Platelet ADP receptors contribute to the initiation of intravascular coagulation
Catherine Leon, Meike Alex, Antje Klocke, Eberhard Morgenstern, Christine Moosbauer, Anita Eckly, Michael Spannagl, Christian Gachet, and Bernd Engelmann

While the adenosine 5′-diphosphate (ADP) pathway is known to enhance thrombus formation by recruiting platelets and leukocytes to the primary layer of collagen-adhering platelets, its role for the initiation of coagulation has not been revealed. Ex vivo inhibition of the P2Y12 ADP receptor by clopidogrel administration diminished the rapid exposure of tissue factor (TF), the major initiator of coagulation, in conjugates of platelets with leukocytes established by the contact of whole blood with fibrillar collagen. Under in vitro conditions, the P2Y12 and P2Y1 ADP receptors were both found to be implicated in the exposure of TF in collagen-activated whole blood. Immunoelectronmicroscopy revealed that collagen elicited the release of TF from its storage pools within the platelets. Functional activation of the intravascular TF was reduced by inhibition of the ADP receptors, partially due to the disruption of the platelet-neutrophil adhesions. Injection of collagen into the venous system of mice increased the number of thrombin-antithrombin complexes, indicative for the formation of thrombin in vivo. In P2Y12−/− deficient mice, the ability of collagen to enhance the generation of thrombin was impaired. In conclusion, the platelet ADP pathway supports the initiation of intravascular coagulation, which is likely to contribute to the concomitant formation of fibrin at the site of the growing thrombus. (Blood. 2004;103:594-600)

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Patients, materials, and methods
Materials
The fluorescein isothiocyanate (FITC)-labeled monoclonal antihuman TF antibody was obtained from American Diagnostica (Greenwich, CT; catalog no. 4508). The unlabeled antihuman monoclonal (VIC7) and the polyclonal TF antibodies directed against the extracellular domain of the human TF(22) were

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generously provided by T. Luther (Dresden, Germany). VIC7 recognizes an epitope on the extracellular domain of TF encoded by exon 5 of the TF gene, which is absent from the alternatively spliced form of TF recently described. The monoclonal anti-CD15 antibody was from Biotrend (Cologne, Germany). The FITC-labeled immunoglobulin G1 (IgG1) isotype antibody was from Becton Dickinson (Heidelberg, Germany). The chromogenic substrate S2222 and Beriplex P/N 500 were from Chromogenix (Toulouse, France). Monoclonal anti-CD42b (clone HIP1; PE labeled) were from Pharmingen (San Diego, CA). Adenosine, dipyridamole) anticoagulant was from Becton Dickinson Vacutainer Systems. Clopidogrel was from Sanofi-Synthelabo (Toulouse, France). Monoctonal antibodies directed against human CD42b (glycoprotein Iba [GP Ib/IX]; clone S22; phycoerythrin [PE] labeled) and human CD62P (P-selectin, clone CLB-thromb6; FITC labeled) were from Immunotech Couter (Marseille, France). Anti-human CD45 (clone 30-F11; PE labeled), antihuman CD66b (FITC labeled), and antihuman CD42b (clone HPI1; PE labeled) were from Pharmingen (Meylan, France). Enzyme TAT was from Behringwerke (Marburg, Germany). The monoclonal anti-TF antibody (VIC7) and, respectively. MRS-2179 (2'-deoxy-N^6-methyladenosine-3',5'-bisphosphoric acid) was from Sigma-Aldrich (Deisenhofen, Germany). AR-C69931MX was from ASTRA Charn/biotech (Freiburg, Germany). The microbeads conjugated with either anti-CD14 or anti-CD15 antibodies and the positive selection column were from Miltenyi Biotec (Bergisch Gladbach, Germany). Collagen (type I) was obtained from Nycorner (Munich, Germany). Anesthetic drugs xylazine and ketamine were from Bayer (Puteaux, France) and Merial (Lyon, France), respectively. MRS-2179 (2'-deoxy-N^6-methyladenosine-3',5'-bisphosphoric acid) was from Sigma-Aldrich (Deisenhofen, Germany). AR-C69931MX was from ASTRA Charnwood (Lagiborough, United Kingdom). CTAD (citrate/citric acid, theophylline, adenosine, diprydamole) anticoagulant was from Becton Dickinson Vacutainer Systems. Clopidogrel was from Sanofi-Synthelabo (Toulouse, France). Monocyonal antibodies directed against human CD42b (glycoprotein Iba [GP Ib/IX]; clone S22; phycoerythrin [PE] labeled) and human CD62P (P-selectin, clone CLB-thromb6; FITC labeled) were from Immunotech Coulter (Marseille, France). Anti-human CD45 (clone 30-F11; PE labeled), antihuman CD66b (FITC labeled), and antihuman CD42b (clone HPI1; PE labeled) were from Pharmingen (Meylan, France). Enygnost TAT was from Behringwerke (Marburg, Germany).

