Increased Platelet Binding to Circulating Monocytes in Acute Coronary Syndromes

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Background—Present therapies for acute coronary syndromes aim toward limiting platelet–platelet adhesion and aggregation processes. However, platelet–leukocyte interactions may contribute importantly to disease progression in the arterial wall. Recent studies suggest that prevention of platelet–leukocyte binding via P-selectin glycoprotein ligand-1 (PSGL-1) may be beneficial in animal models of vascular injury.

Methods and Results—P-selectin–PSGL-1 interactions were found to account for most platelet–monocyte binding observed in peripheral blood samples from healthy donors. However, a significant component of observed adhesion was calcium independent, involving neither PSGL-1 nor P-selectin. Platelet–monocyte interactions were examined in 52 patients admitted within 14 hours of symptom onset, with acute coronary syndromes defined as unstable angina (n=12) and acute myocardial infarction (n=13) or noncardiac chest pain (n=27). When compared with patients with noncardiac chest pain, significantly elevated levels of platelet–monocyte binding were found in patients with acute myocardial infarction (70.1±15.4% versus 45.4±23.3%; P<0.01) and unstable angina (67.4±12.9% versus 45.4±23.3%; P>0.01). Calcium-independent platelet–monocyte binding was significantly elevated in myocardial infarction patients alone (14.7±7.7% versus 6.1±5.96%; P<0.001).

Conclusions—There is evidence for a significant P-selectin–independent molecular component to the platelet–monocyte conjugation observed in peripheral blood. Patients with myocardial infarction and unstable angina demonstrate increased total binding of platelets to monocytes. Additionally, calcium-independent adhesion was significantly elevated in patients with evidence of myocardial infarction. These findings demonstrate that novel cation-independent adhesion mechanisms may mediate platelet–monocyte binding, representing a new therapeutic target after vascular injury associated with myocardial infarction. (Circulation. 2002;105:2166-2171.)

Key Words: cell adhesion molecules ▪ leukocytes ▪ platelets ▪ myocardial infarction ▪ coronary disease

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cute coronary syndromes (ACS), defined as myocardial infarction (MI) with ST elevation, non-ST elevation MI, and unstable angina (UA), cause significant morbidity and mortality in industrialized societies. Although the underlying cellular and molecular mechanisms of disease progression are complex, an early event in vessel injury is the adhesion of platelets and leukocytes to the damaged arterial wall. Activated platelets deposit at sites of unstable plaque rupture, precipitating or exacerbating coronary vascular obstruction. Additionally, mechanical interventional treatment strategies for ACS, such as percutaneous coronary intervention, can be compromised acutely by vessel dissection and chronically by a restenosis response. In both processes, inflammatory and thrombotic mechanisms combine to obstruct or occlude the vascular lumen. Activated platelets potentiate thrombus formation and ultimately promote restenosis. Present therapy in ACS aims to limit platelet aggregation mechanisms by use of glycoprotein (GP) IIb/IIIa receptor antagonists or thienopyridines. However, their incomplete effectiveness suggests that the role of alternative platelet adhesion pathways may be important.

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Although interactions between activated platelets and leukocytes may accelerate restenosis, the contribution of leukocyte–platelet interactions to atheromatous plaque instability and to the acute progression of ACS is unknown. Previous in vitro studies have demonstrated interaction of activated platelets with monocytes and neutrophils. Binding of platelets via P-selectin expressed on the surface of activated platelets to the leukocyte counter-receptor P-selectin GP ligand-1 (PSGL-1) may alter leukocyte recruitment and activation patterns. Indeed, platelet–neutrophil binding occurs in UA and after myocardial injury and angioplasty. Disruption of platelet–neutrophil interactions is beneficial in animal models of vascular injury, emphasizing the importance of
platelet–leukocyte interactions in vascular disease processes. More recently, studies in patients with ACS have demonstrated increased platelet–monocyte binding compared with control subjects, indicating that this phenomenon is a sensitive platelet activation marker.

