Role of Collagen-Adherent Platelets in Mediating Fibrin Formation in Flowing Whole Blood

By Daniel Kirchhofer, Thomas B. Tschopp, Beat Steiner, and Hans R. Baumgartner

Activated platelets provide assembly sites for coagulation enzyme complexes and in this way can mediate coagulation during hemostasis and thrombosis. In this study, we examined the procoagulant activity of platelets adhering directly to fibrillar collagen, a main thrombogenic constituent of subendothelium. For this purpose, we used a human ex vivo thrombosis model in which collagen-coated coverslips were exposed to flowing anticoagulated blood (shear rate, 65/s) for 5.5 minutes, which led to the deposition of adherent platelets, platelet thrombi, and fibrin. To examine the procoagulant activity of adherent platelets only, a selective antagonist of the platelet GPIIb-IIIa complex, Ro 44-9883, was infused via a mixing device, resulting in a complete abrogation of platelet thrombus formation but leaving the collagen-adherent platelet layer intact. This platelet layer generated increased postchamber fibrinopeptide A (FPA) levels (203 ± 33 ng/mL) as compared with control experiments without infusion of inhibitor (95 ± 13 ng/mL). Concomitantly, fibrin deposition measured by morphometric analysis of cross-sections was also increased, as was the platelet adhesion to collagen. An immunochromatographic staining of fibrin fibers further showed that the adherent platelets formed the nuclei for fibrin fiber formation. This increase in fibrin deposition was mediated by the intrinsic factor X (F.X) activation complex on adherent single platelets, because almost complete inhibition of FPA generation (9 ng/mL) and fibrin deposition (0.4% ± 0.2% coverage) was achieved upon confusion of the GP IIb-IIIa antagonist and active site-inhibited F.IXa. The large platelet thrombi that were deposited in control experiments contained no significant amounts of immunodetectable fibrin except at the thrombus base, where adherent platelets anchored the thrombi to the collagen surface. These results suggest that the collagen-adherent platelets are important promoters of coagulation during the initial phase of thrombogenesis by providing assembly sites for the F.X activation complex.

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PLATELETS ACCUMULATE in significant numbers at the site of vascular injury, when subendothelium becomes exposed to the blood stream.2,3 Fibrillar collagen is a major thrombogenic component of subendothelium,2 and it triggers platelet thrombus formation in flowing human blood.3,4 The formation of a platelet thrombus is dependent on the platelet glycoprotein (GP) IIb-IIIa, which binds to its bivalent ligand fibrinogen and thereby cross-links the platelets.5 In addition, activated platelets participate in coagulation by providing assembly sites for the factor VIIIa (F.VIIIa)/F.IXa and F.Va/F.Xa complexes.6,8 This results in the generation of thrombin on the platelet surface and the formation of a fibrin network. The importance of platelet procoagulant activity in normal hemostasis is exemplified in the Scott syndrome. This bleeding disorder is characterized by the inability of platelets to promote coagulation9-11 because of impaired translocation of acidic phospholipids to the outer membrane leaflet,12 resulting in reduced F.X and prothrombin converting activities.10,12 Little is known about the procoagulant activity of platelets that are in direct contact with subendothelial collagen as compared with platelets that form the main body of an adherent platelet thrombus. A unique feature of collagen-adherent platelets is that they are activated by collagen as well as by soluble agonists, such as thrombin, whereas the platelets comprising the main body of the attached platelet thrombi are only activated by soluble agonists but not by collagen. This difference might bear consequences for platelet procoagulant activity. In support of this view, Bevers et al13 showed that, when exposed to a collagen-thrombin cosumulus, purified platelets developed procoagulant activity within a much shorter time period as compared with a thrombin stimulus alone. Further indications for the importance of collagen-adherent platelets were provided by experiments in which collagen-containing subendothelium was exposed to blood of thrombasthenic patients11; despite the absence of platelet thrombus formation, due to the genetic defect in these patients, fibrin formation occurred, presumably involving the procoagulant activity of the substratum-adherent platelets.

