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Characterization of static adhesion of human platelets in plasma to protein surfaces in microplates

Running head: Platelet adhesion to proteins in microplates

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Abstract

Platelet adhesion is a complex and important event for prevention of blood loss after vessel injury. This study investigated fundamental adhesive mechanisms occurring in an *in vitro* assay developed for the measurement of static adhesion of human platelets in plasma. The aim was to gain methodological knowledge that could be used for interpretations of results from other studies using this specific assay. Involvement of adhesive receptors was investigated by the use of various antibodies as well as therapeutic drugs (abciximab, eptifibatid and tirofiban). Inhibitors of adenosine 5'-diphosphate (ADP)-receptors (cangrelor, MRS2179) and of thromboxane A₂ (TXA₂)-signaling (BM-531) were used to estimate the role of autocrine activation. Adhesion to collagen was found to be mainly mediated by $\alpha_2\beta_1$ and to some extent by $\alpha_{IIb}\beta_3$. Adhesion to fibrinogen was mediated by $\alpha_{IIb}\beta_3$. Also, ADP-induced adhesion to albumin was dependent on $\alpha_{IIb}\beta_3$. Furthermore, experiments with cangrelor and BM-531 showed that the majority of the adhesive interactions tested were dependent on ADP or TXA₂. We conclude that the mechanisms of adhesion measured by the static platelet adhesion assay are in accordance with the current knowledge regarding platelet activation and adhesion. Despite its simplicity, we suggest that this adhesion assay could be used as a screening device for the study of the influence of various surfaces and soluble substances on platelet adhesion.

Keywords: platelet adhesion, platelet assay, adhesion receptor, autocrine signaling, antiplatelet agents

Introduction

Platelet adhesion is an important initial event in primary hemostasis. Consequently, a lot of research has been performed to investigate which platelet receptors are involved in this complex process. An important part of the extracellular matrix of blood vessels consists of collagen [1], which acts as an attachment surface for platelets in wounded vessels. Platelet receptors for collagen include the $\alpha_2\beta_1$ -integrin [2-4] and the GPVI-receptor belonging to the immunoglobulin receptor family [5]. It is generally accepted that $\alpha_2\beta_1$ primarily acts as an adhesive receptor for collagen, while GPVI is responsible for platelet activation [6, 7]. Another structure important for platelet adhesion is the platelet receptor GPIb-IX-V, which binds subendothelial von Willebrand factor (vWf) during conditions of high shear stress [8]. Finally, the $\alpha_{IIb}\beta_3$ -integrin present on platelets is also of importance for the hemostatic process. $\alpha_{IIb}\beta_3$ binds an Arg-Gly-Asp-sequence (RGD) present in several different proteins such as fibronectin, laminin and fibrinogen [9]. Interactions between $\alpha_{IIb}\beta_3$ and fibrinogen are especially important since it results in platelet aggregation [10]. It is clear that the adhesive process is dependent on different contributions from the different receptors. In general, temporary interactions between GPIb-IX-V and vWf supports a rolling phenomenon, which allows binding of GPVI to collagen [7]. The GPVI-collagen interaction as well as thrombin from plasma and substances such as adenosine 5'-diphosphate (ADP) and thromboxane A₂ (TXA₂) released from platelets all contribute to platelet activation. Furthermore, firm adhesion to collagen is achieved by $\alpha_2\beta_1$. The details of the process during flow conditions *in vivo*, especially regarding the relative contributions from $\alpha_2\beta_1$ and GPVI, are debated. An early model suggested that the initial interaction between platelets and collagen occurs through the $\alpha_2\beta_1$ -receptor [11]. However, a second model claims that initial

interaction between GPVI and collagen must occur in order for $\alpha_2\beta_1$ to be activated and to be able to support firm adhesion [12]. Finally, a third model combines the previous two by assuming that some platelets act according to the first model and some according to the second [13]. This study investigated the adhesive mechanisms occurring in a modified version of the assay described by Bellavite et al. [14], further developed for measuring adhesion of platelets in plasma to protein surfaces in microplates [15]. This assay is simple to use and, since it is performed in 96-well microplates, it is well suited for screening purposes in order to measure several aspects of platelet function simultaneously. The assay has earlier been used for investigation of platelet activity both in basic studies [16, 17] and in clinical research [18, 19]. The aim of this study was to investigate fundamental adhesion events occurring in this particular assay. Such methodological knowledge is always necessary when trying to interpret laboratory results. In this assay, platelets are added as platelet rich plasma (PRP), which makes it especially important to investigate whether platelets adhere to the coated protein or if the surface is influenced by plasma proteins.

Methods

Blood sampling

Blood was collected consecutively from healthy blood donors at the Blood Transfusion Centre, University Hospital, Linköping, Sweden. The study conforms to the Declaration of Helsinki (1975) and later revisions and was approved by the local ethics committee. Only blood from donors declaring that they had not used any anti-platelet medication for two weeks prior to the study was used. Donors were also included only if they declared that they during the previous three months had not: suffered fever after visiting malaria-region; suffered medical treatment-required conditions; been pregnant; used acupuncture, tattoo or piercing; been treated by dentist the previous 14 days; or had been vaccinated or suffered infection the previous month. Blood was drawn into two 6 mL sodium heparin tubes for each donor. Thereafter, 8 mL of blood was transferred to a single plastic centrifuge tube and centrifuged for 20 min at 220×g. Approximately 2/3 of the PRP in the supernatant was then removed and diluted 4 times with 0.9% NaCl. Dilution was necessary since undiluted plasma interferes with the below described spectrophotometric measurements of platelet amount [15].

Microplate coating

Ninety-six well microplates (Nunc Maxisorp, Roskilde, Denmark) were coated by the addition of 100 µL/well of 2 mg/mL human albumin (Octapharma AB, Stockholm, Sweden), 0.1 mg/mL bovine collagen I (RnDSystems, Abingdon, UK) or 2 mg/mL human fibrinogen (American Diagnostica Inc., Greenwich, CT, USA). The microplates were then left at 4°C at least overnight but for a maximum of 7 days to allow protein adsorption to the wells.

