Role of ADP Receptor P2Y\textsubscript{12} in Platelet Adhesion and Thrombus Formation in Flowing Blood

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Abstract—ADP plays a central role in regulating platelet function. It induces platelet aggregation via the activation of 2 major ADP receptors, P2Y\textsubscript{1} and P2Y\textsubscript{12}. We have investigated the role of P2Y\textsubscript{12} in platelet adhesion and thrombus formation under physiological flow by using blood from a patient with a defect in the gene encoding P2Y\textsubscript{12}. Anticoagulated blood from the patient and from healthy volunteers was perfused over collagen-coated coverslips. The patient’s thrombi were smaller and consisted of spread platelets overlaying platelets that were not spread, whereas control thrombi were large and densely packed. Identical platelet surface coverage, aggregate size, and morphology were found when a P2Y\textsubscript{12} antagonist, N\textsuperscript{6}-(2-methylthioethyl)-2-(3,3,3-trifluoropropylthio)-β,γ-dichloromethylene ATP (also known as AR-C69931 MX), was added to control blood. The addition of a P2Y\textsubscript{1} antagonist (adenosine-3’-5’-diphospate) to control blood resulted in small, but normally structured, thrombi. Thus, the ADP-P2Y\textsubscript{12} interaction is essential for normal thrombus buildup on collagen. The patient’s blood also showed reduced platelet adhesion on fibrinogen, which was not due to changes in morphology. Comparable results were found by using control blood with AR-C69931 MX and also with adenosine-3’-5’-diposphosphate. This suggested that P2Y\textsubscript{12} and P2Y\textsubscript{1} were both involved in platelet adhesion on immobilized fibrinogen, thereby revealing it as ADP dependent. This was confirmed by complete inhibition on the addition of creatine phosphate/creatine phosphokinase. (Arterioscler Thromb Vasc Biol. 2002;22:686-691.)

Key Words: ADP receptors ■ platelet adhesion ■ collagen ■ fibrinogen ■ thrombus formation under flow
P2Y₁₂. The platelets of patient M.L. showed impaired ADP-dependent platelet aggregation, had a much reduced ADP binding capacity, and lacked the ability to inhibit cAMP levels in response to ADP.¹²

Until now, studies involving the role of ADP in platelet thrombus formation under physiological flow conditions have been performed either (1) in vivo, with the use of mice with a knockout for P2Y₁,¹³,¹⁴ and in animal models of arterial thrombosis, with the use of N-(2-methylthioethoxy)-2-(3,3,3-trifluoropropylthio)-β,γ-dichloromethylene ATP (also known as AR-C69931 MX),¹⁵ or (2) in perfusion models ex vivo, with the use of blood from healthy volunteers treated with clopidogrel¹⁶ and, in a preliminary report, with the use of whole blood in the presence of the P2Y₁₂ antagonist, AR-C69931 MX, and the P2Y₁ antagonist, adenosine-3′,5′-diphosphate (A³P₅P).¹⁷ However, no studies have been reported on platelet adhesion and thrombus formation in flow with the use of human blood deficient in the ADP receptor P2Y₁₂. In the present study, we have investigated the role of the P2Y₁₂ receptor in platelet adhesion to fibrinogen and platelet thrombus formation on collagen by using blood from patient M.L. compared with control blood in the presence or absence of ADP receptor antagonists by using our ex vivo perfusion model.¹⁸

The data indicate for the first time that P2Y₁₂-dependent activation of platelets by ADP is involved in platelet adhesion to fibrinogen as well as in collagen-induced formation of densely packed large thrombi under physiological flow conditions.

Methods

Patient

The patient (M.L.), with a familial bleeding disorder linked to a defective interaction between ADP and its receptor on platelets, was first described in detail by Nurden et al.¹² In brief, the patient was a 67-year-old man who had shown excessive bleeding mainly after surgery or trauma. Bleeding times were consistently prolonged. Furthermore, a much decreased activation of aggregation with medium to high doses of other agonists was observed, whereas ADP-induced aggregation always showed low maximal intensity and rapid reversibility of aggregation at all doses of ADP, whereas aggregation with medium to high doses of other agonists was normal. Furthermore, a much decreased activation of adenylyl cyclase was found in response to ADP. ADP-induced lowering of cAMP levels was absent for prostaglandin E₁ (PGE₁)-treated platelets from the patient. The binding of [³H]2-methylthio-ADP to his platelets was severely decreased.

