

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

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# 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, 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 composition. 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 lumenally 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;36:692-699. 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.<sup>1</sup> Key vascular constituents triggering this process are collagen fibers and tissue factor (TF), serving as potent platelet- and coagulation-stimulating agents, respectively.<sup>2</sup> 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.<sup>3</sup> Yet, the complex role of thrombin in the formation of a platelet-fibrin thrombus under flow is at best incompletely studied.

Murine 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 FeCl<sub>3</sub>, resulting in endothelial denudation, leads to collagen-dependent platelet activation with an additional role of TF, particularly upon milder FeCl<sub>3</sub> injury.<sup>4-7</sup> Thrombin and fibrin generations are elicited on procoagulant platelets exposing phosphatidylserine.<sup>8,9</sup> Platelets, furthermore, control the coagulation process by secreting procoagulant and anticoagulant proteins, whereas there is ample evidence for a role of platelet-exposed TF.<sup>10,11</sup> On the other hand, in most laser-induced injury models, thrombus formation is primarily triggered by thrombin that is generated via vascular TF.<sup>12,13</sup> Severe laser injury, however, also leads to collagen exposure and collagen-dependent platelet activation.<sup>14,15</sup>

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**Nonstandard Abbreviations and Acronyms**

<b>RedYM</b>	reduced Young modulus
<b>TF</b>	tissue factor

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,<sup>16</sup> and thrombin/fibrin-rich areas in the thrombus core region.<sup>17,18</sup> 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.<sup>19,20</sup> 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 enforces the fibrin network<sup>21</sup> and that blood flow supports the alignment of stiff fibrin fibers.<sup>22,23</sup> Macroscopic thromboelastic studies have not come much further than demonstrating that, in patients with dilutional coagulopathy, the overall clot strength is predictive for the risk of perioperative bleeding.<sup>24</sup>

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**

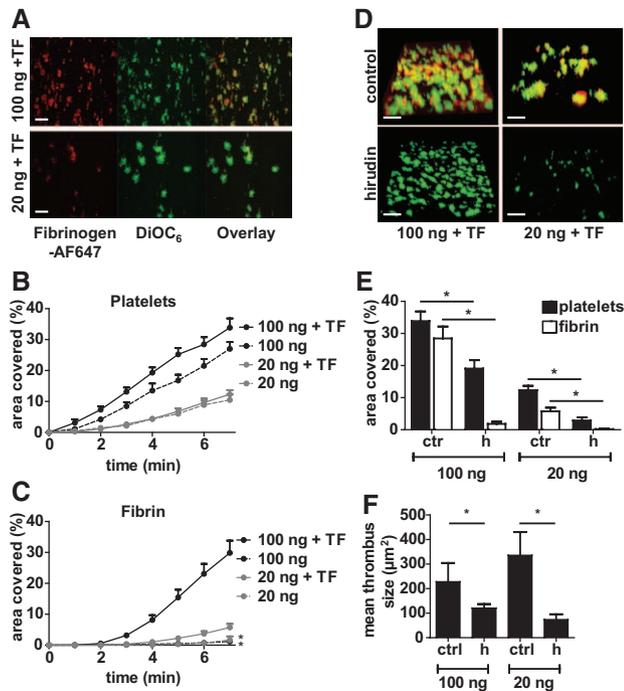
To study the roles of platelets and coagulation in whole-blood thrombus formation under flow, we applied a previously developed microspot method of thrombus formation in parallel-plate flow chambers,<sup>25</sup> which was adapted to operate under strictly controlled coagulant conditions. The changes included (1) preparation of microspots with defined amounts

of collagen and TF, (2) coinfusion 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'-dihexyloxycarbocyanine iodide (DiOC6) and Alexa Fluor (AF) 647 fibrinogen as probes that were preadded to the blood samples (Figure I in the online-only Data Supplement).

