Platelet Microparticles Promote Platelet Interaction With Subendothelial Matrix in a Glycoprotein IIb/IIIa–Dependent Mechanism

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Background—Platelets, on activation, release vesicular particles called platelet microparticles. Despite their procoagulant activity, their functional role in platelet–vessel wall interactions is not known.

Methods and Results—We examined the binding of microparticles to vessel wall components in vitro and in vivo. Microparticles bound to fibrinogen-, fibronectin-, and collagen-coated surfaces. Compared with activated platelets, we observed minimal binding of microparticles to vitronectin and von Willebrand factor. The glycoprotein IIb/IIIa (GP IIb/IIIa) inhibitors abciximab and eptifibatide (Integrilin) inhibited the binding to fibrinogen and fibronectin but had minimal effect on binding to collagen. Furthermore, monoclonal antibodies to GP Ib or anionic phospholipid-binding proteins (β₃-glycoprotein I or annexin V) had no effect in these interactions. Microparticles did not bind to monolayers of resting or stimulated human umbilical vein endothelial cells (HUVECs), even in the presence of fibrinogen or von Willebrand factor. However, under similar conditions, microparticles bound to extracellular matrix produced by cultured HUVECs. Abciximab inhibited this interaction by ≈50%. In a rabbit model of arterial endothelial injury, the infusion of ⁵¹Cr-labeled microparticles resulted in a 3- to 5-fold increase of microparticle adhesion to the injured site compared with the uninjured site (P<0.05%). Furthermore, activated platelets bound to surface-immobilized microparticles in a GP IIb/IIIa–dependent mechanism. This binding increased in the presence of fibrinogen by ≈30%.

Conclusions—Platelet microparticles bind to subendothelial matrix in vitro and in vivo and can act as a substrate for further platelet binding. This interaction may play a significant role in platelet adhesion to the site of endothelial injury. (Circulation. 1999;99:2577-2582.)

Key Words: platelets • microparticles • endothelium • glycoproteins

A fter activation of platelets with certain stimuli, there is a release of vesicular particles called platelet microparticles. These microparticles have been shown to accelerate thrombin generation.¹⁻⁵ The membranes of microparticles contain platelet GPs Ib, IIb, and IIIa as well as P-selectin and thrombospondin.⁶,⁷ Elevated levels of microparticles have been detected in patients with disseminated intravascular coagulation,⁸ unstable angina,⁹ myocardial infarction,¹⁰ coronary angiography,¹¹ transient ischemic attacks,¹² and diabetes mellitus¹² and during cardiopulmonary bypass.¹³ Conversely, a deficiency of platelet microparticle generation leads to a bleeding disorder with isolated prolonged bleeding time.¹⁴

In addition to their procoagulant effect, microparticles may interact with components of the vessel wall, which contributes to their prothrombotic activities. Thus, microparticles were suggested to promote platelet adhesion to subendothelium, but the precise mechanism was not elucidated.¹⁵ Our findings demonstrate that platelet microparticles adhere to subendothelial matrix and provide a substrate for subsequent binding of platelets in a GP IIb/IIIa–dependent manner.

Methods

Materials
Anti-CD42b (IgG1 mouse, clone SZ2, Immunotech) binds to platelet GP Ib. Abciximab (Reopro) was purchased from Eli Lilly. CP8 is a murine monoclonal antibody (mAb) to GP IIb/IIIa and was a generous gift from Dr Zaverio Ruggeri (Scripps Clinic, La Jolla, Calif). Integrilin (a gift from Cor-therapeutics, San Francisco, Calif) is a cyclic heptapeptide that specifically blocks GP IIb/IIIa. Anti–GP IIb/IIIa (IgG1 mouse, clone B79.7), anti–GP IIb (IgG1 mouse, clone G1.9), and rabbit anti–polyclonal GP IIb/IIIa have been described previously.¹⁶ Control mAb K4.40 is a murine IgG1 directed against platelet GP IV. Peroxidase-conjugated goat anti-mouse IgG1 was purchased from Calbiochem. Peroxidase-conjugated protein A was obtained from Promega, and phycoerythrin-labeled anti–GP IIIa was obtained from Pharmingen. β₃-Glycoprotein (β₃GPI),¹⁷ annexin V,¹⁸ fibrinogen,¹⁹ fibronectin,²⁰ vitronectin,²¹ and von Willebrand factor (vWF)²² were isolated as previously described. Thrombin receptor peptide (SFLLRNA) was synthesized by Dr T.C. Liang (University of Texas, Houston). Recombinant human interleukin-1β was pur-
Platelet/Microparticle Preparation

