Endothelial von Willebrand factor recruits platelets to atherosclerosis-prone sites in response to hypercholesterolemia

Gregor Theilmeyer, Carine Michiels, Erik Spaepen, Ingrid Vreys, Désiré Collen, Jos Vermylen, and Marc F. Hoylaerts

Platelets are thought to play a causal role during atherogenesis. Plateletendothelial interactions in vivo and their molecular mechanisms under shear are, however, incompletely characterized. Here, an in vivo platelet homing assay was used in hypercholesterolemic rabbits to track platelet adhesion to plaque predilection sites. The role of platelet versus aortic endothelial cell (EC) activation was studied in an ex vivo flow chamber. Pathways of human platelet immobilization were detailed during in vitro perfusion studies. In rabbits, a 0.125% cholesterol diet induced no lesions within 3 months, whereas lesions were detailed during in vitro perfusion studies. In rabbits, a 0.125% cholesterol diet induced no lesions within 3 months.

Introduction

Platelets can be identified in atherosclerotic lesions at all stages. A contribution of platelets to the early stages of atherosclerotic lesion development has been postulated. Experimental evidence demonstrating platelet recruitment to the endothelial layer at lesion-prone sites in response to atherogenic stimuli is, however, lacking.

Plateletendothelial interactions have been characterized in static assays and in venous shear conditions, but the adhesion pathways involved in interactions requiring higher tensile strength, as prevailing in the arterial vasculature, remain elusive. In static adhesion assays, the integrin αvβ3, bridging with various partners on endothelial cells (ECs), among which intercellular adhesion molecule 1 (ICAM-1), αvβ3 integrin, and possibly glycoprotein Ibα (GPIbα), played the major role in mediating platelet adhesion to activated human umbilical vein endothelial cells (HUVECs). In arterial shear conditions, even at sites of turbulent flow, the contribution of individual adhesion pathways may vary.

The interaction of platelets with subendothelial matrix in high shear conditions has been well characterized and is primarily mediated by engagement of platelet GPIbα with von Willebrand factor (VWF), present in the subendothelial matrix of healthy and diseased vessels. VWF is synthesized by ECs, stored in the subendothelial matrix of healthy and diseased vessels. In vitro, endothelial VWFplatelet glycoprotein (GP) Ib and platelet P-selectinendothelial P-selectin glycoprotein ligand 1 interactions accounted for 83% of translocation and 90% of adhesion. In platelet GPIbα, whereas platelet GPIbα/Illa contributed 20% to arrest. In conclusion, hypercholesterolemia primes platelets for recruitment via VWF, GPIbα, and P-selectin to lesion-prone sites, before lesions are detectable.

Materials and methods

Reagents and materials

Cell culture reagents, Hanks balanced salt solution (HBSS), phosphate-buffered saline (PBS), trypsin/EDTA, fetal bovine serum (FBS), and Funds for Research, Belgium).

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penicillin/streptomycin (10 000 U/mL and 10 000 µg/mL) were purchased from Gibco, Life Technologies (Paisley, United Kingdom). Formaldehyde, paraformaldehyde, and microscopic glass coverslips were from VEL (Haarssode, Belgium), sterilized in 70% propanol, washed in PBS, and coated with tissue culture grade calf skin collagen I from Boehringer Mannheim (Mannheim, Germany). Tissue culture dishes were from Becton Dickinson Labware (Maylan Cedex, France). Uncoated Petri dishes were from Sterilin (Staffordshire, United Kingdom). For flow chamber experiments, rinsing of HUVECs or Ea.hy926 monolayers and reconstitution of perfusate with packed red blood cells (Red Cross, Leuven, Belgium) to a hematocrit of 20%, was done with 1% bovine serum albumin (Boehringer Mannheim), 2 mM CaCl₂, and 2 mM MgCl₂ (Sigma, St Louis, MO), added to HBSS. For the preparation of pH 6.5 acid-citrate-dextrose (ACD), Na₃ citrate (75 mM) and dextrose (100 mM) were purchased from VEL. Citric acid (38 mM) was obtained from Sigma. 2',7'-bis-(2-carboxyethyl)-5-(6-carboxyfluorescein-acetoxyethyl) ester (BCECF-AM), cell tracker green (3-chloromethylfluorescein [CTG]), and cell tracker red (5-chloromethylrhodamine [CTR], Molecular Probes Europe, Leiden, The Netherlands) were dissolved in dimethyl sulfoxide (DMSO), aliquoted, and stored at ~20°C until use. The human 14-aminoo acid form (SFLLRNPNDKYEPF; single-lan letter amino acid codes) of thrombin receptor-activating peptide (TRAP) was custom synthesized by Eurogentec (Seraing, Belgium).