Pilot data indicated that coating 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.<sup>26</sup> Perfusion of blood samples at a shear rate of  $1000\text{ s}^{-1}$  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,  $12.3\% \pm 1.4\%$  versus  $33.8\% \pm 3.0\%$ ;  $P=0.005$ ) 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,<sup>27</sup> 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 showed that hirudin significantly reduced the size of individual thrombi on the high-collagen surface (from  $228 \pm 76$  to  $121 \pm 16\ \mu\text{m}^2$ ;  $P=0.007$ ), a reduction that was even more pronounced on the low collagen surface (from  $335 \pm 94$  to  $74 \pm 21\ \mu\text{m}^2$ ;  $P<0.001$ ; Figure 1F).

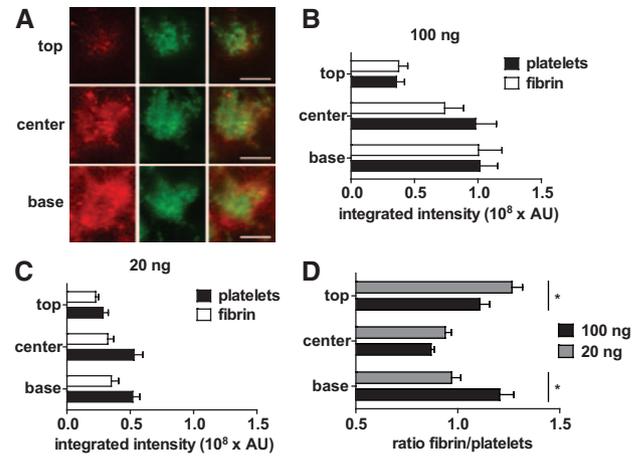
As microscopic images showed a gradual contraction of platelets in fibrin-containing thrombi, we aimed to quantify this process by addition of 3% DiOC<sub>6</sub>-labeled washed platelets to the blood and then monitoring the movement of adhered platelets in time. It seemed that the overall displacement rate of platelets was high on microspots with collagen/TF during the time of fibrin formation ( $1.06 \pm 0.11 \times 10^{-3}\ \mu\text{m/s}$ ), whereas it was non-significantly changed ( $0.87 \pm 0.10 \times 10^{-3}\ \mu\text{m/s}$ ) in the presence of



**Figure 1.** Collagen–tissue factor (TF) coating determines the buildup of platelet–fibrin thrombus formed under flow. Citrated whole blood was perfused during recalcification over microspots with collagen (20 or 100 ng) in the absence or presence of TF (10 pg) for 7 minutes at a wall shear rate of  $1000\text{ s}^{-1}$ . Blood samples were prelabeled with DiOC<sub>6</sub> to label platelets (green) and with AF647 fibrinogen to monitor fibrin formation (red). Control samples were pretreated with hirudin (h,  $3\text{ }\mu\text{g}/\text{mL}$ ), where indicated. Two-color microscopic confocal images were recorded in real time at a frequency of 1 Hz. **A**, Representative fluorescence images from collagen/TF microspots after 7 minutes of flow (bar,  $50\text{ }\mu\text{m}$ ). Time-dependent accumulation of DiOC<sub>6</sub>-labeled platelets (**B**) and AF647-fibrin(ogen) during blood flow (**C**). **D**, Representative stacks of thrombi in 3D (z step,  $1\text{ }\mu\text{m}$ ) of platelets (green) and fibrin(ogen) (red) on collagen/TF microspots after 7 minutes of flow (bar,  $50\text{ }\mu\text{m}$ ). **E**, Fluorescence area covered by platelets and fibrin on collagen/TF microspots after 7 minutes of flow. **F**, Average thrombus size on spots as determined by morphometric image analysis. Mean $\pm$ SE (n=6–15); \* $P$ <0.05.

