, spiked blood samples were pre-treated with Gly-Pro-Arg-Pro (GPRP) or hirudin.

Two-color confocal fluorescence microscopy and image analysis
During or after blood perfusion, two-colored images were recorded using a fast line-scanning Zeiss LSM7 system, equipped with OPSS lasers and a 40× oil-immersion objective (numerical aperture 1.30) (Carl Zeiss, Oberkochen, Germany). Confocal fluorescence images (16 bit, 1024 × 1024 pixels, single channel plus overlay) were obtained at both 488 and 635 nm excitation. Fluorescence was detected with an ultra-sensitive CCD line camera after selection by optical emission filters of 495-555 nm and 665-750 nm (pinhole 1 AU). For time series, fluorescence images were taken at 1 s intervals; for z-stacks, fluorescence images were taken at 1 μm z-steps.

Image analysis of morphometry and fluorescence was performed with Live7 Metamorph software, version 7.5.0.0 (MDS Analytical Technologies, Sunnyvale, Canada) and ImageJ (version 1.48g: Rasband, NIH, Bethesda, MD, USA), as described elsewhere. For the quantification of fibrin and platelet fluorescence, images were thresholded to eliminate background. Integrated fluorescence intensity as well as percentage area covered were calculated per image. For co-localization of two-color fluorescence, binary images were created based on threshold to determine overlap percentages. As a standard, integrated fluorescence intensity was compared to surface area coverage of fluorescence features. Considering the overall similarity of these two analyses (Supplemental Figure 1), for most conditions data of surface area coverage are given only.

For the quantification of (fluorescent) fibrin formation, threshold levels of fibrinogen binding to platelets was determined under conditions where coagulation was inhibited. Image intensities above this threshold were considered as originating from fibrin fibers. The presence of fluorescent fibrin fibres was always confirmed from brightfield images. Times to onset of fibrin formation were the moments at which the fluorescence threshold was passed, as confirmed by visual inspection of brightfield images.

For measurement of platelet contraction in a thrombus, time series of DiOC₆-labeled images were collected during flow. Movement towards each other of labeled platelets within thrombi was analyzed off-line via tracking analysis, as described. In brief, individual images of time-lapse series were thresholded to a binary images. Using the Mtrack2 plugin displacement was measured by including objects (platelets) of <5 pixels which were present >60 s. Objects were rejected when the displacement was >25 pixels s⁻¹ (indicating noise or platelets not part of one thrombus). Calculated was per condition the average movement of platelets in time (μm/s).

Dilution and reconstitution of whole blood samples
Citrated blood was diluted in vitro with saline medium, consisting of 154 mM NaCl, 10.8 mM trisodium citrate, 2 mM CaCl₂ and 2 mM MgCl₂, in order to keep equal concentrations of free Ca²⁺ and Mg²⁺ in all diluted samples. Washed platelets and/or red cells were added to diluted blood samples to restore original cell counts, as described before. Amounts of fluorescent labels, DiOC₆ and AF647-fibrinogen, were adjusted according to the extent of dilution and reconstitution.
Measurement of thrombin generation on microspots in flow chamber
Fluorogenic thrombin substrate Z-Gly-Gly-Arg-AMC (0.5 mM: Thrombinscope, Maastricht, The Netherlands) was added to blood samples where indicated, and used to measure thrombin generation on preformed thrombi under static conditions.\cite{7} Real-time fluorescence accumulation due to substrate cleavage was recorded at 447/30 nm emission every 30 s for at least 5 min, using an inverted fluorescence microscope equipped with an Olympus 60 × oil-immersion objective (numerical aperture 1.35), equipped with a 360 nm led cube (EVOS, Life Technologies, Bleiswijk, The Netherlands).\cite{5} Temporal analysis of fluorescence was performed using ImageJ.