Materials

Human albumin was purchased from ICN Biomedicals B.V. Human fibrinogen (plasminogen, von Willebrand factor, and fibrinectin-free fibrinogen) was from Kordia Life Sciences. Pentasaccharide was a generous gift from Organon B.V. Phe-Pro-Arg chloromethyl ketone (PPACK) was purchased from Bachem AG. The ADP receptor P2Y₁ antagonist (the ATP analogue AR-C69931 MX) was a kind gift from AstraZeneca (Loughborough, UK). The P2Y₁ inhibitor A³P₅P and human placenta collagen type III were purchased from Sigma-Aldrich Chemicals B.V. Creatine phosphate (CP) and creatine phosphokinase (CPK) were from Boehringer-Mannheim.

Blood Collection

Whole blood, obtained from patient M.L. (platelet count 180 000/μL) and from healthy volunteers (platelet counts 150 000 to 250 000/μL), who denied having taken aspirin or other platelet function inhibitors in the preceding week, anticoagulated with 1/10 (vol/vol) of 200 U/mL of a synthetic derivative of the pentasaccharide containing the minimal binding region of heparin to antithrombin III in 0.15 mol/L NaCl in combination with 50 μmol/L PPACK, an inhibitor of α-thrombin. Thrombin generation was inhibited for at least 4 hours after blood collection. Perfusion studies using pentasaccharide/PPACK anticoagulated blood were performed within 4 hours of blood collection.

Perfusion Studies

Perfusion experiments were performed over Theranox coverslips (Nunc), which were sprayed with collagen type III or coated with fibrinogen (100 μg/mL) by using a single-pass small perfusion chamber with a slit height of 0.1 mm and a slit width of 2 mm.¹⁸ Blood was prewarmed to 37°C for 5 minutes, treated or not with ADP receptor antagonists, and subsequently drawn through the chamber for the indicated time period. After perfusion, the coverslips were fixed, dehydrated, and stained as described.²⁰ Platelet deposition and thrombus formation were evaluated as platelet surface coverage and thrombus area by using light microscopy (Leitz Diaplan, Leica) and computer-assisted analysis with OPTIMAS 6.0 software (Watershed module, DVS). Thrombus volume analysis was measured in real time by using confocal laser scanning microscopy as described previously.²

Statistical Analysis

Unless otherwise stated, results obtained with control blood (with or without antagonist) were expressed as mean±SEM for data obtained from ≥3 separate experiments, each performed at least in triplicate. The collagen results for patient M.L. are based on the results from 2 experiments, each performed at least in triplicate (expressed as means±SD). One-way ANOVA was used to compare statistical significance between groups. A value of P<0.05 was considered significant. No probability values are reported for data from patient M.L. because it concerns only 1 patient.

Results

Role of P2Y₁₂ in Thrombus Formation on Collagen in Flowing Blood

Role of ADP in Thrombus Formation

When whole blood from control donors was treated with the ADP scavenging system, CP/CPK (50 mmol/L/40 μg/mL), and subsequently perfused over collagen type III at a wall shear rate of 1600 s⁻¹, platelet surface coverage was strongly reduced (please see online Table I, which can be accessed at http://atvb.ahajournals.org). This decrease in surface coverage at 5 minutes was due to reduced platelet attachment (adhesion) to the collagen, a reduced spreading, and inhibited thrombus formation, as is shown in the Figure.

Perfusion Experiments With Blood From Patient Deficient in P2Y₁₂

The contribution of the interaction of ADP with P2Y₁₂ to thrombus formation on collagen under conditions of flow was investigated by using blood from patient M.L., who was deficient in the ADP receptor P2Y₁₂. Perfusion with the patient’s blood, compared with blood from control donors, showed a rapid coverage of the surface (3 minutes, online Table I), with a small increase in the surface covered with platelets after 5 minutes of perfusion (online Table I). This increase in surface coverage was not due to an increased thrombus size, as shown by computer analysis. The bulk of thrombi (89.3±11.4%) formed with blood from the patient covered areas <400 μm², whereas control thrombi were...
Representative light micrographs of thrombi formed on collagen after perfusion of blood from patient M.L. or a control donor in the presence (+) or absence of the ADP receptor antagonists (AR-C69931 MX, A3P5P, and CP/CPK). Surface coverage from these perfusion experiments is presented in online Table I. Original magnification ×400. Bar=100 μm.

much larger (only 24.0±2.0% of the thrombi had thrombus areas <400 μm²). Control thrombi were large and tightly packed, and individual platelets could hardly be distinguished (Figure). In contrast, the patient’s thrombi consisted of a layer of spread platelets that adhered as a patchwork on the collagen surface, whereas on top of this monolayer, nonspread (contact) platelets were attached (Figure). The decrease in thrombus size was confirmed with 3D thrombus volume analysis measured after 1, 3, and 5 minutes of perfusion with the use of confocal laser scanning microscopy. The results showed impaired thrombus growth with the patient’s blood compared with the control blood (at 1, 3, and 5 minutes, 10.9±1.8, 14.4±2.0, and 16.5±1.3, respectively, for control versus 7.7±1.1, 9.6±0.76, and 12.7±0.6, respectively, for the patient; data are the mean×10⁴ μm³±SD of a single experiment performed in duplicate). No destabilization was observed.