Gly-Pro-Arg-Pro, inhibiting fibrin polymerization, and significantly lowered in the presence of hirudin ( $0.64\pm 0.05\times 10^{-3}\text{ }\mu\text{m}/\text{s}$ ;  $P=0.0064$ ). Hence, under the present flow conditions, platelet contraction did occur in the absence of thrombin or fibrin but was enhanced by the formation of either. This resolves a dispute in the literature that platelet–fibrin retraction is considered a thrombin-dependent event<sup>28</sup> but can still occur under conditions where thrombin is blocked.<sup>29</sup>

By lowering the wall shear rate from  $1000$  to  $150\text{ s}^{-1}$ , platelet deposition on high or low collagen was reduced (Table I in the online-only Data Supplement), as expected because of a diminished role of von Willebrand factor.<sup>25</sup> On the other hand, total fibrin formation did not reduce at a lower shear rate (Figure II in the online-only Data Supplement). Colocalization analysis of fluorescence from platelets and fibrin indicated major extension of the fibrin fibers outside of platelet aggregates especially at low shear conditions ( $150\text{ s}^{-1}$ ) and to a lesser extent at the higher shear rate (Figure II in the online-only Data Supplement). Jointly, these data indicate that limitation of the platelet-adhesive collagen surface resulted in



**Figure 2.** Collagen–tissue factor (TF) coating determines distribution of fibrin through a thrombus. Platelet–fibrin thrombi were formed by blood flow over microspots of collagen (20 or 100 ng) plus TF (10 pg) for 7 minutes, as described in Figure 1. Confocal images of DiOC<sub>6</sub> (green) and AF647-fibrin(ogen) (red) fluorescence in 3D were captured. **A**, Representative fluorescence images of optical slices at base, center, and top regions of thrombus on collagen (100 ng)/TF microspots (bar,  $20\text{ }\mu\text{m}$ ). **B** and **C**, Integrated fluorescence intensity (arbitrary units [AU]) of platelet (green) and fibrin (red) fluorescence from z stacks at base ( $<10\text{ }\mu\text{m}$ ), center ( $10\text{--}20\text{ }\mu\text{m}$ ), and top ( $>20\text{ }\mu\text{m}$ ) regions of thrombi formed. **D**, Ratio of platelet/fibrin fluorescence intensity at base, center, and top regions of thrombi. Mean $\pm$ SE (n=6–7); \* $P$ <0.05.

an overall reduced platelet deposition, which was partly compensated by a larger thrombus size because of the activity of TF-triggered thrombin generation. Lowering of the shear rate also reduced platelet accumulation but in a relative way promoted fibrin formation.

### 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 fluorescently 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 ( $0\text{--}10\text{ }\mu\text{m}$ ) and center regions ( $10\text{--}20\text{ }\mu\text{m}$ ) of the thrombi. On microspots with low collagen, overall thrombus volume was reduced from  $1.10\pm 0.20$  to  $0.46\pm 0.10\times 10^6\text{ }\mu\text{m}^3$  ( $P<0.001$ ; 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 showed a significantly higher ratio of fibrin to platelet fluorescence ( $P=0.022$ ) compared with those on high collagen (Figure 2D). In contrast, the base 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 ( $28.8\%\pm 1.9\%$  of fibrin-positive pixels) than at the center and top regions of the thrombus ( $8.9\%\pm 2.1\%$  and  $5.3\%\pm 1.8\%$  of

fibrin-positive pixels, respectively;  $P < 0.001$ ). Fibrin outgrowth was further restricted on low collagen microspots, where only  $9.7\% \pm 2.8\%$ ,  $2.3\% \pm 0.6\%$ , and  $5.2\% \pm 1.1\%$  of the fibrin fluorescence were detected outside the platelet aggregates at the base, center, or top regions of thrombi, respectively.