Static platelet adhesion

Unattached proteins were removed from coated microplates by washing twice in 0.9% NaCl by plate inversion. Thereafter, 50 μ L diluted PRP was added to each well together with soluble platelet activators and inhibitors. The platelet activators used were ADP and lysophosphatidic acid (LPA) from Sigma-Aldrich (St Louis, MO, USA), adrenaline from Merck NM AB (Stockholm, Sweden) and ristocetin from Diagnostica Stago (Asnières-sur-Seine, France). This study aimed to investigate the adhesive mechanisms by using drugs and antibodies that inhibits platelet function in different ways. The inhibiting drugs used were cangrelor (P2Y₁₂-antagonist also called AR-C69931MX, which was a kind gift from The Medicines Company, Parsippany, NJ, USA) and MRS2179 (P2Y₁-antagonist), BM-531 (combined TP-receptor antagonist and thromboxane synthase inhibitor) and indomethacin (cyclooxygenase inhibitor) from Sigma-Aldrich. Antibodies used were AK7 (anti- $\alpha_2\beta_1$), AK2 (anti-GPIb-IX-V), PM6/13 (anti- $\alpha_{IIb}\beta_3$), PM6/248 (anti- $\alpha_{IIb}\beta_3$) and W3/25 (negative isotype control) from AbD Serotec (Oxford, UK) as well as the therapeutic $\alpha_{IIb}\beta_3$ -antagonists abciximab (Centocor B.V., Leiden, The Netherlands), tirofiban (Merck Sharp & Dohme B.V., Haarlem, The Netherlands) and eptifibatide (Glaxo Operations UK Ltd, Durham, UK). To maximize the effects of cangrelor, BM-531 or indomethacin, diluted PRP was preincubated with these substances before addition to the wells unless otherwise stated. Initial experiments with the antibodies indicated that preincubation was not necessary and the antibodies were therefore added directly to the wells. Also, since Mg²⁺ can affect the levels of platelet adhesion to all surfaces tested in this study [15, 20], addition of PRP and platelet activators/inhibitors were performed both in the presence or absence of 5 mmol/L MgCl₂. The final volume of PRP mixed with activators/inhibitors were 100 μ L

in each well, regardless of the platelet inhibitors used or whether $MgCl_2$ was present or not. The microplates were then left in room temperature (RT) for 1 hour in order to allow platelet attachment to the surfaces.

Spectrophotometric detection of adhered platelets

Adhesion of platelets was performed as described above and unattached platelets were removed by washing twice in 0.9% NaCl by plate inversion. 140 μ L of a sodium citrate/citric acid buffer (0.1 mol/L, pH 5.4) containing 0.1% Triton X-100 and 1 mg/mL *p*-nitrophenyl-phosphate (Sigma-Aldrich) was then added to all wells. On a separate microplate, 140 μ L of the sodium citrate/citric acid buffer was mixed with 50 μ L 0.9% NaCl or 50 μ L diluted PRP. These wells served as measures of 0% and 100% platelet adhesion respectively. Background absorbance was measured at 405 nm using a Spectramax microplate reader (Molecular Devices, Sunnyvale, CA, USA). This was performed for all microplates, including the microplate measuring 0% and 100% adhesion. The microplates were then incubated for 40 min in RT during constant shaking. This incubation allowed the occurrence of an enzymatic reaction between added *p*-nitrophenyl-phosphate and platelet acid phosphatase which resulted in a soluble product. After the 40 min incubation, 100 μ L of 2 mol/L NaOH was added to all wells, which stopped the reaction and resulted in a colour change of the developed product. The microplates were then subjected to another absorbance measurement at 405 nm. Background absorbance was subtracted and percentage adhesion was calculated.

Platelet visualization with fluorescence microscopy

Adhesion of platelets was performed as described above and unattached platelets were removed by washing twice in 0.9% NaCl by plate inversion. 50 μ L (6.3 μ g/mL) of anti-fibrinogen-FITC (Diapensia, Linköping, Sweden) was added followed by incubation for 20 min at RT. The microplates were then manually washed 2 times in phosphate buffered saline (PBS) followed by addition of 4% paraformaldehyde. After incubation for 10 min in RT, the microplates were once again washed manually 2 times with PBS followed by addition of 50 μ L 0.1% Triton X-100. The microplates were then incubated for 5 min in RT, washed manually twice with PBS and platelets were stained for actin by addition of 50 μ L (66 nmol/L) of Alexa fluor 546 Phalloidin (Invitrogen Corporation, Carlsbad, CA, USA). After incubation for 20 min in RT, the microplates were washed manually twice with PBS followed by microscopical examination using a Zeiss AxioObserver inverted fluorescence microscope.

Statistics

Statistical analyses were performed as either paired t-tests or Repeated Measures ANOVA followed by Dunnett's or Bonferroni's post hoc tests. Results were considered significant at $p < 0.05$. The concentration of cangrelor that inhibited 50 % of ADP-induced adhesion (IC_{50}) was calculated from sigmoidal dose-response curves developed by non-linear regression using GraphPad Prism version 4.03 (GraphPad Software, Inc., La Jolla, CA, USA). The equation used was $Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + 10^{\text{LogEC}_{50} - X})$. Bottom equals the Y-value at the bottom plateau and Top equals the Y-value at the top plateau.

Results

Adhesion to fibrinogen, in the presence of 5 mmol/L MgCl₂, was significantly inhibited by the $\alpha_{IIb}\beta_3$ -antibody PM6/248 and by the $\alpha_{IIb}\beta_3$ -inhibiting drugs abciximab, eptifibatide and tirofiban (Figs. 1A and 1B). However, adhesion to fibrinogen was not affected by the antibody PM6/13, which is also directed against $\alpha_{IIb}\beta_3$ (not shown). Neither the negative isotype control W3/25 nor the $\alpha_2\beta_1$ -antibody AK7 affected platelet adhesion to fibrinogen (Fig. 1A). AK7 was used at 10 μ g/mL, which was enough to significantly inhibit platelet adhesion to collagen (see below). Furthermore, the GPIIb-IX-V-antibody AK2 induced a small but significant increase in platelet adhesion to fibrinogen (mean adhesion for solvent were 14.1% compared to 17.9 % for adhesion in the presence of 40 μ g/mL AK2, $p < 0.01$, $n = 4$, not shown).

Platelet adhesion to collagen, in the presence of 5 mmol/L MgCl₂, was significantly inhibited by the antibody AK7 directed towards $\alpha_2\beta_1$ (Fig. 2A). The $\alpha_{IIb}\beta_3$ -inhibitors abciximab (10 μ g/mL), eptifibatide (1 μ g/mL) and tirofiban (1 μ g/mL) had minor but significant inhibiting effects on platelet adhesion to collagen (Fig. 2B). The concentrations chosen for abciximab, eptifibatide and tirofiban were used since they resulted in maximal block of adhesion to fibrinogen (Fig. 1B). Elevating their concentrations to 40 μ g/mL did not increase the inhibiting effects on platelet adhesion to collagen (not shown). Furthermore, adhesion to collagen was neither affected by the $\alpha_{IIb}\beta_3$ -antibodies PM6/13 (not shown) and PM6/248 (Fig. 2A) nor by the negative isotype control W3/25 (Fig. 2A) or by AK2 directed against GPIIb-IX-V (not shown).