Perfusion Experiments With Blood in the Presence of AR-C69931 MX
Perfusions over collagen with control blood in the presence of a selective P2Y₁₂ antagonist, the ATP analogue AR-C69931 MX (1 μmol/L), showed no significant differences in platelet surface coverage compared with the control without the antagonist after 3 and 5 minutes of perfusion (online Table I). However, 2D computer analysis confirmed that the thrombus area was reduced and was similar to that observed with the patient’s blood (82.5±7.1% [control+AR-C69931 MX] versus 89.3±11.4% [patient] of thrombus areas <400 μm²) and significantly reduced compared with control (control was 24.0±2.0%; P<0.001). Thrombi formed in the presence of AR-C69931 MX had a morphology identical to that obtained with the patient’s blood: an initial layer of spread platelets with single nonspread platelets on top of these (Figure). Increasing the concentration of AR-C69931 MX up to 10 μmol/L did not change the results. No significant additive effect of AR-C69931 MX on platelet deposition and morphology was observed when the antagonist was added to the patient’s blood. Although the thrombi were loosely packed in the presence of AR-C69931 MX compared with the control, no emboli were observed, with the results of 3D thrombus growth analysis being 7.6±2.3, 10.3±2.8, and 9.7±2.0 (×10⁴ μm³±SD) after 1, 3, and 5 minutes of perfusion, respectively.

Perfusion Experiments With Blood in the Presence of A3P5P
The contribution of the ADP receptors P2Y₁₂ and P2Y₁ to thrombus formation was investigated with use of the patient’s blood, which was treated with a selective P2Y₁ antagonist, A3P5P (300 μmol/L). These thrombi showed a morphology in terms of platelet packing that was similar to that obtained with the patient’s blood alone (Figure), although the surface covered with platelets was decreased at each time point (online Table I), and the thrombus area was reduced (99.5±0.9% [patient] versus 7.1% [control] thrombus areas <400 μm²). When AR-C69931 MX and A3P5P were added simultaneously to control blood, similar results for thrombus area were obtained (99.8±0.3% [control+AR-C69931 MX+A3P5P] versus 82.5±7.1% [control+AR-C69931 MX] thrombus areas <400 μm², P=0.05) as well as for morphology and surface coverage (online Table I and Figure).

The addition of A3P5P alone to control blood did not significantly affect platelet surface coverage (online Table I) but decreased thrombus size (92.0±5.2% [control+A3P5P] versus 24.0±2.0% [control] thrombus areas <400 μm², P<0.001). The small platelet aggregates were not as densely packed as with the control blood, but no single contact platelets were observed on top of the thrombi, as was found when P2Y₁₂ was inhibited (Figure). Increasing the concentration of A3P5P up to 1 mmol/L did not change the observed results from those at 300 μmol/L.

Effect of AR-C69931 MX and A3P5P on Platelet Adhesion to Collagen
To study the effects of AR-C69931 MX and A3P5P on the (primary) adhesion of platelets to collagen, we have performed platelet adhesion studies in the presence of d-Arginyl-glycyl-L-aspartyl-L-tryptophan (dRGDW; 50 μmol/L). dRGDW completely inhibits aggregate formation without inhibiting platelet adhesion.²¹ The presence of dRGDW in
control blood resulted in the adhesion of single platelets to the collagen without the formation of aggregates (surface coverage 39.8±6.7%). The addition of AR-C69931 MX or A3P5P to control blood in the presence of dRGDW did not significantly alter platelet adhesion compared with control (32.1±4.4% [AR-C69931 MX] and 34.7±8.5% [A3P5P], P>0.05). Data are the mean±SEM of 3 independent experiments, each performed in triplicate.

Role of P2Y$_{12}$ in Platelet Adhesion to Fibrinogen in Flowing Blood

Role of ADP in Platelet Adhesion

When whole blood is perfused at a wall shear rate of 300 s$^{-1}$ over immobilized fibrinogen, platelets adhere in an α$_{IIb}$β$_{3}$-dependent manner without the formation of thrombi. After perfusion, a single layer of adhered platelets is detectable on the fibrinogen surface. These consist of contact, dendritic, and completely spread platelets. On the addition of the ADP-scavenging system (CP/CPK), platelet adhesion was completely inhibited after 5 minutes of perfusion (please see online Table II, which can be accessed at http://atvb.ahajournals.org), suggesting that platelet adhesion to fibrinogen requires ADP.