**Subjects**

To evaluate the contribution of the P2Y12 receptor for the activation of coagulation by the intravascular TF ex vivo, 8 healthy volunteers (4 men, 4 women; age range, 22 to 35 years) received a single dose of clopidogrel (300 mg) on day 1, followed by a daily dose of 75 mg for an additional 3 days. After a washout phase of 3 weeks, the same donors were supplemented with a daily dose of aspirin (100 mg) for a total of 4 days. Blood was drawn immediately before and at day 5 after the intervention.

**Preparation of cells**

For the isolation of platelets, venous blood from healthy volunteers (age range, 23 to 32 years) was drawn into sodium citrate (0.38% final concentration) and subsequently centrifuged at 190g for 15 minutes. The upper two thirds of the supernatant representing the platelet-rich plasma (PRP) was supplemented with apyrase (0.2 U/mL). The PRP was centrifuged at 330g for 10 minutes, and the platelet pellet was recovered and resuspended in a buffer composed of 145 mM NaCl, 10 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), 5 mM KCl, 1 mM MgCl2, and 5 mM glucose (pH 7.4; resuspension buffer). When ioprost (10 ng/mL) was included into the PRP and the isolated platelets were additionally washed twice with the resuspension buffer, similar results for the platelet-dependent factor Xa formation were obtained as without ioprost and the washing step. The suspensions of the isolated platelets thus obtained contained less than 0.05% of total leukocytes.

Human neutrophils were prepared by incubation of freshly obtaineduffy coats with microbeads coupled to anti-CD15 antibodies (Miltenyi Biotec) for 15 minutes at 8°C. The suspensions were thereafter applied onto the positive selection column, and neutrophils were eluted with the antibody buffer (phosphate-buffered saline [PBS] supplemented with 0.1% EDTA and 0.5% bovine serum albumin). The purity of the suspensions thus obtained was 92%. Monocytes were isolated by buoyant density centrifugation and subsequent purification with anti-CD14 antibodies, as described earlier.

**Flow cytometry**

For the flow cytometric determinations in whole blood, immediately after collection, 70 µL blood anticoagulated with citrate was preincubated for 2 minutes at 37°C without agitation with or without MRS-2179 (1 mM) and AR-C69931MX (10 µM). Blood was stimulated for 10 minutes at 37°C with collagen (10 µg/mL). A 5-µL aliquot of blood was then incubated with 45 µL Tyrode buffer (137 mM NaCl, 2 mM KCl, 12 mM NaHCO3, 0.3 mM NaH2PO4, 2 mM CaCl2, 1 mM MgCl2, 5.5 mM glucose, 5 mM HEPES, pH 7.3) containing the labeled monoclonal antibodies (1:10 final dilution). The samples were incubated at room temperature for 20 minutes, diluted, and fixed with 0.5% (vol/vol) formaldehyde saline before the flow cytometry measurements. All parameters were acquired on a logarithmic scale. The forward scatter and the side scatter parameters were used to first delineate the leukocyte and the isolated platelet populations. To determine the TF exposure associated with the leukocyte fractions, the percentage of TF-positive cells was recorded in the total population of CD45+ cells by using the FITC-labeled anti-TF antibody. Platelet-leukocyte aggregates were determined as the percentage of CD62P+ events in the leukocyte population. The labeled isotype control antibodies were used to delineate the background staining. P-selectin exposure was determined as the percentage of CD62P+ events in the platelet (CD45+) population. Flow cytometry was performed using a FACSScan flow cytometer (Becton Dickinson).