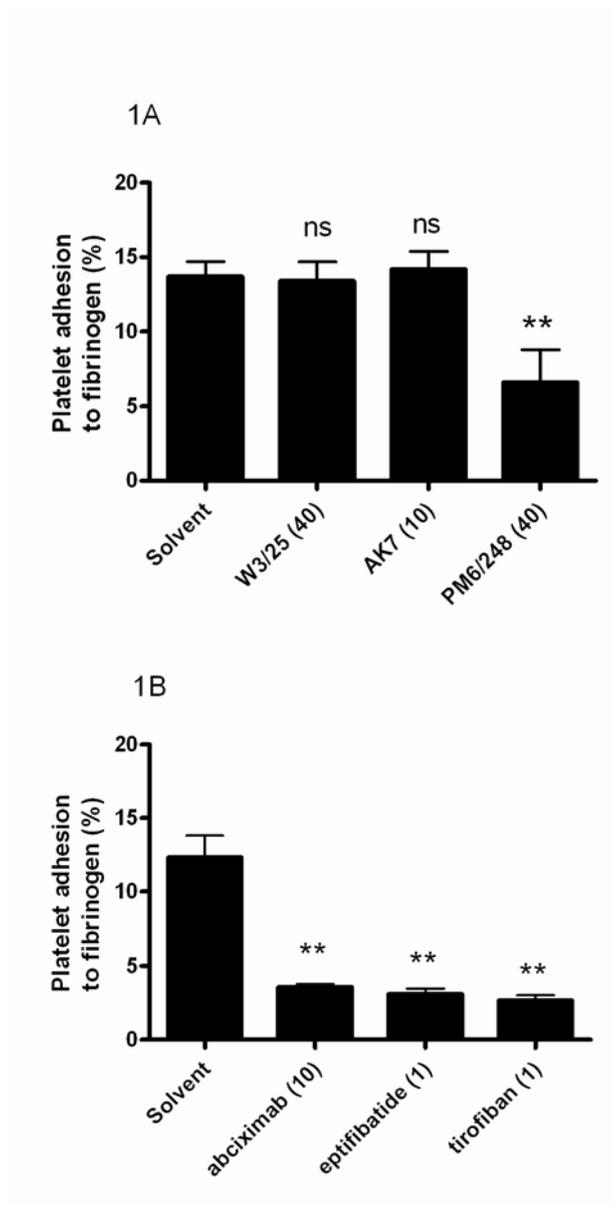


Fig. 1. Platelet adhesion to fibrinogen was inhibited by the $\alpha_{IIb}\beta_3$ -antibody PM6/248 (1A, n=6) and the $\alpha_{IIb}\beta_3$ -inhibitors abciximab, eptifibatid and tirofiban (1B, n=4) but not by the $\alpha_2\beta_1$ -antibody AK7 (1A). W3/25, which is an isotype-control antibody for PM6/248 and AK7, had no effect on platelet adhesion (1A). Numerals in brackets indicate concentration in $\mu\text{g/mL}$. Stars and ns indicate difference compared to solvent. Data are presented as mean + SEM. ** $p < 0.01$, ns = not significant.

We have earlier reported adhesion of activated platelets to albumin [15, 16], and confirm these findings in this study by using ADP (Fig. 3A), as well as adrenaline alone or in combination with ristocetin (Table 1), as platelet activators. We next wanted to

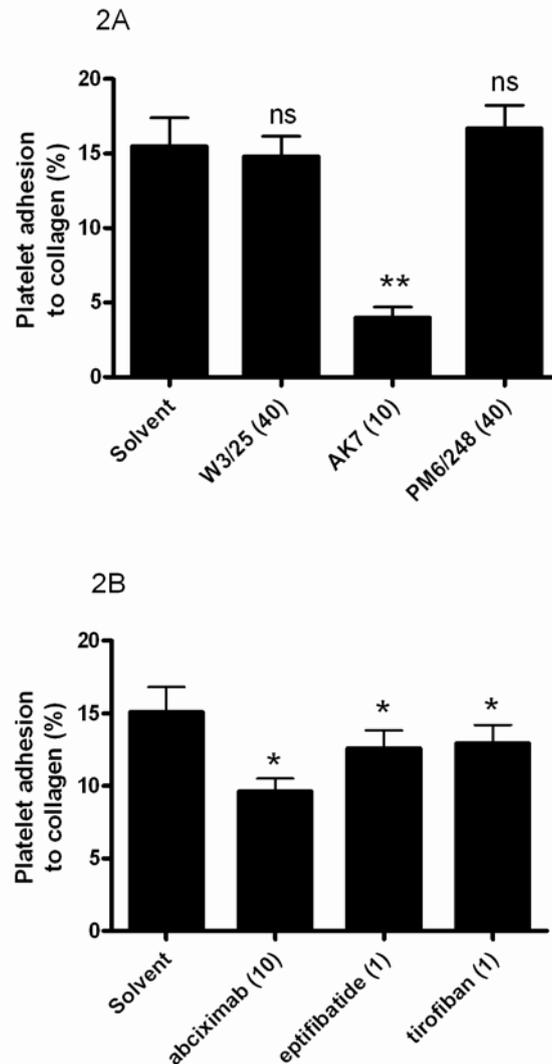


Fig. 2. Platelet adhesion to collagen was inhibited by the $\alpha_2\beta_1$ -antibody AK7 (2A, $n=6$), the $\alpha_{IIb}\beta_3$ -inhibiting drugs abciximab, eptifibatide and tirofiban (2B, $n=6$) but not by the $\alpha_{IIb}\beta_3$ -antibody PM6/248 (2A). W3/25, which is a negative isotype-control antibody for PM6/248 and AK7, had no effect on platelet adhesion (2A). Numerals in brackets indicate concentration in $\mu\text{g/mL}$. Stars and ns indicate difference compared to solvent. Data are presented as mean + SEM. * $p<0.05$, ** $p<0.01$, ns = not significant.

investigate the receptor dependency for this phenomenon. Platelet adhesion to albumin induced by ADP was significantly inhibited by the $\alpha_{IIb}\beta_3$ -antibody PM6/248 and by the $\alpha_{IIb}\beta_3$ -inhibitors abciximab, eptifibatide and tirofiban but not by W3/25, AK2, AK7

Table 1. The influence of ADP- or TXA₂-signaling on platelet adhesion.