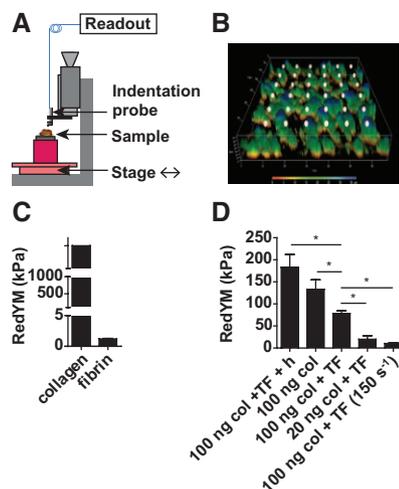
Interestingly, when compared with a high shear rate, at a low shear rate of  $150 \text{ s}^{-1}$ , overall thrombus volume reduced from  $1.10 \pm 0.20$  to  $0.78 \pm 0.02 \times 10^6 \mu\text{m}^3$  ( $P = 0.032$ ) on high collagen and from  $0.46 \pm 0.10$  to  $0.29 \pm 0.04 \times 10^6 \mu\text{m}^3$  ( $P = 0.026$ ) 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). Uncoated (not shown) or collagen-coated coverslips without thrombi gave a high RedYM of  $>1000 \text{ kPa}$  (Figure 3C). In contrast, coverslips covered with coagulated, fibrin-containing plasma produced a low RedYM of  $1.22 \pm 0.1 \text{ kPa}$ , thus pointing to high microelasticity of the fibrin network. Nanoindentation measurements of thrombi formed on high collagen ( $78.2 \pm 5.9 \text{ kPa}$ ) and low collagen ( $20.0 \pm 8.1 \text{ kPa}$ ) 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  $1000 \text{ s}^{-1}$  to  $150 \text{ s}^{-1}$  resulted in a lower RedYM ( $P < 0.0001$ ) 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  $133.1 \pm 21.6 \text{ kPa}$ . In the presence of hirudin to block thrombin activity, and hence fibrin formation, the RedYM even further increased to  $182.9 \pm 29.3 \text{ kPa}$  (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.

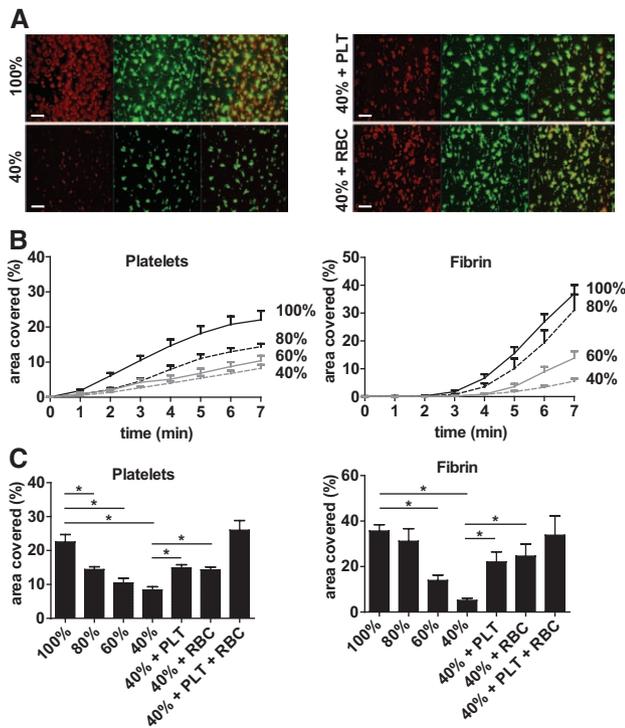
### Formation of Platelet–Fibrin Thrombus Under Conditions of Impaired Hemostasis

Using several approaches, we further assessed the roles of platelets and other blood components in the formation of



