Circulating Monocyte-Platelet Aggregates Are a More Sensitive Marker of In Vivo Platelet Activation Than Platelet Surface P-Selectin

Studies in Baboons, Human Coronary Intervention, and Human Acute Myocardial Infarction

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Background—Platelet surface P-selectin is considered the “gold standard” marker of platelet activation. Degranulated, P-selectin–positive platelets, however, aggregate with leukocytes in vitro and rapidly lose surface P-selectin in vivo.

Methods and Results—Flow cytometric tracking of autologous, biotinylated platelets in baboons enabled us to directly demonstrate for the first time in vivo that (1) infused degranulated platelets very rapidly form circulating aggregates with monocytes and neutrophils, and (2) 30 minutes after infusion of the degranulated platelets, the percentage of circulating monocytes aggregated with infused platelets persist at high levels, whereas the percentage of circulating neutrophils aggregated with infused platelets and the platelet surface P-selectin of nonaggregated infused platelets return to baseline.

We therefore performed 2 clinical studies in patients with acute coronary syndromes. First, after percutaneous coronary intervention (n=10), there was an increased number of circulating monocyte-platelet (and to a lesser extent, neutrophil-platelet) aggregates but not P-selectin–positive platelets. Second, of 93 patients presenting to an Emergency Department with chest pain, patients with acute myocardial infarction (AMI) (n=9) had more circulating monocyte-platelet aggregates (34.2±10.3% [mean±SEM]) than patients with no AMI (n=84, 19.3±1.4%, P<0.05) and normal control subjects (n=10, 11.5±0.8%, P<0.001). Circulating P-selectin–positive platelets, however, were not increased in chest pain patients with or without AMI.

Conclusions—As demonstrated by 3 independent means (in vivo tracking of activated platelets in baboons, human coronary intervention, and human AMI), circulating monocyte-platelet aggregates are a more sensitive marker of in vivo platelet activation than platelet surface P-selectin. (Circulation. 2001;104:1533-1537.)

Key Words: platelets ■ leukocytes ■ tests ■ coronary disease ■ receptors

Platelet activation resulting from plaque disruption is important in the pathogenesis and clinical outcome of acute coronary syndromes.1 There is therefore great interest in the measurement of in vivo platelet activation.2,3 P-selectin (CD62P) is a component of the α-granule membrane of resting platelets that is expressed only on the platelet surface during and after platelet degranulation and secretion.4 Platelet surface P-selectin is considered to be the “gold standard” marker of platelet activation.2,3,5,6 In vitro, the activation-dependent increase in platelet surface P-selectin is not reversible over time.7 We8 and others,9 however, have recently demonstrated that in vivo circulating degranulated platelets rapidly lose their surface P-selectin yet continue to circulate and function. Furthermore, degranulated platelets aggregate with monocytes and neutrophils in vitro, initially via platelet surface P-selectin binding to its PSGL-1 counterreceptor on the leukocyte surface,10 and increased numbers of monocyte-platelet and neutrophil-platelet aggregates have been found to circulate in patients with coronary artery disease.11–14 The in vivo kinetics of circulating monocyte-platelet and neutrophil-platelet aggregate formation and clearance and the temporal relationship of this formation and clearance to the loss of platelet surface P-selectin on degranulated platelets, however, are unknown.

In the present study, the temporal and quantitative relationship between circulating monocyte-platelet aggregates, neutrophil-platelet aggregates, and nonaggregated P-selectin–positive platelets was examined in 3 in vivo models of

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Platelet activation: (1) tracking of autologous, degranulated platelets in baboons, (2) human percutaneous coronary intervention (PCI), and (3) human acute myocardial infarction (AMI). These 3 independent models establish that circulating monocyte-platelet aggregates are a more sensitive marker of in vivo platelet activation than platelet surface P-selectin.

Methods

Baboon Studies

Baboons

Studies were performed in baboons (adult male Papio anubis) because these primates are hemostatically similar to humans. The animals were housed at and cared for according to the standard protocols of the Animal Care Facility of Boston Medical Center. The protocol for this study was approved by the Institutional Animal Care and Use Committee. Before all procedures, the animals (average weight 35 kg) were sedated with ketamine hydrochloride 250 mg IM and anesthetized with sodium pentobarbital 50 to 75 mg IV.