	Albumin without Mg		Fibrinogen with Mg		Collagen with Mg		Collagen without Mg	
	Control	cangrelor / BM-531	Control	cangrelor / BM-531	Control	cangrelor / BM-531	Control	cangrelor / BM-531
Solvent	5.3 / 5.3	5.0 / 4.9	12.0 / 15.5	10.4 / 14.5	17.2 / 15.3	10.9*** / 12.9**	2.8 / 5.4	3.2 / 5.2
ADP 10	14.8 / 16.9 ◆◆◆	4.2*** / 13.6***	26.8 / 31.6 ◆◆◆	11.8*** / 29.3* ◆◆◆	23.9 / 26.0 ◆◆◆	10.7*** / 21.7***	16.7 / 19.7 ◆◆◆	3.2*** / 16.3***
Adr 0.1	7.0 / 6.7	6.6 / 6.2	nd	nd	nd	nd	4.3 / 6.3	3.9 / 6.1
Adr 1	6.8 / 8.4 ◆	5.5 / 7.1	24.6 / 27.5 ◆◆◆	21.8* / 25.5	22.4 / 21.9 ◆◆◆	19.9* / 19.3**	7.2 / 9.3 ◆	5.3 / 7.3**
Risto 1	3.6 / 5.8	3.7 / 5.7	28.1 / 26.4 ◆◆◆	26.9 / 25.2	31.0 / 30.2 ◆◆◆	29.5 / 28.7	8.8 / 8.8 ◆	6.2* / 8.4
Adr 0.1+Risto 1	12.3 / 8.2 ◆	6.4*** / 6.9	nd	nd	nd	nd	21.7 / 19.7 ◆◆◆	14.2*** / 18.1

The effects of 0.1 µmol/L cangrelor or 10 µmol/L BM-531 on platelet adhesion to albumin, collagen and fibrinogen were investigated in the absence (n=6 for cangrelor and n=8 for BM-531) or presence (n=6 for cangrelor and n=4 for BM-531) of different platelet activating stimuli. The results are presented as mean percentage adhesion. Two values are shown in each column since the cangrelor (bold)- and BM-531 (plain)-experiments were performed on blood from separate donors followed by pairwise statistical analysis. Significantly decreased adhesion for cangrelor or BM-531 compared to control is indicated as *p<0.05, **p<0.01 and ***p<0.001. Absence of stars means that the difference is not significant. Tilted squares (◆p<0.05, ◆◆◆p<0.001) in the control columns show significantly increased platelet adhesion induced by platelet activators compared to solvent (analysis performed on combined data from the cangrelor- and BM-531-experiments). Absence of tilted squares means lack of significantly increased platelet adhesion. All numerals associated with platelet activators describe concentration in µmol/L except for ristocetin where the unit of concentration is mg/mL. Abbreviations are: Adr = adrenaline, Risto = ristocetin, nd = not determined.

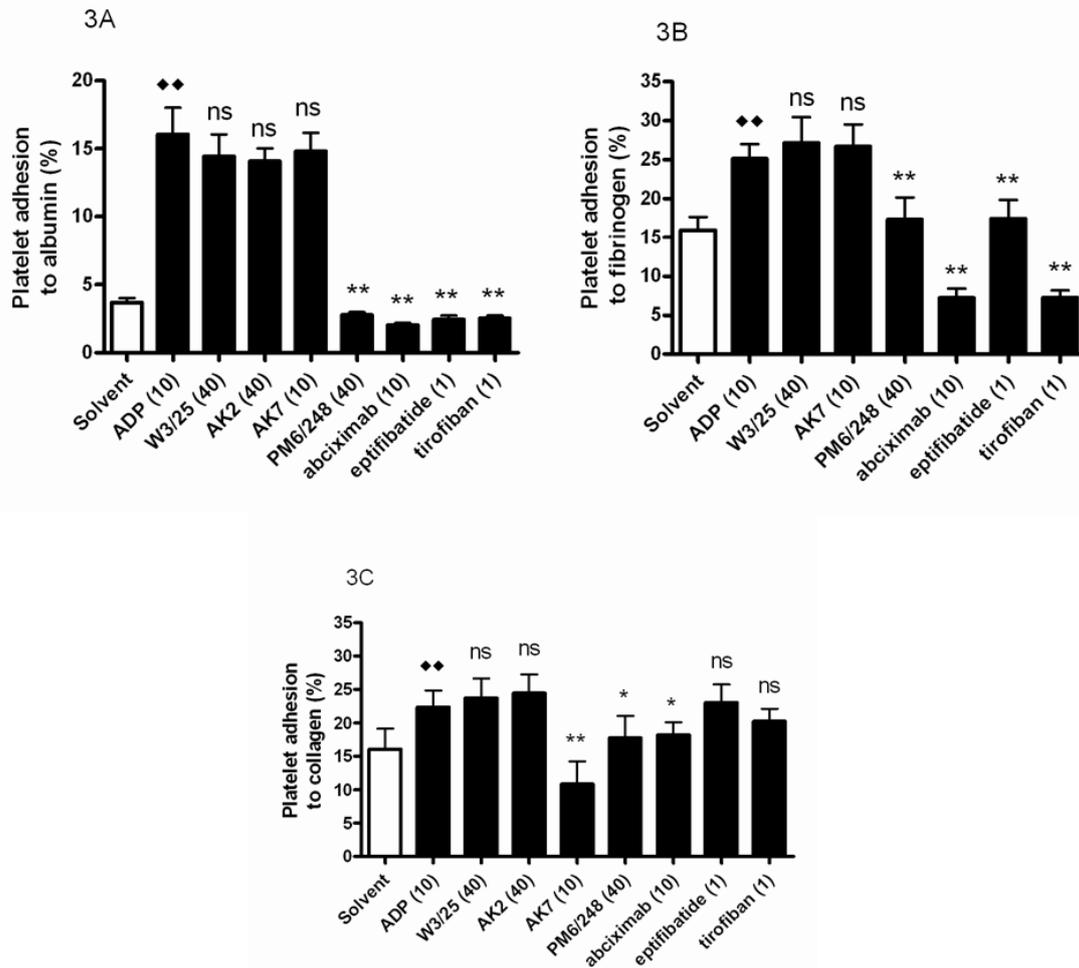


Fig. 3. Platelet adhesion to albumin (3A) induced by 10 $\mu\text{mol/L}$ ADP was inhibited by the $\alpha_{\text{IIb}}\beta_3$ -inhibitors abciximab, eptifibatide, tirofiban and PM6/248 but not by the $\alpha_2\beta_1$ -antibody AK7 or the GPIb-IX-V antibody AK2 ($n=4$). Similarly, platelet adhesion to fibrinogen (3B) induced by 10 $\mu\text{mol/L}$ ADP was inhibited by the $\alpha_{\text{IIb}}\beta_3$ -inhibitors abciximab, eptifibatide, tirofiban and PM6/248 but not by the $\alpha_2\beta_1$ -antibody AK7 ($n=4$). Platelet adhesion to collagen (3C) induced by 10 $\mu\text{mol/L}$ ADP was inhibited by the $\alpha_{\text{IIb}}\beta_3$ -inhibitors PM6/248 and abciximab and by the $\alpha_2\beta_1$ -antibody AK7 but not by eptifibatide, tirofiban or AK2. For all three surfaces, W3/25 (negative isotype control for AK2, AK7 and PM6/248) had no effect on platelet adhesion. Numerals in brackets indicate concentration in $\mu\text{g/mL}$. Stars and ns indicate difference compared to ADP-induced adhesion. * $p<0.05$, ** $p<0.01$, ns = not significant. Significant difference for ADP-induced adhesion compared to solvent is denoted by tilted squares (♦) ♦♦ $p<0.01$. All filled bars represent presence of 10 $\mu\text{mol/L}$ ADP, while open bars represent basal adhesion. Data are presented as mean + SEM.