**Figure 3.** Collagen–tissue factor (TF) coating determines microelasticity of platelet–fibrin thrombus formed under flow. Platelet–fibrin thrombi were formed on microspots with collagen $\pm$ TF, as described in Figure 1. Flow experiments were carried out at a shear rate of  $1000 \text{ s}^{-1}$ , except where indicated otherwise ( $150 \text{ s}^{-1}$ ). After careful rinse with HEPES buffer pH 7.5, coverslips with thrombi were subjected to nanoindentation (6 $\times$ 6 indentations per microspot). Control coverslips were used for scanning electron microscopy. **A**, Schematic presentation of nanoindenter, featuring the indentation probe, sample, and automated  $x$ – $y$  stage. **B**, Schematic grid pattern of preset 6 $\times$ 6 indentations per microspot with height gradient. **C**, Reduced Young modulus (RedYM, kPa), a parameter inversely related to the microelasticity, in control measurements with a collagen-coated surface or with fibrinated plasma ( $n = 16$ ). **D**, RedYM assessed for thrombi formed on indicated collagen/TF surfaces ( $n = 36$ – $72$ ). Mean $\pm$ SE; \* $P < 0.05$ . h indicates hirudin.

fibrin-containing thrombi. Considering the diminished clotting activity of patients with perioperative dilutional coagulopathy,<sup>30,31</sup> we first determined the consequences of blood dilution in vitro. Blood samples were flowed at a shear rate of  $1000 \text{ s}^{-1}$  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  $14\% \pm 2\%$  ( $P = 0.046$ ),  $10\% \pm 3\%$  ( $P = 0.004$ ), and  $8\% \pm 3\%$  ( $P < 0.001$ ), 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  $150 \text{ s}^{-1}$  (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).



**Figure 4.** Restoration of platelet–fibrin thrombus formation by reconstitution of diluted blood. Platelet–fibrin thrombi were formed on microspots with collagen (100 ng) and tissue factor (TF; 10 pg), as described in Figure 1. Blood samples were diluted to indicated percentages with saline (keeping fixed concentrations of citrate,  $MgCl_2$  and  $CaCl_2$ ). Specific samples were reconstituted with autologous washed platelets (PLT) or red blood cells (RBC), added to the original counts. **A**, Representative fluorescence images of DiOC<sub>6</sub>-labeled platelets (green) and AF647-fibrin(ogen) (red) after 7 minutes of flow using 100% or 40% blood with(out) reconstitution (bar, 50  $\mu$ m). **B**, Time-dependent accumulation of fluorescence from platelets (green) and fibrin (red) during perfusion. **C**, Fluorescence area covered after 7 minutes of flow by platelets and fibrin. Mean $\pm$ SE (n=5–12); \* $P$ <0.05.

As a second approach, blood was used from various patients with hemostatic insufficiencies and a bleeding risk. Blood samples were obtained from 3 patients with dilutional coagulopathy because of massive fluid infusion during surgery. In this case, only few platelet aggregates of small size were formed and no fibrin was generated during the measured time of 7 minutes (Figure 5A). With blood samples from 2 thrombocytopenic patients, platelet deposition was comparably low, although measurable amounts of fibrin were formed (Figure 5A and 5C), such in accordance with normal coagulation activity in this condition. A requirement for platelet procoagulant activity (phosphatidylserine exposure) was investigated with blood samples from a Scott patient, whose platelets lack this property.<sup>32</sup> Whereas platelet deposition and aggregate size were not affected, fibrin formation was essentially absent in the Scott blood samples. Similarly, impaired coagulation, as in blood from 2 patients with hemophilia B ( $\approx$ 5% factor IX), led to normal platelet aggregation under flow but an impaired fibrin formation (Figure 5A and 5C).

Because the flow of thrombocytopenic blood over high collagen microspots still gave limited fibrin formation, the 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 ( $12\pm 8\%$  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  $34\pm 14\%$  and  $6\pm 1\%$ , respectively. Jointly, these results pointed to platelet control of fibrin formation and distribution in thrombi formed on collagen/TF surfaces, relying on phosphatidylserine exposure.