Several observations indicate that platelets bind specifically to monocytes in peripheral blood collected from healthy donors and that these interactions are mediated in a divalent cation–dependent manner. Isolation of peripheral blood monocytes results in the co-isolation of bound platelets. Accordingly, most isolation techniques include steps that minimize this phenomenon, such as low-speed centrifugation in divalent cation–free buffers or EDTA inclusion in isolation buffers.

In the present study, 2-color flow cytometry was used to examine platelet–leukocyte binding in freshly drawn peripheral blood samples from healthy donors. Although platelets were found to bind to monocytes in a P-selectin–PSGL-1–dependent manner, similar levels of platelet binding to PSGL-1–expressing granulocytes were not observed, suggesting that alternative molecules mediate platelet binding to monocytes. Furthermore, consistent residual levels of cation-independent platelet–monocyte binding were noted, again suggesting alternative and potentially novel platelet–monocyte–binding mechanisms. In addition, we examined platelet–monocyte interactions in 52 patients admitted with ACS within 14 hours of symptom onset. Significantly elevated platelet–monocyte–binding levels were observed in patients with acute MI and UA compared with patients with noncardiac chest pain. Moreover, a significant increase in cation-independent platelet–monocyte binding occurred in patients with MI as opposed to UA or noncardiac chest pain. The physiological significance of monocyte–platelet binding is presently unknown, but our data suggest that monocyte–platelet binding may be useful in predicting the extent of vascular injury. Because platelet–monocyte interactions are unaffected by GP Ib/IIa antagonists, we would speculate that inhibitors of PSGL-1 function might be a useful therapeutic adjunct to present treatment strategies after vascular injury associated with MI. Furthermore, PSGL-1 binding to P-selectin may induce intracellular signals in monocytes, raising the possibility that bound platelets influence monocyte recruitment and differentiation programs.

Methods

Blood Sampling and Patient Inclusion Criteria

Blood from consenting healthy volunteers was drawn by venipuncture via 19-gauge needles, collected into 15-mL polypropylene conical tubes containing 10 U/mL heparin sodium (PUM-HEP, Leo Laboratories Ltd), and gently inverted several times to minimize cell activation. All patients presenting with chest pain to the Edinburgh Royal Infirmary with ischemic symptoms at rest and ECG changes of ischemia (Braunwald Class IIb) or infarction (with or without troponin elevation at presentation) were included in the clinical study. Patients hospitalized for chest pain at rest or MI within 3 months of their present presentation or who had undergone coronary artery bypass grafting or percutaneous coronary intervention within 6 months of their present presentation were excluded, as were patients taking antiplatelet aggregation drugs other than aspirin. Ethical approval was obtained from the Lothian Research Ethics Committee, and written consent was obtained before collecting patient blood into Sarstedt Monovette Li-Heparin 5.5-mL tubes within 14 hours of symptom onset. Patient age, sex, history of hypertension, diabetes, smoking habit, use of lipid-lowering drugs, family history of coronary artery disease, previous MI, previous angioplasty or coronary artery bypass graft, medication use, and medical history were recorded.

Antibodies and Other Reagents

All chemicals were obtained from Sigma Chemical Company unless otherwise stated. Monoclonal antibodies (mAbs) directly conjugated to fluorochromes used in this study were purchased from the following sources: FITC-conjugated CD42a (GRP-P, IgG1), CD62P (1.2B6, IgG1); and control IgG1 were obtained from Serotec Ltd (Oxford, UK). PE-conjugated CD14 (Tak-4, IgG2a) and IgG2a control were obtained from Dako Ltd (Buckinghamshire, UK). Function-blocking CD62P mAb CLB-thromb/6 (IgG1) was obtained from CLB (Amsterdam, the Netherlands). PSGL-1 mAb (PL 1 and PL 2, both IgG1) was supplied by Beckman-Coulter (High Wycombe, UK). Abxicimab, the “humanized” Fab portion of mAb 7E3 directed against the GP Ib/IIa receptor, was supplied by Lilly (Hampshire, UK).

