Platelets and Blood Cells

Glycoprotein Ibα inhibition and ADP receptor antagonists, but not aspirin, reduce platelet thrombus formation in flowing blood exposed to atherosclerotic plaques

Sandra M. Penz, Armin J. Reininger, Orsolya Toth, Hans Deckmyn, Richard Brandl, Wolfgang Siess

Introduction

Arterial thrombosis plays an important role in the pathogenesis of acute coronary syndromes, myocardial infarction and ischemic stroke, today the most common causes of mortality in developed countries. Excessive accumulation of platelets at sites of atherosclerotic plaque rupture is one of the key pathogenic events precipitating arterial thrombus formation and thereby fatal vessel occlusion (1).

The composition of human atherosclerotic plaques is not comparable with the normal arterial intima, and plaques, in particular their atheromatous core regions, are known to be more thrombogenic than healthy vascular tissue or collagen-rich matrix isolated from stable sclerotic lesions (1–4). The generally held view is that tissue factor (TF) found in plaques plays the key role in arterial thrombus formation (2, 5). This implies that coagulation is activated first and that platelets are only stimulated in a second step through thrombin generated by the coagulation system. However, our previous studies have shown that lipid-rich atherosclerotic plaques can cause directly platelet activation, independently of TF-induced thrombin formation (6–8).

Summary

Anti-platelet drugs are used to prevent intra-arterial thrombus formation after rupture of atherosclerotic plaques. Until now, the inhibitory effect of present and future anti-platelet drugs such as aspirin, ADP receptor P2Y1/P2Y12 antagonists and glycoprotein (GP) Ibα inhibitors on the interaction of platelets with human plaques is not known. To study those effects we obtained human atherosclerotic plaques by surgical endarterectomy. Plaques induced maximal platelet aggregation in hirudinized platelet-rich plasma (PRP) and blood that was effectively inhibited by aspirin, the P2Y12 antagonist MRS2179 and the P2Y1-antagonist AR-C69931MX, but not by GPIbα blockade with the mAB 6B4. Inhibition of platelet aggregation by MRS2179 was 74 ± 37% and 68 ± 20%, by AR-C69931MX 94 ± 7% and 80 ± 6%, and by aspirin 88 ± 19% and 64 ± 28%, in PRP and blood, respectively (mean ± SD; n=6–12 with plaques from 6 patients). The combination of both ADP receptor antagonists completely inhibited plaque-induced platelet aggregation in hirudinized PRP and blood. Under arterial flow conditions (1,500 s−1), blockade of platelet GPIbα resulted in a strong decrease of plaque-stimulated platelet adhesion/aggregation formation of 77 ± 5% (mean ± SD; n=4). Furthermore, MRS2179, AR-C69931MX and their combination reduced plaque-dependent platelet aggregate formation by 35 ± 14%, 32 ± 13% and 58 ± 12% (mean ± SD; n=5), respectively. Aspirin was without significant effect. In conclusion, a GPIbα-blocking antibody, as well as P2Y1 and P2Y12 receptor antagonists, alone or in combination, reduce in contrast to aspirin human plaque-induced platelet thrombus formation under arterial flow. Although these new anti-platelet agents inhibit platelet thrombus formation after plaque rupture, more efficient platelet blockers are required.

Keywords

ADP receptor antagonists, aspirin, GPIb, plaque, platelet thrombus formation


Financial support: The study was supported by the grants from the Deutsche Forschungsgemeinschaft (Graduate Program GK 438 „Vascular Biology in Medicine”; Si-274/9) (S.M.P.; W.S.), the Friedrich-Baur foundation and the August-Lenz foundation (O.T.).

Correspondence to:
Dr. Wolfgang Siess
Institut für Prophylaxe und Epidemiologie der Kreislaufkrankheiten
Klinikum Innenstadt, Ludwig-Maximilians Universität München
Pettenkoferstr. 9, D-80336 München, Germany
Tel.: +49 89 5160 4380, Fax: +49 89 5160 4382
E-mail: wsiess@med.uni-muenchen.de

Received July 29, 2006
Accepted after resubmission December 29, 2006
Prepublished online February 8, 2007
doi:10.1160/TH06–07–0415
Atherosclerotic plaques contain various platelet-activating molecules such as different types of collagens, von Willebrand factor (VWF), thrombospondin, fibronectin, vitronectin, fibrin/fibrinogen, oxidized low density lipoprotein (LDL), lysophosphatidic acid (LPA), SDF-1, and cholesterol sulphate (6–15). Within these numerous platelet stimuli, our studies have identified the two main platelet-activating plaque components and elucidated their platelet activation mechanisms (6–8, 16). We were able to show that the interaction of LPA molecules in the plaque lipid-rich core with their corresponding G-protein-coupled LPA receptors induced platelet shape change, and that the interaction of collagen type I- and type III-positive plaque constituents with glycoprotein VI (GPVI) stimulated platelet adhesion, aggregation and thrombus formation under arterial flow conditions (6–8, 16).