To gain further insight into the procoagulant activity of collagen-adherent platelets we used a parallel plate perfusion system14 that allowed us to quantify platelet-mediated coagulation in human nonanticoagulated blood at venous blood flow. The procoagulant activity of collagen-adherent platelets was differentiated from that of platelet thrombi by the use of a potent and selective antagonist of GPIIb-IIIa, Ro44-9883. This compound was infused into normal blood via a mixing device,15 resulting in a complete inhibition of platelet thrombus formation but without a concomitant inhibition of platelet adhesion to the collagen surface. The results obtained provide evidence for the importance of collagen-adherent single platelets and the platelet F.X activation complex in mediating coagulation in flowing human blood.

MATERIALS AND METHODS

Antibodies, proteins, and inhibitors. Monoclonal antifibrin antibody was from American Diagnostica (Greenwich, CT). The antibody used for immunogold-silver staining of platelets was the monoclonal antibody (MoAb) pl-62 directed against the platelet-specific GPIIb-IIIa. Pl-62 is a complex-specific antibody and recognizes GPIIb-IIIa on resting as well as on activated platelets.16,17 Bovine F.IXa, which was blocked at its active site by dansyl-Glu-Gly-Arg-

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chloromethylketone (F.IXai), was obtained from Dr. David Stern (Columbia University, New York, NY). Human collagen type III was purified by salt precipitation, as described previously.15 Fibril formation was induced by dialyzing a solution of 1 mg/mL of collagen type III in 0.1 mol/L acetic acid against 20 mmol/L NaOH, pH 7.5, at 4°C for 24 hours. The activity of the preparations were tested in the aggregometer using human plasma and they were stored at 4°C until used in the perfusion experiments. Ro 44-9883 (molecular weight [MW] 468.5) was used as an inhibitor of platelet aggregation. The structure and its antiaggregatory activities were described previously.16,18 Briefly, this compound is a potent and reversible, nonpeptide antagonist of the platelet fibrinogen receptor GPIIb-IIIa19 and inhibits platelet aggregation in plasma with an IC₅₀ of 30 nmol/L.20 Ro 44-9883 is highly selective for GPIIb-IIIa and does not inhibit other Arg-Gly-Asp (RGD)-dependent receptors of the integrin family, including the closely related vitronectin receptor αvβ₃.18 Currently, Ro 44-9883 (lamifibra) is in the clinical development stage and showed promising results in patients with unstable angina.21

Perfusion experiments with infusion of the GPIIb-IIIa antagonist to normal nonanticoagulated human blood. Human collagen type III was sprayed in fibrillar form onto Thermaxan plastic coverslips (Miles Lab, Naperville, IL) at a concentration of 20 μg/cm². The collagen-coated coverslips were dried for several hours at room temperature, washed with 0.9% NaCl solution over a period of 1 hour, and kept in NaCl solution containing 0.1% bovine serum albumin (BSA) until they were used for the perfusion experiments. The coverslips were positioned in the three parallel plate perfusion chambers and the entire system, including the tubing, mixing devices, and parallel plate chambers, was filled with phosphate-buffered saline (PBS)-0.1% bovine serum albumin (BSA). The experimental system was described recently.25 Blood was then drawn from the antecubital perfusion device without interrupting the flow. The mixing device was then disconnected from the distribution block without interrupting the flow. 1 mL/min was maintained. The mixing device was then disconnected from the parallel plate perfusion devices, which were subsequently perfused at 1 mL/min for 2 minutes with 3% paraformaldehyde in PBS (for immunohistochemical staining) after a brief interruption of flow (approximately 5 seconds). For morphometrical examinations, we used 2.5% glutaraldehyde in 0.1% cacodylate buffer, pH 7.4, containing 2.5 mmol/L CaCl₂ and 0.9 mmol/L MgCl₂ instead of 3% paraformaldehyde.

The coverslips were then removed from the chambers and incubated in fresh fixative for an additional 30 minutes and stored in PBS-0.03% azide and in cacodylate buffer containing 7% sucrose for immunohistochemical staining and morphometrical analysis, respectively.