Immunolabeling and Flow Cytometry

Blood (100 μL) was labeled within 30 minutes of collection by incubation with specific antibodies for 15 minutes at room temperature with or without EDTA (final concentration, 5 or 10 mmol/L) before the addition of 0.5 μL FACSLyse solution (Becton-Dickinson). Samples were run through either a Becton-Dickinson FACSCalibur or a Beckman-Coulter XL2 flow cytometer, and data analysis was performed using CellQuest (Becton-Dickinson) or EXPO32 (Beckman-Coulter) software, respectively. Monocyte–platelet conjugation levels did not vary with altered cytometer flow rates. Samples were initially analyzed with the flow cytometer triggered on forward scatter and then again by triggering on FL-2 to select CD 14–PE–positive monocytes. For molecular mechanism studies of platelet–monocyte binding, samples were preincubated with saturating concentrations of inhibitory mAb for 15 minutes before labeling. Platelet–monocyte adhesion was then determined using directly conjugated CD14-PE and CD42a-FITC mAb, as described below. Leukocyte/platelet fluorescence levels using these antibodies were unaltered by EDTA treatment.

Statistical Analysis

Numerical values are represented by mean ± SD. Data from normal volunteers were analyzed by unpaired t tests or by one-way ANOVA. Comparisons between patient groups were made using ANOVA with Tukey’s post test using GraphPad InStat (Graph Pad Software). Patient data were found to be normally distributed using the Kolmogorov and Smirnov method. P<0.05 was considered statistically significant.

Results

Platelet–Leukocyte Interactions in Normal Peripheral Blood

The extent of platelet–leukocyte interactions in peripheral blood from healthy volunteers was assessed using 2-color flow cytometry, which allowed leukocyte subpopulations to be distinguished by their distinct laser scatter properties (Figure 1A). Monocytes were identified using CD14, eliminating the possibility that large lymphocytes with similar laser scatter properties had been included in the analysis (Figure 1B). Preliminary experiments revealed that although samples were fixed immediately after antibody labeling, the extent of monocyte–platelet interactions was found to increase with prolonged storage, and all subsequent analyses were performed within 2 hours of venepuncture. Chelation of calcium with EDTA inhibited the percentage of monocyte-
platelet conjugates observed, but no significant differences with heparin and hirudin or aspirin were found (data not shown). In all subsequent analyses, platelet binding to leukocytes was determined in peripheral blood anticoagulated in heparin. The divalent cation-dependent nature of platelet-monocyte adhesion is demonstrable by a distinct population of CD14/CD42a-positive cells. This population is clearly observed in the absence of EDTA (Figure 1C) but is no longer evident in the presence of EDTA (Figure 1D). These data illustrate that monocyte–platelet interactions are readily reversed after divalent cation chelation and also exclude the unlikely possibility that the FITC signal was not simply an artifact attributable to inappropriate compensation settings (set using isotype controls and CD14-FITC or CD14-PE alone). Using this assay, the extent of platelet–monocyte binding in peripheral blood from healthy volunteers was determined (57±5.1% in the absence of EDTA compared with 6.5±0.5% in the presence of EDTA, n=20, mean±SEM) (Figure 2). The same samples were used to examine the extent of platelet binding to the more abundant granulocytes present in whole blood. The overall percentage of platelet–granulocyte binding was lower (12±1.3% in the absence of EDTA versus 4.5±0.4% in the presence of EDTA, n=16, mean±SEM), demonstrating preferential platelet binding to monocytes. Evidence that individual monocytes adhere to multiple platelets can be seen using indirect immunofluorescence microscopy (Figure 4). Although observed platelet CD42a expression was relatively homogeneous, it should be noted that detectable fluorescence extended over a log decade. Thus, a stepwise increment in CD42a fluorescence for monocytes that have bound multiple platelets was not observed.