**Monoclonal antibodies**

The neutralizing monoclonal anti-P-selectin antibody CLB-Thromb/6 (mouse IgG1, Immunotech, Marseille, France) was used at 2.5 µg/mL. The anti-CD34 control antibody, Bima-K3 (mouse IgG1, Dako, Glostrup, Denmark) was used at concentrations corresponding to those of the blocking antibodies. The GPIIb/IIIa blocking antibody MA-16N7C2¹⁵ and the GPIIb/IIIa blocking antibody G9H10 were raised and characterized in our laboratory, as described elsewhere.¹⁶ A nonblocking anti-GPIIb antibody raised in the laboratory was also used as a control. The VWF-A1 domain-blocking antibody AlVW-2 was from Ajinomoto (Yokohama, Japan).¹⁷ The monoclonal anti-P-selectin antibody WAPS12.2 was purchased from American Type Culture Collection (Rockville, MD).

**Animal protocols**

All animal procedures have been approved by the local Institutional Review Board. New Zealand white rabbits were placed on a chow sprayed with ether-dissolved pure cholesterol to achieve a concentration of 0.125% (wt/wt) cholesterol. Control rabbits received the same chow without cholesterol. The diet regimen was maintained for the indicated times. For injection of anesthetics and labeled platelets in the homing assays, a 22-gauge catheter was inserted into the ear vein of conscious rabbits. For withdrawal of blood, the ear artery was instrumented with a 20-gauge catheter. Lipid profiles were determined in arterial blood in the university hospital routine laboratory. To euthanize, rabbits were sedated with ketamine (10 mg/kg) and xylazine (10 mg/kg) intramuscularly. Pentobarbital (5 mg/kg) was injected intravenously to maintain adequate anesthesia.

**Platelet isolation**

Rabbit or human whole blood was drawn on 0.1 vol ACD and centrifuged at 150g for 10 minutes to obtain platelet-rich plasma (PRP), which was diluted 1:1 with ACD and centrifuged at 600g for 10 minutes. The resulting pellet was resuspended in HBSS, and 0.3 vol ACD was added before the final washing step at 600g for 10 minutes. Platelets were counted, resuspended in HBSS, and stored at room temperature for use within 3 hours.

**Platelet homing assay**

Rabbits used as recipients for the homing assays (ie, control and 3-month groups), were age-matched. Pooled autologous rabbit platelets were adjusted to 300 000/µL and CTG was added at 1 µM for 30 minutes at 37°C. Platelets were washed at 600g, resuspended in HBSS, and left resting at 37°C for 20 minutes to facilitate sulfatation and cytosolic entrapment of the dye. These platelets aggregated comparably to unlabeled washed platelets for labeling concentrations of CTG up to 1 µM (data not shown). Circulating labeled platelets were detectable until 48 hours after injection (data not shown). Then, 1 × 10⁹ CTG-labeled platelets/kg body weight were slowly injected intravenously. Aortas were harvested 72 hours after platelet injection. Low-molecular-weight heparin (500 IU) was injected to avoid artificial postmortem platelet adhesion. The chest was opened and 10 mL blood was drawn from a left ventricular cannula for lipid profiles. Then, 0.9% saline containing 1 IU heparin/mL was infused at 80 mm Hg from a pressure bag until no blood was flowing from a caval venotomy at the level of the renal veins. The aorta was dissected from the arch until the bifurcation. Adventitial tissue was carefully removed and the vessel was opened longitudinally. To facilitate recognition of the endothelial cell plane, the whole aorta was counterstained in 10 mL HBSS containing 1 µM CTR for 45 minutes. The vessel was carefully rinsed and placed in binding buffer until examined by confocal scanning laser microscopy on the same day (LSM510, Zeiss, Oberkochen, Germany). All ostia of segmental arteries as well as the superior and inferior mesenteric artery ostia were examined for green- and red-labeled platelets. The total number of platelets per aorta as well as the number of ostia that did or did not recruit platelets was recorded.