Determination of fibrinopeptide A (FPA) levels. FPA levels were measured in the blood leaving the perfusion device (postchamber). For that purpose, a second mixing device was positioned between the parallel plate perfusion chambers containing noncoated or collagen-coated coverslips and the roller pumps. This second mixing device served to mix the blood with an anticoagulant cocktail (32 mg/mL trisdium-citrate, 1,000 IU/mL heparin, and 1 TIU/mL aprotinin) to prevent further FPA generation. The anticoagulant cocktail was supplied by an additional roller pump distal to the perfusion chambers at a flow rate of 0.1 mL/min resulting in a mixing ratio of 1:10 (anticoagulant cocktail/blood). The blood flow rate at the distal end of the mixing device was 1.1 mL/min, which resulted in a blood flow rate of 1 mL/min (shear rate, 65/s) over the cover slip, consistent with the perfusion experiments described above. The anticoagulated blood was collected from the roller pumps into polypropylene tubes over a period of 4.5 minutes. After centrifugation, the platelet poor plasma was stored at -20°C until the FPA concentrations were determined according to the manufacturer's instructions (enzyme-linked immunosorbent assay [ELISA] FPA; Boehringer Mannheim GmbH, Mannheim, Germany). For some experiments, a T-branching was used instead of the distribution block and mixing device and was positioned upstream to the perfusion chambers. The T-branching served to mix the buffer and fixative.

Quantiﬁcation of platelets, platelet thrombi and fibrin on cross sections. After the perfusion experiments, the coverslips were embedded in Epon (Fluka Chemie, Buchs, Switzerland) and semithin sections perpendicular to the blood flow direction were prepared as described previously.26 The cross-sections were stained with 0.01% toluidine blue and 0.01% fuchsin and the deposition of platelets, platelet thrombi, and fibrin was determined at 10-μm intervals along the length (8 mm) of the sections using a Zeiss Axioscop microscope.27 Fibrin coverage was measured as the total of both the fibrin deposited on the collagen surface and on adherent platelets. Platelet adhesion was measured as the area covered by the both the spreading platelets or the nonspreading platelets in contact with collagen (contact platelets) and the platelets spread on collagen (spread platelets). Platelet thrombi were defined as platelet aggregates that were greater than 5 μm. The measurements were from a coverslip section 1 mm downstream from the flow entrance. In certain experiments, the height of the fibrin layer was also determined. For this purpose, we used coverslip sections further downstream at a position 7 mm from the flow entrance, because the fibrin deposition at this position showed less variation between individual coverslips.

Immunogold-silver staining of fibrin and platelets. After the perfusion experiments, the coverslips were incubated with either 2.5 μg/mL of monoclonal anti–GPIIb-IIIa antibody PI-62 (for platelet staining) or with 10 μg/mL of monoclonal anti–GPIIb-IIIa antibody I-62 (for platelet staining) in PBS. After washing with PBS, the coverslips were incubated at room temperature for 30 minutes with 5 nm gold-labeled antiamouse antibody (Auro Probe LM; Amersham, Amersham, UK) diluted 1:50 in PBS for fibrin staining and diluted 1:10 for platelet staining, respectively. The coverslips were then washed with PBS, treated for 10 minutes with 2% glutaraldehyde in PBS, and washed with PBS and distilled water. After incubation with silver enhancer for 10 to 15 minutes (IntenSE M; Amersham), the coverslips were fixed with Rapidfix (Eastman Kodak, Rochester, NY) and thoroughly washed in distilled water. After air-drying, the coverslips were embedded in Merckoglass (Merck, Darmstadt, Germany) and examined under the microscope (Zeiss Axioshot; Carl Zeiss, Oberkochen, Germany).

RESULTS

Activation of coagulation by the flow system. Homogeneous distribution of the platelet inhibitor Ro 44-9883 in the flowing blood required the positioning of a Plexiglas distr-
distribution block and a mixing device in front of the perfusion chambers containing the collagen-coated coverslips. This resulted in an appreciable activation of the coagulation system when compared with conditions in which the distribution block and mixing device was omitted and replaced with a T-branching. Postchamber FPA levels increased from 33.9 ± 9.4 ng/mL (13 donors) with the T-branching setting to 95.4 ± 13.2 ng/mL (21 donors) when distribution block and mixing device were included. Similarly, fibrin coverage increased from 44.3% ± 14.6% (8 donors) to 98.7% ± 1.2% (13 donors).