(Fig. 3A) or PM6/13 (not shown). The experiments, shown in Fig. 3A, with the albumin-surface were performed both in the presence (n=2) and absence (n=2) of externally added Mg^{2+} . All subjects showed obvious dependence on $\alpha_{IIb}\beta_3$ indicating that this receptor is important for adhesion to albumin independently of Mg^{2+} -levels. In a similar way adhesion to fibrinogen in the presence of ADP and Mg^{2+} was inhibited by PM6/248, abciximab, eptifibatide and tirofiban but not by the $\alpha_2\beta_1$ -antibody AK7 or the negative isotype control W3/25 (Fig. 3B). We also investigated the receptor dependency for ADP-induced adhesion to collagen. Since presence of Mg^{2+} has a large impact on basal platelet adhesion to collagen [15], we investigated ADP-induced adhesion to collagen both in the presence and absence of Mg^{2+} . Adhesion in the presence of 5 mmol/L $MgCl_2$ was inhibited by the $\alpha_2\beta_1$ -antibody AK7 as well as by PM6/248 and abciximab directed against $\alpha_{IIb}\beta_3$ but not by W3/25, AK2, eptifibatide and tirofiban (Fig. 3C). Furthermore, AK7 but not PM6/248, AK2 or W3/25 inhibited ADP-induced platelet adhesion in the absence of Mg^{2+} (not shown).

Initial experiments with cangrelor, which is an antagonist of the ADP-binding P2Y₁₂-receptor, was aimed at investigating relevant doses of this compound for inhibition of ADP-dependent adhesion. Thus, for this purpose we wanted to use surfaces where platelet adhesion is distinctly increased by ADP. This criterion was met in the absence of Mg^{2+} for albumin and collagen. Cangrelor was found to effectively reduce ADP-induced platelet adhesion on both surfaces. IC₅₀-values for inhibition of platelet adhesion induced by 1 and 10 $\mu\text{mol/L}$ ADP were 1.2 and 9.2 nmol/L on albumin (Fig. 4A) and 0.26 and 2.5 nmol/L on collagen (Fig. 4B). Adhesion was completely inhibited at 0.1 $\mu\text{mol/L}$ (Figs. 4A and 4B) and this concentration was therefore used in all further