Determination of micro-elasticity of thrombi on microspots by nanoindentation technology
Micro-elasticity of formed thrombi on coverslip was analyzed with a recently developed Pluma nanoindenter (Optics11, Amsterdam, The Netherlands), which features a calibrated nanoindentation probe consisting of a cantilever fabricated on top of a ferruled optical fiber. Recent developments in indentation probe fabrication have resulted in probes that can measure soft (biological) materials, e.g. cartilage and vascular models. These probes measure the material hardness in the Pascal to kilo-Pascal range.\cite{8} In atomic force microscopy a cantilever tip is used that is repelled from the surface by minute forces. In contrast, in nanoindentation methods a spherical, cylinder or Berkovich shaped tip is used that is forced into the sample. The employed Optics11 Pluma Nanoindenter system has been validated before with biological samples.\cite{9,11}

In brief, a probe with a defined cantilever of 1.39 N/m and calibrated spherical tip of 56.5 μm was gradually brought into contact with the sample surface, up to a depth of maximum 10% of the sample thickness.\cite{12} The counter-force of the sample on the tip results in cantilever bending, which is linear with the load on the cantilever. The result is a load-displacement curve of both loading (indenting) and unloading (retraction) of the probe. The probe, fixed on a closed-loop z-piezoelectric translator, was equipped with an integrated strain gage sensor and controlled by a closed-loop controller. From the slope of the unloading curve, the maximum load and the indentation area (tip surface area), a reduced Young's modulus (RedYM) can be calculated as a proxy measure of micro-elasticity.\cite{13} The modulus is the amount of force per unit area (stress), needed to reach a certain amount of deformation (unit = Pa). Hence, a higher RedYM indicates that the given material is harder to deform. This measurement outcome is similar to atomic force microscopy.\cite{14}

As a conservative rule of thumb, the indentation value is independent of the height and size of a sample, provided that the indentation depth is smaller than the sample radius and <10% of the sample height.\cite{12,15} With these criterions met for the present conditions, the RedYM reflects the elastic modulus of platelet-fibrin thrombi. The attractiveness of the nanoindentation technique is that the mecano-elastic properties of a microscopic sample can directly be determined without any further sample knowledge. A drawback is the lack of visual feedback of the probe location above the thrombus sample. To overcome this, we have indented all samples (old and new conditions) 36 times in a grid-like pattern. With an optical magnifier, we further confirmed that the right spots on the sample were indented.

Coverslips with thrombi were carefully washed and incubated with Heps buffer pH 7.45. The diameter of the contact area of indentation tip with thrombi was ~20 μm². Per microspot, 6 x 6 indentations were performed following a gridding pattern with a step-size of 50 μm. All indents were depth controlled (1 μm to a maximum of 5 μm). Loading and unloading times were set at 3 s, data were collected for 1 s per step. The unloading curves were used to calculate slopes using the compliance method of Oliver and Pharr in order to calculate the RedYM.\cite{13,16} This is a measure of the micro-rigidity of the indented spot, which is inversely related to the micro-elasticity. A high RedYM reflects high micro-rigidity or stiffness of the indented sample and, hence, low micro-elasticity. Of the grid nanoindentations, only curves reflecting thrombi on glass were included. As negative and positive controls bare collagen surfaces and macroscopic fibrin clots were used, which showed a very low and high micro-elasticity, respectively. Furthermore, scanning electron
microscopy pictures were obtained for direct comparison of the dimensions of tip and thrombi.

**Statistics**

Statistical significance of differences between two independent groups was determined using the independent samples $t$ test. Differences with $P$ values <0.05 were considered significant. Error bars indicate inter-individual variability, except for patient data, where the variation of independent measurements is shown; values outside reference ranges of controls (mean ± 2SD) were considered to be different.

**References**


Platelet-dependent control of fibrin distribution and micro-elasticity in thrombus formation under flow

Frauke Swieringa1, Constance C.F.M.J. Baaten1*, Remco Verdoold1*, Tom G. Mastenbroek1, Niek Rijnveld2, Koen O. van der Laan2, Ernst J. Breeël2, Peter W. Collins3, Marcus D. Lancé4, Yvonne M.C. Henskens5,6, Judith M.E.M. Cosemans1, Johan W.M. Heemskerk1, Paola E.J. van der Meijden1

1Department of Biochemistry, Cardiovascular Research Institute Maastricht (CARIM), Maastricht University, Maastricht, The Netherlands; 2Optics11, Amsterdam, The Netherlands; 3Arthur Bloom Haemophilia Centre, School of Medicine, Cardiff University, Cardiff, United Kingdom; Departments of Anaesthesiology4 and Internal Medicine5 and Central Diagnostic Laboratory6, Maastricht University Medical Center, Maastricht, The Netherlands
*Equal contribution

SUPPLEMENTAL MATERIAL AND METHODS

Healthy volunteers
Blood was taken from healthy volunteers after informed consent was obtained in accordance with the Declaration of Helsinki. Studies were approved by the local medical ethics committee (METC 10-3-023). All donors were free from antiplatelet and anticoagulant medication for at least 2 weeks.