Fibrin deposition and FPA generation mediated by the collagen-adherent platelet layer. Platelet thrombus formation was prevented by blocking the platelet fibrinogen receptor GPIIb-IIIa with the synthetic inhibitor Ro 44-9883, allowing us to specifically determine the procoagulant activity of the collagen-adherent platelets. This compound inhibits ADP-induced platelet aggregation in plasma with an IC50 value of 30 nmol/L. It was shown that the inhibitor did not induce conformational changes on GPIIb-IIIa, and further studies showed that the compound neither affected basal intracellular calcium levels or pH of unstimulated platelets nor the calcium and pH increases in response to agonists. The GPIIb-IIIa antagonist Ro 44-9883 was infused at a concentration that completely inhibits platelet aggregation (500 nmol/L) into normal human blood under venous flow conditions (shear rate, 65/s). This resulted in a complete inhibition of platelet thrombus formation as shown on cross-sections (Fig 1A and B) and by a platelet-specific immunostaining (Fig 1C and D). Nevertheless, platelets were found to avidly adhere to the collagen surface as shown on cross-sections and by a platelet-specific immunogold staining on en face preparations (Fig 1B and D). Moreover, despite inhibition of platelet thrombus formation, fibrin was deposited on the collagen surface, as shown on cross-sections (Fig 1B) and on en face preparations after fibrin-specific immunostaining (Fig 1F). The fibrin deposited on top of the adherent platelets also contained single platelets and erythrocytes (Fig 1B). This explains the heterogeneous platelet distribution pattern on stained en face preparations, because the staining visualized all platelets, including collagen-adherent and fibrin-associated platelets (Fig 1D).

A quantitative analysis on cross-sections showed that infusion of the GPIIb-IIIa antagonist reduced the coverage of the collagen layer with platelet thrombi from 10.7% to 0% (Fig 2a). Conversely, fibrin formation determined as FPA levels in the postchamber blood increased from 95.4 ± 13 ng/mL to 203.1 ± 33.1 ng/mL when platelet aggregation was inhibited (Fig 2b). Similarly, platelet adhesion (contact + spread platelets) determined as the percentage of coverage of the collagen surface increased from 19.9% ± 2.2% to 31.8% ± 2.4%. The coverage with contact platelets increased as well from 0.4% ± 0.07% to 2% ± 0.4%. Thus, the ratio of contact to spread platelets increased from 2% in control to 6%. Platelet adhesion was measured on cross-sections 1 mm downstream from the flow entrance on the coverslip. Further downstream, at a position 7 mm distal to the entrance, platelet adhesion in control experiments was only 8.9% ± 0.4% and increased to 35.1% ± 2.3% by infusion of the GPIIb-IIIa antagonist. The lower platelet adhesion of controls at the downstream position is probably due to the higher consumption of platelets from the boundary layer by the upstream deposited platelet thrombi. This finding is in full agreement with the previously reported axial-dependence of platelet deposition in flowing, nonanticoagulated blood.

We next examined whether the increased procoagulant activity measured as elevated FPA levels was paralleled by an increased fibrin deposition on the coverslips. Table 1 shows that, in controls, about 50% of the coverslip surface was covered with a fibrin layer of less than 5 µm and only 10% was covered with a layer greater than 10 µm. In contrast, in the absence of platelet thrombus formation, i.e., with infusion of the GPIIb-IIIa antagonist, the surface coverage with a fibrin layer greater than 10 µm was up to 45% and only 7% was covered with fibrin less than 5 µm in height (Table 1).