Biotinylation of Platelet Concentrates

Baboon platelet concentrates were prepared and stored at 22°C for not more than 18 hours under standard blood bank conditions. Platelet concentrates (~50 mL containing ~1.5 x 10^10 platelets/mL) were biotinylated by addition of 300 μg/mL biotin–X–NHS (Calbiochem) by a modification of the method of Dale et al. Sterile preparations of biotin–X–NHS were introduced directly into the platelet storage bag and incubated at 22°C for 15 minutes. (All incubations in this study were at 22°C unless otherwise stated.) Platelets were then washed as previously described and either (1) resuspended in Plasma-Lyte (Baxter Health Care) or (2) resuspended in modified Tyrode’s buffer, pH 7.4, incubated for 10 minutes with sodium citrate Vacutainer (Becton Dickinson) (after the first 2 mL of blood had been discarded). Samples were immediately fixed for 10 minutes and diluted 25-fold in HEPES–Tyrode’s buffer, pH 7.4. Aliquots were incubated for 20 minutes with (1) 0.25 μg/mL phycoerythrin (PE)-conjugated GP IIb–specific monoclonal antibody SB12 (Dako), (2) 12.5 μg/mL streptavidin-RED670, and (3) either 2 μg/mL FITC-conjugated P-selectin–specific monoclonal antibody PB1.3 (Cytel) or 2 μg/mL isotype-matched FITC-conjugated murine IgG1. Samples were then diluted 20-fold with HEPES–Tyrode’s buffer, pH 7.4. Three-color whole-blood flow cytometry in an EPICS XL was used to simultaneously (1) identify platelets by gating on their characteristic forward and orthogonal light scatter using a 575-nm band-pass filter to discriminate the binding of the PE-conjugated GP IIb–specific monoclonal antibody SB12; (2) distinguish the infused platelets by their RED670 fluorescence; and (3) determine the platelet surface expression of P-selectin on individual platelets by the binding of FITC-PB1.3. P-selectin–specific FITC fluorescence was expressed as the percentage of a preinfusion maximally activated thrombin (10 U/mL) control sample. Appropriate color compensation was determined in singly labeled samples and matched nonspecific antibody controls. In these experiments and in those measuring platelet recovery (below), 5000 biotinylated platelets per sample were analyzed.

Platelet Recovery

At the indicated times after the infusion of autologous biotinylated platelets, peripheral blood was drawn into a Vacutainer and immediately fixed with 1% formaldehyde (Polysciences) in 1.4 × Hanks balanced saline solution (Gibco), then diluted 4.6-fold with distilled water to lyse the erythrocytes. Aliquots (500 μL) of the fixed/lysed blood samples were concentrated by centrifugation (400g, 5 minutes), and the resuspended pellet was incubated for 20 minutes with 5 μg/mL of the fluorescein isothiocyanate (FITC)–conjugated glycophorin (GP) IIIa–specific monoclonal antibody Y2/51 (Dako) and 12.5 μg/mL streptavidin-RED670 (Gibco), then diluted with 500 μL HEPES–Tyrode’s buffer (mmol/L: HEPES 10, NaCl 137, KCl 2.8, MgCl2 1, NaHCO3 0.4, and glucose 5.5, plus 0.35% BSA), pH 7.4, before flow cytometric analysis. Isotype-matched mouse IgG-FITC (Dako) and preinfusion samples containing no biotinylated platelets served as negative controls. Flow cytometry was performed in an EPICS XL (Coulter) equipped with an argon ion laser. Neutrophils and monocytes were identified by their characteristic forward and orthogonal light scatter properties. Circulating neutrophils and monocytes with adherent infused platelets were identified by positivity for both RED670 with a 670-nm band-pass filter (ie, the infused biotinylated platelets) and FITC with a 525-nm band-pass filter (ie, the leukocyte binding of the platelet-specific anti–GP IIIa monoclonal antibody Y2/51). Data are expressed as a percentage of all neutrophils or monocytes positive for infused platelets. In these experiments and those in humans (below), 2000 monocytes and 20 000 neutrophils per sample were analyzed.