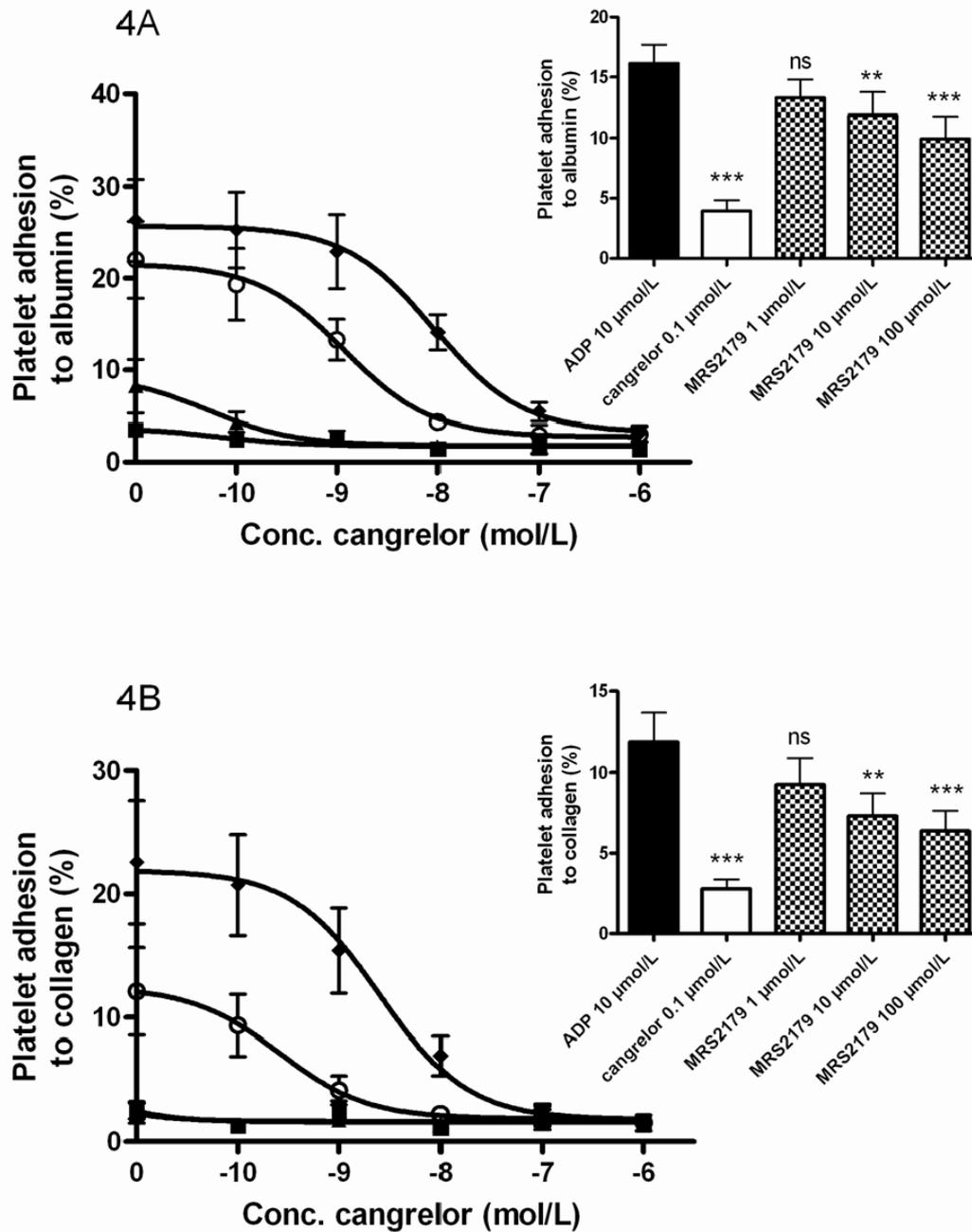


Fig. 4. Cangrelor inhibited platelet adhesion to albumin (4A, $n=4$) and collagen (4B, $n=4$) induced by 1 (O) and 10 (◆) $\mu\text{mol/L}$ ADP in the absence of Mg^{2+} . Absence of ADP and ADP at 0.1 $\mu\text{mol/L}$ are denoted by (■) and (▲) respectively. Insets show comparisons between the effects of cangrelor and MRS2179 ($n=4$, no preincubation) on ADP-induced adhesion to albumin (Inset 4A) and collagen (Inset 4B). The bars represent adhesion induced by 10 $\mu\text{mol/L}$ ADP (filled bars) as well as adhesion induced by 10 $\mu\text{mol/L}$ ADP in the presence of cangrelor (open bars) and MRS2179 (patterned bars). Mean values for basal adhesion was 5.0 and 4.7 % for albumin and collagen respectively. Stars in insets show significant differences compared to adhesion induced by 10 $\mu\text{mol/L}$ ADP. Data for insets are presented as mean + SEM. ** $p<0.01$, *** $p<0.001$, ns = not significant. Data for the main figures are presented as mean \pm SEM.

experiments. We also compared the effect of cangrelor with the effect of MRS2179, which antagonizes the ADP-receptor P2Y₁. These experiments were performed without preincubation and showed that 10 µmol/L MRS2179 was able to induce a significant decrease in platelet adhesion to albumin and collagen induced by 10 µmol/L ADP (Insets of Figs. 4A and 4B). For both surfaces, adhesion induced by 10 µmol/L ADP was significantly lower in the presence of 0.1 µmol/L cangrelor compared to 100 µmol/L MRS2179 ($p < 0.01$ on collagen, $p < 0.001$ on albumin). We further studied the effect of cangrelor on adhesion to albumin, collagen and fibrinogen in the presence of different activating stimuli (Table 1). Beyond platelet adhesion induced by ADP, activating stimuli such as collagen, ristocetin, adrenaline as well as adrenaline combined with ristocetin were significantly dependent on ADP for inducing platelet adhesion. However, the data shows great differences in ADP-dependency for different activators with smallest effects of cangrelor occurring when stimulating platelets with adrenaline or ristocetin alone (Table 1).

We further wanted to establish the role of released TXA₂ in this platelet adhesion assay. For this we used BM-531, which is a combined TP-receptor antagonist and thromboxane synthase inhibitor [21]. Investigating platelet adhesion to albumin, collagen and fibrinogen in the presence of ADP, adrenaline or ristocetin showed that BM-531 induced small but significant effects at 10 µmol/L (Table 1). The adhesion to a collagen surface without Mg²⁺ induced by the combination of adrenaline and ristocetin was not affected by BM-531. However, we wanted to further clarify the possible TXA₂-dependency for this stimulus by using the cyclooxygenase inhibitor indomethacin (10

$\mu\text{mol/L}$) instead of BM-531. We then found indomethacin to decrease adhesion from 19.7% (SEM = 6.3) to 14.6% (SEM = 3.4) ($p < 0.01$, $n = 4$, not shown).

Complementary to the detection of adhered platelets by spectrophotometry, platelet adhesion was also investigated by fluorescence microscopy. Platelet adhesion, as visualized by fluorescence microscopy, appeared to correlate well with percentage adhesion as measured spectrophotometrically. It was also clear that adhered platelets were homogeneously spread over the surface. Representative examples of adhesion to albumin, collagen and fibrinogen are shown in Fig. 5.

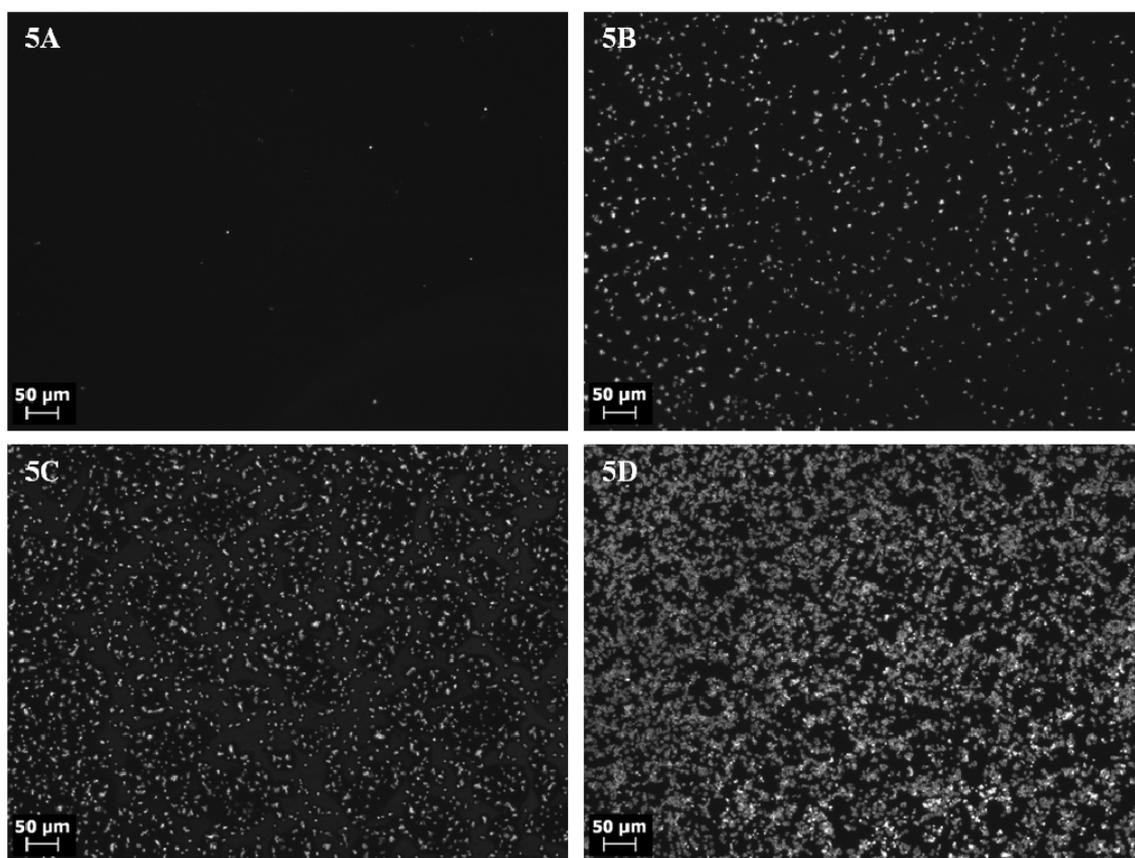


Fig. 5. Platelets visualized by actin-staining, attached to surfaces coated with albumin, collagen and fibrinogen. Adhesion to collagen was in the presence of 5 mmol/L MgCl_2 , while adhesion to albumin and fibrinogen was visualized in the absence of MgCl_2 . These conditions were chosen in order to facilitate adhesion to all surfaces, which would increase the likelihood of achieving measurable platelet adhesion. The respective images show basal platelet adhesion to albumin (5A), adhesion to albumin in the presence of 10 $\mu\text{mol/L}$ ADP (5B), basal adhesion to collagen (5C) and basal adhesion to fibrinogen (5D). In parallel with the actin-staining, a separate microplate was used for spectrophotometrical quantification of platelet adhesion. Corresponding adhesion values for the respective figures were: 5A = 5.1%, 5B = 15.6%, 5C = 18.4%, 5D = 26.9%. Scale bars show the length of 50 μm .