Based on these results and on present knowledge (1), the following sequence of events might lead to thrombus formation after plaque rupture. Under arterial flow conditions, VWF endogenously present in the plaque (11), or circulating VWF adsorbed onto plaque collagen structures, induces initial platelet tethering and transient adhesion through the interaction with its corresponding platelet receptor GPIbα (1). Subsequently, stable platelet adhesion and platelet activation are mediated by the interaction of collagen-like structures and LPA molecules in the plaque with their corresponding receptors (6–8). This initiates a cascade of events leading to platelet secretion, fibrinogen receptor (integrin αIIbβ3) activation, spreading and aggregation. Finally, thrombus growth is mainly propagated by the release of the positive feedback mediators adenosine-5’-diphosphate (ADP) and thromboxane A$_2$ (TxA$_2$) (1, 8, 16).

Platelet inhibition has become an important strategy for the prevention of thrombus formation in patients with cardio- and cerebrovascular diseases (17, 18). Among the clinically administered anti-platelet agents the cyclooxygenase-1 inhibitor aspirin was the first and continues to be the most widely used drug in primary and secondary prevention of atherothrombotic diseases (19–21). However, aspirin is only effective in about one quarter of high-risk patients and fails to prevent the majority of serious vascular events (21, 22). Therefore additional anti-platelet drugs are needed to inhibit plaque-induced platelet thrombus formation. We and others have previously shown that inhibition of GPVI completely blocked plaque-induced platelet thrombus formation under arterial flow conditions (8, 23).

The effect of aspirin and future anti-platelet drugs such as specific P2Y$_1$/P2Y$_12$ receptor antagonists and inhibitors of the GPIb-α-VWF axis on the interaction of platelets with human plaque tissue is not known. We have recently validated a new device, called Multiplate®, to measure platelet aggregation in whole blood (24), and developed a novel in-vitro flow model for direct real time visualisation of platelet adhesion and aggregate formation on surfaces coated with human atheromatous plaques (8). In the present study the platelet GPIbα blocking antibody 6B4 and two novel anti-platelet drugs, the P2Y$_1$ antagonist MRS2179 and the P2Y$_12$ antagonist AR-C69931MX, alone or in combination, were compared with aspirin concerning their inhibition of plaque-induced platelet aggregation in platelet-rich plasma and whole blood, as well as on plaque-induced thrombus formation under arterial flow conditions.

Materials and methods

Materials

Tri-sodium citrate dihydrate was obtained from Merck (Darmstadt, Germany), recombinant hirudin (Refudan®) from Schering (Berlin, Germany). Collagen (Horm®) was purchased from Nycomed Pharma (Munich, Germany). ADP, apyrase (grade VII), the P2Y$_1$-receptor antagonist MRS2179 (N$^6$-methyl-2’-deoxyadenosine-3’,5’-bisphosphate), acetylsalicylic acid (aspirin, ASA), bovine serum albumin (essentially fatty acid free), mouse IgG1 and mepacrine dihydrochloride came from Sigma (Taufkirchen, Germany). The P2Y$_{12}$-receptor antagonist AR-C69931MX (N$^6$–2-(methylthioethyl)-2-(3,3,3-trifluoropropylthio)-β,y-dichloromethylene ATP) was generously provided by Astra-Zeneca R&D Charnwood (Loughborough, UK). The inhibitory mAb 6B4 against platelet GPIbα was generated as previously specified (25). If not mentioned separately all chemicals were obtained from Merck (Darmstadt, Germany).

Isolation of human carotid atherosclerotic plaque material

Atherosclerotic tissue specimens were obtained from patients who underwent surgery for high-grade carotid artery stenosis as described previously (8). Patient consent was obtained as approved by the Ethics Committee of the Faculty of Medicine of the University of Munich. The carotid plaque tissue was endarterectomised by careful operative technique preserving the plaque structure en bloc and leaving the arterial media and adventitia in the patient (26). Only advanced lipid-rich plaque specimens, classified as type V atheroma (plaque with a lipid-rich and/or necrotic core surrounded by fibrous tissue), were collected for further studies. The atheromatous plaques were carefully dissected from other regions of the atherosclerotic tissue specimens such as the distal collagen-rich plaque shoulder and the proximal region containing connective tissue, foam cells, and lipid deposits (26). After infra-operative removal, the tissue was immediately frozen in liquid nitrogen and stored at –80°C. Under sterile conditions, individual plaque samples were weighed, homogenized, and the homogenates were kept at –80°C until their final experimental usage. The atheromatous plaque concentration was adjusted to 50 mg/ml wet weight, and protein concentrations ranged between 0.5–2.5 mg/ml. The plaque homogenates contain various LPA molecules and morphologically heterogeneous collagen type I- and type III-positive structures (7, 8). Plaque homogenates were used for platelet aggregation studies of platelet-rich plasma and whole blood. Moreover, plaque homogenates were coated onto glass cover slips for flow studies (see below).

