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INCREASED PLATELET REACTIVITY AND CIRCULATING MONOCYTE-PLATELET AGGREGATES IN PATIENTS WITH STABLE CORONARY ARTERY DISEASE

BY

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Abstract

Background

Platelet-dependent thrombosis and leukocyte infiltration into the vessel wall are characteristic cellular events seen in atherosclerosis. We examined whether patients with stable coronary artery disease (CAD) have increased platelet reactivity and an enhanced propensity to form monocyte-platelet aggregates.

Methods and Results

Anticoagulated peripheral venous blood from 19 patients with stable CAD and 19 normal controls was activated with or without various platelet agonists and analyzed by whole blood flow cytometry. Circulating degranulated platelets were increased in patients with CAD compared to controls (2.1 ± 0.2 [mean ± S.E.M.] vs. 1.5 ± 0.2, % P-selectin positive platelets, $p<0.01$) and were more reactive to stimulation with ADP 1 μM (28.7 ± 3.9 vs. 16.1 ± 2.2, $p<0.01$), ADP/epinephrine 1 μM (51.4 ± 4.6 vs. 37.5 ± 3.8, $p<0.05$), or thrombin receptor agonist peptide (TRAP) 5 μM (65.7 ± 6.8 vs. 20.2 ± 5.1, $p<0.01$). Patients with stable CAD also had increased circulating monocyte-platelet aggregates compared to controls (15.3 ± 3.0 vs. 6.3 ± 0.9, % platelet-positive monocytes, $p<0.01$). Furthermore, patients with stable CAD formed more monocyte-platelet aggregates than controls when their whole blood was stimulated with ADP 1 μM (50.4 ± 4.5 vs. 28.1 ± 5.3, $p < 0.01$), ADP/epinephrine 1 μM (60.7 ± 4.3 vs. 48.0 ± 4.8, $p < 0.05$), or TRAP 5 μM (67.6 ± 5.7 vs. 34.3 ± 7.0, $p<0.01$). Compared to controls, patients with stable CAD did not have a significant increase in circulating neutrophil-platelet aggregates or circulating activated monocytes.

Conclusion

Patients with stable CAD have circulating activated platelets, circulating monocyte-platelet aggregates, increased platelet reactivity, and an increased propensity to form monocyte-platelet aggregates.

Abbreviated Abstract

As determined by whole blood flow cytometry, patients with stable coronary artery disease have circulating activated platelets, circulating monocyte-platelet aggregates, increased platelet reactivity, and an increased propensity to form monocyte-platelet aggregates.

Key Words: platelets, coronary disease, flow cytometry, leukocytes, monocytes, P-selectin.

Background

The pathophysiology of atherosclerosis involves thrombosis and inflammation,[1-3] conditions in which cellular activation plays a predominant role. Traditionally these two cellular processes have been studied independently of each other. However, the heterotypic interaction between platelets and leukocytes has recently become a focus of attention.[4-10]

It is well established that serological markers of platelet activation are present in acute coronary artery syndromes.[2, 11-13] However, direct evidence of hyperreactive or "primed" platelets in stable coronary artery disease is lacking. Furthermore, recent evidence points to a major role for leukocytes[14, 15] and humoral inflammatory mediators in the pathogenesis of coronary atherosclerosis.[1, 16-18]

P-selectin (also known as CD62P, previously known as GMP-140 and PADGEM protein), a component of the platelet α granule membrane, is translocated to the surface membrane upon platelet activation.[19, 20] P-selectin mediates the binding of platelets to leukocytes.[21, 22] Leukocytes have been associated with platelets at sites of hemorrhage[23] and during atherogenesis.[1] P-selectin-mediated circulating leukocyte-platelet aggregates have been noted after exposure to oxidized LDL[24] and cigarette smoke,[25] both of which are known risk factors for the development of atherosclerosis. Recent reports have suggested an increase in leukocyte-platelet interactions in patients with unstable angina[10] and following percutaneous transluminal coronary angioplasty (PTCA).[9] Currently no data are available concerning the presence or formation of monocyte-platelet aggregates or neutrophil-platelet aggregates in stable coronary artery disease.

In this study, we used whole blood flow cytometry to investigate platelet activation and leukocyte-platelet aggregation in patients with stable coronary artery disease (CAD). Whole blood flow cytometry has the advantage of directly analyzing individual cells in their native milieu with minimal artifactual cellular activation.[26]

Materials and Methods

Study Population

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 undergoing outpatient cardiac catheterization at the University of Massachusetts Medical Center for symptomatic stable angina. Sampling occurred prior to administration of heparin or contrast agents. Characteristics of the study population are displayed in Table 1. The control population consisted of healthy adult volunteers who had not taken any anti-platelet agent for at least 10 days prior to sampling.

Monoclonal Antibodies

S12 and G1(Centocor, Malvern, PA) are directed against different epitopes on P-selectin.[22] S12 was biotinylated as previously described.[27] Y2/51 (DAKO, Carpenteria, CA) is directed against glycoprotein (GP) IIIa[28] and was purchased conjugated to fluorescein isothiocyanate (FITC). 7E3 (a generous gift of Dr. Barry Coller, Mt. Sinai Medical School, New York, NY) is directed against the GPIIb-IIIa complex[29] and was FITC-conjugated with QUICK TAG Kit (Boeringer Mannheim, Indianapolis, IN). BEAR1 (Immunotech, Westbrook, ME) is directed against the M chain of CD11b (MAC-1)[30] on leukocytes and was purchased FITC-conjugated. S12, G1, Y2/51, 7E3, and BEAR1 are all IgG murine antibodies.