**Flow cytometry**

P-selectin on platelets from hypercholesterolemic or control rabbits was stained using monoclonal antibody (mAb) Psel.KO.2.10, kindly provided by Dr. Pizcueta (Barcelona, Spain). Hence, 10 µL PRP, supplemented with CaCl₂ to 1 mM and with the GPIIb/IIIa antagonist G4120 (Genentech, San Francisco, CA) to 10 µg/mL in a volume of 25 µL, was added directly to an equal volume of spent medium of this antibody or was first activated for 15 minutes with 50 µg/mL equine tendon collagen (Horn collagen, Nycomed Arzneimittel, München, Germany). Platelets were washed via centrifuga
tion after 30 minutes and resuspended in 50 µL tris(hydroxymethyl) aminomethane–buffered saline (TBS). Then 2.5 µL secondary goat anti-mouse Ig antibodies, conjugated to fluorescein isothiocyanate (FITC; Dako) were added for 15 minutes, following which samples were 10-fold diluted in TBS. Gated by forward versus side scatter, the percentage of fluorophor-labeled platelets was determined by flow cytometry (FACScan Calibur, Becton Dickinson) at wavelength 488 nm and the mean fluores
cence calculated as a marker of platelet activation.

**Sudan black staining**

To determine lesion coverage of aortic surface, vessels were fixed in 4% formaldehyde overnight, stored in PBS, and transferred to 70% ethanol for 2 hours before staining. Vessels were incubated in a saturated and filtered solution of Sudan black B (Merck, Darmstadt, Germany) in 70% ethanol for 90 minutes and then washed repeatedly in 70% ethanol. The aortas were scanned en face. The total vessel area and the stained area were measured using NIH-Image 1.62. Data are presented as percentage lesion coverage.

**Scanning electron microscopy**

To exclude that platelets in the ex vivo experiments were recruited to subendothelial matrix at sites where ECs had been removed, segments after flow chamber experiments were examined by scanning electron micros
copy. After completion of the superfusion, the vessel segment was washed with buffer in the flow chamber. Immersion fixation was achieved by perfusing the chamber with cacodylate buffer (0.1 M, pH 7.4) containing 1.5% glutaraldehyde for 20 minutes at 4°C; then the vessel was cut into 1-cm segments and placed in fresh cacodylate-glutaraldehyde buffer overnight at 4°C. Vessels were dehydrated for 15 minutes in 30%, 50%, 70%, and 90% acetone at 4°C followed by a 100% acetone step at room temperature. Tissues were critical point dried, mounted on an aluminum stub, and covered with a thin layer of gold (20 nm). Specimens were examined with a scanning electron microscope (Philips, XL-20, Eindhoven, The Netherlands).¹⁸

**Platelet-endothelial interactions in an ex vivo flow chamber model**

The descending thoracic aorta of control and 3-month rabbits was dissected, freed from adventitial tissue, and opened longitudinally. The vessel was

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Results

Triglycerides were not affected by the diet (Table 1). In rabbits that were on a control diet or on a cholesterol diet for 3 months (3 mo) no lesions were found in the aortic arch in hematoxylin-eosin-stained microscopic sections. The diet proved, however, to be atherogenic, because in rabbits that were maintained on the cholesterol diet for 12 months (12 mo), rather large fatty streak lesions were found in the arch (Figure 1A). In en face stainings, 21% ± 6% of the surface of 12 mo aortas (n = 3) was covered with Sudan black staining lesions, typically spreading out from arterial branching points. Only 2.7% ± 0.9% of 3 mo (n = 4, P < .05 versus 12 mo) and 1.6% ± 0.5% of control aorta surfaces (n = 4, P < .05 versus 12 mo, P = NS versus 3 mo) were stained by Sudan black (Figure 1B).

Platelet homing assay

The platelet suspension used for homing assays contained less than 1% leukocytes. Virtually all platelets were detectable in fluorescence microscopy and flow cytometry (data not shown). At the time of death, 72 hours later, adherent platelets were exclusively detected in the immediate vicinity of segmental arteries (Figure 1C). No platelets were found adherent to the endothelium overlaying the anterior aspect of the aorta, which is not considered a plaque predilection site, except for the ostia of the 2 large mesenteric arteries, where platelets had been recruited in some rabbits. On average, 17 ostia were examined. In rabbits fed the cholesterol chow for 3 months, 14 ± 2.6 platelets were detected on all segmental artery ostia (P = .015) compared to 3.3 ± 0.85 platelets on all segmental artery ostia of control aortas (n = 4) (Figure 1d). One to 3 platelets were found per ostium. In normal rabbits only 18% of 63 examined ostia had recruited platelets compared to 56% of 89 ostia examined in hyperlipidemic rabbits (P < .0001; Figure 1E).