Blood collection

Blood was obtained from healthy volunteers by venipuncture using a 19-Gauge needle and plastic syringe containing either 1/10 volume sodium citrate (final concentration in blood 0.31% wt/vol; 12.9 mM) or 1/10 volume recombinant hirudin (final concentration in blood ~200 U/ml; 13 µg/ml) after informed consent in accordance with the Helsinki protocol was obtained. The first 3 ml of blood were discarded. All volunteers denied taking any medication affecting platelet function for at least two weeks.
preceeding the experiments. Sodium citrate, as well as recombinant hirudin were dissolved in 20 mM HEPES, 138 mM NaCl, 2.9 mM KCl, 1 mM MgCl₂, 6 mM H₂O₂, 0.36 mM NaH₂PO₄, pH 7.4. For some experiments, acetylsalicylic acid was dissolved in sodium citrate solution or hirudin-containing buffer solution and drawn together with the anticoagulants into a syringe to obtain a final concentration of 1 mM aspirin in the collected blood sample. Aggregation measurements were performed between 45 minutes (min) and 2 hours (h), flow experiments between 45 min and 4 h after venipuncture.

**Platelet aggregation in platelet-rich plasma**

Platelet-rich plasma (PRP) was prepared from citrated or hirudinized blood by centrifuging the blood at 160 x g for 20 min at room temperature (RT). Platelet-poor plasma (PPP) was obtained by centrifugation of PRP at 1,000 x g for 10 min at RT. Platelet aggregation in response to collagen (0.5 µg/ml) or human plaque homogenates (0.83 mg/ml) was determined turbidimetrically at 37°C using stirred PRP (1,100 rpm) in a LABOR®-aggregometer (Fresenius, Bad Homburg, Germany) as described before (8). Light transmission was defined 0% for PRP and 100% for PPP. For inhibition of the platelet ADP receptors P2Y₁ and P2Y₁₂, PRP was preincubated for 3 min at 37°C with MRS2179 (100 µM) and AR-C69931MX (1 µM), respectively. TxA₂ biosynthesis was prevented by collecting the blood directly into acetylsalicylic acid (final concentration 1 mM)-containing citrate- or hirudin-solutions.

**Whole blood platelet aggregation**

Whole blood platelet aggregation was determined by impedance aggregometry as recently described by Tóth et al. (24). The multiple platelet function analyser “Multiplate®” from Dynabyte Medical (Munich, Germany) was used to detect the electrical impedance change due to the adhesion and aggregation of platelets on two independent electrode-set surfaces in the test cuvette. A 1:2 mixture of 0.9% NaCl and whole blood was stirred at 37°C for 3 min in the test cuvettes, ADP (5 µM), collagen (0.5 µg/ml), ristocetin (0.5 mg/ml) or plaque homogenates (0.83 mg/ml) were added and the increase in electrical impedance was recorded continuously for 5 min. The mean values of the two independent determinations were expressed in arbitrary “aggregation units” (AU). Platelet P2Y₁ and P2Y₁₂ were inhibited by a 3 min preincubation of citrated or hirudinized blood at 37°C with different concentrations of MRS2179 and AR-C69931MX, respectively. TxA₂ biosynthesis was prevented by collecting the blood directly into aspirin (final concentration 1 mM)-containing citrate- or hirudin-solutions.