Blood collection and preparation
Blood was collected in 1:10 (v/v) 3.2 % trisodium citrate, and used for whole blood perfusion experiments within 4 h. Nanoindentation measurements and scanning electron microscopy were performed afterwards, where indicated.

Formation of platelet-fibrin thrombi under conditions of coagulation and flow
Thrombus formation under flow conditions was determined by perfusion of citrated whole blood over a collagen-tissue factor coated glass coverslip in a transparent parallel-plate perfusion chamber (width 3 mm, depth 50 µm, length 300 mm). Coverslips were coated with microspots containing 20 or 100 ng Horm type I collagen (Nycomed Pharma, Munich, Germany) in the absence or presence of 10 pg recombinant human tissue factor (Innovin, Dade Behring, Deerfield IL, USA). After coating, surfaces were blocked with 1 % bovine serum albumin (BSA, Sigma, St. Louis MO, USA) in Hapes buffer pH 7.45 (10 mM Hapes, 136 mM NaCl, 2.7 mM KCl, 2 mM MgCl2), and used within 4 h. Immediately before use, blood samples were pre-labeled with the membrane probe DiOC6 (f.c. 0.5 µg/mL, Anaspec, Reeuwijk, The Netherlands) to identify platelets (green) and with Alexa fluor (AF)647-fibrinogen (f.c. 16.5 µg/mL, Molecular Probes, Life Technologies, New York NY, USA) to detect fibrinogen and fibrin (red). Using two pulse-free micro-pumps (Model 11 Plus, 70-2212, Harvard apparatus, Holliston MA, USA) and a Y-shaped flattened mixing tube, blood samples in a 1 mL plastic syringe (Becton Dickinson, Franklin Lakes NJ, USA) were mixed with coagulation medium (Hapes buffer pH 7.45 supplemented with 32 mM MgCl2 and 63 mM CaCl2) also in 1 mL syringe, at a volume ratio of 10:1 (push mode). This resulted in complete mixing and physiological (mM) concentrations of Ca2+ and Mg2+. Where indicated, blood samples were pre-treated with 3 µg/mL hirudin (Refludan, Schering, Berlin, Germany)
Two-color confocal fluorescence microscopy and image analysis
During or after blood perfusion, two-colored images were recorded using a fast line-scanning Zeiss LSM7 system, equipped with OPSS lasers and a 40× oil-immersion objective (numerical aperture 1.30) (Carl Zeiss, Oberkochen, Germany). Confocal fluorescence images (16 bit, 1024 × 1024 pixels, single channel plus overlay) were obtained at both 488 and 635 nm excitation. Fluorescence was detected with an ultra-sensitive CCD line camera after selection by optical emission filters of 495-555 nm and 665-750 nm (pinhole 1 AU). For time series, fluorescence images were taken at 1 s intervals; for z-stacks, fluorescence images were taken at 1 μm z-steps.

Image analysis of morphometry and fluorescence was performed with Live7 Metamorph software, version 7.5.0.0 (MDS Analytical Technologies, Sunnyvale, Canada) and ImageJ (version 1.48g: Rasband, NIH, Bethesda, MD, USA), as described elsewhere. For the quantification of fibrin and platelet fluorescence, images were thresholded to eliminate background. Integrated fluorescence intensity as well as percentage area covered were calculated per image. For co-localization of two-color fluorescence, binary images were created based on threshold to determine overlap percentages. As a standard, integrated fluorescence intensity was compared to surface area coverage of fluorescence features. Considering the overall similarity of these two analyses (Supplemental Figure I), for most conditions data of surface area coverage are given only.

For the quantification of (fluorescent) fibrin formation, threshold levels of fibrinogen binding to platelets was determined under conditions where coagulation was inhibited. Image intensities above this threshold were considered as originating from fibrin fibers. The presence of fluorescent fibrin fibres was always confirmed from brightfield images. Times to onset of fibrin formation were the moments at which the fluorescence threshold was passed, as confirmed by visual inspection of brightfield images.

