Activation of Platelets in Blood Perfusing Angioplasty-Damaged Coronary Arteries

Flow Cytometric Detection

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Fluorescence-activated flow cytometry has been used to investigate platelet activation in blood flowing through atherosclerotic coronary arteries after sustaining mechanical damage induced by percutaneous transluminal angioplasty (PTCA). For flow cytometry, platelets and platelet-derived microparticles were identified by biotinylated anti-glycoprotein (GP) Ib monoclonal antibody (mAb) and a fluorophore, phycoerythrin-streptavidin. Activated platelets were detected by using a panel of fluoresceinated mAbs specific for activation-dependent platelet epitopes, including 1) activated GPIIb-IIIa complex (PAC1); 2) fibrinogen bound to platelet GPIIb-IIIa (9F9); 3) ligand-induced binding sites on GPIIIa (anti-LJBSl); and 4) P-selectin, an α-granule membrane protein expressed on the platelet surface after secretion (SI2). The binding of antibodies to platelets was determined in blood that was sampled continuously via heparin-coated catheters from the coronary sinus in 1) patients before, during, and for 30 minutes after PTCA and 2) control patients undergoing coronary angiography without PTCA. Platelets in coronary sinus blood showed significant binding of mAbs that specifically detect activation epitopes associated with the GPIIb-IIIa complex (PAC1, anti-LJBSl, and 9F9) during and for 30 minutes after angioplasty in four of the five patients. The relative proportion of platelets positive for PAC1 and anti-LJBSl increased from baseline values of 2.0±0.5% (mean±SD) to 18±14% and 28±14%, respectively, during PTCA or 30 minutes after PTCA (p<0.01 in both cases). Binding with 9F9 was less prominent. The expression of P-selectin was detected in one of the five patients. By contrast, activation-specific mAbs failed to bind detectably with platelets obtained from 1) the peripheral blood during coronary angiography in eight patients or 2) coronary sinus blood obtained via catheter throughout control catheterization procedures in three patients or before PTCA in five. We conclude that circulating platelets become activated while flowing through PTCA-damaged stenotic coronary arteries and that this process of platelet activation is readily demonstrated by measuring the expression of activation-specific membrane GP epitopes by flow cytometric analysis. (Arteriosclerosis and Thrombosis 1992;12:1475–1487)

KEY WORDS • activated platelets • platelet membrane glycoproteins • activation-specific antiplatelet monoclonal antibodies • platelet receptors • flow cytometry • percutaneous transluminal coronary angioplasty

Successful percutaneous transluminal coronary angioplasty (PTCA) mechanically disrupts stenosing atheromatous plaques.1 The resultant exposure of flowing blood to the highly thrombogenic constituents that compose subendothelial structures and atherosclerotic lesions gives rise to the adhesion and activation of platelets.2 Concurrently, thrombin is produced at the site of vascular injury via sequential zymogen activation of serine proteases comprising both extrinsic and intrinsic pathways of coagulation.3–5 Locally deposited platelets further amplify thrombin generation.6 Thus, PTCA-initiated thrombus formation may lead to thrombo-occlusion in some patients7–9 and may give rise to subsequent intimal proliferative lesion formation, both directly by liberating platelet-derived growth factor and indirectly by amplifying the generation of thrombin, an important mitogenic factor at sites of vascular injury.10,11

The sequence of events underlying platelet recruitment includes 1) adhesion of nonactivated platelets with subendothelial cytoadhesive proteins (collagen, von Willebrand factor [vWF], fibronectin, laminin, thrombospondin, and vitronectin), 2) activation of adherent platelets and their release of proaggregatory ADP and thromboxane A2 (TXA2), 3) thrombin production, 4) activation (by thrombin, ADP, and TXA2) and receptor expression for adhesive molecules by ambient platelets, and 5) platelet accumulation through platelet–ligand interactions (primarily binding of fibrinogen and vWF to...
TABLE 1. Expression of Activation-Specific Platelet Epitopes and Glycoprotein IIb-IIIa in Whole Blood From Patients Undergoing PTCA, Control Patients, and Normal Subjects, as Evaluated by Flow Cytometric Measurement