**Analysis of platelet adhesion and thrombus formation in flowing whole blood**

For flow chamber experiments glass cover slips were coated with homogenized carotid plaque (5 mg/ml) dissolved in PBS containing 15 µM (0.1%) fatty acid-free albumin and prior to experimentation blocked with PBS-albumin (45 µM; 0.3%) for 16 h at 4°C to inhibit non-specific binding. The immobilized plaque homogenate was subsequently shown to contain morphologically diverse collagen type I- and type III-positive plaque structures (8). Cover slips were rinsed carefully with phosphate-buffered saline (PBS) and then inserted into a parallel-plate flow chamber. Perfusion at wall shear rates of 500 and 1,500 s⁻¹ was obtained by aspiration of the blood through the chamber via a syringe pump (Harvard Apparatus, Holliston, MA, USA). The perfusion chamber was mounted on the stage of an upright microscope (Axioskope 2 plus, Carl Zeiss, Jena, Germany) for real-time visualization of platelet adhesion and aggregation in flowing blood. The platelets were stained with mepracine (10 µM) and observed by fluorescence microscopy. Platelet deposition was recorded continuously on S-VHS videotape at the acquisition rate of 25 frames/second using an AVT BC-71 camera (AVT Horn, Aalen, Germany) connected to a VCR (Panasonic NV-HS930, Osaka, Japan). For inhibition of platelet P2Y₁ and P2Y₁₂ or platelet GPIbα, blood was preincubated for 10 min at 37°C with MRS2179 (100 µM), AR-C69931MX (1 µM) and the combination of both antagonists or 5 µg/ml 6B4 (25) and mouse IgG1 (control). To prevent TxA₂ formation blood was directly collected into an aspirin (final concentration 1 mM)-containing hirudin-solution. Control and aspirin-treated blood were also prewarmed to 37°C for 10 min prior to the flow experiments. Digitizing and analyzing images was performed off-line using the Matrox Inspector software package (Matrox Electronix Systems Ltd., Canada). During minutes 4 to 5 (only for experiments with mAB 6B4) and during the last 3 min of each 10-min flow experiment, the superfused area was scanned and 10 to 15 images (visual fields) of the surface were obtained (40 x objective, zoom 0.8). In each field the plaque-areas and respective aggregate-areas were measured and related as “% plaque covered with platelet aggregates”.

**Statistical analysis**

Results are reported as mean ± SD from 4–12 experiments conducted with PRP or whole blood from at least three different donors. Statistical significance was assessed by paired and unpaired Student’s t-test. Differences were considered significant when p was < 0.05 and indicated were applicable.

**Results**

**Effects of ADP receptor antagonists and aspirin on human plaque-induced platelet aggregation in PRP and blood**

First, we compared the anti-aggregatory effects of ADP receptor antagonists and aspirin on human plaque-induced platelet aggregation in citrate- and hirudin-anticoagulated PRP and whole blood. Dose-response experiments on the inhibition of ADP (5 µM)-induced platelet aggregation in blood revealed 1 µM AR-C69931MX (P2Y₁₂ antagonist) and 100 µM MRS2179 (P2Y₁ antagonist) as the antagonist concentrations which completely (AR-C69931MX) or to more than 90% (MRS2179) blocked ADP-dependent platelet aggregation (data not shown; [16]). For aspirin, conditions and concentrations (1 mM) were used, which are known to completely inhibit platelet cyclooxygenase-1 (16, 27).

Human atheromatous plaque material (0.83 mg tissue/ml) induced platelet aggregation of 101 ± 11 and 99 ± 19% light transmission (mean ± SD; n=6–7) in citrated and hirudinized PRP, respectively, which was slightly higher than platelet aggregation after collagen (0.5 µg/ml) stimulation (Fig. 1A). Platelet pre-
treatment with aspirin significantly reduced plaque-dependent platelet aggregation to $19 \pm 10$ and $11 \pm 15\%$ light transmission (mean $\pm$ SD; $n=6$) in citrated and hirudinized PRP, respectively. However, aspirin decreased collagen-stimulated aggregation somewhat more efficiently than plaque-induced aggregation to $9 \pm 10$ and $5 \pm 8\%$ light transmission in citrated and hirudinized PRP (mean $\pm$ SD, $n=6–12$), respectively. As determined by impedance aggregometry with the Multiplate®, plaque (0.83 mg/ml)-induced platelet aggregation was more pronounced in hirudinized compared to citrated whole blood (Fig. 1B, C). This difference was even stronger, when collagen (0.5 µg/ml) was used as platelet stimulus, indicating clear dissimilarities between

Figure 1: Effects of MRS2179, AR-C69931MX and aspirin on platelet aggregation in citrated or hirudinized PRP (A) and blood (B, C) induced by individual human plaques or collagen. A, B) Bar diagrams of plaque- (left) and collagen- (right) induced platelet aggregation. Data are presented as absolute values and mean $\pm$ SD (n=4–12 with blood of at least 3 different donors). *p<0.05 treatment versus control. †p<0.05 citrated versus hirudinized PRP/blood. C) Representative impedance aggregation tracings of plaque-induced platelet aggregation in citrated (left) and hirudinized (right) blood.
plaque material and fibrillar collagen. Aspirin reduced plaque-induced platelet aggregation from 55 ± 1 to 28 ± 17 AU in citrated blood and from 69 ± 27 to 24 ± 16 AU in hirudinized blood (mean ± SD; n=4–13) (Fig. 1B, C). As already observed in PRP, collagen-stimulated platelet aggregation in citrated and hirudinized whole blood was even more reduced by aspirin from 67 ± 15 to 23 ± 19 AU and from 132 ± 36 to 17 ± 14 AU (mean ± SD; n=6–12), respectively. This indicates again a clear difference between plaque- and collagen-dependent platelet activation.