Determination of micro-elasticity of thrombi on microspots by nanoindentation technology
Micro-elasticity of formed thrombi on coverslip was analyzed with a recently developed Piuma nanoindenter (Optics11, Amsterdam, The Netherlands), which features a calibrated nanoindentation probe consisting of a cantilever fabricated on top of a ferruled optical fiber. Recent developments in indentation probe fabrication have resulted in probes that can measure soft (biological) materials, e.g. cartilage and vascular models. These probes measure the material hardness in the Pascal to kilo-Pascal range. In atomic force microscopy a cantilever tip is used that is repelled from the surface by minute forces. In contrast, in nanoindentation methods a spherical, cylinder or Berkovich shaped tip is used that is forced into the sample. The employed Optics11 Piuma Nanoindenter system has been validated before with biological samples.

In brief, a probe with a defined cantilever of 1.39 N/m and calibrated spherical tip of 56.5 μm was gradually brought into contact with the sample surface, up to a depth of maximum 10% of the sample thickness. The counter-force of the sample on the tip results in cantilever bending, which is linear with the load on the cantilever. The result is a load-displacement curve of both loading (indenting) and unloading (retraction) of the probe. The probe, fixed on a closed-loop z-piezoelectric translator, was equipped with an integrated strain gage sensor and controlled by a closed-loop controller. From the slope of the unloading curve, the maximum load and the indentation area (tip surface area), a reduced Young’s modulus (RedYM) can be calculated as a proxy measure of micro-elasticity. The modulus is the amount of force per unit area (stress), needed to reach a certain amount of deformation (unit = Pa). Hence, a higher RedYM indicates that the given material is harder to deform. This measurement outcome is similar to atomic force microscopy.

As a conservative rule of thumb, the indentation value is independent of the height and size of a sample, provided that the indentation depth is smaller than the sample radius.
and <10% of the sample height. With these criterions met for the present conditions, the RedYM reflects the elastic modulus of platelet-fibrin thrombi. The attractiveness of the nanoindentation technique is that the mechano-elastic properties of a microscopic sample can directly be determined without any further sample knowledge. A drawback is the lack of visual feedback of the probe location above the thrombus sample. To overcome this, we have indented all samples (old and new conditions) 36 times in a grid-like pattern. With an optical magnifier, we further confirmed that the right spots on the sample were indented.

Coverslips with thrombi were carefully washed and incubated with Hepes buffer pH 7.45. The diameter of the contact area of indentation tip with thrombi was ~20 μm². Per microspot, 6 x 6 indentations were performed following a gridding pattern with a step-size of 50 μm. All indents were depth controlled (1 μm to a maximum of 5 μm). Loading and unloading times were set at 3 s, data were collected for 1 s per step. The unloading curves were used to calculate slopes using the compliance method of Oliver and Pharr in order to calculate the RedYM. This is a measure of the micro-rigidity of the indented spot, which is inversely related to the micro-elasticity. A high RedYM reflects high micro-rigidity or stiffness of the indented sample and, hence, low micro-elasticity. Of the grid nanoindentations, only curves reflecting thrombi on glass were included. As negative and positive controls bare collagen surfaces and macroscopic fibrin clots were used, which showed a very low and high micro-elasticity, respectively. Furthermore, scanning electron microscopy pictures were obtained for direct comparison of the dimensions of tip and thrombi.

Dilution of whole blood samples
Citrated blood was diluted in vitro with saline medium, consisting of 154 mM NaCl, 10.8 mM trisodium citrate, 2 mM CaCl₂ and 2 mM MgCl₂, in order to keep equal concentrations of free Ca²⁺ and Mg²⁺ in all diluted samples. Amounts of fluorescent labels, DiOC₆ and AF647-fibrinogen, were adjusted according to the extent of dilution.

Scanning electron microscopy
Thrombi were formed on microspots in a perfusion chamber, as described above, and prepared for scanning electron microscopy. In short, after disassembly of the perfusion chamber, coverslips were removed and gently washed with Hepes buffer pH 7.45. Fixation was with 2.5% glutaraldehyde in 0.1 M K-phosphate buffer pH 7.4 at 4 °C. Following a rinsing step with 0.1 M phosphate buffer, samples were dehydrated in graded ethanol series, then critical point dried, mounted with silver paint on specimen stubs, and coated with gold. Thrombi were visualized with a Phillips XL30 scanning electron microscope at 10 kV (Eindhoven, The Netherlands).