Perfusion Experiments With Blood From the Patient

The specific role of the ADP receptor P2Y$_{12}$ in this process was studied by performing perfusion experiments over immobilized fibrinogen with blood from the patient. Online Table II shows reduced platelet adhesion after 2 minutes as well as after 5 minutes of perfusion. The density of adhered platelets was manually counted from 15 representative images of nonoverlapping fields on each coverslip and showed that this reduction in surface coverage was not due to changes in morphology of the adhered platelets but was due to a decreased number of platelets adhering to the immobilized fibrinogen (data not shown). The control platelets and the patient’s platelets showed contact platelets as well as dendritic and completely spread platelets that had adhered to the immobilized fibrinogen.

Perfusion Experiments With Blood in the Presence of AR-C69931 MX and A3P5P

The addition of the P2Y$_{12}$ antagonist AR-C69931 MX to control blood resulted in a delayed platelet deposition: after 2 minutes of perfusion, platelet deposition was significantly reduced, but this was normalized when the blood was perfused for 5 minutes (online Table II). The addition of AR-C69931 MX to control blood did not influence the morphology of the adhered platelets. No additive effect was observed when AR-C69931 MX was added to the blood from the patient (online Table II).

Inhibition of P2Y$_{12}$ with A3P5P in control blood significantly decreased the adhesion to fibrinogen after 2 and 5 minutes of perfusion (online Table II) but did not influence platelet morphology. The addition of A3P5P to the patient’s blood did not further reduce platelet adhesion after 2 minutes of perfusion compared with the control (online Table II). The addition of A3P5P plus AR-C69931 MX to control blood resulted in a significant reduction in platelet adhesion after 2 minutes as well as after 5 minutes of perfusion (online Table II)

Discussion

We report on the crucial role of ADP in platelet adhesion and thrombus formation in flowing blood. The presence of the ADP-scavenging system, CP/CPK, resulted in a reduced platelet adhesion to collagen, whereas thrombus formation was completely inhibited. In agreement are early perfusion studies involving subendothelium and collagen that reported reduced platelet deposition with blood from patients deficient in the contents of dense granules (where ADP is stored in platelets) and with control blood to which an ADP-using enzyme system was added. In addition, in vivo studies reported the effects of infusing ADP-scavenging systems on the bleeding time from rat mesenteric arteries. The contribution of the interaction of ADP with P2Y$_{12}$, the target of the ADP antagonists (ticlopidine, clopidogrel, and the AR-C compounds AR-C66096 MX, AR-C67085 MX, and AR-C69931 MX), was investigated by using blood from control donors and from patient M.L. Until now, 2 unrelated patients have been fully characterized whose platelets, on exposure to ADP, have normal shape change but show a reversible aggregation and do not exhibit the normal inhibition of PGE$_{1}$-stimulated adenylyl cyclase. The clinical profile and platelet functions of these patients are comparable to those observed with humans or animals who have received thienopyridine drugs, suggesting that the patients have a P2Y$_{12}$ deficiency, which was confirmed by genetic analysis for patient M.L. Northern blotting showed that platelets from this patient contain no mRNA for P2Y$_{12}$, and Western blotting has confirmed the absence of P2Y$_{12}$ protein (Dr P.B. Conley, unpublished data, 2001). Therefore, the availability of this patient provides a unique way of investigating the role of P2Y$_{12}$ in thrombus formation.