Role of platelet versus EC activation for platelet adhesion ex vivo

Perfusion experiments yielded individual platelets adhering to the endothelial monolayer of the aortas. The endothelial layer was intact until after the perfusions as evidenced by scanning electron microscopy (data not shown). At the time of death, 72 hours later, adherent platelets were exclusively detected in the immediate vicinity of segmental arteries (Figure 1C). No platelets were found adherent to the endothelium overlaying the anterior aspect of the aorta, which is not considered a plaque predilection site, except for the ostia of the 2 large mesenteric arteries, where platelets had been recruited in some rabbits. On average, 17 ostia were examined. In rabbits fed the cholesterol chow for 3 months, 14 ± 2.6 platelets were detected on all segmental artery ostia (P = .015) compared to 3.3 ± 0.85 platelets on all segmental artery ostia of control aortas (n = 4) (Figure 1d). One to 3 platelets were found per ostium. In normal rabbits only 18% of 63 examined ostia had recruited platelets compared to 56% of 89 ostia examined in hyperlipidemic rabbits (P < .0001; Figure 1E).

Table 1. Effect of the 0.125% cholesterol diet on plasma lipid profiles

<table>
<thead>
<tr>
<th>Diet</th>
<th>Total cholesterol (mg/dL)</th>
<th>LDL cholesterol (mg/dL)</th>
<th>HDL cholesterol (mg/dL)</th>
<th>Triglycerides (mg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>26 ± 2</td>
<td>2 ± 1.5</td>
<td>17 ± 2</td>
<td>49 ± 7</td>
</tr>
<tr>
<td>3 mo</td>
<td>140 ± 35*</td>
<td>87 ± 30*</td>
<td>42 ± 8*</td>
<td>49 ± 9</td>
</tr>
<tr>
<td>12 mo</td>
<td>110 ± 12*</td>
<td>45 ± 9*</td>
<td>49 ± 7*</td>
<td>54 ± 24</td>
</tr>
</tbody>
</table>

* P < .05 versus control.
observed compared to the control/control combination. Translocation and adhesion of 12 mo platelets superfused over 3 mo aortic segments was similarly increased.

Although hypercholesterolemia was associated ex vivo with increased rolling of platelets over endothelium, flow cytometry did not detect an increase of platelet P-selectin expression on the surface of platelets circulating in rabbits fed a cholesterol-rich diet for more than 6 months.

Role of endothelial VWF for platelet recruitment

To elucidate the adhesion pathway responsible for platelet recruitment to aortic endothelium, studies using the VWF-A1 domain-blocking mAb AJvW-2 were carried out in the ex vivo flow chamber. When present during the experiment, the antibody dramatically reduced the number of 3 mo platelets translocating on and adhering to 3 mo aortic endothelium to 25% and 30% of that observed in 3 mo/3 mo experiments (P < .01, n = 4; Figure 3A). To test whether VWF presented by the endothelium at predilection sites could contribute to augmented platelet recruitment, we performed autoradiographs on the aortas to probe for VWF. The iodine-labeled anti-VWF mAb AJvW-2 revealed pronounced VWF staining at segmental artery branching points in aortas of rabbits on the diet for 3 months, whereas no signal enhancement was observed in control aorta branching points (Figure 3B).

In vitro flow chamber studies—characterization of the model

Because a wide range of adhesion molecules cannot readily be blocked in rabbits and also to extend our observations to the adhesion pathways relevant in man, we modeled the in vivo activation of platelets and ECs, as observed in the ex vivo flow chamber system, in vitro. Platelets do not readily interact with ECs if resting.13 Contact with the subendothelial lining will readily activate platelets. Platelet reactivity is enhanced in hypercholesterolemia.21 Thrombin plays a role in platelet activation during atherogenesis.3 We used platelets activated with TRAP, the synthetic thrombin receptor–activating peptide, to reproduce the observed diet-induced platelet activation. The ex vivo studies had also pointed to EC activation as a major contributor to platelet recruitment. LPC is an important bioactive component of oxidatively modified LDL and activates ECs through the platelet-activating factor (PAF) receptor as has previously been shown.20 We therefore induced EC preactivation by incubating HUVECs or the HUVEC-derived cell line Ea.hy926 overnight with palmitoyl-LPC.