Whole Blood Flow Cytometry

Platelet Activation State

The method has been previously described in detail.[31] There were no centrifugation, gel filtration, vortexing, or stirring steps that could artifactually activate

platelets. Blood from patients and controls was drawn into a sodium citrate Vacutainer (Becton Dickinson, Rutherford, NJ). Within 15 minutes of drawing, the blood was diluted 1:4 in modified HEPES-Tyrode's buffer (137 mM NaCl, 2.8 mM KCl, 1 mM $MgCl_2$, 12 mM $NaHCO_3$, 0.4 mM Na_2HPO_4, 0.35% bovine serum albumin, 10 mM HEPES, 5.5 mM glucose), pH 7.4, containing the peptide glycyl-L-prolyl-L-arginyl-L-proline (GPRP, Calbiochem, San Diego, CA) at a concentration of 2.5 mM to prevent fibrin polymerization[27] and either: a) no agonist; b) ADP 1 μM or 10 μM (BIO/DATA, Hatboro, PA); c) ADP 1 μM with epinephrine 1 μM (BIO/DATA) or ADP 10 μM with epinephrine 10 μM; d) thrombin receptor activating peptide (TRAP) 5 μM or 30 μM (Calbiochem); or e) purified human α-thrombin (provided by Dr. John W. Fenton II, New York Department of Health, Albany, NY) 2 U/ml.

After 10 minutes, all samples were fixed at 22°C for 20 minutes with formaldehyde 1% (final concentration). After fixation, samples were diluted 10-fold in modified Tyrode's buffer, pH 7.4. The samples were then incubated at 22°C for 20 minutes with a near saturating concentration of FITC-conjugated monoclonal antibody Y2/51 and a saturating concentration of biotinylated monoclonal antibody S12, followed by an incubation at 22° for 20 minutes with 30 μg/ml of phycoerythrin-streptavidin (Jackson ImmunoResearch, West Grove, PA).

As previously described,[27] samples were analyzed in an EPICS Profile flow cytometer (Coulter Cytometry, Miami, FL). The flow cytometer was equipped with a 500 mW argon laser (Cyonics, San Jose, CA) operated at 15 mW with an emission wavelength of 488 nm. The fluorescence of FITC and phycoerythrin was detected using 525 nm and 575 nm band pass filters, respectively. After identification of platelets by gating on both Y2/51-FITC positivity (i.e. GPIIIa-positivity) and their characteristic light scatter, binding of the biotinylated monoclonal antibody S12 was determined by analyzing 5,000 individual platelets for phycoerythrin fluorescence. Ther percentage of P-selectin-positive platelets was defined as the percentage of platelets that had phycoerythrin fluorescence greater than

99% of platelets incubated with purified biotinylated mouse IgG (Boehringer Mannheim) rather than biotinylated S12.

Monocyte-Platelet Aggregates/Neutrophil-Platelet Aggregates

Blood was diluted 1:4 in modified HEPES-Tyrode's buffer, pH 7.4 containing the monoclonal antibody 7E3 2 μg/ml and either: a) no agonist; b) ADP 1 μM or 10 μM; c) ADP 1 μM with epinephrine 1 μM or ADP 10 μM with epinephrine 10 μM; d) TRAP 5 μM or 30 μM; or e) purified human α-thrombin 2 U/ml. After 10 minutes, the samples were fixed at 22° for 10 minutes with 1.1% formaldehyde and 1.4X Hanks balanced saline solution (GIBCO, Grand Island, NY), then diluted 4.6-fold with distilled H_20.

The samples were then analyzed in an EPICS Profile flow cytometer. The fluorescence of FITC was detected using a 525 nm band pass filter. Monocytes and neutrophils were identified by their characteristic light scatter properties (Fig 1, upper panel). Platelet-positive monocytes and neutrophils were identified by binding of the FITC-conjugated GPIIb-IIIa-specific monoclonal antibody 7E3 (Fig 1, lower panel). The percentage of platelet-positive monocytes and neutrophils was defined as the percentage of monocytes and neutrophils that had a FITC fluorescence greater than 99% of monocytes and neutrophils incubated with FITC-mouse IgG (Boehringer Mannheim) rather than FITC-7E3. One thousand monocytes and 5,000 neutrophils were counted.

The monocyte-platelet and neutrophil-platelet aggregates were P-selectin-dependent because, consistent with previous studies,[22] the formation of these aggregates was completely blocked by the P-selectin-specific monoclonal antibody G1 (data not shown).

Monocyte Activation

Blood was diluted 1:4 in modified HEPES-Tyrode's buffer, pH 7.4 containing a saturating concentration of the FITC-conjugated CD11b-specific monoclonal antibody BEAR1 and fixed at 22° for 10 minutes with 1% formaldehyde. The samples were then

analyzed in an EPICS Profile flow cytometer. Monocytes were identified by their characteristic light scatter properties The fluorescence of FITC-BEAR1 was detected using a 525 nm band pass filter. The percentage of CD11b-positive monocytes was defined as the percentage of monocytes that had FITC fluorescence greater than 99% of monocytes incubated with FITC mouse IgG (Boehringer Mannheim) rather than FITC-BEAR1.