MRS2179 and AR-C69931MX reduced plaque-induced platelet aggregation to 93 ± 11 and 51 ± 17% light transmission in citrated PRP and to 27 ± 31 and 7 ± 8% in hirudinized PRP (mean ± SD; n=6–7), respectively (Fig. 1A). The inhibitory effects of the ADP-receptor antagonists were significantly more pronounced in hirudin- compared to citrate-anticoagulated PRP. Furthermore, AR-C69931MX was more effective than MRS2179 in reducing plaque-stimulated platelet aggregation. The combination of both ADP receptor antagonists inhibited platelet aggregation more efficiently than each antagonist alone, and resulted in 27 ± 18 and 2 ± 4% light transmission (mean ± SD; n=6) in citrated and hirudinized PRP, respectively. The combination of MRS2179, AR-C69931MX and aspirin could not completely inhibit plaque-stimulated platelet shape change and aggregation in citrated PRP although the effect compared to both ADP receptor antagonists alone was increased. However, in hirudinized PRP the combination of all three platelet inhibitors completely blocked platelet shape change and aggregation.

In citrated whole blood, MRS2179 did not significantly reduce plaque-dependent platelet aggregation, but it significantly decreased plaque-stimulated aggregation from 69 ± 27 to 22 ± 25 AU (mean ± SD; n=5) in hirudinized whole blood (Fig. 1B, C). Blood pretreatment with AR-C69931MX reduced platelet aggregation from 55 ± 16 to 32 ± 15 AU and from 69 ± 27 to 17 ± 10 AU (mean ± SD; n=6–12) in citrated and hirudinized blood, respectively, with AR-C69931MX again being more effective than MRS2179. Dose-response experiments on the inhibition of plaque-induced platelet aggregation showed that 100 µM MRS2179 and 1 µM AR-C69931MX already maximally inhibited platelet aggregation in citrated and hirudinized whole blood (Fig. 2A, B). Interestingly, lower ADP receptor antagonist concentrations were also effective in reducing plaque-stimulated platelet aggregation in blood, but could, as mentioned before, not maximally block ADP-induced platelet aggregation. The reason for this could be that compared to the ADP concentration (5 µM) added exogenously, less ADP is released by plaque-stimulated platelet activation and aggregation. Notably, both ADP receptor antagonists worked more efficiently in hirudinized than in citrated whole blood. The combination of MRS2179 and AR-C69931MX reduced plaque-stimulated aggregation to 19 ± 1 AU and to 3 ± 2 AU in hirudinized blood (mean ± SD; n=5–11). An almost complete inhibition of plaque-stimulated platelet aggregation could be obtained by the combination of MRS2179, AR-C69931MX and aspirin. The additional inhibitory effect of aspirin was more pronounced in citrated compared to hirudinized blood. In summary, inhibition of plaque-induced platelet aggregation by all studied anti-platelet agents was less pronounced in blood compared to PRP and weaker when citrate instead of hirudin was used as anticoagulant.

**Figure 2**: Effect of increasing concentrations of MRS2179 (A) and AR-C69931MX (B) on plaque-induced platelet aggregation in citrated (dashed line) and hirudinized (solid line) whole blood. Dose-response curves of the effects of MRS2179 and AR-C69931MX on plaque-induced platelet aggregation. In all experiments the same plaque-pool of three different plaque homogenates was added as platelet agonist. Data are presented as absolute values and mean ± SD (n=5 with blood of 5 different donors). *p < 0.05 treatment versus control.

**Role of VWF and platelet GPIbα in human plaque-induced platelet thrombus formation under arterial flow conditions**

The interaction of VWF endogenously present in plaques (11) or of circulating VWF in blood with GPIbα could play a crucial role in the initial steps of plaque-induced thrombus formation under flow. We analyzed the effect of the potent inhibitory monoclonal anti-GPIbα antibody 6B4 on plaque-stimulated platelet aggregation and thrombus formation under arterial flow conditions with low (500 s⁻¹; present in medium sized arteries) and high (1,500 s⁻¹; present in moderately stenosed arteries) wall shear rates, as well as in stirred blood using the Multiplate®. In contrast to ristocetin (0.5 mg/ml)-induced platelet aggregation, which was completely abolished by 1 µg/ml 6B4, 6B4 concentrations...
up to 5 µg/ml had no effect on plaque-stimulated platelet aggregation in stirred hirudinized blood (data not shown).