**Western blot**

Isolated platelets that had been either activated for 15 minutes with collagen (8 µg/mL) and thrombin (0.5 U/mL) or treated with buffer alone were solubilized with 1% Triton X-100. After determining the protein contents of the samples, extracts containing the same amount of protein were loaded onto the gel (sodium dodecyl sulfate–polyacrylamide gel electrophoresis [SDS-PAGE]; with a 12% separating gel). After electroblotting, the membranes were exposed to the monoclonal anti-TF antibody (VIC7) and, subsequently, to a horseradish peroxidase–conjugated antimouse IgG.

**TF-induced factor Xa formation**

The isolated platelets (10^7) and neutrophils (10^5) were incubated for different time intervals in 170 µL resuspension buffer at 37°C: 50 µL portions of the suspensions were added to 100 µL of 0.88 U/mL factor VII present in a coagulation factor concentrate, which also contained factors II, IX, and X (Beriplex P/N 500; Aventis-Behring, Marburg, Germany). Moreover, 50 µL of a 8 mM CaCl2 suspension and 125 µg/mL (final concentrations) of the chromogenic substrate S2222 (Chromogenix) were added. After incubation for 1 minute at room temperature, the increase in the optical density at 405 nm was registered in 5 subsequent 360-second intervals in an enzyme-linked immunosorbent assay (ELISA) reader (Dynatech MR 7000). Each value was determined in triplicate. A standard curve was prepared using dilutions of recombinant TF.

**Electron microscopy**

For immunoelectronmicroscopy of neutrophils, human blood was drawn into anticoagulant plus fixative solution (3% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2; 50 µL/mL) to prevent activation of the cells. After centrifugation, the platelet-rich plasma was removed, and the cell pellet was covered with the fixative solution and incubated for 30 minutes at 4°C. Then, the upper layer mainly containing leukocytes and platelets was recovered and cut into small blocks. In parallel, platelets were isolated and stimulated for 10 minutes with collagen (12 µg/mL) and thrombin (0.1 U/mL). Thereafter, the activated platelets and their microvesicles were incubated with fixative and equally cut into blocks. After washing, the blocks were suspended in cacodylate buffer (containing 2.3 M sucrose) and plunge-frozen in liquid propane at −180°C (KF80, Reichert-Jung, Vienna, Austria). The frozen samples were dehydrated...
with ethanol under progressive lowering of the temperature and embedded at −30°C in Lowicryl HM20 (Chemische Werke Lowi, Waldkraiburg, Germany). Ultrathin serial sections of the blood components fixed onto Pololform-coated Ni-grids were incubated for 60 minutes at room temperature with the primary antibody (rabbit polyclonal antihuman TF). After washing, the grids were incubated with a gold-labeled secondary antibody (goat antirabbit 10 nm, Arians, Wageningen, The Netherlands) and investigated in series with an EM 109 electron microscope (Carl Zeiss, Oberkochen, Germany). Incubations with the secondary antibody alone showed no labeling.

For the detection of fibrin in the pulmonary vasculature of mice, the lungs were fixed by immersion in fixative solution (2.5% glutaraldehyde in 0.1 mM Na+ cacodylate buffer, containing 2% sucrose, pH 7.3) for 60 minutes at room temperature. The tissues were then rinsed, cut into small pieces, and postfixed for 60 minutes at 4°C with 1% osmium tetroxide in cacodylate buffer. Subsequently, they were washed in the same buffer, dehydrated in graded ethanol solutions, and embedded in epoxy. The resin was allowed to polymerize at 50°C for 2 days. Ultrathin sections (100 nm) were stained with lead citrate and uranyl acetate and examined under a Philips CM 120 Biotwin transmission electron microscope.