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Coronary intervention</th>
<th>Mean fluorescence intensity (arbitrary units)* of FITC-conjugated mAbs bound per platelet</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PAC1 (act. GPIIb-IIIa)</td>
<td>S12 (GMP-140)</td>
</tr>
<tr>
<td>1</td>
<td>Before</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>During/After</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>Before</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>During/After</td>
<td>23</td>
</tr>
<tr>
<td>3</td>
<td>Before</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>During/After</td>
<td>30</td>
</tr>
<tr>
<td>4</td>
<td>Before</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>During/After</td>
<td>14</td>
</tr>
<tr>
<td>5</td>
<td>Before</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>During/After</td>
<td>19</td>
</tr>
<tr>
<td>Total</td>
<td>Before</td>
<td>6.8±2.8†</td>
</tr>
<tr>
<td></td>
<td>During/After</td>
<td>17.6±10.5</td>
</tr>
<tr>
<td>Control patients (n=3)†</td>
<td>Before</td>
<td>5.7±1.2†</td>
</tr>
<tr>
<td></td>
<td>During/After</td>
<td>6.0±2.0</td>
</tr>
<tr>
<td>Normal subjects (n=5)</td>
<td>Before</td>
<td>5.5±1.9†</td>
</tr>
</tbody>
</table>

PTCA, percutaneous transluminal coronary angioplasty; FITC, fluorescein isothiocyanate; mAbs, monoclonal antibodies; act., activated; GP, glycoprotein; GMP, α-granule membrane protein; LIBS, ligand-induced binding sites; Fg, fibrinogen.

*Listed are baseline and peak levels of log platelet membrane immunofluorescence intensity, as obtained for the different mAbs in serial whole-blood samples that were collected before, during, and after termination of coronary intervention procedures, including coronary angiography and PTCA (patients 1–5) or coronary angiography only (control patients).

†Mean±SD.

‡Patients for whom PTCA was considered but not performed.

glycoprotein [GP] IIb-IIIa), and 6) stabilization of platelet thrombus by thrombin-catalyzed fibrin formation.6 Adhesion of circulating platelets to thrombogenic surfaces is mediated by the interaction of several platelet functional receptors, including GPIb-IX, GPⅠa-ⅠⅡa, GPIIIa-Ⅱa, GPIIb-IIIa (IV), and the vitronectin receptor complexes; platelet aggregate formation depends primarily on the GPIIb-IIIa complex.6-12 Under high-shear flow conditions that are characteristic of stenosed coronary arteries,13 the binding of platelets to subendothelial vWF via the GPIb–IX complex may be important in mediating platelet accumulation.12 Since platelet GPIb also binds with thrombin,14 GPIb may have an additional role in the activation of circulating platelets.15

The recruitment of platelets after denuding vascular injury depends on the expression by activated platelets of a functional receptor for adhesive ligand molecules in the GPIIb-IIIa complex.16 GPIIb-IIIa, a member of the integrin superfamily of adhesion receptors,17 interacts via the recognition sequence Arg-Gly-Asp common to a variety of adhesive molecules, including fibronogen, fibronectin, vitronectin, and vWF.18,19 Other events may occur at the platelet surface on activation, including conformational changes in receptors occupied by ligands, as well as in platelet-bound ligands themselves, and expression of membrane constituents after granule secretion, with the fusion of granule membranes to the platelet plasma membrane.20-23 The changes at the activated platelet surface may be evaluated by specific murine monoclonal antibodies (mAbs) raised against activation-dependent platelet epitopes and bound ligands by flow cytometry.20 Fluorescence-activated flow cytometry (FAFC) detects platelet activation by identifying qualitative or quantitative changes of membrane GPs on small samples of whole blood.24-27 Using flow cytometric analysis, we now report that platelets undergo detectable activation during PTCA procedures in humans.