Flow studies were conducted with a concentration of 5 µg/ml 6B4. At low shear rates of 500 s⁻¹ the anti-GPIbα antibody did not significantly reduce plaque-stimulated platelet aggregate formation, either in the first 4 to 5 min or after 10 min of flow (Fig. 3). In contrast, with high shear rates (1,500 s⁻¹), preincubation of blood with 6B4 resulted compared to the control (100%) in a strong reduction of platelet aggregate formation of about 77 ± 5 to 84 ± 3% at the time points 4 to 5 and 7 to 10 min after start of the flow (mean ± SD; n=4–5), respectively. Not only the number of aggregate-covered plaque areas was lower, but also the height of the individual platelet aggregates was decreased by 6B4. These results indicate that GPIbα is not only involved in platelet adhesion to atherosclerotic plaque material, but also in subsequent platelet-platelet interaction in the growing thrombus.

**Effect of ADP receptor antagonists and aspirin on human plaque-induced platelet thrombus formation under arterial flow conditions**

The effects of aspirin and ADP receptor antagonists on human plaque-induced platelet aggregation and thrombus formation under arterial flow conditions are shown in Figure 4. After perfusion of hirudinized blood over plaque-coated surfaces for 10 min at 37°C with high shear rates (1,500 s⁻¹) 60 ± 13% (mean ± SD; n=13) of the plaque area was covered with platelet aggregates (Fig. 4A). Addition of ADP receptor antagonists reduced plaque-induced platelet aggregate formation. Compared to the control (100%), MRS2179, AR-C69931MX and the combination of MRS2179 and AR-C69931MX significantly inhibited platelet adhesion/aggregate formation by 35 ± 14%, 32 ± 13% and 58 ± 12% (mean ± SD; n=5), respectively. However, aspirin was with 17 ± 13% inhibition (mean ± SD; n=8) without significant effect. Also, aspirin in combination with both ADP receptor antagonists showed no additional reducing effect on plaque-stimulated platelet coverage. Platelet inhibition by all platelet inhibitors was much less pronounced under flow than in stirred PRP and blood. Video-microscopic analysis showed that platelet adhesion to plaque, which started 0.5–3 min after start of flow, was not affected by MRS2179 and AR-C69931MX.

Furthermore, we compared the antithrombotic effect of the combination of all three anti-platelet drugs under flow at lower shear rates (500 s⁻¹) with those at higher shear rates (1,500 s⁻¹) (Fig. 4B). Under low shear rates 75 ± 13% (mean ± SD, n=4) of the coated plaque surface was covered with platelet aggregates after 10 min of flow at 37°C. At lower shear rates, the combination of MRS2179, AR-C69931MX and aspirin was significantly less effective in reducing plaque-dependent platelet thrombus formation than at higher shear rates.

**Discussion**

Although anti-platelet drugs are used to prevent intra-arterial thrombus formation leading to acute coronary syndromes and ischemic stroke, surprisingly little is known about the effects of those agents on the interaction of platelets with human atherosclerotic plaques. In general, the effects of anti-platelet drugs are studied after stimulation of human platelets with defined platelet stimuli in vitro, or in animal thrombosis models in vivo. Good animal models for rupture of atherosclerotic plaques do not exist (28). Moreover, plaque composition in humans and animal models is very different (28). Last but not least, animal platelets fail to respond to certain platelet stimuli such as LPA found in human material is visualized by autofluorescence (light-grey; ▲), platelets by meracrine fluorescence (white; ▲). Bar: 50 µm. Right, bar diagram of the effects of 6B4 or mouse IgG1 on platelet aggregate formation 4–5 (grey bars) and 7–10 min (black bars) after start of the flow with 500 and 1,500 s⁻¹. Values are mean ± SD (n=4–5 with blood of at least 4 different donors). *p < 0.05 treatment versus control.

**Figure 3:** Comparison of the effect of GPIbα inhibition on human plaque-induced platelet aggregate formation under arterial flow conditions with lower (500 s⁻¹) and higher (1,500 s⁻¹) wall shear rates. Hirudinized blood was perfused over plaque-coated surfaces for 10 min at 37°C with shear rates of 500 or 1,500 s⁻¹. In all experiments the same plaque-pool of three different plaque homogenates was immobilized on the glass cover slips. Blood was pre-treated with 6B4 (5 µg/ml) or the same amount of mouse IgG1 (control). Left, representative flow images 7–10 min after start of the flow; plaque material is visualized by autofluorescence (light-grey; ▲), platelets by mepacrine fluorescence (white; ▲). Bar: 50 µm. Right, bar diagram of the effects of 6B4 or mouse IgG1 on platelet aggregate formation 4–5 (grey bars) and 7–10 min (black bars) after start of the flow with 500 and 1,500 s⁻¹. Values are mean ± SD (n=4–5 with blood of at least 4 different donors). *p < 0.05 treatment versus control.
plagues, and therefore animal studies will not resemble arterial thrombus formation after plaque rupture in humans (29–31). Our experimental approach, i.e. the exposition of human atherosclerotic plaque material to the blood under arterial flow conditions is novel and aimed to imitate as close as possible platelet thrombus formation in vivo upon plaque rupture.