Animal model of systemic thromboembolism

Wild-type and P2Y1-deficient mice were produced as described previously,19 and both genotypes were on the same C57BL/6 background. Clopidogrel treatment was performed by oral administration of clopidogrel solubilized in arachic gum, the day before and 2 hours before the experiment. Arabic gum alone was given orally to the control mice group. Male mice weighting 20 to 30 g were anesthetized, and the jugular vein was exposed surgically. Thereafter, collagen (type I; 0.3 mg/kg) plus epinephrine (0.06 mg/kg) was injected within an infusion time frame of 3 to 4 seconds. Because variations from batch to batch have been observed, all thromboembolism experiments were performed with the same batch number of collagen. Blood was drawn from the abdominal aorta 2 minutes after the challenge for platelet count and plasma thrombin-antithrombin (TAT) determinations, as described previously.24

Statistics

The results were subjected to statistical analysis by paired t test or one-way analysis of variance (ANOVA) for multiple comparisons, where appropriate. All mean values are given ± SD.

Results

**ADP receptors mediate rapid exposure of intravascular TF**

We initially analyzed whether ADP participated in the rapid presentation of TF in human blood, which is promoted by collagen. In blood challenged for 10 minutes with fibrillar collagen (type I), TF was exposed in association with CD14+ and CD15+ leukocytes, in agreement with our earlier results.20 Because the TF exposure was common to different leukocyte fractions, we used a cell surface marker equally expressed in neutrophils and monocytes (CD45) for the detection of the overall TF presentation in blood. Collagen augmented the TF presentation associated with CD45+ cells by 6.1-fold (Figure 1A). In the presence of the selective P2Y1, ADP receptor antagonist MRS-2179 (1 mM), the TF presentation was reduced by 56%. Moreover, AR-C69931MX (10 μM), a specific antagonist of the P2Y12 ADP receptor, decreased the TF presentation by 64% (Figure 1A). Essentially no further inhibition was evoked when the 2 ADP receptor antagonists were added together. In parallel experiments, we found that the collagen-elicited TF presentation was exclusive for neutrophils and monocytes that stained positive for the platelet-specific cell surface marker CD42b30 (not shown), indicating that TF was exposed in platelet-leukocyte conjugates. To determine whether the effect of the ADP pathway on the TF exposure was dependent on the activation of the integrin GPIIb/IIIa, we performed experiments using the anti-GPIIb/IIIa antibody abciximab, which inhibits platelet aggregation by preventing fibrinogen binding. At the concentration used, abciximab decreased fibrinogen binding by 85% in whole blood stimulated by collagen (10 μg/mL) and reduced by more than 90% collagen-induced aggregation. Abciximab inhibited collagen-induced TF exposure in whole blood by 50% (Figure 1B), indicating that GPIIb/IIIa engagement is needed to obtain maximal TF exposure. The inhibitory effect of abciximab was not further enhanced by MRS-2179. In contrast, AR-C69931MX further reduced the TF exposure compared with the presence of abciximab alone (Figure 1B), suggesting that the P2Y12 receptor was involved in the activation of the TF presentation in the absence of the integrin-mediated outside-in signaling.

To evaluate whether the secretory response of the platelets was elicited under the same experimental conditions of collagen stimulation of whole blood, α-granule release was estimated by the P-selectin exposure. Collagen caused a substantial increase in the P-selectin exposure, which was reduced by 48% and 90% by blocking the P2Y1 and P2Y12 ADP receptors, respectively (Figure 2). In the presence of both antagonists, the P-selectin appearance was suppressed by 92%. Thus, under the experimental conditions applied, the platelet secretory response was nearly completely suppressed by the ADP receptor antagonists.