Methods

Patients

We studied five patients undergoing PTCA, three patients for whom PTCA was considered but not performed, and five normal control subjects. Mean patient age was 64 (range, 50–71) years. All patients undergoing PTCA suffered from coronary artery disease and demonstrated at least one lesion under consideration for dilation with a diameter of stenosis of 70% or more, as estimated by visual examination. Written informed consent according to the guidelines of the Human Subjects Committee of Scripps Clinic and Research Foundation was obtained from all individuals participating in this study.

Drug Regimens and Angioplasty Procedures

Patients received standard periprocedural medications, including aspirin (325 mg administered orally the morning of the procedure), heparin (intravenous bolus injection of 10,000 units before PTCA and an additional bolus of 5,000 units after 1 hour when the procedures were still ongoing), low-molecular-weight dextran (500 ml of 10% dextran 40), verapamil (5 mg), and lidocaine (75 mg). Nitroglycerin (200 μg) was given intravenously immediately before dilation.

The dilation procedure was performed using the steerable technique.28 In all patients a successful dila-
FIGURE 1. Quantification of an activated-platelet subpopulation by flow cytometric analysis in a patient undergoing percutaneous transluminal coronary angioplasty (PTCA). Platelets in whole blood were doubly labeled with biotinylated PAC1 monoclonal antibody (mAb; reacts with activated glycoprotein [GP] IIb-IIIa), followed by phycoerythrin-streptavidin and a fluorescein isothiocyanate (FITC)-conjugated anti-GPⅠb mAb, LJ-P3. Panel A: Bidimensional contour plot representing baseline binding levels of PAC1 and LJ-P3. An analytical marker (vertical line) was set in the red fluorescence channel to define 2% of the platelet population with the highest membrane fluorescence (quadrant 2, upper right). Panel B: Contour plot analysis of a blood sample collected during PTCA. Note the increase in fluorescence intensity (quadrant 2), corresponding to activated platelets. Quantification of this subpopulation revealed a sevenfold increase in particle number and a fivefold increase in PAC1 binding compared with baseline data. Binding of FITC-LJ-P3 to this platelet subpopulation remained unchanged, as determined by mean fluorescence intensity (115 arbitrary units in panel A and 112 in panel B). Contour lines represent numbers of platelets, with the innermost contours corresponding to the highest numbers of platelets.

Membrane Immunofluorescence

Membrane Immunofluorescence

mAbs

LJ-P4, an immunoglobulin G murine mAb specific for the GPIIb-IIIa complex on resting and activated platelets, was prepared and characterized as the previously reported anti-GPIIb-IIIa mAbs, LJ-P5 and LJ-P9.20 LJ-P4 is of the “complex-specific” variety and does not inhibit platelet aggregation. LJ-P3, a murine immunoglobulin G anti-GPⅠb mAb, which was obtained and characterized as described,30,31 partially inhibits binding of vWF to platelets.30

To identify and quantify activated circulating platelets, a panel of murine mAbs directed to distinct activation-dependent epitopes on the platelet membrane was used, including 1) PAC1, specific for the activated GPIIb-IIIa complex;21 2) 9F9, specific for surface-bound fibrinogen;32 3) anti-LIBS1 for ligand (e.g., fibrinogen)-induced binding sites on GPIIIa;33 and 4) S12 for P-selectin (GMP-140), a 140-kd α-granule membrane protein expressed on the platelet surface after secretion.22 These mAbs were generously provided by Dr. Sanford J. Shattil, Hospital of the University of Pennsylvania, Philadelphia, Pa. (PAC1); Dr. Andrei Z. Budzynski, Department of Biochemistry, Temple University School of Medicine, Philadelphia, Pa. (9F9); Drs. Mark H. Ginsberg and Edward F. Plow, The Scripps Research Institute, La Jolla, Calif. (anti-LIBS1); and Dr. Rodger P. McEver, Oklahoma Medical Research Foundation, Oklahoma City, Okla. (S12). Antibody preparations were pure, as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (data not shown).