Fibrin fiber deposition mediated by adherent single platelets. To better visualize fibrin associated with adherent platelets, the standard perfusion period of 5.5 minutes was reduced to 3.5 minutes, resulting in reduced fibrin density. A fibrin-specific immunostaining strongly suggested that fibrin formation under control conditions was platelet-associated (Fig 3A). Similarly, with infusion of the GPIIb-IIIa antagonist, the single platelets that adhered to collagen formed the nuclei for fibrin fiber formation (Fig 3B). The greater density of fibrin fibers as compared with controls (Fig 3A) can be attributed to the increased platelet density on the collagen surface. Note that the growth direction of fibrin fibers originating from single platelets is not parallel to the blood flow direction (Fig 3A). This was most likely due to blood flow disturbances caused by the nearby deposited platelet thrombus.

Role of the intrinsic F.X activation complex in fibrin formation. To further establish that fibrin formation was dependent on adherent platelets, we used F.IXai as a selective inhibitor of the F.X activation complex. Coinfusion of the GPIIb-IIIa antagonist and F.IXai to the flowing blood resulted in a complete inhibition of fibrin deposition (Fig 4C) as compared with infusion of the GPIIb-IIIa antagonist alone (Fig 1F). Platelets adhered to the collagen surface as a dense monolayer (Fig 4A and B), and, frequently, leukocytes were bound to single platelets (Fig 4A). Most significantly, fibrin deposition on the coverslips determined by morphology as well as postchamber FPA levels were inhibited by more than 90% (Table 2), indicating that fibrin formation by single, adherent platelets is dependent on the intrinsic F.X activation complex.

The observed inhibitory effect of F.IXai allowed us to reduce fibrin density for immunostainings with a standard perfusion period of 5.5 minutes. For that purpose, the GPIIb-IIIa antagonist Ro 44-9883 was coininfused with a low concentration (12.5 nmol/L) of F.IXai. The result showed that, similar to the findings using a shorter perfusion period and infusion of Ro 44-9883 alone (Fig 3B), the fibrin fibers originated from deposited single, adherent platelets (data not shown).

**DISCUSSION**

This report shows that collagen-adherent platelets play a key role in promoting coagulation in flowing human blood.
Fig 1. Fibrin and platelet deposition to exposed collagen in the presence of infused GPIIb-IIIa antagonist. Using a parallel plate perfusion system, collagen-coated coverslips were exposed for 5.5 minutes to normal human nonanticoagulated blood supplemented with 500 nmol/L of GPIIb-IIIa antagonist (B, D, and F) or with buffer (control; A, C, and E). After washing and fixation, the coverslips were used for immunostaining or for preparing cross-sections after embedding in Epon. (A and B) Cross-sections stained with fuchsin-toluidine solution (arrowheads indicate spread platelets). (C and D) Immunogold-silver staining of platelets and platelet thrombi (en face view) using a GPIIb-IIIa-specific MoAb. (E and F) Immunogold-silver staining of fibrin (en face view) using a fibrin-specific MoAb. L, leukocyte; T, platelet thrombus; F, fibrin fibers; P, single platelet; E, erythrocyte. Arrows indicate spread platelets. The bar in (A) represents 20 μm (for A and B) and that in (C) represents 50 μm (for C through F). In (C) through (F), the blood flow direction was from left to right.

This conclusion is based on results obtained from measuring postchamber FPA levels and fibrin deposition to the exposed collagen surface mediated by the collagen-adherent platelet layer in the presence or absence of concomitant platelet thrombus formation. Infusion of the GPIIb-IIIa antagonist Ro 44-9883 (lamifiban), which prevented platelet thrombus formation, resulted in the deposition of a collagen-adherent platelet layer that retained the full capacity to promote coag-
ulation. In fact, both FPA and fibrin deposition were increased and correlated with the surface coverage of adherent platelets. This suggested that coagulation was dependent on the collagen-adherent platelets rather than the presence of deposited platelet thrombi. The reason for the increased platelet adhesion with infusion of the GPIIb-IIIa antagonist is most likely due to a higher concentration of platelets in the boundary layer as compared with the situation in control experiments in which the platelet concentration near the collagen surface was reduced by the forming platelet thrombi. Evidence for such mechanisms is provided by experiments with a similar flow system, in which collagen was exposed to blood containing the platelet aggregation inhibitor clopidogrel.