Results

Circulating Activated Platelets and Circulating Leukocyte-Platelet Aggregates

Unstimulated peripheral blood from patients with stable coronary artery disease contained increased numbers of surface P-selectin-positive platelets compared with peripheral blood from normal controls (2.1 ± 0.2 [mean ± S.E.M.] vs. 1.4 ± 0.2, $p< 0.01$) (Fig 2). Furthermore, peripheral blood from patients with stable CAD as compared to normal controls contained increased numbers of circulating monocyte-platelet aggregates (15.3 ± 3.0 vs. 6.3 ± 0.9, $p< 0.01$) (Fig 2) but no significant increase in the presence of circulating neutrophil-platelet aggregates (6.8 ± 1.2 vs. 5.6 ± 1.0, $p=0.10$) (Fig 2).

Platelet Reactivity

As determined by the platelet surface expression of P-selectin, platelets from patients with stable CAD were significantly more reactive to *ex vivo* agonist stimulation than were platelets from normal controls (Fig 3). Platelets from patients with stable CAD were more likely than controls to degranulate in response to stimulation with a low concentration of ADP (1 μM) (28.7 ± 3.9 vs. 16.1 ± 2.2, $p<0.01$), a low concentration of ADP (1 μM) and concomitant epinephrine (1 μM) (51.4 ± 4.6 vs. 37.5 ± 3.8, $p<0.05$) and a low concentration of TRAP (5 μM) (65.7 ± 6.8 vs. 20.2 ± 5.1, $p < 0.01$) (Fig 3). No significant differences between patients with stable CAD and controls were seen with high concentrations of agonists (ADP 10 μM, ADP 10 μM and epinephrine 10 μM, TRAP 30 μM) (Fig 3) demonstrating that the differences observed with the use of low agonist concentrations was not due to an increased amount of available platelet P-selectin in the patients.

Propensity to Form Leukocyte-Platelet Aggregates

In addition to the increased monocyte-platelet aggregates present in the peripheral circulation of patients with stable CAD, monocyte-platelet aggregates were more likely to form in patients with stable CAD than in controls in response to *ex vivo* agonist stimulation (Fig 4). Stimulation of peripheral whole blood from patients with stable CAD by a low concentration of ADP (1 μM) resulted in significantly more monocyte-platelet aggregates than in the normal controls (50.4 ± 4.5 vs. 28.1 ± 5.3, $p<0.01$). Similar results were seen when peripheral whole blood from patients with stable CAD and normal controls was stimulated with a low concentration of ADP (1 μM) and concomitant epinephrine (1 μM) (60.7 ± 4.3 vs. 48.0 ± 4.8, $p<0.05$) or a low concentration of TRAP (5 μM) (67.6 ± 5.7 vs. 34.3 ± 7.0, $p<0.01$) (Fig 4). As was observed with platelet reactivity (Fig 3), no significant differences between patients with stable CAD and controls were seen in the propensity to form monocyte-platelet aggregates when high concentrations of agonists (ADP 10 μM, ADP 10 μM and epinephrine 10 μM, TRAP 30 μM) were used (Fig 4).

No significant differences between patients and controls were noted in the propensity to form neutrophil-platelet aggregates in response to stimulation with any of the following agonists: ADP 1 μM, ADP 10 μM, ADP 1 μM and epinephrine 1 μM, ADP 10 μM and epinephrine 10 μM, TRAP 5 μM, TRAP 30 μM (Fig 5).

Monocyte Activation

There was no significant difference in the binding of the activation-dependent anti-CD11b monoclonal antibody BEAR1 to the surface of monocytes from patients with stable CAD compared with controls (Fig 6).

Discussion

Platelet Degranulation in Patients with Stable CAD

This study directly demonstrates circulating degranulated platelets and circulating monocyte-platelet aggregates in patients with stable CAD. Previous studies of platelet function in patients with coronary atherosclerosis have focused on patients with acute coronary artery syndromes and active ischemia presumably due to thrombosis within the coronary vessels.[11, 32-34] These studies[11, 32-34] measured platelet secretory products and the assays used were prone to artifactual platelet activation.[35] Flow cytometry is a direct method for the measurement of platelet function, avoids *in vitro* artifactual platelet activation and assesses the activation state of individual platelets.[26, 31] Flow cytometry has been used to study platelets from patients with CAD undergoing angioplasty[36-39] and to a limited extent in patients with unstable angina.[40] The present study supports the usefulness of flow cytometry in studying platelets from patients with stable CAD.

Our study not only demonstrates the presence of circulating degranulated platelets in patients with stable CAD, but also provides evidence of enhanced platelet reactivity as demonstrated by the increased platelet surface expression of P-selectin in response to stimulation with low concentrations of agonists (Fig 3). In patients with stable CAD, full stimulation with ADP, ADP/epinephrine or TRAP resulted in a similar number of P-selectin positive platelets as controls. Thus, the increased platelet reactivity in patients with stable CAD is not due to an increased amount of available platelet P-selectin, but to an alteration in the intracellular platelet environment that makes them more responsive to agonist stimulation. The cellular mechanisms responsible for these "primed" platelets remain unknown, but may be relate to alterations in platelet nitric oxide production.[41] Alterations in platelet surface receptors are unlikely to be involved in the mechanism because similar results were obtained with ADP, epinephrine and TRAP, each of which is known to stimulate platelets via a different receptor.