mAbs were conjugated to fluorescein isothiocyanate (FITC, Calbiochem, La Jolla, Calif.) by standard techniques at a fluorescein to protein molar ratio of 7 to 1. PAC1, S12, and LJ-P3 were also prepared as biotinylated conjugates by standard techniques.34 Phycoerythrin-streptavidin (PE-SA, Becton-Dickinson, San Jose, Calif.) was used to quantify biotinylated antibody that was bound to platelets. mAbs were used at saturating concentrations determined previously.21,22,29,31-33

Specimen Collection

Blood was continuously withdrawn via a heparin-coated catheter (Baxter Laboratories) placed into the coronary sinus, and serial samples (n=8) were obtained.
FIGURE 2. Relation between blood cells exhibiting positive immunofluorescence and flow rate. Whole blood was incubated with a fluorescein isothiocyanate-conjugated anti-glycoprotein IIb-IIIa monoclonal antibody, LJ-P4, and analyzed for fluorescence intensity (FL1) and forward light scatter (FSC) at various flow rates. Platelets were differentiated from larger red (RBC) and white (WBC) blood cells by their characteristic light scatter and specific membrane immunofluorescence. At a flow rate of 1,000 cells per second (inset A), two distinct cell populations are identified by using a bidimensional contour plot of the distribution of FL1 versus FSC: quadrant 1 (upper left), containing cells with high FL1 and relatively low FSC (representing platelets), and quadrant 4 (lower right), containing cells with low FL1 and relatively high FSC (representing RBCs and WBCs). Under this condition, quadrant 2 (upper right) revealed a few cells with background immunofluorescence and relatively high FSC. Mean fluorescence intensity and particle number in quadrant 1 (open circles) and quadrant 2 (closed circles) were recorded at various flow rates. Insets B and C show contour plots of the same preparation analyzed at flow rates of 10,000 and 20,000 cells per second, respectively. Note the decrease of cells in quadrant 1 and the concomitant increase of cells with the same level of fluorescence intensity but higher FSC in quadrant 2. Cells in quadrant 2 of inset B are clearly separated with regard to particle size from the platelet population depicted in quadrant 1. However, at higher flow rate, fusion of both populations occurred (inset C). These changes were reversible with decreased flow rate, indicating their relation to coincidental detection of platelets and other blood elements rather than to formation of platelet aggregates. Each plot represents an analysis of 20,000 cells.

Before, during, and up to 30 minutes after PTCA, the catheter was inserted via jugular venous access immediately before the coronary interventional procedure. To ensure sampling ease, the catheter was connected externally with a 25-cm length of Teflon tubing, 3.2 mm in internal diameter (Silastic, Dow Corning Corp., Midland, Mich.), and a pump system (Harvard Apparatus Co. Inc., Dover, Mass.) set at a withdrawal rate of 1.0 ml/min. As described in detail previously, the Teflon-Silastic tubing, sterilized by autoclaving before placement, does not detectably activate platelets or thrombin. To assess the possibility of platelet activation by the heparin-coated catheter in the coronary sinus, peripheral venous blood samples were also drawn before PTCA from all patients by clean venipuncture with a 21-gauge butterfly infusion set (Miniset, Baxter, Deerfield, Ill.) with minimal tourniquet. The initial 2 ml of blood was discarded. Peripheral venous blood collections obtained by separate venipunctures were repeated at various time points during and after the coronary intervention procedure to allow comparisons between coronary sinus and peripheral blood specimens with respect to detection and quantification of activation-dependent platelet membrane epitopes. In all instances, blood specimens were obtained by using a multipletube sampling technique. The first 2.5 ml of blood, collected in disodium EDTA, was used for determination of platelet concentration and hematocrit by using a JT Baker model 9000 (Allentown, Pa.) whole-blood analyzer; the next 0.9 ml of blood was collected into a polypropylene syringe containing 0.1 ml of 3.8% trisodium citrate and used for flow cytometric analyses. In
addition, serial coronary sinus blood samples were collected and processed for radioimmunologic determination of plasma levels of β-thromboglobulin (βTG), platelet factor 4 (PF4), and fibrinopeptide A (FPA) as described previously.37-41