If in this model TRAP-activated platelets were superfused over the HUVEC-derived immortalized EC line Ea.hy926 that had been activated with LPC, translocation increased to 23 ± 1 platelets/50 s compared to 10 ± 0.9 resting platelets/50 s (P < .001, n = 8/7, Figure 4). Firm adhesion was 2.8-fold increased (49 ± 5.6 versus 138 ± 16.4 platelets/0.9 mm², P < .001, n = 8/7). Very similar results were obtained if primary ECs (HUVECs) were examined: HUVECs activated with LPC recruited 36 ± 3 TRAP-activated human platelets for translocation (n = 5). Translocation translated into 1300 ± 270 firmly adhering platelets/0.9 mm² (n = 4, not shown).

Role of VWF

Blocking the A1 domain of VWF with mAb AJvW-2 decreased translocation on Ea.hy926 cells by 60% and their adhesion by 70% (Figure 4; P < .001, n = 4). On HUVECs, the VWF-blocking mAb AJvW-2 reduced translocation by 47% and adhesion by 75% (n = 4, P < .01).

Role of P-selectin

Inhibition of P-selectin reduced translocation of activated platelets on Ea.hy926 cells by 52% and adhesion by 70% (Figure 4; P < .001, n = 4). On HUVECs, P-selectin inhibition reduced translocation by 62% and adhesion by 48% (n = 3, P < .05).
that had been labeled with BCECF-AM. (A) Hypercholesterolemia itself already
activation in response to hyperlipidemia. Endothelial and platelet activation were
aortas from cholesterol-fed rabbits demonstrated an independent role for endothelial
not further augment platelet translocation. Superfusion of control platelets over
morphologic changes, as evidenced by the rougher endothelial surface after
perfused at 24 dynes/cm² with reconstituted blood spiked with 10 000 platelets/μL
that had been labeled with BCECF-AF. (A) Hypercholesterolemia itself already
induced a more irregular appearance of the endothelial monolayer (upper panels).
The endothelial monolayer remained intact but the perfusion protocol induced slight
malignant cells already expressed the VWF-GPIb axis and P-selectin. (B,C) Hypercholesterolemia caused platelet activation as evidenced by
increased translocation and firm adhesion of platelets isolated from rabbits on the
diet for 3 months (3 mo) compared to control platelets on control aortas. Interestingly,
the presence of lesions in the arterial tree of rabbits on the diet for a year (12 mo) did
not further augment platelet translocation. Superfusion of control platelets over
aortas from cholesterol-fed rabbits demonstrated an independent role for endothelial
activation in response to hyperlipidemia. Endothelial and platelet activation were
additive in augmenting the interaction ("P < .05, ""P < .01).

Role of GPIb and GPIIb/IIIa

Inhibition of GPIb reduced translocation on the cell line by 45% (P < .01, n = 4); adhesion was reduced by 20% (P < .05, n = 4). No effect of GPIIb/IIIa inhibition on adhesion of resting platelets
was observed (data not shown). A nonsignificant trend for decreased translocation of platelets onto Ea.hy926 cells was observed when GPIIb/IIIa was blocked. On the contrary, the adhesion of activated platelets was reduced by 20% (Figure 4; P < .05, n = 4).

Combined inhibition of VWF, P-selectin, GPIb, and GPIIIb/IIIa

Combined VWF and P-selectin inhibition reduced translocation of TRAP-activated platelets on the cell line by 83% and adhesion by 90% (P < .001 versus TRAP-activated platelets and P < .05 versus AJvW-2 alone, n = 4; Figure 5). Combined inhibition of GPIIb/IIIa and VWF resulted in an additional 53% inhibition of platelet adhesion on Ea.hy926 cells compared to AJvW-2 alone (P < .05, n = 4). Inhibition of GPIIb in addition to VWF inhibition significantly reduced translocation by 56% (P < .05, n = 4) and firm adhesion by 65% (P < .01, n = 4) compared to AJvW-2 alone. Use of the irrelevant control antibody against CD34 did not result in significant inhibition in either EC type (Figure 5).