Because platelets were previously shown to store TF in their α-granules,26 we analyzed whether the intraplatelet TF was released by activation with collagen. As visualized by immunoelectronmicroscopy, TF was indeed localized on the plasma membrane of the activated platelets, in particular on the plasmalemmal extensions forming the pseudopodium (Figure 3Ai, arrows). Platelet TF not participating in the surface exposure was allowed to polymerize at 50°C in Lowicryl HM20 (Chemische Werke Lowi, Waldkraiburg, Germany). For the detection of fibrin in the pulmonary vasculature of mice, the lungs were fixed by immersion in fixative solution (2.5% glutaraldehyde in 0.1 mM Na+ cacodylate buffer, containing 2% sucrose, pH 7.3) for 60 minutes at room temperature. The tissues were then rinsed, cut into small pieces, and postfixed for 60 minutes at 4°C with 1% osmium tetroxide in cacodylate buffer. Subsequently, they were washed in the same buffer, dehydrated in graded ethanol solutions, and embedded in epoxy. The resin was allowed to polymerize at 50°C for 2 days. Ultrathin sections (100 nm) were stained with lead citrate and uranyl acetate and examined under a Philips CM 120 Biotwin transmission electron microscope.

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By Western blotting using a monoclonal antibody that binds to an epitope of the extracellular domain of TF absent from the soluble form (see “Patients, materials, and methods”), we detected the 47-kDa full-length form of TF in the isolated platelets (data not shown). In parallel experiments, we recovered the total leukocyte fraction from the blood and evaluated whether the neutrophils contained TF. This rapid preparation method was preferred over the lengthy isolation of single leukocyte subfractions, because the TF expression might be induced during the isolation procedure.
Consistently, the neutrophils included in the total leukocyte preparations were found to exhibit very low intracellular TF labeling (Figure 3B). Moreover, no TF was present on their cell surface.

Functional activation of blood-based TF requires platelet ADP pathway

Subsequently, we evaluated whether the platelet ADP receptors were required for the procoagulant competence of the blood-based TF. The TF presented on the platelet surface was functionally silent, because collagen-activated platelets barely supported the formation of the factor Xa (Figure 4A). In view of the association of TF with the platelet-leukocyte conjugates, neutrophils were added to the platelet suspensions. Neutrophils were preferred over monocytes because neutrophils had previously been shown to be devoid of TF after their isolation, while, on the other hand, TF synthesis might be induced in the monocytes during the preparation procedure. In the presence of the neutrophils, the factor Xa generation was markedly stimulated when compared with the isolated platelets or isolated neutrophils alone (Figure 4A). The factor Xa formation in the platelet suspensions supplemented with the neutrophils was nearly completely prevented by the monoclonal anti-TF antibody, indicating TF as initiating stimulus. In contrast, the isotype-control antibody was ineffective (Figure 4A). In the presence of 0.5 μM and 10 μM of the P2Y12 antagonist AR-C69931MX, the TF activity of the blood cell suspensions was lowered by 46% and 65% (Figure 4B). Moreover, the P2Y1, antagonist MRS-2179 (100 μM) reduced the TF activity by 53%. No further reduction of the TF activity was noted in the simultaneous presence of both inhibitors. Because neutrophils were thus required for the activation of the platelet-exposed TF, we analyzed whether the adhesive interactions between the 2 blood components were necessary for the stimulation of the intravascular TF. An antibody against platelet P-selectin, the major mediator of the initial tethering of platelets to neutrophils, reduced the TF activity of the blood cell suspensions by 40% (Figure 5A). In contrast, the control antibody was ineffective (not shown). Under similar experimental conditions, the anti–P-selectin antibody diminished the number of collagen-elicited platelet-neutrophil conjugates by 39%, the control antibody being ineffective (Figure 5B). Moreover, the anti–P-selectin antibody also suppressed the functional activation of TF in suspensions of activated platelets and monocytes (from 21.8 ± 6.8 [without antibody] to 11.3 ± 3.8 mU/mL [with antibody]; means ± SD, n = 3, P < .05).