Statistics
Statistical significance of differences between two independent groups was determined using the independent samples t test. Data are expressed as means ± SE, and P values <0.05 were considered significant. The error bars indicate inter-individual differences.
Supplemental Figure I. Quantification of platelet-fibrin thrombus formation on collagen/TF surfaces. Citrated whole blood was perfused during recalcification over microspots with collagen (100 ng) and TF (10 pg) for 7 min at a wall-shear rate of 1000 s⁻¹. Blood samples were pretreated with DiOC₆ to label platelets (green) and with AF647-fibrinogen (red). (A, B) Analysis of integrated fluorescence intensity (open) and surface area coverage above background (close) from deposited platelets (A) and fibrin (B). Note high similarity of either analysis parameter.
**Supplemental Figure II. Effect of wall shear rate in platelet-fibrin thrombus formation on collagen-tissue factor surfaces.** Platelet-fibrin thrombi were formed by blood perfusion over microspots with collagen (100 ng) and TF (10 pg), as for Figure 1. Wall-shear rate was 150 or 1000 s⁻¹. (A) Representative images after 7 min of flow of DiOC₆-labeled platelets (green) and AF647-fibrin(ogen) (red) (bars = 50 µm). (B) Time-dependent deposition of fluorescent-labeled platelets and fibrin during blood perfusion. (C) Quantification of platelet-fibrin co-localization at indicated shear rates. Means ± SE (n = 5-10), *P<0.05.
Supplemental Figure III. Nanoindentation load-displacement curves for the assessment of micro-elasticity of platelet-fibrin thrombi. Platelet-fibrin thrombi were formed by blood perfusion over microspots with collagen ± TF, as for Figure 1. Microspot surfaces were subjected to nanoindentation, as for Figure 4. Shown are representative load-displacement curves for thrombi formed on (A) 100 ng collagen, (B) 100 ng collagen plus 10 pg TF, or (C) 20 ng collagen plus 10 pg TF. Indicated is the loading curve during indentation, the maximum load and the unloading curve with slope (= RedYM). The RedYM represents a measure of the micro-rigidity of the sample, which is inversely related to micro-elasticity.
Supplemental Figure IV. Effects of blood dilution on formation of platelet-fibrin thrombus at low shear rate. Platelet-fibrin thrombi were formed by perfusion of blood over microspots with collagen (100 ng) and TF (10 pg) at low shear rate of 150 s⁻¹ for 7 min. Whole blood samples were undiluted or diluted to indicated % with saline (see Figure 5). (A) Time-dependent accumulation of fluorescence from platelets (green) and fibrin (red) during blood perfusion. (B) Fluorescence area covered by platelets and fibrin after 7 min of flow. Means ± SE (n = 6). *P<0.05.
Supplemental Figure V. Scanning electron microscopy of platelet-fibrin thrombus formed on different surfaces. Platelet-fibrin thrombi were formed by perfusion of blood over microspots with collagen ± TF at shear of 150 or 1000 s$^{-1}$. Thrombi on microspots were fixed, stained and visualized with a scanning electron microscope at 10 kV. Representative (n = 3) images are shown from thrombi formed on: (A) 20 ng collagen + TF; (B) 100 ng collagen + TF; (C) 100 ng collagen; (D) 100 ng collagen + TF, with hirudin in blood; (E) 100 ng collagen + TF, perfusion at 150 s$^{-1}$; (F) 100 ng collagen + TF, 40% whole blood. Bar = 20 μm. Upper part shows schematic representation of the spherical nanoindentation cantilever to scale.
Table I. Collagen-tissue factor surface determines platelet-fibrin thrombus buildup under flow.