P-selectin is rapidly cleared from the surface of circulating degranulated platelets.[42] Thus, the detection of circulating P-selectin-positive platelets in patients with stable coronary artery disease must be due to a process of ongoing platelet degranulation since the sampling of the patients' blood was not temporally related to an ischemic event nor did it occur immediately distal to an injured vessel.

Monocyte-platelet Aggregates in Patients with Stable CAD

In addition to identifying increased circulating degranulated platelets and increased platelet reactivity in patients with stable CAD, we demonstrate that this patient group has increased circulating monocyte-platelet aggregates and an increased propensity to form monocyte-platelet aggregates (Figs 2 & 4). Platelet adhesion to monocytes and neutrophils is mediated by the platelet surface expression of P-selectin[21, 22] which binds to P-selectin glycoprotein ligand-1 (PSGL-1) on leukocytes.[43-45] Thus the increased circulating monocyte-platelet aggregates observed in this study may simply reflect the presently described increased platelet degranulation in patients with stable CAD. Leukocyte-platelet aggregates have also been identified in whole blood of patients undergoing cardiopulmonary bypass[8] or hemodialysis.[46] More recently, neutrophil-platelet aggregates have been identified in the peripheral blood of patients with unstable angina[10] and monocyte-platelet and neutrophil-platelet aggregates in the coronary sinus of patients undergoing coronary angioplasty.[9]

The exact physiologic significance of monocyte-platelet aggregation is unknown, but it may represent targeting of both cell types to appropriate inflammatory or hemostatic sites.[6] Functional changes in one cell type by another have also been demonstrated. For example, platelets have been shown to supply cholesterol to monocytes which may then mature into lipid-laden macrophages.[47] Palabrica et. al.[48] have demonstrated that P-selectin-mediated platelet adhesion to leukocytes is important in promoting fibrin deposition within a growing thrombus. More recently, Weyrich et. al.[49] have shown that platelet

surface P-selectin can regulate monocyte chemokine synthesis in concert with the platelet chemokine RANTES (regulated upon activation normal T cell expressed presumed secreted). Our study did not demonstrate enhanced formation of neutrophil-platelet aggregates in patients with stable CAD, a finding consistent with recent reports that monocyte-platelet aggregates form more easily than neutrophil-platelet aggregates.[6-8]

Study Limitations

A limitation of this study is that platelets and leukocyte-platelet aggregates were sampled in the peripheral circulation, not at the diseased vessel wall where cellular activation presumably occurs. Thus, the circulating degranulated platelets, circulating monocyte-platelet aggregates, increased platelet reactivity, and increased propensity to form monocyte-platelet aggregates may not be wholly reflective of local events within the coronary vasculature. Furthermore, our control population consisted of healthy adults without significant cardiac risk factors. Therefore, the differences in platelet function and monocyte-platelet aggregation between the patients with stable CAD and the controls may not be caused by the presence of atherosclerosis in the patients, but may be due in part to the presence of hypertension, hyperlipidemia, diabetes or the use of beta blockers, angiotensin converting enzyme inhibitors or calcium channel blockers.

Summary

In this study we demonstrate that patients with stable CAD have circulating degranulated platelets, circulating monocyte-platelet aggregates, increased platelet reactivity and an increased propensity to form monocyte-platelet aggregates. The question as to whether the degree of increased platelet reactivity and monocyte-platelet aggregation correlates with specific clinical events in these patients is currently being addressed in our laboratory.

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Figure Legends

Figure 1. Whole blood flow cytometric analysis of monocyte-platelet and neutrophil-platelet aggregates in normal donors. Monocytes and neutrophils were identified by their characteristic light scatter properties (upper panel). Platelet-positive monocytes and neutrophils were identified by binding of FITC-conjugated GPIIb-IIIa-specific monoclonal antibody 7E3 (lower panels). Addition of thrombin 2 U/ml in the presence of glycyl-L-prolyl-L-arginyl-L-proline 2.5 mM (an inhibitor of fibrin polymerization) resulted in platelet binding to most monocytes and neutrophils

Figure 2. Platelet surface P-selectin, monocyte-platelet aggregates, and neutrophil-platelet aggregates circulating in the peripheral blood of patients with stable CAD and controls as determined by whole blood flow cytometry. Data are mean ± S.E.M., n=19. * = $p<0.01$.

Figure 3. Platelet reactivity of patients with stable CAD and controls, as determined by the platelet surface expression of P-selectin in response to exogenous agonist stimulation. Data are mean ± S.E.M., n=19, * = $p<0.01$. † = $p<0.05$.

Figure 4. Monocyte-platelet aggregates in the blood of patients with stable CAD and controls, as determined by whole blood flow cytometry after *ex vivo* agonist stimulation. Data are mean ± S.E.M., n=19, * = $p<0.01$. † = $p<0.05$.

Figure 5. Neutrophil-platelet aggregates in the blood of patients with stable CAD and controls, as determined by whole blood flow cytometry after *ex vivo* agonist stimulation. Data are mean ± S.E.M., n=19. p = not significant.

Figure 6. Circulating CD11b-positive monocytes in patients with stable CAD and controls, as determined by whole blood flow cytometry. Data are mean ± S.E.M., n=19. p = not significant.