In the present study, with 84% inhibition GPIbα blockade was very effective in reducing plaque-stimulated platelet thrombus formation under flow conditions with high shear rates of 1,500 s⁻¹, whereas blockade by the combination of P2Y₁⊂ and P2Y₁₂-receptor antagonists was less effective with 58%. Surprisingly, aspirin failed to significantly inhibit plaque-induced thrombus formation under arterial flow conditions. Also, aspirin in combination with both ADP receptor antagonists showed no additional anti-platelet effect under flow compared to MRS2179 plus AR-C69931MX. In contrast, under static conditions, both ADP receptor antagonists plus aspirin completely blocked platelet aggregation in blood, and also in PRP. It is likely that during platelet thrombus formation under flow platelet-released ADP and TxA₂ will not readily accumulate at the site of platelet adhesion to the plaque, and the ability of these substances to act as positive feedback mediators and recruit circulating platelets into the growing aggregates will thereby be limited. The discrepancy of our results and the proven protective effect of aspirin in patients with acute coronary syndromes or patients at a risk of major cardiovascular events might probably be explained by a

---

**Figure 4: Effects of MRS2179, AR-C69931MX and aspirin on human plaque-induced platelet aggregate formation under arterial flow.** Hirudinized blood was perfused over plaque-coated surfaces for 10 min at 37°C at shear rates of 500 or 1,500 s⁻¹. Blood was pre-treated with MRS2179 (100 µM), AR-C69931MX (1 µM) and aspirin (1 mM). A) Left, representative flow images 7–10 min after start of the flow; right, bar diagram (values are mean ± SD of 5 different experiments with 4 different plaques). Plaque material is visualized by autofluorescence (light-grey; ▲), platelets by mepacrine fluorescence (white; ▲). Bar: 50 µm. B) Comparison of two shear rates (500 and 1,500 s⁻¹) on plaque-induced platelet thrombus formation and inhibition by anti-platelet drugs. Left, representative flow images 7–10 min after start of the flow. Bar: 50 µm. Right, bar diagram (mean ± SD of 4 different experiments with 2 different plaques). *p < 0.05 treatment versus control.
role of platelet adhesion, cyclooxygenase-1 and TXA2 formation in the early stages and progression of atherosclerosis, rather than in acute thrombus formation after plaque rupture (32, 33).

It was previously demonstrated that VWF is endogenously present in human plaques (11) and probably mediates direct platelet adhesion to plaque material under flow, similar to platelet adhesion onto VWF-coated surfaces. Platelet adhesion on immobilized VWF is markedly inhibited by blockade of GPIbα under flow conditions with different shear rates ranging from 50 to 1,500 s\(^{-1}\) (34). However, circulating VWF-dependent platelet adhesion and aggregation formation on collagen-coated surfaces is abolished by inhibition of GPIbα under flow with higher wall shear rates of 1,500 s\(^{-1}\), but not with lower shear rates of 500 s\(^{-1}\) (35, 36). In our work, the inhibitory anti-GPIbα antibody 6B4 did not significantly reduce plaque-induced platelet aggregation in hirudinized whole blood under static conditions and arterial flow conditions with wall shear rates of 500 s\(^{-1}\). However, with higher shear rates of 1,500 s\(^{-1}\) the number of aggregate-covered plaque areas was strongly decreased by 6B4, indicating that platelet adhesion to plaque resembles platelet adhesion on circulating VWF adsorbed onto collagen. We therefore conclude that circulating VWF, rather than endogenous plaque VWF, might play a role in mediating plaque-induced thrombus formation at 1,500 s\(^{-1}\). Interestingly, at this wall shear rate platelet thrombus growth was also reduced by blocking GPIbα. The inhibition not only of platelet adhesion, but also of platelet aggregate formation on plaque surfaces and collagen at 1,500 s\(^{-1}\) might be explained by the inhibition of GPIbα-signalling to the integrin α\text{IIb}β\text{3} (25) and a reduced platelet-platelet interaction through GPIbα/VWF (37–39). Thus, VWF seems to play an important role in platelet thrombus formation after plaque rupture.