Platelet microparticles were isolated from blood obtained by venipuncture from healthy adult volunteers in a modification of previously described methods. For the preparation of microparticles, washed platelets were resuspended in medium M199 supplemented with 1 mMol/L CaCl2 and then activated with calcium ionophore A23187 (25 µmol/L) at 37°C for 10 minutes. After activation, the platelets were stirred in siliconized cuvettes for 15 minutes and then sedimented twice at 1000g for 5 minutes. The resulting supernatant, containing microparticles, was sedimented and washed twice at 10 000g for 10 minutes. The protein content of microparticles was determined by the protein assay of Bradford according to the manufacturer’s instructions (Bio-Rad). Flow cytometric analysis of the microparticles was performed with a FACScan flow cytometer.

Platelet binding to surface-immobilized microparticles

Wells of 96-well microtiter plates (MaxiSorp F96, Nunc) were incubated with 0.2 µg protein of microparticles in 50 µL M199 per well at 37°C for 1 hour. After washing, the plate was blocked with TBS containing 3% BSA at 4°C for 1 hour. The binding of platelets to the surface-bound microparticles was examined in 4 different experiments: (1) “resting” platelets added in the presence of 100 µL PGE2, (2) “activated” platelets prepared by incubation with 10 µmol/L thrombin receptor peptide (SFLRNRNA) at room temperature 10 minutes before their addition; (3) addition of fibrinogen (300 µg/mL) before the activation of platelets; and (4) addition of activated platelets incubated at room temperature with either abciximab (50 µg/mL), Integlin (50 µg/mL), CP8 (50 µg/mL), SZ2 (20 µg/mL), β3 (200 µg/mL), annexin V (20 µg/mL), or control mAb K4.40 (50 µg/mL) 10 minutes before their addition to the wells. In these experiments, the platelets were added at 107 to 108 platelets in 100 µL M199 to each well and incubated at 37°C for 1 hour.

In dose-response assays, microparticles were coated at concentrations varying from 0.25 to 5 µg protein of microparticles per well. After washing, the plate was blocked with 3% BSA for 1 hour, and 100-107 activated platelets were added to each well. The subsequent steps were the same as described for microparticles. Because the polyclonal anti–GP IIb/IIIa antibodies recognize both platelets and microparticles, the adsorbance due to antibodies bound to surface-coated microparticles was subtracted from the total adsorbance. Each of these experiments was performed at least 3 times.

SDS-PAGE and Immunoblotting

Substrate-bound microparticles were solubilized in boiling TBS containing 2% SDS and 5% mercaptoethanol, subjected to SDS-PAGE, and transferred electrophoretically to PVDF membranes, and the platelet GP IIb was identified by immunoblotting with an mAb to GP IIb (G1.9).

Radioactive Labeling of Microparticles

Microparticles isolated from 1 U blood were resuspended in 250 µL PBS containing 250 µCi 51Cr and incubated for 60 minutes. The 51Cr-labeled microparticles were then washed 3 times by centrifugation at 10 000g and resuspended in HEPES-buffered saline. The radioactivity of the labeled microparticles was assessed by a gamma-counter, LKB-Wallac Clini Gamma 1272 (Wallac Oy). The labeling efficiency of microparticles with 51Cr was 7%.

Adhesion of Microparticles in a Rabbit Model of Endothelial Injury

New Zealand White rabbits (~3 kg) were anesthetized with ketamine and xylazine percutaneously, and the abdominal aorta and iliac arteries were surgically exposed in a modification of previously
A balloon catheter with a balloon length of 20 mm was introduced into the abdominal aorta ~1 cm above the aortic bifurcation. The catheter was forwarded to place the balloon just below the aortic bifurcation. After 3 minutes, the rabbits were killed, and ~1-cm pieces of the injured and the contralateral uninjured iliac arteries were removed. After 3 washings with physiological saline solution, the radioactivity of each piece was assessed by gamma-counter. These data were analyzed by ANOVA followed by the Bonferroni test.