Table 1. Patient Characteristics

Age (mean ± S.E.M.)	62 ± 3 yrs
Male	13 (68%)
Hypertension	17 (89%)
Diabetes	6 (32%)
Current Smoker	0
Hypercholesterolemia (>230 mg/dl)	6 (32%)
Family history of CAD	4 (21%)
Prior MI	6 (32%)
Prior PTCA	5 (26%)
Average Ejection Fraction	55%
Extent of CAD	
Single vessel	16 (84%)
Double vessel	3 (16%)
Medications	
Aspirin	19 (100%)
Beta Blocker	19 (100%)
Calcium Channel Blocker	14 (79%)
Oral nitrates	11 (58%)
ACE inhibitor	6 (32%)

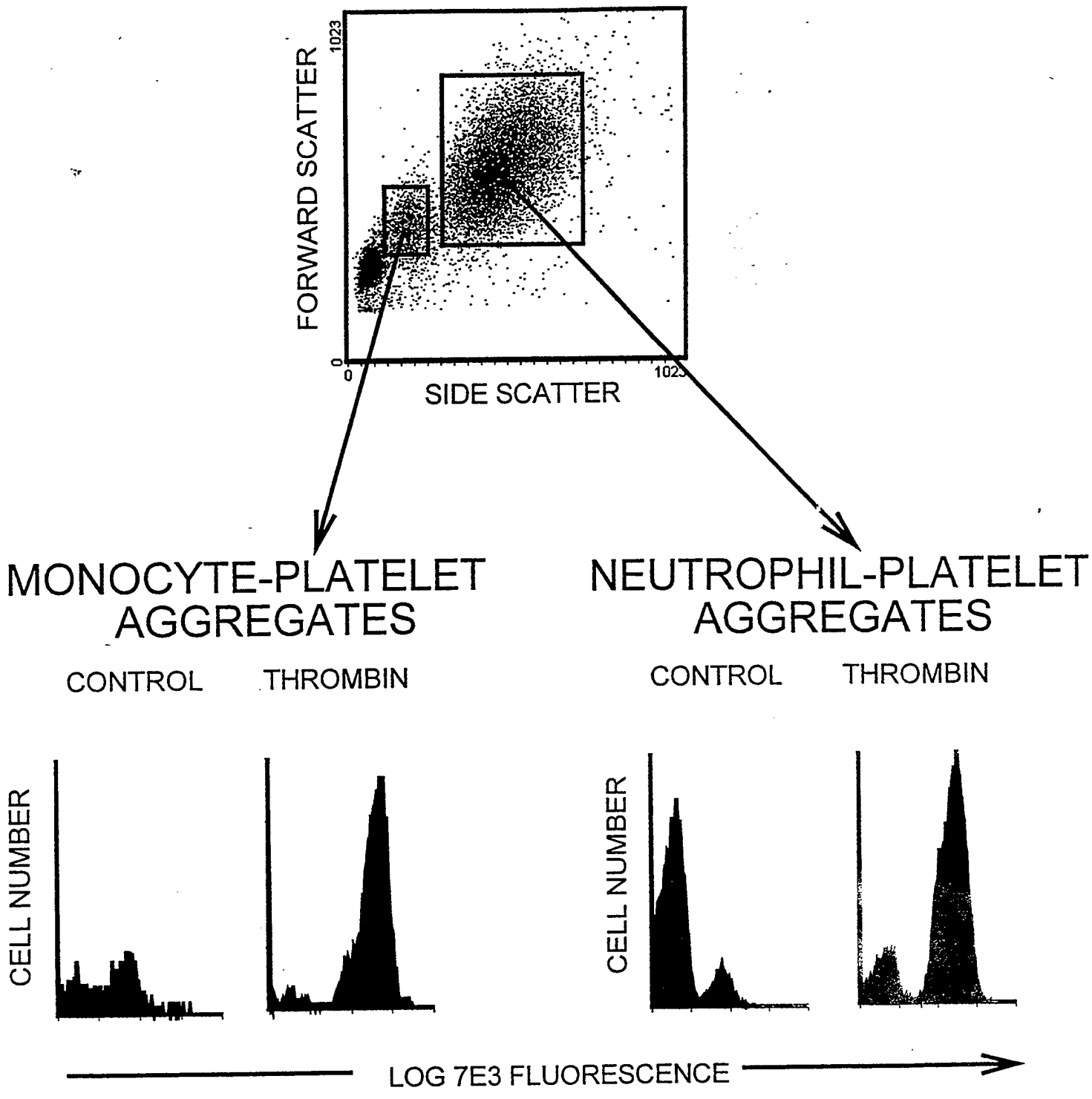
FORWARD SCATTER
1023
0
0
1023
SIDE SCATTER
MONOCYTE-PLATELET AGGREGATES
CONTROL
THROMBIN
NEUTROPHIL-PLATELET AGGREGATES
CONTROL
THROMBIN
CELL NUMBER
CELL NUMBER
LOG 7E3 FLUORESCENCE