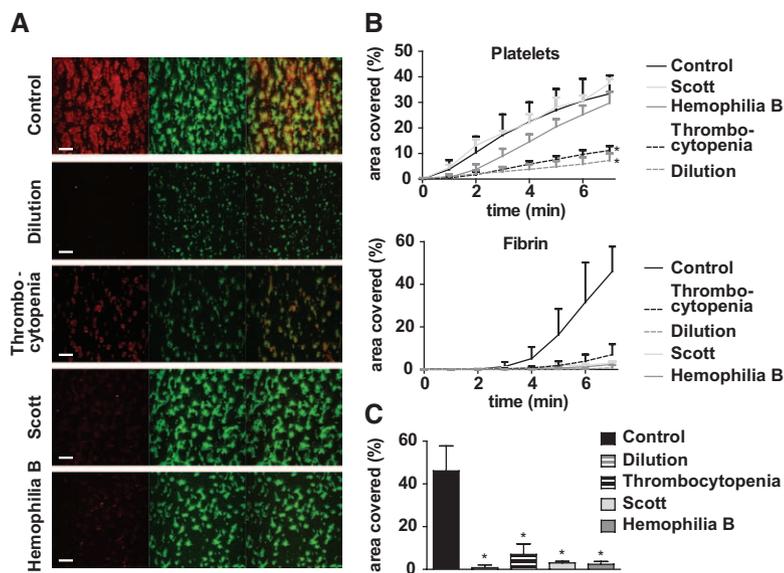
## Discussion

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 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 phosphatidylserine exposure. This procoagulant surface greatly promotes the assembly of coagulation factors culminating in the formation of factor Xa and thrombin.<sup>2</sup> 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 phosphatidylserine exposure.<sup>3</sup> 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 phosphatidylserine 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.<sup>33</sup> In this study, we have

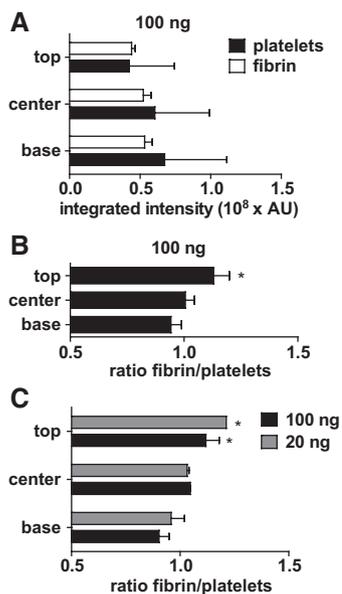


**Figure 5.** Impaired platelet–fibrin thrombus formation using blood from patients with impaired hemostasis. Blood was obtained from 7 healthy control subjects, 3 patients with dilutional coagulopathy because of massive fluid infusion during cardiothoracic surgery, 2 patients with acquired thrombocytopenia, 1 Scott syndrome, or 2 patients with hemophilia B. At least 3 blood samples were analyzed per condition. Platelet–fibrin thrombi were formed in a standard way by blood flow at 1000 s<sup>-1</sup> over microspots with collagen (100 ng) and TF (10 pg), as described for Figure 1. **A**, Representative images of DiOC<sub>6</sub>-labeled platelets (green) and AF647-fibrin(ogen) (red) after 7 minutes (bar, 50 μm). **B**, Time-dependent accumulation of platelet and fibrin fluorescence during blood perfusion. **C**, Fluorescence area covered by fibrin after 7 minutes of flow. Mean±SD (n=6–9); \* significantly different from reference range of control blood samples.

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.<sup>20,34</sup>

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 microelasticity is determined by the distribution pattern of fibrin.