Results

Microparticle Binding to Extracellular Matrix Proteins

Microparticles bound to surface-bound fibrinogen, fibronectin, and collagen I and III (Figure 1, Ia through Ic). Compared with binding studies with activated platelets under similar conditions (Figure 1, Ia through Ic), we found that microparticles bound minimally to vitronectin and vWF (Figure 1, Id and Ie). The binding of microparticles to fibrinogen and fibronectin was inhibited by abciximab (50 µg/mL), the Fab fragment of chimeric mouse/human mAb against GP IIb/IIIa, to ~95% and 90%, respectively (Figure 1, Ia and Ib). The cyclic heptapeptide Integrilin (50 µg/mL) inhibited this interaction by ~95% and 90%, respectively (Figure 1, Ia and Ib). There was only minimal inhibition of microparticle binding to collagen I and III by abciximab (Figure 1, Ic). The mAb to GP Ib SZ2 (20 µg/mL) or a control mAb (50 µg/mL) did not affect binding of microparticles to all the examined extracellular matrix (ECM) proteins (Figure 1). Furthermore, the anionic phospholipid-binding proteins annexin V (20 µg/mL) and β2GPI (200 µg/mL) had no effect, suggesting that anionic phospholipids do not play a role in this interaction (Figure 1).

Microparticle Binding to Endothelium

We examined the binding of microparticles to intact monolayers of HUVECs. The bound microparticles were detected with an mAb to GP IIb/IIIa (CP8). No significant binding of microparticles to a monolayer of resting HUVECs was detected, even in the presence of fibrinogen (300 µg/mL) or vWF (2 µg/mL) (Figure 2). After pretreatment of the endothelial monolayer with thrombin receptor peptide (SFL-RNA, 100 µmol/L), microparticles did not bind to the endothelial cells in the presence of fibrinogen (300 µg/mL) or vWF (2 µg/mL) (Figure 2). Similar results were obtained after stimulation with recombinant interleukin-1β (100 pg/mL). Also, using an mAb to GP Ib (SZ2) to detect microparticle binding gave similar results. To further support these findings, the binding of microparticles was also assessed by means of SDS-PAGE and immunoblotting with a GP Ib mAb (G1.9). In these immunoblot studies, we could not demonstrate binding of microparticles to endothelial cells, whereas binding of microparticles to fibrinogen or fibronectin could be shown under similar conditions (Figure 3).

Microparticle Binding to Subendothelial Matrix

To determine whether microparticles bind to subendothelial matrix, we studied the binding of microparticles to ECM produced by cultured HUVECs. As shown in Figure 2, microparticles bound to subendothelial matrix produced by HUVECs. This interaction was inhibited by abciximab to ~50%, suggesting that microparticles bound to subendothelial matrix at least partly in a GP IIb/IIIa–dependent manner.

Adhesion of Microparticles to the Site of Endothelial Injury in a Rabbit Model

To determine whether microparticles will interact with the subendothelium in vivo, the endothelium of one iliac artery of a rabbit was injured with a balloon catheter.

Figure 1. Binding of microparticles (I) and activated platelets (II) to fibrinogen (A), fibronectin (B), collagen I and III (C), vitronectin (D), and vWF (E). Wells of 96-well plates were coated with either fibrinogen (0.5 µg/well), fibronectin (0.5 µg/well), collagen (2 µg/well), vitronectin (2 µg/well), or vWF (2 µg/well) overnight. After blocking with 3% BSA, microparticles or activated platelets were added in various concentrations and incubated for 1 hour. Samples were then washed and fixed with paraformaldehyde. Bound microparticles or activated platelets were quantified by ELISA using polyclonal antibody to GP IIb/IIIa. Microparticles or activated platelets were incubated with various agents before addition to wells. •, Medium 199 only; □, abciximab (50 µg/L); △, Integrilin (50 µg/mL); ○, CP8 (50 µg/mL); ×, SZ2 (20 µg/mL); ●, β2GPI (200 µg/mL); *, annexin V (20 µg/mL); +, control mAb (50 µg/mL); C; binding to collagen I; ■, binding to collagen III; □, binding to collagen I with abciximab (50 µg/mL); △, binding to collagen III with abciximab.
New Zealand White rabbits (n=3) was injured by repetitive balloon inflation. 51Cr-labeled microparticles were infused into the abdominal aorta above the aortic bifurcation. There was a 3- to 5-fold increase in radioactivity associated with the injured site compared with the contralateral uninjured site (P<0.05%) (Figure 4).