Fig 1

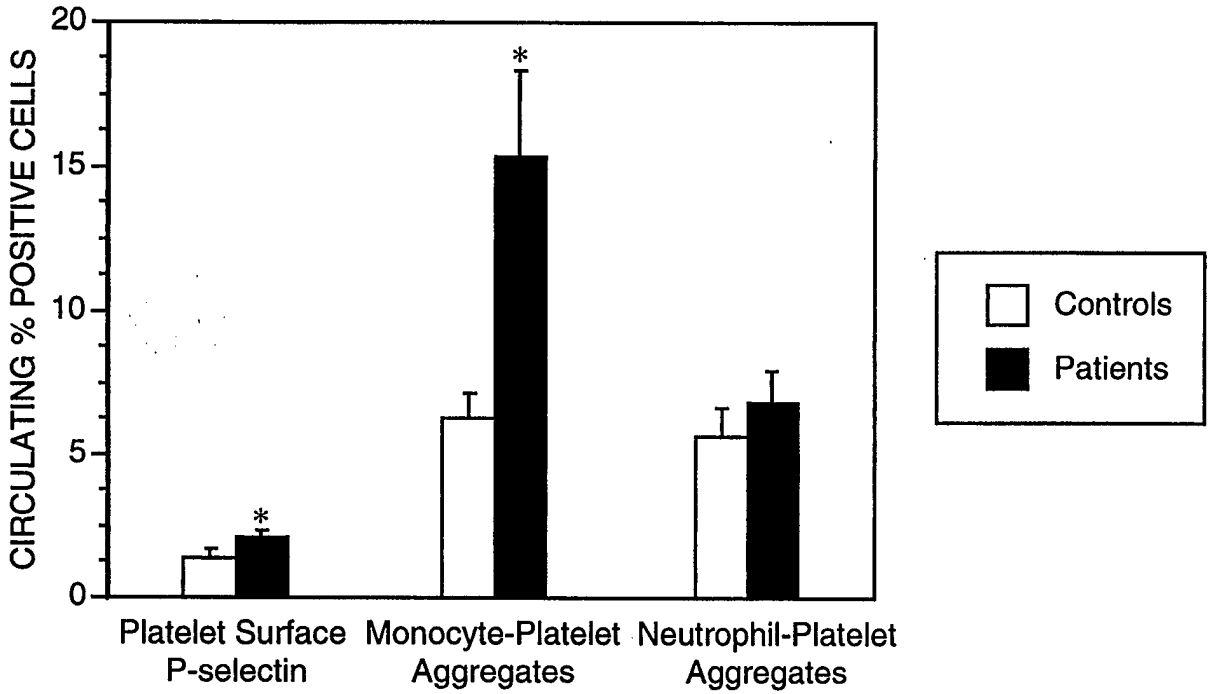
20
15
10
5
0
CIRCULATING % POSITIVE CELLS
*
*
Platelet Surface P-selectin
Monocyte-Platelet Aggregates
Neutrophil-Platelet Aggregates
Controls
Patients

Fig 2

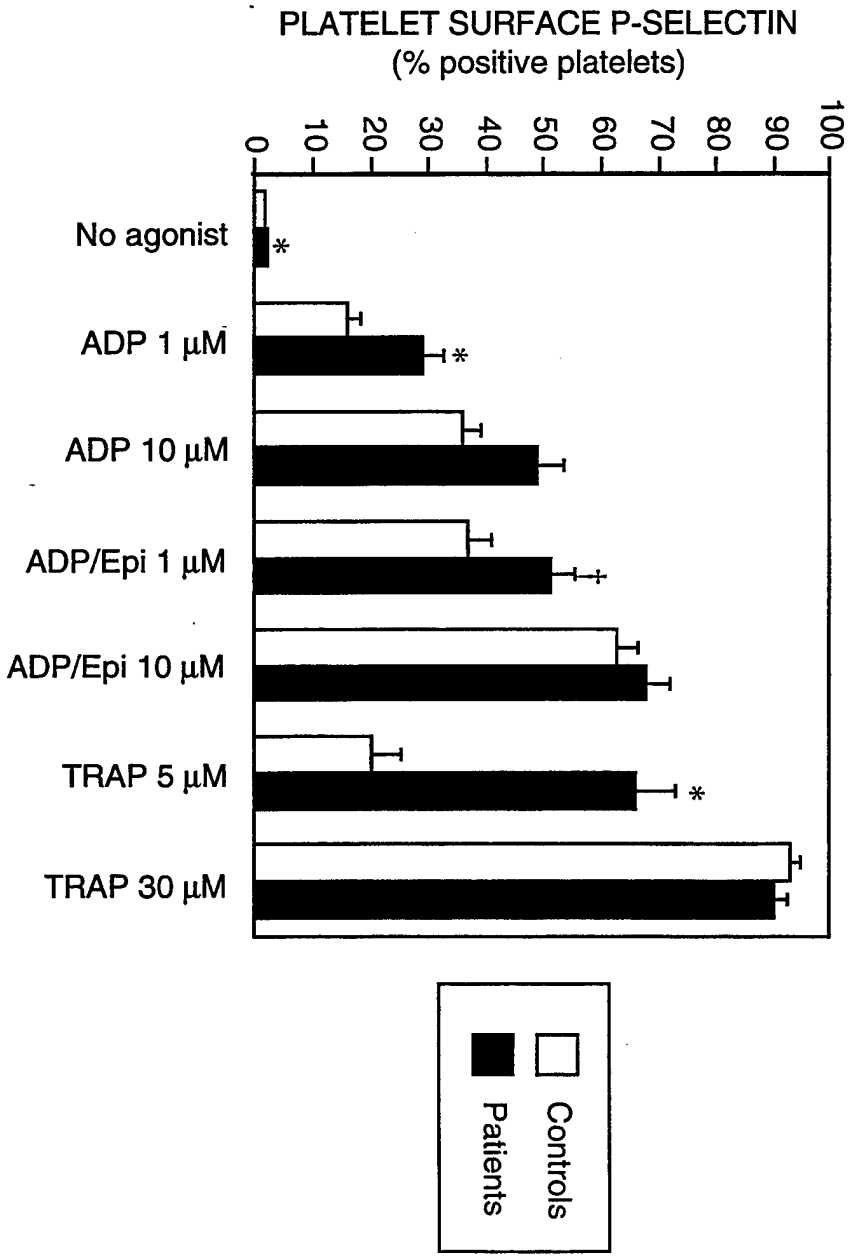
PLATELET SURFACE P-SELECTIN
(% positive platelets)
0
10
20
30
40
50
60
70
80
90
100
No agonist
ADP 1 µM
ADP 10 µM
ADP/Epi 1 µM
ADP/Epi 10 µM
TRAP 5 µM
TRAP 30 µM
*
*
*
Controls
Patients