Discussion

Mild LDL hypercholesterolemia specifically induced in vivo platelet recruitment to segmental artery ostia that represent plaque predilection sites before lesions become histologically detectable. No platelets were found at the anterior aspect of the aorta remote from vessel branching points. The absolute number of detectable platelets and the likelihood for individual predilection sites to recruit platelets were increased by the mild cholesterol diet. Platelet adhesion to aortic endothelium was due to increased VWF expression on the endothelial surface at lesion-prone sites. In vivo, platelet and endothelial activation contributed equally to increased platelet-endothelial interactions in response to hyperlipidemia and were additive. In vitro, the main adhesion pathways involved in the recruitment of TRAP-activated platelets to LPC-activated endothelial cells in flow were the VWF-GPIb axis and P-selectin.

The prominent role of platelets for thrombus formation and vessel occlusion on plaque rupture is well established. Platelets may, however, also play a pivotal role in early atherogenesis before plaque fissuring or rupturing occurs. This has been suggested by the histologic identification of platelets in atherosclerotic lesions at almost all stages. Platelets carry inflammatory mediators, chemokines, and growth factors such as platelet-derived growth factor (PDGF) and are capable of generating vasoactive and proaggregatory substances such as thromboxane A₂.3,4 The findings presented here place the platelet at the level of endothelial injury where release of proatherogenic substances could aggravate or perpetuate endothelial injury and accelerate lesion development. Resident platelets are able to recruit leukocytes from the bloodstream and could thereby facilitate their extravasation to the subendothelial space. In addition, joint release of chemotactic substances by platelet and endothelium may augment monocyte adhesion to the endothelium, which is considered the first step in atherosclerotic lesion development.

Our ex vivo data support the view that activation of ECs and of circulating platelets and leukocytes occurs in the presence of atherogenic stimuli. Platelet and EC activation induced by hypercholesterolemia were additive in augmenting platelet margination and firm adhesion. The interaction of platelets from hypercholesterolemic rabbits with the aortic endothelium at an arterial shear rate of 24 dynes/cm² was to a large extent mediated by VWF as evidenced by an 80% inhibition of translocation and adhesion on VWF neutralization. Evidence that patients with bleeding disorders
due to the lack of functional VWF may be protected from atherosclerosis is inconclusive. 

Studies in cholesterol-fed pigs with VWF disease have likewise produced controversial results. VWF may bind to the endothelial surface and mediate platelet rolling in inflamed venules of the mesenteric circulation. Neither GPIbα nor a number of integrin receptors mediate VWF immobilization on endothelium, suggesting that the molecule might be held in place by endothelial heparan sulfate binding sites. 

André and coworkers observed transient VWF expression on the endothelial surface following inflammatory activation; we, however, demonstrate sustained increase of VWF expression at arterial branching points in hypercholesterolemic but not in control aortas. VWF expressed during hypercholesterolemia appears to be accessible for blood-borne platelets. VWF immobilized on the platelet surface and engaging ligands on the endothelial surface may additionally contribute to platelet recruitment. A recent study by Methia et al has shown that murine VWF is involved in the development of atherosclerosis in LDLR−/− mice and that half of all lesions were located at the branch points of the renal and mesenteric arteries, whereas lesions in these areas were not as prominent in VWF−/− mice. Hence, our own and these findings show in 2 different animal models that VWF is up-regulated at branch points and is involved in platelet recruitment, in association with lesion development at these sites.

To characterize the adhesion pathways in more detail, we superfused in vitro human TRAP-activated platelets over LPC-activated ECs. LPC, a phospholipid moiety contained in oxidatively modified LDL, activates ECs through engagement of the PAF receptor, leading to Ca++ signaling, vascular cell adhesion molecule 1 (VCAM-1), and ICAM-1 expression and monocyte recruitment and has thus proven to be a good activation mode for in vitro studies. Studies with mAb AJvW-2 that blocks the VWF-A1 domain, confirmed the major contribution of VWF to platelet-endothelial interactions, as had been found ex vivo.

Simultaneous inhibition of VWF and the VWF-binding domain of GPIbα suggested that a minor portion of the GPIb-mediated adhesion is independent of VWF. Mice lacking VWF still experience arterial thrombosis on vascular injury. The GPIb partner for this event is unknown. GPIb can engage P-selectin. Preliminary evidence suggests that GPIb can also engage thrombospondin-1 to realize platelet adhesion at high shear. Our finding that Ea.hy926 cells express low amounts of GPIbα could suggest that endothelial GPIbα may interact with rolling platelets through platelet ligands other than VWF.