Platelet Surface P-Selectin

Peripheral blood samples were fixed in 1% formaldehyde for 20 minutes and diluted 25-fold in HEPES–Tyrode’s buffer, pH 7.4. Aliquots were incubated for 20 minutes with (1) 0.25 μg/mL phycoerythrin (PE)-conjugated GP IIb–specific monoclonal antibody SB12 (Dako), (2) 12.5 μg/mL streptavidin-RED670, and (3) either 2 μg/mL FITC-conjugated P-selectin–specific monoclonal antibody PB1.3 (Cytel) or 2 μg/mL isotype-matched FITC-conjugated murine IgG1. Samples were then diluted 20-fold with HEPES–Tyrode’s buffer, pH 7.4. Three-color whole-blood flow cytometry in an EPICS XL was used to simultaneously (1) identify platelets by gating on their characteristic forward and orthogonal light scatter and using a 575-nm band-pass filter to discriminate the binding of the PE-conjugated GP IIb–specific monoclonal antibody SB12; (2) distinguish the infused platelets by their RED670 fluorescence; and (3) determine the platelet surface expression of P-selectin on individual platelets by the binding of FITC-PB1.3. P-selectin–specific FITC fluorescence was expressed as the percentage of a preinfusion maximally activated thrombin (10 U/mL) control sample. Appropriate color compensation was determined in singly labeled samples and matched nonspecific antibody controls. In these experiments and in those measuring platelet recovery (below), 5000 biotinylated platelets per sample were analyzed.

Human Studies

Percutaneous Coronary Intervention

The protocol was approved by the Committee for the Protection of Human Subjects in Research at the University of Massachusetts Medical Center. PCI was performed by standard techniques, with 9 patients receiving an intracoronary stent and 1 patient balloon angioplasty only.

Chest Pain Study

The protocol was approved by the Committee for the Protection of Human Subjects in Research at the University of Massachusetts Medical Center. The study population consisted of patients presenting to the Emergency Department at the University of Massachusetts Medical Center with a chief complaint of chest pain. The control population for the chest pain study consisted of healthy, nonsmoking adult volunteers who had not taken any antiplatelet agents for ≥10 days and were not on any other medication. The diagnosis of coronary artery disease was excluded in this control population by self-reported lack of symptoms suggestive of coronary artery disease or a documented cardiac event. Peripheral blood samples were drawn in the Emergency Department at the time of hospital presentation. Of the 100 patients enrolled in the study, complete data on circulating P-selectin–positive platelets and monocyte-platelet aggregates are available for 93. Nine patients were ruled in for AMI with a serum creatine kinase-MB isoenzyme level >3 times the upper limit of normal. There were no significant differences between patients with AMI and those without AMI with respect to sex, time between onset of symptoms and phlebotomy, history of coronary artery disease, congestive heart failure, valvular heart disease, hypertension, diabetes, hyperlipidemia, hepatic disease, renal disease, medications, or tobacco use. Patients with unstable angina were defined as having Braunwald class III to IV.

Leukocyte-Platelet Aggregates

Leukocyte-platelet aggregates were analyzed by whole-blood-flow cytometry in a Becton Dickinson FACSCalibur for the PCI study and in a Coulter XL for the chest pain study) as described above,
with some modifications. Briefly, peripheral blood was drawn into either a sodium citrate Vacutainer (PCI study) or Diatube-H Vacutainer containing citrate, theophylline, adenosine, and dipyridamole (chest pain study); fixed with 1.1% paraformaldehyde in 1.4X Hanks balanced saline solution; labeled with monoclonal antibodies CD14-PE (Becton Dickinson) and Y2/51-FITC; diluted 4.6-fold with distilled water to lyse the erythrocytes; and further diluted in an equal volume of HEPES–Tyrode’s buffer, pH 7.4. Monocytes and neutrophils were identified by their bright and dim, respectively, staining with CD14-PE and by their characteristic orthogonal light scatter. Monocytes and neutrophils with adherent platelets were identified by Y2/51-FITC positivity.

**Platelet Surface P-Selectin**

Platelet surface P-selectin was analyzed by whole-blood-flow cytometry (in a Becton Dickinson FACScalibur for the PCI study and in a Coulter XL for the chest pain study) as described above, with some modifications. Briefly, peripheral blood was drawn into either a sodium citrate Vacutainer (PCI study) or Diatube-H Vacutainer (chest pain study). In the PCI study, the samples were fixed immediately in 1% paraformaldehyde, then labeled with the PE-conjugated P-selectin–specific monoclonal antibody AC1.2 (Becton Dickinson) and the FITC-conjugated GP IIIa–specific monoclonal antibody 7F12 (Becton Dickinson). In the chest pain study, the samples were labeled with the FITC-conjugated P-selectin–specific monoclonal antibody S12 (Centocor) and the PE-conjugated GP IIb/IIIa–specific monoclonal antibody 10E5 (Centocor), and then fixed within 1 hour of the blood draw. The percentage of P-selectin–positive platelets was defined as the percentage of platelets that had a FITC or PE fluorescence greater than a threshold determined by 99% of platelets incubated with purified FITC- or PE-conjugated mouse IgG isotypic controls. Five thousand platelets per sample were analyzed.