**Molecular Basis of Platelet–Monocyte Adhesion**

Both EDTA and EGTA dramatically reduced the percentage of monocytes bound to platelets (Figure 3), demonstrating that adhesion was primarily dependent on the presence of extracellular calcium. Because EGTA was equally effective in blockade, monocyte integrins (eg, α1β1 or β2 integrins) are unlikely to mediate the observed adhesion. Several adhesion receptors exhibit calcium dependence, including GP IIb/IIIa and selectins.20 It should be noted that a small percentage of monocytes bind platelets in the absence of divalent cations, implying that other receptors play a role in platelet adhesion. mAbs were therefore used to examine the molecular pathways involved in platelet–monocyte adhesion. GP IIb/IIIa receptor blockade with abciximab resulted in a potent inhibition of platelet–platelet aggregation (data not shown) but failed to inhibit monocyte–platelet interactions (Figure 3), suggesting that platelet GP IIb/IIIa does not play a major role in monocyte–platelet interactions and that other molecules mediate adhesion. Platelet–monocyte binding was found to be

**Figure 1.** Platelets bind specifically to monocytes in peripheral blood. Monocytes were labeled with CD14-PE and platelets with CD42a-FITC. Specific platelet–monocyte binding was assessed using flow cytometry of fixed/lysed whole blood. A, Representative forward and side scatter properties of leukocytes present in samples. B, Laser scatter properties of blood sample monocytes identified by CD14-PE. Platelet binding to CD14-positive monocytes shown by two-color immunofluorescence using platelet-specific CD42a mAb with (C) or without (D) 5 mmol/L EDTA illustrates divalent cation–dependent adhesion.

**Figure 2.** Platelets preferentially bind to intravascular monocytes. Two-color flow cytometry on whole blood from healthy volunteers showed marked platelet–monocyte binding (57.3±1.34%, mean±SEM, n=20). This decreased significantly after cation-chelation using 5 mmol/L EDTA (6.6±0.46%, mean±SEM, P<0.0001). Binding to granulocytes occurred at much lower levels (12±1.3% in control buffer compared with 4.5±0.4% in EDTA, mean±SEM, n=16).
significantly reduced by saturating concentrations of function-blocking PSGL-1 mAb PL1 but not by the functionally inert, isotype-matched PSGL-1 mAb PL2 (Figure 3). It is interesting to note that blockade of P-selectin consistently produced greater inhibition than PSGL-1 mAb, indicating involvement of other monocyte receptors. P-selectin mAb failed to inhibit monocyte–platelet binding to the same extent as divalent cation chelation, indicating involvement of other selectin receptors.

Clinical Study

Because P-selectin mobilizes to the surface of activated platelets and platelet activation may be associated with vascular injury, the extent of platelet–monocyte adhesion was examined in the peripheral blood of 52 patients admitted with suspected ACS. On the basis of history, electrocardiographic data, and cardiac enzyme profiles, 13 of these patients had acute MI and 12 had UA (Table). Twenty-seven patients had noncardiac chest pain. For this study, samples were labeled and analyzed as described in the previous mAb inhibition studies, at a time point within 14 hours of symptom onset.

<table>
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<tr>
<th>Characteristics of Patients Admitted With Chest Pain</th>
<th>Noncardiac</th>
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<th>Myocardial</th>
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<td>62.93±4.35</td>
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<tr>
<td>Time from onset of pain, h</td>
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<td>11.77±1.71</td>
<td>10.77±1.82</td>
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<td></td>
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<td>8/12 (67)</td>
<td>7/13 (54)</td>
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<td>7/12 (58)</td>
<td>6/13 (46)</td>
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<td>1/12 (8)</td>
<td>5/13 (38)</td>
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<td>Previous PCI/CABG</td>
<td>5/27 (18.5)</td>
<td>3/12 (25)</td>
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Values are mean±SEM or n (%).

IHD indicates ischemic heart disease; PCI, percutaneous coronary intervention; and CABG, coronary artery bypass graft.
Platelet–monocyte binding was significantly increased in both MI (70.1±15.4% SD; P<0.001) and UA (67.4±12.9% SD) patient groups compared with those with noncardiac chest pain (45.4±23.3% SD; P<0.01) (Figure 5). It is interesting that there was also a significant increase in cation-independent adhesion for patients subsequently shown to have MI compared with patients with noncardiac chest pain (14.7±7.7% SD versus 6.1±5.96% SD; P<0.001). This observation raises the intriguing possibility that myocardial injury leads to monocyte activation and the engagement of additional adhesion receptors that mediate platelet binding.