In accordance with the view that the collagen-adherent platelets are the predominant mediators of coagulation, immunostaining experiments showed that adherent single platelets formed the nuclei for fibrin fiber formation under control conditions as well as with infusion of the GPIIb-IIIa antagonist. In control experiments, fibrin fibers also originated from small platelet aggregates, whereas large platelet thrombi, in most cases, did not contain much stained fibrin (Figs 3A and 1E). However, fibrin was detectable at the thrombus base anchoring the thrombi to the collagen surface (data not shown). This finding could be interpreted to be in agreement with in vitro experiments in that the strong collagen stimulus for these anchoring platelets and adherent single platelets shortened their lag phase to acquire a procoagulant surface and promote fibrin formation, whereas platelets contained in the thrombi and not exposed to collagen required more time for this process. Therefore, the time period of 5.5 minutes of the experiments may have been too short for the main body of platelet thrombi to develop a procoagulant surface. Besides receiving a collagen stimulus, the anchoring platelets were exposed the longest to soluble agonists such as thrombin, which may also have contributed to an earlier procoagulant activity as compared with platelets contained in the thrombi.

The fibrillar collagen surface represented the primary stimulus for platelets to adhere and to acquire a procoagulant state. In addition, the thrombin produced in this system, which was indirectly measured as the generated FPA, may

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**Table 1. Fibrin Deposition Mediated by Collagen-Adherent Platelets**

<table>
<thead>
<tr>
<th>Height of Fibrin Layer</th>
<th>Control (% coverage)</th>
<th>GPIIb-IIIa Antagonist (% coverage)</th>
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<tbody>
<tr>
<td>Fibrin layer &lt;2.5 μm</td>
<td>18.8 ± 5.0</td>
<td>0.8 ± 0.5</td>
</tr>
<tr>
<td>Fibrin layer 2.6-5 μm</td>
<td>33.2 ± 3.1</td>
<td>6.6 ± 2.4</td>
</tr>
<tr>
<td>Fibrin layer 5.1-10 μm</td>
<td>34.9 ± 4.9</td>
<td>47.1 ± 5.8</td>
</tr>
<tr>
<td>Fibrin layer &gt;10 μm</td>
<td>10.4 ± 2.5</td>
<td>45.4 ± 7.5</td>
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</table>

Collagen-coated coverslips were exposed for 5.5 minutes to flowing normal human blood (control) and to blood supplemented with the GPIIb-IIIa antagonist Ro 44-9983 to prevent thrombus formation, resulting in the deposition of a collagen-adherent platelet layer. Cross-sections were prepared as described in the Materials and Methods and the surface coverage with fibrin layers of different height was measured morphometrically. The values are the mean ± SEM of 6 donors (GPIIb-IIIa antagonist) and 13 donors (control).
have functioned as an amplifying costimulus. In vitro experiments showed that a combined stimulus of collagen and thrombin enhanced the rate of F.Xa generation by the platelet F.X activation complex as compared with thrombin alone. To examine whether fibrin deposition mediated by the collagen-adherent platelet layer involved the F.X activation complex, we coinjected F.IXa, which competitively inhibits the binding of F.IXa to activated platelets. This resulted in a complete inhibition of FPA generation and fibrin deposition, suggesting that coagulation was entirely mediated by the intrinsic F.X activation complex on adherent platelets, most of which were spread on the collagen surface. It seems very unlikely that contact platelets accounting for less than 6% of the adherent platelets significantly contributed to fibrin deposition. Consistent with these results, we previously showed that fibrin formation in control experiments without infusion of the GPIIb-IIIa antagonist is also entirely dependent on F.IXa enzymatic activity.

Our experimental conditions with infusion of the GPIIb-IIIa antagonist that resulted in the blockade of GPIIb-IIIa function may resemble thrombasthenic conditions, which are characterized by reduced or functionally inactive expression of GPIIb-IIIa on platelets. Weiss et al showed that, on exposure of collagen-containing subendothelium to blood of thrombasthenic patients, an adherent platelet monolayer was formed and fibrin deposition was slightly increased, results similar to our findings. In contrast to our results, platelet adhesion was not increased, which was most likely related to the higher shear rate (650/s) and the use of subendothelium instead of collagen. Based on our data, we suggest that the procoagulant activity of the platelet monolayer represents the underlying mechanism for the fibrin deposition observed in the study of Weiss et al.