**Statistics**

Differences in selected categorical variables between the respective comparison groups were analyzed with the χ² test of statistical significance. Paired t tests and ANOVA were used to examine differences in continuous variables overall and at each time point under study in the different comparison groups.

**Results**

**Baboon Studies**

**Monocyte-Platelet and Neutrophil-Platelet Aggregates**

Autologous biotinylated baboon platelets were activated with thrombin ex vivo and reinfused, and peripheral blood samples were drawn for flow cytometric analysis of the biotinylated platelets. Within 1 minute, the thrombin-activated infused (biotinylated) platelets formed circulating monocyte-platelet and neutrophil-platelet aggregates (Figure 1, top). The percentage of monocytes with adherent infused platelets was greater than the percentage of neutrophils with adherent infused platelets (Figure 1, top). The in vivo half-life of detectable circulating monocyte-platelet aggregates was ≈30 minutes, whereas the in vivo half-life of neutrophil-platelet aggregates was ≈5 minutes (Figure 1, top). By 2 hours after infusion, none of the infused platelets were still adherent to monocytes or neutrophils (Figure 1, top). Infused autologous biotinylated baboon platelets that were not activated with thrombin preinfusion did not form monocyte-platelet or neutrophil-platelet aggregates (Figure 1, bottom).

**P-Selectin**

In the same experiments as above, platelet surface P-selectin was measured by 3-color whole-blood-flow cytometry. After ex vivo activation of the platelet unit with thrombin 1 U/mL, 90% of the platelets became P-selectin–positive, with a platelet surface P-selectin expression of 58.6±14.7% (mean±SEM, n=4) (mean fluorescence intensity [MFI] expressed as percentage of an aliquot of preinfusion baboon peripheral blood that was maximally stimulated with thrombin 10 U/mL). After infusion, there was a rapid decline in the surface P-selectin on infused (biotinylated) platelets (Figure 1, top). By 10 minutes after infusion, surface P-selectin on infused, circulating platelets was only 7.8±3.0% MFI (Figure 1, top). Autologous infused (biotinylated) baboon platelets that were not activated with thrombin ex vivo demonstrated no increase in platelet surface P-selectin before or after infusion (Figure 1, bottom).

**Platelet Recovery**

As previously reported,8,20 platelets that were thrombin-activated before infusion sequestered earlier and longer than platelets not activated before infusion (Figure 2). Nevertheless, despite their aggregation with and disaggregation from circulating monocytes and neutrophils and despite their loss...
of platelet surface P-selectin (Figure 1, top), thrombin-activated infused platelets continued to circulate (Figure 2).

**Human Studies**

**Percutaneous Coronary Intervention**
The number of circulating monocyte-platelet aggregates increased within 5 minutes after PCI (Figure 3, “Post” time point) and was maximal 2 to 4 hours after PCI (Figure 3). There was no significant increase in the number of circulating neutrophil-platelet aggregates within 5 minutes after PCI (Figure 3, “Post” time point). Although circulating neutrophil-platelet aggregates were significantly increased 1 to 2 hours after intervention, this increase was quantitatively far less than the increase in circulating monocyte-platelet aggregates, and the number of circulating neutrophil-platelet aggregates returned to normal earlier (4 hours after PCI) than the number of circulating monocyte-platelet aggregates (24 hours after PCI) (Figure 3). In contrast to monocyte-platelet aggregates and neutrophil-platelet aggregates, PCI did not result in increased numbers of circulating P-selectin–positive platelets (Figure 3).