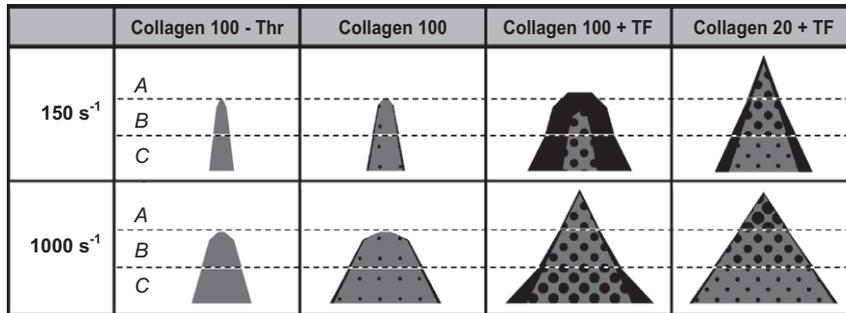


**Figure 6.** Altered fibrin distribution within thrombus formed by thrombocytopenic blood. Platelet–fibrin thrombi were formed by blood flow over microspots with collagen (20 or 100 ng) and tissue factor (TF; 10 pg), as described in Figure 1. Confocal images of DiOC<sub>6</sub> (green) and AF647-fibrin(ogen) staining (red) in 3D were captured. **A**, Integrated fluorescence intensity (arbitrary units [AU]) of platelet (green) and fibrin (red) fluorescence from z stacks at base (>10 μm), center (10–20 μm), and top (>20 μm) regions of thrombi after 7 minutes of blood perfusion on high collagen/TF. Ratio of platelet/fibrin staining at base, center and top of thrombi after 7 minutes on high collagen (**B**) or after 12 minutes (**C**) of blood perfusion on low and high collagen. Mean±SD (n=3); \*P<0.05 vs base.

### 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,<sup>18,35,36</sup> in which thrombin activity is confined to the dense thrombus core. Previous in vivo studies have shown that, upon 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.<sup>6,12,14</sup> 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 (protease-activated receptor 4)-deficient mice, in which fibrin was found to redistribute through the whole thrombus as a consequence of diminished platelet activation.<sup>16</sup>

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



**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. In specific blood samples, thrombin was inhibited with hirudin (–Thr). Multiple parameters of thrombus formation were assessed (full data are available in Table I in the online-only Data Supplement). Cartoons 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  $\mu\text{m}$  from microspot surface; B, 10 to 20  $\mu\text{m}$ ; and C, >20  $\mu\text{m}$ .

removal of thrombin and fibrin monomers.<sup>37</sup> 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.<sup>38</sup>

### 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.<sup>30</sup> 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.<sup>37</sup> Remarkably, in reconstitution experiments, 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.<sup>39</sup>

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.<sup>40</sup> However, current overviews indicate that collagen (TF)-based in vitro flow studies compare well with in vivo arterial thrombosis models, when evaluating the functional effects of genetic knockout in mice.<sup>41,42</sup> Hence, we consider the present test of formation of platelet–fibrin thrombi under flow as a valid method to determine the hemostatic capacity of a blood sample. For fibrin localization, we used fluorescently labeled fibrinogen, and therefore, the distinction between fibrinogen and fibrin cannot be made unambiguously. We minimized the contribution of fibrinogen to the fibrin signal by threshold settings, and the presence of fluorescent fibrin fibers was always confirmed from bright-field images. The advantage is that this method prevents underestimation of the fibrin signal as a consequence of poor permeation in the thrombus when using a fibrin antibody.

In summary, we conclude that under conditions of flow and coagulation, the amount and localization of fibrin are

regulated by the relative abundance of triggers for platelet adhesion/activation (eg, collagen) and coagulation (TF) and that the fibrin distribution is determinative for the clot microelasticity. Thrombin herein influences thrombus growth and fibrin formation, depending on its local concentration.

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### Disclosures

N. Rijnveld, E.J. Breeel, and K.O. van der Laan are employees of Optics11, Amsterdam, The Netherlands. The other authors report no conflicts.

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## Significance

Activation of blood platelets, as well as the coagulation system, is required for a normal hemostatic response. Although it is known that platelet- and fibrin-dependent thrombus formation is regulated by blood flow, the blood composition and vascular triggers, such as collagen and tissue factor, the interactions between these blood-borne and vascular components are not well understood. Our data imply that the relative abundance of vascular triggers of platelet adhesion/activation (collagen) at one side and of coagulation (tissue factor) at the other side is determinative for both thrombus growth and thrombus composition, in particular about the distribution of fibrin. Furthermore, we show that the fibrin distribution is determinative for the thrombus microelasticity as assessed by nanoindentation.