We found a similarly impaired platelet aggregation under physiological flow conditions for blood from the patient and control blood incubated with the P2Y$_{12}$ antagonist AR-C69931 MX. Although primary platelet adhesion on collagen (as shown with dRGDW) was not affected, the thrombi were smaller and morphologically different, consisting of clusters of spread platelets on top of which were single nonspread platelets. This is compatible with a previous observation that the interaction of ADP with P2Y$_{12}$ is responsible for the full activation of α$_{IIb}$β$_{3}$ and, thus, supports the formation and growth of a densely packed thrombus under physiological flow conditions. In agreement are unpublished real-time observations of increased rolling of platelets over spread platelets in the presence of AR-C69931 MX (Dr J.A. Remijn, unpublished data, 2001). The reduced platelet consumption by the growing thrombus apparently resulted in a higher platelet concentration at the collagen surface, which enhances platelet-collagen adhesion and explains the increased surface coverage observed with blood from the patient at early perfusion times. ADP-induced platelet aggregates from patient M.L., formed in suspension and studied by electron microscopy showed similar loosely bound platelets with few contact points, as reported in the present study, and showed similarities with aggregates of platelets from subjects receiving clopidogrel. The formation of loosely packed thrombi was also observed with clopidogrel without decreased platelet adhesion to collagen under conditions of flow in an human ex
vivo perfusion model using non-anticoagulated blood by Roald and Sakariassen. Our results obtained with blood from patient M.L. and AR-C69931 MX are consistent with these flow studies on clopidogrel. One of the interesting aspects of P2Y1; as a potential antithrombotic target is that thrombus growth is inhibited, whereas primary adhesion to collagen is not affected. The latter might explain why patient M.L. does not have major bleeding complications in the absence of trauma or surgery. The ADP-mediated downregulation of cAMP via P2Y1; and/or other unidentified signaling pathways emanating from Gαi and βγ chain dissociation are evidently important for the activation of αmβ3 in the support of thrombus growth but not for the primary platelet-collagen interaction.

Furthermore, inhibition of P2Y1 alone did not affect primary platelet adhesion to collagen but resulted in reduced thrombus area, although these thrombi were still densely packed. Indeed, platelet aggregation studies in P2Y1-null mice showed impaired aggregation at a low dose of collagen, a response that was restored at higher collagen concentrations.13,14 This restoration of normal aggregation by increasing collagen concentrations in combination with the observed dense aggregate packing could be explained by the participation of secreted ADP in the activation of αmβ3 via P2Y12. The latter was inhibited when patient M.L. blood was incubated with A3P5P or when control blood was incubated with a combination of A3P5P and AR-C69931 MX, resulting in reduced platelet surface coverage and further reduced thrombus size, which consisted of loosely bound nonactivated platelets. These results show that both ADP receptors participate in collagen-induced platelet thrombus formation under conditions of flow. However, the inhibition of the separate receptors results in different morphology. A differential participation of P2Y1 and P2Y12 in the activation of αmβ3 may also need to be considered. The quantitative differences in adhesion between the presence of CP/CPK and the residual adhesion when ADP receptors P2Y1 and P2Y12 are both inhibited might be due to an incomplete inhibition of the receptors with recycling of internal pools or due to a role of the largely uncharacterized P2X1 receptor for ATP and ADP. The involvement of ADP interaction with P2Y12 for the activation of αmβ3 was confirmed by the observed reduced platelet adhesion at 2 and 5 minutes of perfusion of the patient’s blood over immobilized fibrinogen, an interaction that is totally αmβ3 dependent at wall shear rates of 300 s−1.22 Similar results to the patient were obtained on the addition of AR-C69931 MX to control blood after 2 minutes of perfusion. However, with this antagonist, platelet adhesion was normal after 5 minutes of perfusion, indicating that platelet adhesion was delayed rather than prevented. The differences in inhibition between the patient’s platelets and control platelets in the presence of AR-C69931 MX could be due to the total absence of P2Y12 on the patient’s platelets; in control blood, AR-C69931 MX probably inhibits the surface-expressed receptors only, whereas any internal receptor pool may not be inhibited because it would be inaccessible to the antagonist. Inhibition of P2Y1 with A3P5P also resulted in decreased platelet adhesion, probably explained by the fact that the ADP-P2Y1 interaction plays a role in the initial rate of activation of αmβ3. The addition of the combination of AR-C69931 MX and A3P5P markedly reduced platelet adhesion after 5 minutes of perfusion but did not abrogate it. Furthermore, the morphology of the adhered platelets was not affected. When the total released ADP was scavenged by CP/CPK, platelet adhesion was totally inhibited, confirming the role of ADP in adhesion to immobilized fibrinogen. Our results regarding ADP dependence in platelet adhesion to immobilized fibrinogen suggest that platelets need to be preactivated to adhere. This is in contrast with previous studies under static or flow conditions, which have suggested that (resting) platelets can adhere to surface-immobilized fibrinogen, presumably via unactivated αmβ3.31–33 These studies were based on observations that platelets treated with PGE1 still adhered to immobilized fibrinogen. Our results performed in whole blood under physiological flow conditions are more consistent with a recent study of the coaggregation of unactivated or ADP-activated platelets with fibrinogen-coated beads in flowing suspensions, which showed that only preactivated platelets can adhere to the fibrinogen-coated surface.34 In the present study, we show that the interactions of ADP with P2Y12 and with P2Y1 are both necessary to obtain platelet adhesion on immobilized fibrinogen under conditions of flow. Under shear conditions using whole blood, ADP could be secreted from red blood cells or from preactivated platelets.

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