Fig 3

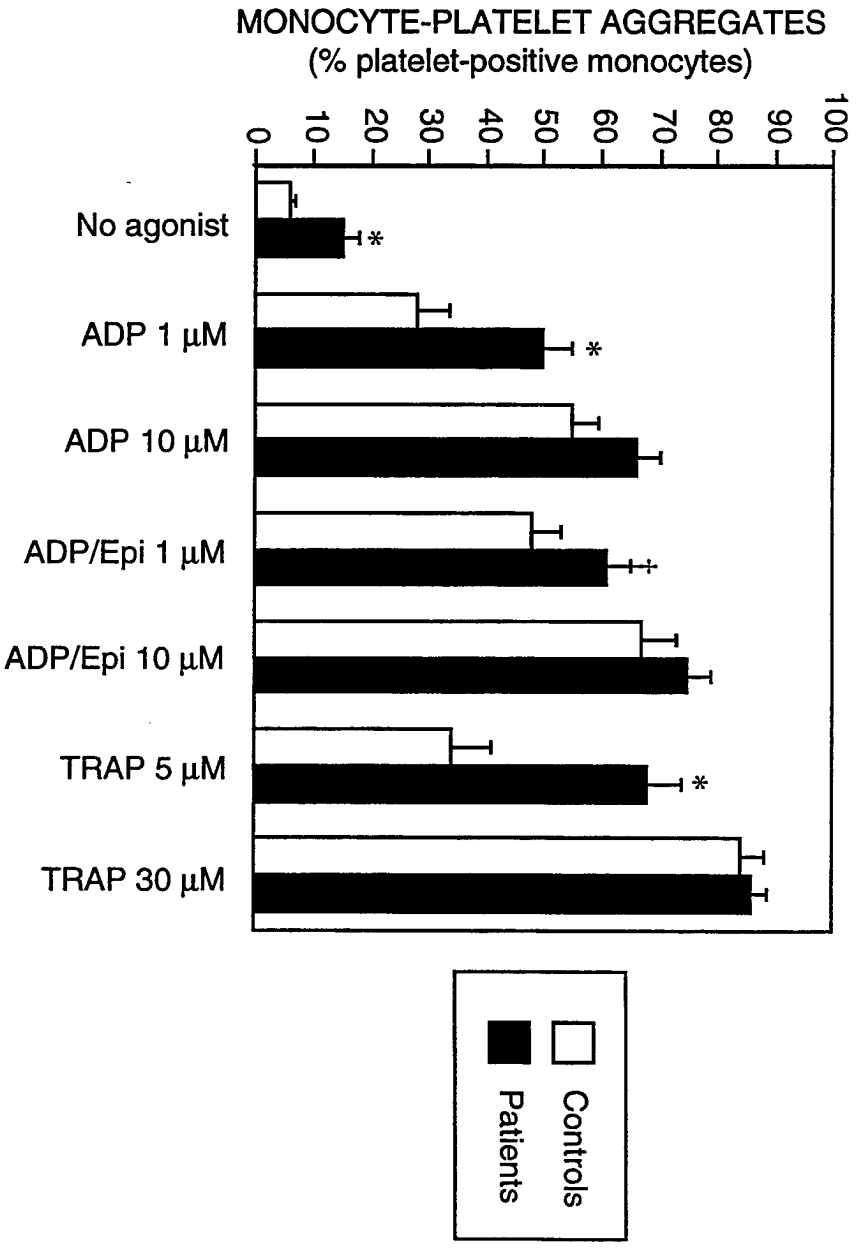
MONOCYTE-PLATELET AGGREGATES
(% platelet-positive monocytes)
0
10
20
30
40
50
60
70
80
90
100
No agonist
ADP 1 µM
ADP 10 µM
ADP/Epi 1 µM
ADP/Epi 10 µM
TRAP 5 µM
TRAP 30 µM
*
*
*
Patients
Controls