The requirement for platelet-neutrophil adhesions led us to evaluate whether the ADP pathway was implicated in the establishment of platelet-leukocyte conjugates per se. As expected, the number of platelet-leukocyte conjugates in whole blood was markedly enhanced in response to collagen (Figure 5C). Inhibition of the ADP-P2Y1 receptor interaction by MRS-2179 decreased the amount of platelet-leukocyte conjugates by 53%. Moreover, the number of conjugates was lowered to the same extent by AR-C69931MX (Figure 5C). In the presence of both antagonists, the
The absence or presence of MRS-2179 (1 mM) and AR-C69931MX (10 μM) induced TF presentation induced by collagen in association with CD14+ cells. At the end of the 4-day supplementation period, the collagen-elicited TF exposure in association with monocytes (CD14+ cells) was reduced by 26% (Figure 6A). The participation of ADP in the procoagulant function of the intravascular TF, in part by supporting the formation of platelet-neutrophil conjugates, was suppressed by 76%. Taken together, these findings demonstrate that the ADP pathway contributes to the procoagulant function of the intravascular TF, in part by supporting the formation of platelet-neutrophil conjugates.

Contribution of ADP receptors to the in vivo activation of coagulation

We next evaluated the role of the P2Y12 receptor for the activation of the blood-based TF under ex vivo conditions. To this purpose, 8 healthy volunteers were supplemented with clopidogrel, a selective inhibitor of this receptor. The volunteers received a single dose of 300 mg clopidogrel, followed by a daily dose of 75 mg for 3 further days. At the end of the 4-day supplementation period, the collagen-induced TF presentation was prevented by inhibition of the ADP receptors. The number of CD42b+ leukocytes among the total fraction of CD45+ cells was reduced by 26% (Figure 6A). The participation of ADP in the formation of platelet-neutrophil conjugates, as addressed by supplementing the volunteers with aspirin. After a washout period of 3 weeks, the volunteers received a daily dose of aspirin (100 mg) for a total of 4 days. Aspirin did not affect the TF presentation in association with CD14+ cells, as analyzed after the 4-day treatment period (Figure 6B). Experiments with the platelet function analyzer performed in parallel indicated that the 4-day supplementation with aspirin significantly delayed the formation of the platelet thrombus (closure time) in response to collagen and epinephrine (B.E., unpublished data, May 2003). While not completely excluding, this makes it rather unlikely that the donors were nonresponders to this drug. In the same donors, we tested whether the 2 amplifier systems of platelet activation were involved in the functional activation of the platelet TF. After 4 days of treatment with clopidogrel alone, the TF-dependent factor Xa activity of the platelet-neutrophil suspensions was lowered by 33% (Figure 6C). No change in the TF activity was observed following the 4-day treatment with aspirin (Figure 6D). The ex vivo data thus substantiated the results of the in vitro experiments, demonstrating that ADP and its P2Y12 receptor participate in the blood-based initiation of coagulation.

To evaluate the role of ADP and its platelet receptors for the activation of coagulation in vivo, an animal model of thrombom eliminated was employed. To achieve this, collagen was injected together with epinephrine into the jugular vein of mice, which is known to cause massive pulmonary thrombemboli. By the use of electron microscopy, we detected fibrin fibers in association with platelet thrombi and adjacent to the injected collagen in several lung vessels (Figure 7A). No fibrin could be observed in the vehicle-treated mice (not shown). The same collagen stimulus augmented the systemic levels of thrombin-antithrombin (TAT) complexes, a quantitative measure for the extent of thrombin generation. Indeed, 2 minutes after injection of collagen into the control mice, the number of TAT complexes in the blood was increased by 3-fold (Figure 7B). Following clopidogrel infusion, the thrombin generation tended to be diminished. In the P2Y1−/− mice, the ability of collagen infusion to generate thrombin was markedly reduced (Figure 7B).