**Chest Pain Study**
Patients with AMI had significantly more circulating monocyte-platelet aggregates than patients without AMI (34.2 ± 10.3% versus 19.3 ± 1.4%, mean ± SEM, P < 0.05) and normal control subjects (34.2 ± 10.3% versus 11.5 ± 0.9%, P < 0.001) (Figure 4A). In contrast to the findings with circulating monocyte-platelet aggregates, patients with AMI had no increase in the number of circulating P-selectin–positive platelets compared with patients without AMI and normal control subjects (Figure 4B). The difference in the numbers of monocyte-platelet aggregates between non-AMI chest pain patients and normal control subjects is not significantly accounted for by patients with unstable angina in the non-AMI group. The number of monocyte-platelet aggregates in chest pain patients with unstable angina but not AMI was 21.2 ± 6.1 (mean ± SEM, n = 13), whereas the number of monocyte-platelet aggregates in chest pain patients with neither unstable angina nor AMI was 17.8 ± 2.2 (n = 71, P = NS). The (not statistically significant) difference in the number of monocyte-platelet aggregates between non-AMI chest pain patients (19.3 ± 1.4, n = 84) and normal control subjects (11.5 ± 0.9, n = 10) was probably accounted for by comorbid diseases in the non-AMI chest patients and/or the fact that the normal control subjects were not sampled under Emergency Department conditions.

**Discussion**
In this study, tracking of autologous infused biotinylated platelets in baboons enabled us to demonstrate for the first time in vivo that (1) platelets degranulated by thrombin very rapidly (within 1 minute) form circulating aggregates with monocytes and neutrophils (Figure 1, top) and (2) the in vivo half-life of detectable circulating monocyte-platelet aggregates (~30 minutes) is longer than both the in vivo half-life of neutrophil-platelet aggregates (~5 minutes) and the previously reported rapid loss of platelet surface

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**Figure 2.** Platelet recovery as determined by flow cytometry. Baboons were infused with autologous, biotinylated platelets that were or were not thrombin-activated before infusion. Peripheral blood was drawn from baboons at times indicated on horizontal axis. Infused platelets were identified in whole blood by light scatter, binding of phycoerythrin-conjugated CD41-specific monoclonal antibody, and binding of streptavidin-RED670. Percent recovery of infused, biotinylated platelets (vertical axis) was determined as described in Methods. Data are mean ± SEM, n = 5.

**Figure 3.** Effect of human PCI on circulating monocyte-platelet aggregates, neutrophil-platelet aggregates, and platelet surface P-selectin. Pre and Post refer to peripheral blood samples drawn within 5 minutes before and within 5 minutes after PCI, respectively. Monocyte-platelet and neutrophil-platelet aggregates are expressed as percentage of all monocytes and neutrophils with adherent platelets. Platelet surface P-selectin is expressed as percentage of platelets that are P-selectin-positive. Data are mean ± SEM, n = 10. *P < 0.05 (paired t test) vs Pre samples.

**Figure 4.** Monocyte-platelet aggregates (A) and percentage of surface P-selectin–positive platelets (B) in peripheral blood of chest pain patients with and without AMI and normal control subjects. Data are mean ± SEM. *P < 0.05 vs chest pain patients without AMI and P < 0.001 vs normal control subjects.
P-selectin (Figure 1, top). All these findings suggested that measurement of circulating monocyte-platelet aggregates may be a more sensitive indicator of in vivo platelet activation than either circulating neutrophil-platelet aggregates or circulating P-selectin–positive nonaggregated platelets.

Platelet activation resulting from plaque disruption is characteristic of acute coronary syndromes. Therefore, to confirm our hypothesis that circulating monocyte-platelet aggregates are a more sensitive marker of in vivo platelet activation than platelet surface P-selectin, we performed 2 clinical studies in patients with acute coronary syndromes. First, we used PCI as an in vivo human model of platelet activation induced by plaque rupture. After PCI-induced plaque rupture, the number of circulating monocyte-platelet aggregates increased, as did the number of circulating neutrophil-platelet aggregates (to a much lesser extent), but the number of circulating P-selectin–positive platelets did not increase (Figure 3). Second, of 93 patients presenting to an Emergency Department with chest pain, those with AMI had higher levels of circulating monocyte-platelet aggregates than those without AMI. The number of circulating P-selectin–positive platelets, however, was not increased in the patients with AMI (or those without AMI) (Figure 4).

Conclusions
Platelet surface P-selectin is generally considered to be the gold standard marker of platelet activation. In this study, however, we demonstrate by 3 independent methods (in vivo tracking of activated platelets in baboons, human PCI, and human AMI) that circulating monocyte-platelet aggregates are a more sensitive marker of in vivo platelet activation than platelet surface P-selectin.

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