Platelet Binding to Surface-Immobilized Microparticles

A previous study suggested that microparticles promote subsequent platelet binding to subendothelium.15 To deter-

mine whether surface-immobilized microparticles mediate this interaction, we studied the binding of platelets to surface-

immobilized microparticles. Our results demonstrate that

resting platelets bound minimally to microparticles (Figure 5A). After activation, however, there was significantly in-

creased (4-fold) binding of platelets to surface-immobilized

microparticles in a dose-dependent manner (Figure 5A and

5B). This binding was inhibited by abciximab (~70%), CP8

(~75%), or Integrilin (~25%) (Figure 6), suggesting that

activated platelets bind to surface-bound microparticles in a

GP IIb/IIIa–dependent manner. In contrast, mAb to GP Ib,

control antibody, or anionic phospholipid-binding proteins

(annexin V or β,GPI) did not have an effect in this interaction

(Figure 6).

Discussion

In this study, we showed that platelet-derived microparticles

bound to adhesive glycoproteins, fibrinogen, fibronectin, and
collagen I and III in a saturating manner, similar to platelets. Abciximab and Integrilin inhibited binding of microparticles to fibrinogen and fibronectin, suggesting a GP IIb/IIIa-dependent binding mechanism. However, there was only minimal inhibition of microparticle binding to collagen I and III by abciximab, suggesting that collagen is not a major ligand for platelet GP IIb/IIIa. This minimal inhibitory effect could have been due to transdominant inhibition of a collagen-binding integrin. Unlike platelets, microparticles showed only minimal binding to vitronectin and vWF. This functional difference could be the result of conformational changes of GP IIb/IIIa in microparticle membranes.

Microparticles also bound to subendothelial matrix, but not to resting or stimulated intact monolayers of HUVECs, under similar experimental conditions. Using flow cytometric analysis, Gawaz et al.\(^\text{27}\) showed that microparticles adhered to thrombin-activated endothelial cells, which involved the GP IIb/IIIa receptor. The difference in our study results could be due to the ELISA and immunoblot techniques used may not be as sensitive as flow cytometric analysis. Alternatively, the removal of the endothelial monolayer and separation into a single-cell suspension by repetitive pipetting, as done by Gawaz et al.,\(^\text{27}\) could have exposed the ECM, which could have resulted in the apparent binding of microparticles to endothelial cells. Furthermore, we could show that in a rabbit model of arterial endothelial injury, \(^{31}\)Cr-labeled microparticles localized to the site of endothelial injury, emphasizing that a similar interaction might even occur in vivo under flow conditions.

A previous study suggested that microparticles promote platelet adhesion to subendothelium.\(^\text{15}\) The precise mechanism involved in this process, however, is not clear. To test the hypothesis that ECM-bound microparticles may provide a substrate for further platelet binding, we used surface-immobilized microparticles to study platelet binding. Resting platelets bound only minimally to surface-immobilized microparticles, whereas activated platelets showed significant binding. This binding was further increased in the presence of fibrinogen and was inhibited by the GP IIb/IIIa inhibitors abciximab and Integrilin. The increased binding in the presence of fibrinogen may be partly due to the formation of platelet aggregates. These findings suggest that activation of platelets is a prerequisite for binding to microparticles, probably because platelet GP IIb/IIIa undergoes conformational changes on activation that provide binding sites for soluble fibrinogen. Fibrinogen may then act as a bridging molecule between platelet and microparticle GP IIb/IIIa. In support of this, recent investigations showed that microparticles bind fibrin\(^\text{28}\) and soluble fibrinogen and coaggregate with platelets.\(^\text{29}\) This interaction of microparticles with platelets may even lead to further platelet activation.\(^\text{30}\)

Platelets activated by high shear stress in arteries with severe stenosis may lead to elevated levels of microparticles in circulation.\(^\text{31}\) Microparticles would be farther in the periphery of the blood stream than platelets because of their smaller size, according to the concept of size-dependent radial distribution of particles in flow.\(^\text{32,33}\) However, their membrane function seems to be comparable to that of activated platelets. Therefore, they would be more likely to bind initially to exposed subendothelial matrix, thus providing a substrate for further platelet adhesion via GP IIb/IIIa–fibrinogen bridging. This interaction may play a significant role in hemostasis and atherosclerosis.

Acknowledgments

This work was supported by National Institutes of Health/National Heart, Lung, and Blood Institute grants HL-39916, HL-50653, HL-50100, and HL-40860 and a Grant-in-Aid from the American Heart Association.

References


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Circulation. 1999;99:2577-2582
doi: 10.1161/01.CIR.99.19.2577

Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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