Preparation of Whole-Blood Samples for Flow Cytometric Analysis

Immunolabeling of platelets with mAbs was performed using minor modifications of previously published procedures24 that allow either one- or dual-color analysis by flow cytometry. Briefly, immediately after blood collection, 5-μl aliquots were added to polypropylene tubes (Becton-Dickinson) preloaded with 45 μl phosphate-buffered saline (PBS) and saturating concentrations of one of the following FITC-conjugated mAbs (final concentrations: PAC1, 30 μg/ml; 9F9, 40 μg/ml; anti-LIBSl, 25 μg/ml; S12, 10 μg/ml; LJ-P4, 5 μg/ml; and LJ-P3, 5 μg/ml). Nonspecific membrane immunofluorescence was determined by using an anti-human TG mAb. Autofluorescence of platelets was determined in an antibody-unlabeled whole-blood sample processed similarly. Samples were incubated in the dark, without stirring, for 15 minutes at room temperature, then diluted with 1 ml PBS and analyzed by flow cytometry within 2 hours of collection. Immunolabeling for dual-color analysis was performed in one of two ways. First, in initial experiments (patients 1 and 2; Table 1), FITC-LJ-P3 was used to identify platelets and platelet-derived microparticles, whereas PE-SA conjugate was used to detect binding of biotinylated mAbs to activation-dependent epitopes (Figure 1). Second, in patients 3–5, a combination of biotinylated LJ-P3 (followed by PE-SA) and FITC-conjugated activation-specific mAbs was used to correlate functional changes in membrane GP epitopes in activated platelet subpopulations, as determined by one-color analysis. In both cases, 5-μl aliquots of whole blood were incubated with the biotinylated primary mAbs for 15 minutes at room temperature, followed by the simultaneous addition of 50 μl PE-SA and FITC-conjugated mAb at a saturating concentration. After another incubation for 15 minutes at room temperature, samples were diluted with 1 ml PBS and analyzed.

Flow Cytometric Analysis of Platelets in Whole Blood

Blood samples were analyzed with a FACStar flow cytometer (Becton-Dickinson), equipped with a 2-W coherent argon-ion laser set at an excitation wavelength of 488 nm. The flow cytometer was calibrated with standard fluorescent microbeads (Flow Cytometry Standards Corp., Research Triangle Park, N.C.). A neutral-density filter (optical density of 0.65, transmission of 22.39%) was placed in front of the forward-side...
Figure 3. Time course of changes in the expression of activation-dependent platelet epitopes and plasma levels of β-thromboglobulin (βTG) in patients undergoing percutaneous transluminal coronary angioplasty. Binding of activation-specific antibodies, prepared as fluorescein isothiocyanate–conjugates, to the total platelet population was evaluated by one-color flow cytometric analysis in whole blood and expressed as log mean fluorescence intensity. Plasma levels of βTG were determined by radioimmunoassay. Panels A–D show data of patients No. 2–5 (Table 1). As indicated in panel A, S12-positive platelets were detected in several blood samples concurrently with the angiographically suspected dissection of a dilated coronary artery. Note that the expression of P-selectin (GMP-140) was associated with a minor and only transient increase in plasma βTG.

Scattering detector to optimize resolution. FITC and PE fluorescence was detected with a 530/30- and 575/26-nm band-pass filter, respectively, and overlap in the emission spectra of FITC (green) and PE (red) was corrected. Blood samples were analyzed at a flow rate of less than 2,000 cells per second as detailed in “Results.” Platelets and platelet-related microparticles were differentiated from red and white blood cells by their characteristic light scatter and specific membrane immunofluorescence. Data from 5,000 to 10,000 platelets and microparticles were recorded in a logarithmic mode and analyzed on a Hewlett-Packard H-P 217 computer (Palo Alto, Calif.) using a Consort 30 program (Becton-Dickinson).