<table>
<thead>
<tr>
<th></th>
<th>Collagen 100</th>
<th></th>
<th>Collagen 100 + TF</th>
<th></th>
<th>Collagen 20 + TF</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>150 s⁻¹</td>
<td>1000 s⁻¹</td>
<td>150 s⁻¹</td>
<td>1000 s⁻¹</td>
<td>150 s⁻¹</td>
<td>1000 s⁻¹</td>
</tr>
<tr>
<td>SAC* (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>platelets</td>
<td>9.04 ± 1.7</td>
<td>27.0 ± 2.2</td>
<td>19.1 ± 3.2</td>
<td>33.8 ± 3.0</td>
<td>5.62 ± 0.7</td>
<td>12.3 ± 1.4</td>
</tr>
<tr>
<td>fibrin</td>
<td>0.73 ± 0.7</td>
<td>1.19 ± 0.1</td>
<td>49.9 ± 6.8</td>
<td>29.8 ± 4.0</td>
<td>4.30 ± 1.7</td>
<td>5.76 ± 1.1</td>
</tr>
<tr>
<td>Fibrin area outside thrombus (%) of total</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-10 µm (base)</td>
<td>48.8 ± 9.7</td>
<td>36.3 ± 8.4</td>
<td>83.1 ± 0.3</td>
<td>28.8 ± 1.9</td>
<td>69.6 ± 3.0</td>
<td>9.7 ± 2.8</td>
</tr>
<tr>
<td>10-20 µm (center)</td>
<td>54.0 ± 18.0</td>
<td>55.5 ± 8.2</td>
<td>91.4 ± 2.1</td>
<td>8.9 ± 2.1</td>
<td>55.8 ± 5.4</td>
<td>2.3 ± 0.6</td>
</tr>
<tr>
<td>&gt;20 µm (top)</td>
<td>54.1 ± 13.9</td>
<td>75.5 ± 14.0</td>
<td>76.9 ± 4.8</td>
<td>5.3 ± 1.8</td>
<td>57.5 ± 1.3</td>
<td>5.2 ± 1.1</td>
</tr>
<tr>
<td>Thrombus volume (x 10⁶ µm³)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>platelets</td>
<td>0.15 ± 0.02</td>
<td>0.28 ± 0.04</td>
<td>0.16 ± 0.02</td>
<td>0.79 ± 0.1</td>
<td>0.15 ± 0.02</td>
<td>0.40 ± 0.1</td>
</tr>
<tr>
<td>fibrin</td>
<td>0.03 ± 0.007</td>
<td>0.02 ± 0.01</td>
<td>0.72 ± 0.03</td>
<td>0.76 ± 0.2</td>
<td>0.20 ± 0.03</td>
<td>0.24 ± 0.07</td>
</tr>
<tr>
<td>total</td>
<td>0.16 ± 0.02</td>
<td>0.28 ± 0.04</td>
<td>0.78 ± 0.02</td>
<td>1.10 ± 0.2</td>
<td>0.29 ± 0.04</td>
<td>0.46 ± 0.1</td>
</tr>
<tr>
<td>Ratio fibrin / platelets</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-10 µm (base)</td>
<td>0.86 ± 0.07</td>
<td>0.84 ± 0.02</td>
<td>0.97 ± 0.02</td>
<td>1.21 ± 0.1</td>
<td>0.88 ± 0.01</td>
<td>0.97 ± 0.1</td>
</tr>
<tr>
<td>10-20 µm (center)</td>
<td>1.09 ± 0.1</td>
<td>1.20 ± 0.1</td>
<td>1.07 ± 0.02</td>
<td>0.87 ± 0.04</td>
<td>0.98 ± 0.01</td>
<td>0.93 ± 0.08</td>
</tr>
<tr>
<td>&gt;20 µm (top)</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>1.07 ± 0.1</td>
<td>1.18 ± 0.01</td>
<td>1.27 ± 0.1</td>
</tr>
</tbody>
</table>

Citrated whole blood was perfused during recalcification over microspots with collagen (20 or 100 ng) with(out) TF (10 pg) for 7 min at a wall-shear rate of 150 or 1000 s⁻¹. Blood samples were pretreated with DiOC₆ to label platelets and with AF647-fibrinogen to monitor fibrin formation. Two-color confocal microscopic images were recorded in real time at a frequency of 1 Hz. For determination of thrombus volume, confocal z-stacks were recorded after 7 min of perfusion (z-step 1 µm). *SAC, surface area covered; NA, not applicable due to relatively low thrombus height. Means ± SE (n = 6-15).
References