In contrast to the lack of GPIbα-inhibition at lower shear rates (500 s\(^{-1}\)), ADP-receptor antagonists reduced plaque-stimulated platelet aggregate formation at both shear rates, 500 and 1,500 s\(^{-1}\). Interestingly, the combination of the two ADP receptor antagonists inhibited plaque-stimulated platelet aggregation more effective at higher (1,500 s\(^{-1}\)) than at lower (500 s\(^{-1}\)) shear rates, suggesting that ADP, known to play an important role in shear stress-mediated platelet aggregation (40–42), is associated with platelet aggregate formation after plaque rupture. Moreover, our results demonstrate that each ADP receptor antagonist alone significantly reduced plaque-induced platelet aggregate formation at 1,500 s\(^{-1}\) to a similar degree. Under all experimental conditions the combination of both ADP receptor antagonists was more effective than each antagonist alone. This indicates that for effective inhibition of plaque-induced aggregation and thrombus formation both ADP receptors which activate different signalling pathways in platelets should be inhibited: P2Y\text{1} and P2Y\text{12}, which couples to G\text{q/11} and inhibits the adenyl cyclase (43). Blockade of plaque-dependent platelet aggregate formation under flow by combined inhibition of platelet P2Y\text{1} and P2Y\text{12} was also recently shown by Cosemans et al. (23). Previous studies have shown conflicting results of ADP receptor blockade on collagen-induced platelet deposition. In one study blockade of both ADP receptors P2Y\text{1} and P2Y\text{12} was required for efficient inhibition (41), whereas in others P2Y\text{1} or P2Y\text{12} blockade alone showed significant inhibition (44–47).

In aggregation experiments with stirred PRP or whole blood, both ADP receptor antagonists significantly reduced platelet aggregation. When the two different ADP receptor antagonists were compared, AR-C69931MX was more effective than MRS2179 in inhibiting plaque-induced platelet aggregation in hirudinized and citrated PRP, as well as in citrated blood. In hirudinized blood AR-C69931MX and MRS2179 were equally effective. Moreover, all anti-platelet agents inhibited more effective in hirudin- than in citrate-anticoagulated PRP and blood. Anticoagulation with hirudin preserves the physiological divalent cation concentrations in blood, and therefore the measurements in hirudinized blood might resemble more the situation in vivo.

Aspirin inhibited plaque-dependent platelet aggregation in hirudin-anticoagulated blood only by 64%, whereas inhibition of collagen-stimulated aggregation was with 85% more pronounced (Fig. 1B and [24]). This may be explained by the LPA-component of plaques, which induces aspirin-resistant platelet aggregation (16). Although aspirin inhibited plaque-stimulated aggregation in stimulated hirudinized blood, it did in contrast to AR-C69931MX and MRS2179 not significantly reduce the formation of platelet aggregates under flow. It was also not effective in combination with the two ADP receptor antagonists at both shear rates (500 s\(^{-1}\), 1,500 s\(^{-1}\)). These results imply that TXA2 formation compared to ADP receptor activation plays only a minor role in plaque rupture-dependent platelet aggregation and thrombus formation under arterial flow conditions. A previous study on collagen-induced thrombus formation has demonstrated that the effect of aspirin on platelet thrombus formation is shear-rate dependent. Aspirin showed only a moderately inhibition at 2,600 s\(^{-1}\) and no anti-platelet effect at lower (650 s\(^{-1}\)) and higher wall shear rates (10,500 s\(^{-1}\)) (20). Moreover, shear-induced VWF-assisted platelet activation and aggregation was demonstrated to be aspirin-resistant and to require released ADP (42). These results support our findings of the lack of aspirin to inhibit plaque-induced platelet thrombus formation under arterial flow conditions. However, we found both ADP receptor antagonists to be effective, indicating an important role of the shear/VWF/ADP-axis in plaque induced platelet aggregate formation under flow.

In conclusion, we show in the present study that the anti-GPIbα antibody 6B4, as well as the P2Y\text{1}– and P2Y\text{12}-receptor antagonists MRS2179 and AR-C69931MX significantly reduced plaque-induced platelet thrombus formation under arterial flow conditions, whereas aspirin was ineffective. Our study, however, demonstrates that the inhibitory effect of these drugs is limited. Previously, inhibition of platelet GPVI has been shown to be very effective in reducing plaque-induced thrombus formation in mouse and man (8, 23). The combination of different anti-platelet drugs (P2Y\text{1}/P2Y\text{12}-receptor antagonists, inhibitors of GPIbα or GPVI) might improve the prevention of human plaque-induced thrombus formation after plaque rupture.

Acknowledgement

The technical assistance of Nicole Wilke is greatly appreciated.
References


32. Moake JL, Turner NA, Stathopoulos NA, et al. Shear-induced platelet aggregation can be mediated by vWF released from platelets, as well as by exogenous large or unusually large vWF multimers, requires adenosine diphosphate, and is resistant to aspirin. Blood 1998; 71: 1366–1374.