It has been suggested that GPIb/IIIa mediates the adhesion of platelets to cultured ECs in the absence of shear forces. GPIb/IIIa will, however, not support high shear interactions prevailing at the arterial wall. Adhesion through GPIb/IIIa indeed disappears at shear rates more than 600 s⁻¹. Fibrinogen-dependent GPIb/IIIa interaction occurs after high tensile strength interactions between the matrix-immobilized VWF-A1 domain and platelet-GPIbα have slowed down the platelet. In the present study the VWF-dependent interaction was only marginally mediated through GPIb/IIIa, which accounted for only about 20% of platelet tethering, but did contribute to arrest of translocating platelets. When the VWF-A1 domain was blocked by AJvW-2, GPIb/IIIa inhibition further reduced platelet adhesion, presumably via the distinct Arg-Gly-Asp site located in the VWF-C1 domain.

Figure 3. Endothelial VWF recruits platelets to the vessel wall. (A) In the ex vivo flow chamber inhibition of the VWF-A1 domain by the blocking mAb AJvW-2 reduced translocation and firm adhesion almost to control levels (⁎ P < .01). (B) In autoradiographs for VWF expression with the iodine-labeled antibody AJvW-2, control arteries (n = 2) did not show localized enhancement of radioactive signal. Aortas from rabbits on a diet for 3 months (n = 6, right panel) expressed VWF primarily at segmental artery ostia, indicating that hyperlipidemia induces endothelial VWF expression before lesions develop.

Figure 4. Identification of the adhesion molecules mediating the interaction between activated platelets and activated endothelium. Translocation of TRAP-activated platelets was increased 2.5 times in comparison to that of resting platelets in the in vitro flow chamber and was largely mediated by endothelial VWF-platelet GPIbα interactions and platelet P-selectin–P-selectin glycoprotein ligand 1 interactions. Platelet GPIb/IIIa contributed for 20% to platelet adhesion (⁎ P < .05, ** P < .001).
GPIIb/IIIa-dependent bridging interactions were not tested in this study. In view of the small residual interactions when VWF and GPIIb/IIIa-blocking mAbs were combined, and in view of a role for GPIbα in those interactions, it seems unlikely that ICAM-1 or αβγ plays a major role for platelet immobilization at high shear.

P-selectin is another VWF-independent effector of platelet adhesion. In mice lacking both the apolipoprotein E and the P-selectin gene, atherosclerotic lesion development was significantly retarded. However, because P-selectin plays an important role not only for platelet adhesion but also for leukocyte adhesion to activated endothelium, these data do not constitute proof that platelet recruitment is indispensable for atherosclerotic lesion development. P-selectin is sufficient for mediating tethering of platelets to inflamed endothelium in venous flow conditions. At arterial shear conditions, platelet rather than endothelial P-selectin is responsible for platelet-monocyte conjugate delivery to activated endothelium, because antibody-mediated inhibition of P-selectin reduced platelet translocation by 52%. Previous studies suggested that endothelial P-selectin recruits individual platelets for dynamic interactions. In our hands, activated but not resting platelets were recruited through P-selectin to LPC-activated endothelial cells, indicating that platelet P-selectin mediates platelet-endothelial interactions at high shear. The lack of P-selectin expression on circulating platelets of hyperlipidemic rabbits could suggest that platelets carrying P-selectin are rapidly cleared from the circulation, potentially as a result of platelet-EC interactions, as observed during video microscopy in athrogenic mice. The increased interaction ex vivo of 3 mo diet platelets with normal endothelium then probably is initiated via low membrane levels of platelet P-selectin, below the limit of flow cytometric detection.

In conclusion, the present study demonstrates in hypercholesterolemic rabbits that platelets are recruited to lesion-prone sites of the arterial tree before atherosclerotic lesions are present. Thereby platelets can aggravate or perpetuate endothelial injury. Moreover, platelets adhering to endothelium overlying plaque predilection sites may provide a docking site for monocyte recruitment to the subendothelial space. High tensile strength interactions between platelets and ECs are mainly mediated through VWF, GPIb, and P-selectin. These adhesion pathways may therefore constitute therapeutic targets to prevent development of premature atherosclerosis.

References


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