**Discussion**

Monocytes and macrophages produce cytokines, extracellular matrix molecules, enzymes and other mediators, direct subsequent cellular recruitment, and tissue remodeling, suggesting a central role for monocytes in many inflammatory diseases, including atherosclerosis. The remarkable functional plasticity of peripheral blood monocytes to exogenous signals confers distinct differentiation patterns associated with diverse tissue macrophage phenotypes. Recent monocyte transmigration and differentiation studies suggest that adhesion receptor ligation additionally influences monocyte differentiation patterns. In the present study, we examined platelet binding to circulating intravascular CD14-positive monocytes. Although a distinct subset of CD14<sup>-hi</sup>/CD16<sup>-hi</sup> expressing monocytes may be important in some cardiovascular disease processes, these cells represent a relatively small percentage of circulating cells. In terms of a molecular mechanism, the binding of platelets was rapidly and readily reversed by addition of EDTA, suggesting that platelet–monocyte binding may exhibit similar characteristics to rolling adhesion. Monocyte–platelet adhesion kinetics would thus be consistent with monocytes forming transient conjugates with circulating platelets.

Antibody inhibition studies indicate that monocytes bind platelets primarily via PSGL-1 and P-selectin. It is interesting that use of a mAb against P-selectin consistently produced greater inhibition than PSGL-1 mAb, implying that other monocyte counter-receptors are capable of binding to P-selectin, possibly those that express appropriate carbohydrates. In addition, P-selectin failed to account for all of the calcium-dependent adhesion observed, indicating that other molecules may play a role. P-selectin rapidly translocates from α granules to the platelet membrane on platelet activation, implying that bound platelets represent a relatively minor proportion of the circulating pool when examined by flow cytometry (<0.5%, data not shown). A possible explanation is that low P-selectin expression levels, below the detection threshold for flow cytometry, are sufficient to confer monocyte binding. Activated platelets have an important role in the development of thrombi, and platelet hyperaggregation has been noted in patients with both stable and unstable coronary artery disease. However, recent data suggest that platelets may undergo a form of constitutive death that also results in P-selectin expression. The data presented in the present study suggest that activated or effete platelets exhibit preferential binding to and sequestration by intravascular monocytes.

One concept suggested by our study is that the extent of monocyte–platelet interaction via PSGL-1 could lead to development of a proatherogenic monocyte phenotype. Platelets may inhibit normal monocyte differentiation, such as inhibition of platelet-activating factor acetylhydrolase activity in monocytes. Thrombin-activated platelets induce the expression and secretion of monocyte chemoattractant protein-1 and interleukin-8 from monocytes in a P-selectin/PSGL-1–dependent manner. Furthermore, P-selectin–dependent interactions potentiate tissue factor expression, PAF release, phagocytosis, and superoxide anion generation by monocytes. P-selectin induces altered tyrosine phosphor-
ulation patterns in neutrophil granulocytes, additionally supporting a signaling role for PSGL-1 and suggesting that similar paradigms may apply in monocytes. Whether platelets bound to circulating monocytes influence differentiation patterns associated with acquisition of destructive macrophage phenotypes that occur in unstable plaque is unknown.

A recent study concluded that monocyte–platelet binding levels in patients with myocardial infarction represent a more sensitive platelet activation index within the vasculature than do soluble P-selectin levels. The present study provides additional evidence that increased adhesion of platelets to monocytes is associated with ischemic events and additionally suggests that the mechanism of this adhesion is perturbed after acute myocardial infarction. Platelets bound to the monocyte membrane may directly and indirectly influence recruitment patterns within the circulation. Identification and quantification of platelet–monocyte binding in patients with chest pain may provide key early in vivo evidence of vascular injury responses and offer opportunities for novel therapeutic intervention strategies in the treatment of ACS.

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References

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