Discussion

Amplification of platelet activation by ADP is of substantial relevance for the recruitment of platelets and leukocytes to the developing thrombus, required to prevent the loss of blood from the site of vessel wall perforation. Under in vivo conditions, the ADP-mediated amplification system is mainly triggered by the interaction of platelets with the subendothelial collagen. The potential participation of the platelet ADP receptors for the rapid
presentation of TF, the major initiator of coagulation, in whole blood was evaluated by using the selective P2Y1 receptor antagonist MRS-2179 and the selective P2Y12 antagonist AR-C69931MX.27,28 Both inhibitors partially prevented the collagen-elicited exposure of the intravascular TF in association with platelet-leukocyte conjugates. In view of the ability of collagen to promote the degranulation of the platelet α-granules, we analyzed whether the same stimulus would enable the exposure of TF on the platelet surface. Earlier results suggest that platelets contain TF,10 which might somehow be released into the extracellular medium upon platelet activation.15-17 By means of immunoelectronmicroscopy, we observed that TF was translocated from the α-granules, its major site of intraplatelet storage,3 to the platelet surface. When combined, these findings suggest that the rapid exposure of the intravascular TF in response to collagen depends in part on the activation of the platelet ADP receptors.

The TF exposed on the surface of the activated platelets themselves is functionally only weakly active,3,19 while the TF released into the extracellular medium appears to exhibit greater functional competence.11,29 In particular, allowing the activated platelets to adhere to the neutrophils enabled the factor X cleavage by the platelet TF/VIIa complex. The functional activation (or inactivation) of the platelet TF by the neutrophils could occur by concomitant inactivation of tissue factor pathway inhibitor (TFPI), the antagonist of the TF pathway. TFPI, which is released by the activated platelets, is indeed efficiently cleaved by neutrophil proteases. In the intercellular microenvironment formed within the platelet-neutrophil conjugates, proteolytic inactivation of TFPI by neutrophil proteases could enhance the ability of the platelet TF to initiate coagulation. Our observations confirm earlier findings demonstrating that neutrophils are capable of supporting the intravascular TF activity and to enhance the fibrin formation in the presence of the activated platelets.9,31-33 When the tethering of platelets to the neutrophil surface was inhibited by the anti-P-selectin antibody, the ability of the intravascular TF to initiate coagulation was suppressed. Ex vivo analyses using specific inhibition of the P2Y12 receptor by clopidogrel confirmed that this ADP receptor partially contributes to the exposure and function of the blood-based TF. The inhibitory effect of clopidogrel on the activation of the blood-based TF was less pronounced in the ex vivo experiments compared with the inhibitory influence of AR-C69931MX in vitro. This is most probably due to the incomplete inhibition of ADP-induced platelet activation elicited with the standard dosage of clopidogrel,34 as also used in the present study. Moreover, the formation of platelet-leukocyte conjugates per se was found to require in part the activation of the platelet ADP receptors, in agreement with earlier work.35,36 Thus, in addition to its participation in the TF exposure, the ADP pathway also contributed to the formation of adhesive contacts between the platelets and neutrophils, necessary for the efficient activation of the intravascular TF. These results extend previous findings reporting about the relevant role of the P2Y12 receptor for the ADP-induced formation of platelet-leukocyte conjugates.7,37 In our ex vivo analyses, aspirin (100 mg/d) did not inhibit the initiation of coagulation by the platelet-associated TF. In principal agreement with these observations, previous investigations were also unable to assign a role for thromboxane A2 as activator of the platelet-dependent thrombin formation.38,39