Discussion

The present study investigated the dependency on adhesion receptors and autocrine activation for platelet adhesion to albumin, collagen and fibrinogen in a static platelet adhesion assay (Fig. 6). Platelet adhesion to collagen was shown to be primarily dependent on the $\alpha_2\beta_1$ -receptor with a possible small contribution from $\alpha_{IIb}\beta_3$. The dependence on both $\alpha_2\beta_1$ and $\alpha_{IIb}\beta_3$ for platelet adhesion to collagen has been observed earlier for isolated platelets [22]. The authors suggested that the effect of $\alpha_{IIb}\beta_3$ was derived from platelet aggregation. Although this is also possible in our study, platelet aggregation is probably of minor importance since the assay was performed during conditions known to restrict aggregation, such as avoidance of shaking [2] and presence of Mg^{2+} [23]. Furthermore, surface characteristics are able to influence the structure of adsorbed proteins [24-26] and collagen might contain cryptic RGD-sequences that could be exposed after degradation [27]. Consequently, it could be proposed that RGD-sequences in collagen are exposed in this assay contributing to platelet adhesion. However, $\alpha_{IIb}\beta_3$ was probably of secondary importance since inhibition of platelet adhesion to collagen by $\alpha_{IIb}\beta_3$ -inhibitors was relatively weak and also not observed for all inhibitors. Furthermore, $\alpha_2\beta_1$ continued to be an important receptor for adhesion to collagen even after activating the platelets with ADP. The role of the GPVI-receptor for platelet adhesion to collagen was not directly investigated in this study. However, we found that platelet adhesion to collagen was dependent on either externally added Mg^{2+} or on addition of an external platelet activator such as ADP. The dependence on Mg^{2+} further strengthened the conclusion that $\alpha_2\beta_1$ is the central receptor for platelet adhesion to collagen in this assay since $\alpha_2\beta_1$ is known to be Mg^{2+} -dependent [2-4]. Furthermore, adhesion to collagen in the absence of Mg^{2+} was not observed unless ADP, or another

activator, was added. This suggests that activation of $\alpha_2\beta_1$ and consequent adhesion to collagen can, in this specific assay, be induced by ADP (and possibly other platelet activators not tested in this study) but not by GPVI.

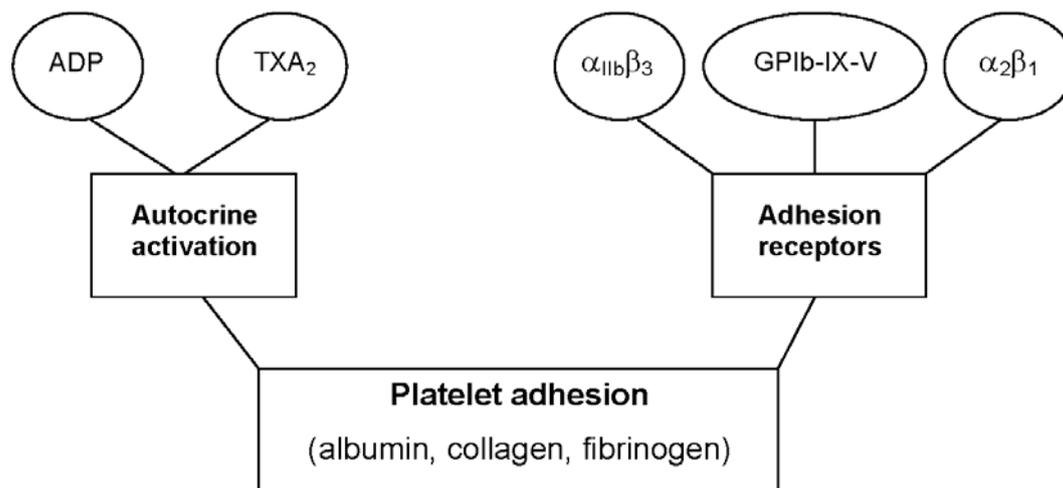


Fig. 6. Schematic diagram summarizing the mechanisms investigated in the current study. The study focused on platelet adhesion to albumin, collagen and fibrinogen in a static assay. The purpose was to evaluate the dependency on adhesion receptors (GPIb-IX-V, $\alpha_2\beta_1$, $\alpha_{11b}\beta_3$) as well as autocrine activation (ADP, TXA_2) for adhesion to the protein surfaces in this specific assay.

In the present assay platelet adhesion to fibrinogen was strongly dependent on the $\alpha_{11b}\beta_3$ -receptor. Irrespective of the presence or absence of ADP there was no effect of the $\alpha_2\beta_1$ -antibody AK7 on platelet adhesion to fibrinogen. This suggests that activating $\alpha_2\beta_1$ with an external activator does not make it adhesive for fibrinogen. Among the $\alpha_{11b}\beta_3$ -inhibitors it was only PM6/13 that was without effect on platelet adhesion to fibrinogen. The absence of effect for the PM6/13 antibody is at first somewhat surprising since this antibody has been shown to inhibit both platelet adhesion [28] and

platelet aggregation [29]. Unlike abciximab, eptifibatide, tirofiban [30] and most probably PM6/248 [28], PM6/13 does not interact with RGD-binding epitope(s) [29]. Thus, we suggest that binding of RGD-sequences is necessary for platelets to adhere to fibrinogen in this assay. A total dependence on RGD-binding in this assay but not in others might explain the discrepancy with previous studies described above for PM6/13. Hornby et al. showed PM6/248 to have a platelet activating effect dependent on binding to both $\alpha_{IIb}\beta_3$ and to Fc γ RII [31]. Regarding platelet aggregation, the activating effect was maximal at 8 μ g/mL. Higher concentrations still resulted in platelet activation but did not result in aggregation suggesting steric hindrance of fibrinogen binding. In our study we found 40 μ g/mL of PM6/248 to inhibit platelet adhesion to fibrinogen in accordance with inhibition of aggregation. However, we could not detect increased adhesion induced by PM6/248, neither by 40 μ g/mL on $\alpha_2\beta_1$ -dependent adhesion to collagen nor by lower concentrations on fibrinogen (not shown). The discrepancy between the study by Hornby et al. and ours is likely due to assay differences since potent inhibition of platelet adhesion to fibrinogen by PM6/248 can only occur if external Mg^{2+} is present (not shown).