GPIIb-IIIa is known to play an essential role in the adhesion of platelets to fibrin. Therefore, it was somewhat unexpected to find numerous single platelets within the fibrin meshwork that covered the collagen-adherent platelet layer when the GPIIb-IIIa antagonist was infused. However, it is possible that platelet adhesion to a dynamically evolving fibrin network in flowing nonanticoagulated blood is different from adhesion to preformed fibrin and may depend on additional mechanisms. Nevertheless, the most plausible explanation for the presence of fibrin-adherent platelets is that they became entrapped in the forming fibrin meshwork in a nonspecific manner. Consistent with this interpretation, we also detected numerous erythrocytes within the fibrin meshwork; their interaction with fibrin, especially under venous flow conditions, is thought to be rather unspecific. These data suggest that, despite the absence of platelet thrombi caused by infusion of the GPIIb-IIIa antagonist, the deposited fibrin together with entrapped single platelets and erythrocytes may form a loosely packed hemostatic plug at the site of vessel damage. It is tempting to speculate that, in regard to the intended use of GPIIb-IIIa antagonists for chronic antithrombotic therapy, the procoagulant activity of single platelets adhering to exposed subendothelial collagen might reduce the antihemostatic impact of such therapies.

Our experimental system caused minor activation of coagulation due to the passage of the blood through the Plexiglas components. In addition, platelet activation measured as β-thromboglobulin levels increased by using the mixing device. This preactivation resulted in a greater fibrin deposition on the collagen-coated coverslips in normal blood as compared with conditions without mixing device (our data) and other reports. By shortening the perfusion period or by infusing low concentrations of F.IXa, the fibrin deposition could be reduced. Immunostainings clearly indicated that, under these conditions, the fibrin deposition was mainly mediated by the collagen-adherent platelet layer, as it was with infusion of the GPIIb-IIIa antagonist, suggesting that the preactivation in our system did not influence the interpretation of our results.
In conclusion, we show that collagen-adherent platelets are essential in promoting coagulation in a human blood flow system. Thrombin generation mediated by collagen-adherent platelets may constitute an important mechanism for further platelet activation as well as fibrin deposition in adherent platelets. We showed further that the procoagulant activity of collagen-adherent platelets depends on F.IXa enzymatic activity. Therefore, the intrinsic F.X activation complex might represent a promising target for developing new classes of antithrombotics.

Table 2. Role of F.IXa in the Procoagulant Activity of Collagen-Adherent Platelets

<table>
<thead>
<tr>
<th>Morphometry and FPA Levels</th>
<th>GPllb-llla Antagonist</th>
<th>GPllb-llla Antagonist + F.IXai</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platelet adhesion (% coverage)</td>
<td>31.8 ± 2.4</td>
<td>36.5 ± 3.3</td>
</tr>
<tr>
<td>Thrombi (% coverage)</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>Fibrin deposition (% coverage)</td>
<td>98.6 ± 0.9</td>
<td>0.4 ± 0.2</td>
</tr>
<tr>
<td>FPA (ng/mL)</td>
<td>203.1 ± 33.1</td>
<td>8.8 ± 2.6</td>
</tr>
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</table>

Using the parallel plate perfusion system, collagen-coated coverslips were exposed for 5.5 minutes to flowing human blood with infusion of the GPllb-llla antagonist Ro 44-9889 (GPllb-llla antagonist) and with or without coinfusion of 50 nmol/L F.IXai (GPllb-llla antagonist + F.IXai). The surface coverage with deposited platelet thrombi (>5 μm in height), platelet adhesion (contact + spread platelets), and fibrin was determined on stained cross-sections as described in the Materials and Methods. FPA levels were measured in plasma derived from postchamber blood using an ELISA. The values represent the mean ± SEM of 11 donors (GPllb-llla antagonist) and 5 to 10 donors (GPllb-llla antagonist + F.IXai).

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