The extent of reduction of the TF exposure, of the TF activity, and of platelet-leukocyte conjugate formation was similar for the 2 ADP receptor antagonists analyzed. Moreover, no clear-cut additive effects were observed under the same conditions, when both antagonists were present. Both ADP receptors thus appear to contribute to the activation of the intravascular TF system. Thus, while for the platelet P-selectin exposure a stronger participation of the P2Y12 receptor could be inferred, no such preference was seen in the stimulation of the intravascular TF. The latter system ultimately depends on platelet-leukocyte adhesions, which apart from P-selectin-mediated initial interactions require the firm interactions supported by integrins. The platelet ADP receptors are known to be coupled to different intracellular signaling pathways. While the signaling route initiated by the P2Y1 receptor involves Gi, P2Y12 is linked to G13, a G protein whose further downstream signaling involves inhibition of the adenyl cyclase activity.15-17 Engagement of the ADP receptors by ADP is known to cause maximal platelet secretion and signal amplification by activation of the integrin GPIIb/IIIa. Our results suggest that GPIIb/IIIa-related outside-in signaling is required, at least in part, for the intravascular TF presentation elicited by ADP in response to collagen stimulation.

Several earlier investigations underline the participation of ADP and its platelet receptors in thrombus formation and in the efficient closure of vessel wall ruptures. Prolongations in bleeding time and resistance against thrombosis were noted in mice deficient in the P2Y1 receptor as well as in patients lacking the P2Y12 receptor.17,20 Moreover, ADP receptor antagonists are currently widely used as antiplatelet agents for the secondary prevention of cardiovascular diseases. In our study, a role for the P2Y1 ADP receptor in the activation of coagulation could be inferred under in vivo conditions, because collagen failed to stimulate the formation of thrombin in the mice deficient in this ADP receptor. Moreover, preventing the ADP binding to this receptor partially suppressed the ability of the isolated platelets to initiate coagulation in vitro. In the whole blood experiments, higher concentrations of the P2Y1 antagonist were needed to prevent the TF exposure, indicating a reduced efficiency of the antagonist under those conditions.
The blood-based TF apparently enables the initiation of physiologic coagulation within the growing thrombus, allowing the generation of thrombin (and fibrin) at the site, where it is necessary for the efficient sealing of the wound. The multilayered thrombus will most likely provide a barrier to the diffusion of coagulation factors previously activated by the vessel wall TF. Because, however, fibrin is essential for the sealing of the developing thrombus, a local fibrin-generating system is apparently required. The intravascular TF system is a likely candidate to fulfill this function. The kinetics of the blood-based TF exposure, which is maximal after 5 to 10 minutes in human blood, also supports the view that the system operates during the growth and the remodeling phase of thrombus development. Under different circumstances, but with principally comparable dynamics, pathological arterial thrombosis are known to increase in size by the stepwise enrichment with platelets and leukocytes. Eventually, this will lead to the complete occlusion of the vessel and thereby cause life-threatening ischemic complications. Under those conditions, the platelet ADP pathway is expected to contribute to the activation of coagulation via stimulation of the intravascular TF, thereby augmenting the resistance of the thrombus against the shear stress of the flowing blood and eventually causing irreversible vessel occlusions.

In conclusion, our observations demonstrate that the interactions of ADP with its P2Y1 and P2Y12 receptors participate in the rapid activation of the blood-based TF. Our results suggest that upon interaction of the platelets with collagen, the intraplatelet ADP secreted into the extracellular environment binds to its cell membrane receptors, thereby enhancing the ω-granule release. In consequence, prestored TF and P-selectin are likely to be exposed. Concomitantly, GPIIb/IIIa (α5β3 integrin) is activated, which further enhances secretion via outside-in signaling. Adhesive interactions between the activated platelets and the neutrophils are subsequently proposed to enable the functional activation of the rapidly exposed TF. By supporting the rapid intravascular TF presentation and by participating in the formation of platelet-neutrophil conjugates, the ADP receptors thus contribute to sustain the functional competence of the intravascular TF. Accordingly, there appears to be a close temporal and spatial coupling between the platelet recruitment during thrombus development and the initiation of coagulation.

References

Platelet ADP receptors contribute to the initiation of intravascular coagulation

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