Platelet adhesion to albumin only occurs in the presence of externally added platelet activators. We have earlier shown that platelet adhesion to albumin in the presence of LPA and adrenaline in this assay is mediated by $\alpha_{IIb}\beta_3$ [16]. In this study we investigated, the receptors which may be important for the adhesion of platelets activated with ADP. There was a striking resemblance between the results obtained on albumin and the results regarding adhesion to fibrinogen. AK2, AK7 and PM6/13 were without effect on both surfaces while PM6/248, abciximab, eptifibatide and tirofiban

inhibited platelet adhesion to albumin as well as fibrinogen. First of all, inhibition observed with inhibitors of $\alpha_{IIb}\beta_3$ but not with $\alpha_2\beta_1$ - or GPIb-IX-V-antibodies shows that adhesion to albumin induced by ADP is mediated by $\alpha_{IIb}\beta_3$. Furthermore, since PM6/13 was the only $\alpha_{IIb}\beta_3$ -inhibitor that was without effect, RGD is probably the binding site common for both surfaces. It is possible that albumin, upon binding to a surface, might change conformation and thereby allow adhesion through $\alpha_{IIb}\beta_3$. It is also possible that fibrinogen is attached to the albumin-coated wells, as earlier studies have shown that fibrinogen can interact with albumin [32], and that platelets in fact adhere to fibrinogen. Fibrinogen might be derived directly from plasma or it might be secreted from activated platelets. We suggest that direct or indirect interactions of platelets with albumin might be important *in vivo* since albumin is present in normal arteries [33] as well as in atherosclerotic plaques [34].

The platelet adhesion assay is developed for measurement of the total amount of platelets attached to a surface. However, more information regarding the adhesive process could possibly be gained if it is combined with morphological evaluation of the adhered platelets. It has previously been shown that adhesion to fibrinogen, but not albumin, induces platelet spreading [35]. This might be important regarding our above discussion about platelet adhesion to an albumin-coated surface. We therefore made an effort to investigate the adhered platelets to both fibrinogen and albumin by fluorescence microscopy. The design of the 96-well microplates makes it difficult to retrieve images of high magnification. However, the visualization by actin-staining clearly showed that platelets were homogeneously attached on the surfaces and were not

present in clusters. Also, when evaluating the platelet-images in parallel with percentage adhesion values obtained from the spectrophotometric measurements, we found good correlation between the assays. This confirms the accuracy of the spectrophotometric estimate of platelet adhesion.

Unlike other antibodies, the GPIb-IX-V-antibody AK2 did not inhibit adhesion to any of the surfaces investigated in this study. Even though vWf is present in the plasma milieu it is not surprising that AK2 was found to be without inhibiting effect in this assay since interactions between GPIb-IX-V and vWf is known to require flow conditions [8, 36]. Instead, we found the GPIb-IX-V-antibody to induce a small but significant increase in platelet adhesion to fibrinogen. It might therefore be possible that the interaction between the antibody and GPIb-IX-V induce intracellular activating events. Furthermore, ristocetin is an interesting compound in this experimental setting since it acts by inducing interactions between vWf and GPIb-IX-V [37]. AK2 was observed to decrease ristocetin-induced platelet adhesion to fibrinogen ($p < 0.01$, $n=4$) and to collagen (non-significant trend, $n=4$) towards the levels of basal adhesion (data not shown). There are two possible explanations for the effect of AK2 on ristocetin-induced adhesion. In the first scenario, AK2 inhibits platelet activation induced by the interaction between GPIb-IX-V and vWf-ristocetin [38] with the secondary consequence of decreased platelet adhesion. In the second scenario, AK2 acts as a direct inhibitor of platelet adhesion mediated by vWf bound to GPIb-IX-V. If the second scenario is true, this opens up the possibility of measuring GPIb-IX-V-dependent adhesion despite the absence of flow. The exact mechanism by which AK2 inhibits ristocetin-induced adhesion must be further investigated in future studies.

The initial experiments performed with cangrelor showed that this substance is very effective in inhibiting ADP-induced platelet adhesion. We have earlier shown that platelet adhesion to albumin induced by LPA combined with adrenaline is dependent on ADP [16], which is most likely released from activated platelets. In this study we antagonized ADP-signalling with cangrelor and showed that ADP is important for several other adhesive interactions as well. Even though adhesion induced by adrenaline or ristocetin as single soluble activators was shown to be significantly inhibited by cangrelor, the effect was relatively weak and probably of no practical importance. However, platelet adhesion induced by simultaneous activation by adrenaline and ristocetin was markedly inhibited by cangrelor. These results suggest that ristocetin and adrenaline both have the capacity to induce ADP-secretion on their own. However, when combined they increase platelet adhesion synergistically, probably by inducing platelet secretion of ADP more effectively.

Even though BM-531 significantly attenuated platelet adhesion for several of the adhesive interactions studied, the effects were very small. These results are in accordance with previous results using this assay showing marginal effects of high doses of ASA on platelet adhesion to collagen [15]. We also found that indomethacin could inhibit adhesion during conditions that were not affected by BM-531.

Consequently, conclusions regarding the influence of TXA₂ on platelet adhesion in this assay may differ depending on the substance used for inhibiting TXA₂-signaling.

Nevertheless, the moderate effects of both BM-531 and indomethacin suggest that release of TXA₂ probably has a relatively minor role for inducing adhesion in this assay.

In conclusion, adhesion to collagen was mainly dependent on the $\alpha_2\beta_1$ -receptor, while the $\alpha_{IIb}\beta_3$ -receptor was necessary for adhesion to fibrinogen. Also, autocrine stimulation mainly by ADP but also by TXA₂ seemed to be important for adhesion during several but not all conditions. Thus, the adhesive mechanisms occurring in the static platelet adhesion assay are in accordance with current knowledge regarding platelet activation. We therefore suggest that the static platelet adhesion assay, despite its simplicity, could generate valuable results regarding platelet activity. Depending on the extensiveness of the chosen protocol, an assay takes between 3 and 4 hours to complete and it might thus be used as a relatively fast screening device for investigating the effects of agonists and/or antagonists on platelet function. For instance, we show in this study that ADP-induced adhesion to albumin is dependent on $\alpha_{IIb}\beta_3$. Such adhesive interactions of platelets and novel surfaces could easily be investigated by using the platelet adhesion assay. Future studies will, in addition to what has already been performed [18, 19], investigate the clinical usefulness of the assay.

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