Fig 4

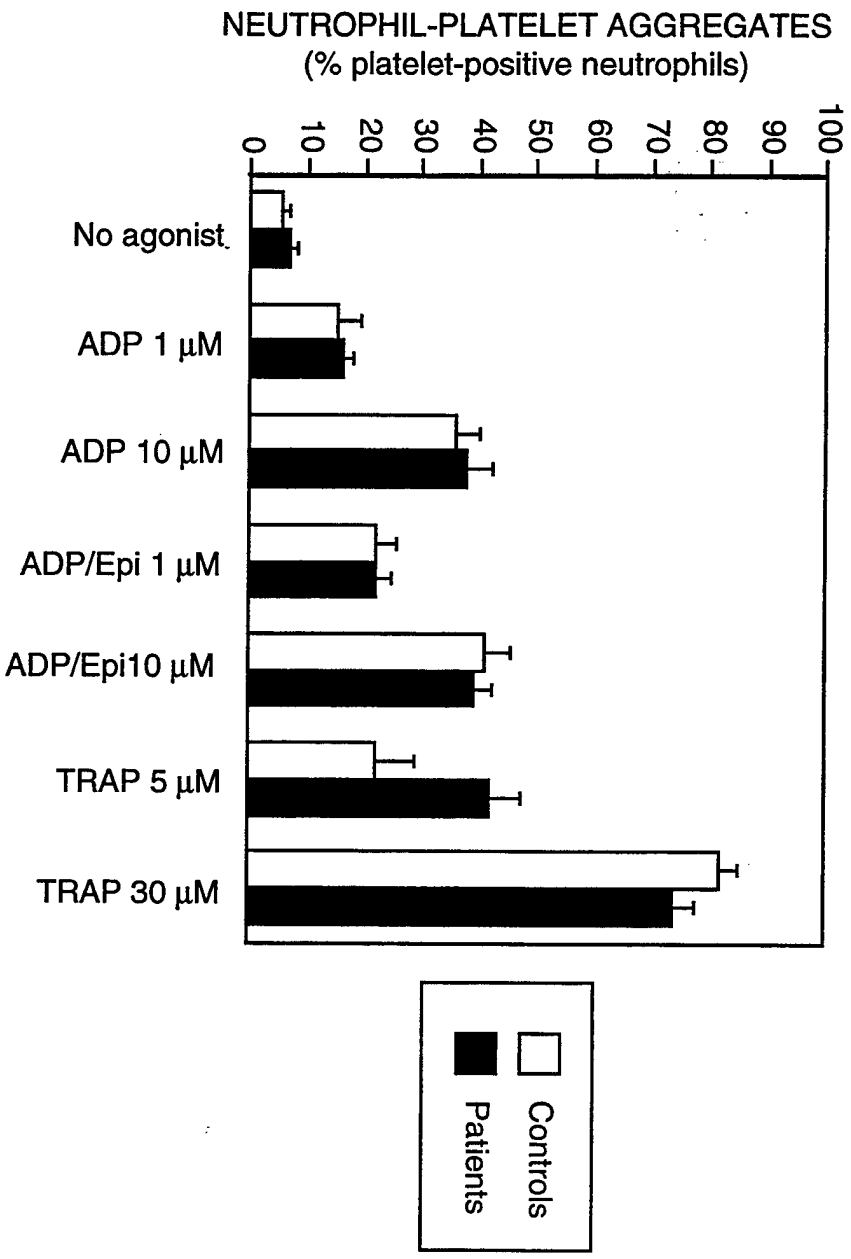
NEUTROPHIL-PLATELET AGGREGATES
(% platelet-positive neutrophils)
0
10
20
30
40
50
60
70
80
90
100
No agonist
ADP 1 µM
ADP 10 µM
ADP/Epi 1 µM
ADP/Epi10 µM
TRAP 5 µM
TRAP 30 µM
Controls
Patients

Fig 5

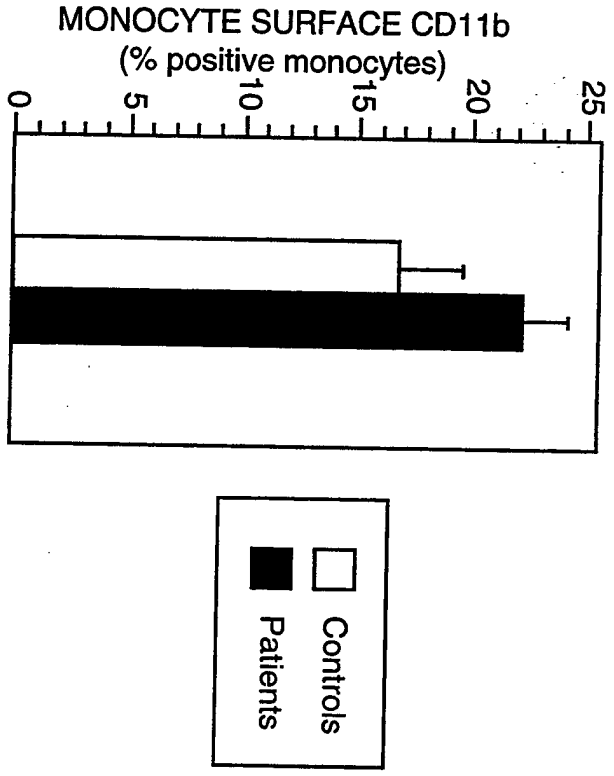
MONOCYTE SURFACE CD11b
(% positive monocytes)
0
5
10
15
20
25
Controls
Patients

Fig 6