Analysis of Activated Platelet Subpopulations and Platelet-Derived Microparticles

Antibody binding was expressed as the mean fluorescence intensity in arbitrary fluorescence units of the analyzed platelet population. To increase resolution of platelet activation, an analytical marker was set within the fluorescence profile for each antibody at baseline level (before PTCA) to define a 2% and 10% platelet subpopulation with the highest membrane immunofluorescence (Figure 1). This marker was used as a threshold to determine the proportion of platelets exhibiting immunofluorescence above these levels in all subsequent samples. Unless otherwise stated, data obtained by one-color analysis were used to quantify platelet activation. Platelet-derived microparticles were detected on the basis of their size, as assessed by forward light scatter and distinct immunofluorescence.32,42,43

Statistical Analyses

Statistical comparisons of the data were performed with the Wilcoxon matched-pairs signed-rank test. All data are given as the mean±SD. Differences were considered significant at p<0.05.

Results

Standardization of FAFC

In initial experiments, several potentially confounding variables for the FAFC analysis were evaluated, including 1) fixation of blood samples with paraformaldehyde; 2) collection of blood in the presence of prostaglandin E1 (PGE1); 3) time intervals between blood sampling, incubation with activation-dependent mAbs, and FAFC analysis; 4) drugs administered to patients undergoing PTCA; and 5) effects of flow rate on FAFC analysis of platelets in whole blood.

The effect of in vitro blood fixation on flow cytometric measurements was evaluated. Binding of LJ-P4, anti-LIBS1, and PAC1 to both resting and stimulated platelets (10 μM ADP or 1 μM phorbol myristate acetate [PMA]) in blood that was fixed with 1% paraformaldehyde before incubation with FITC-conjugated mAbs was significantly reduced compared with unfixed platelets. This inhibitory binding was also observed at paraformaldehyde concentrations as low as 0.1%. Similar observations have been reported by others24,32 studying the binding of PAC1 and S12 to activated platelets fixed with paraformaldehyde. Consequently, fixation of blood samples was avoided in the present study.

Spontaneous activation of platelets in vitro was studied in citrated whole-blood samples collected in the presence or absence of 10 or 100 μM PGE1. The samples were stored at room temperature and at various times up to 90 minutes; aliquots were incubated with fluoresceinated LJ-P4, anti-LIBS1, or PAC1 for 15 minutes at room temperature, diluted 20-fold with 1 ml PBS, and analyzed by flow cytometry. At time zero, no significant difference in mAb binding was observed between the samples. In blood samples collected without PGE1, there was a continuous increase in binding of FITC–LJ-P4 and in the percentage of platelets positive for binding of anti-LIBS1 or PAC1. The increased binding showed a bimodal distribution curve, with slow increases between time zero and 60 minutes (up to 1.2-fold only) and relatively rapid increases between 60 and 90 minutes (up to twofold). PGE1, at either concentration eliminated this effect. Moreover, PGE1, reversed spontaneous in vitro platelet activation (data not shown). Thus, PGE1, was not added to the anticoagulated blood samples from patients undergoing PTCA. Instead, blood aliquots were immediately mixed with antibody containing buffer, incubated for 15 minutes at room temperature, and then diluted 20-fold with PBS. In addition, throughout the study, control samples from healthy volunteers were always run in parallel to assess the possibility of spontaneous platelet activation during the labeling procedure. Increased incubation times to 30 minutes did not result in increased binding of anti-LIBS1 or PAC1. Membrane immunofluorescence levels remained stable for 6 hours, as shown by FAFC analyses performed at various time intervals after processing of blood samples. The relative coefficient of variation obtained for measurements of mean immunofluorescence was 4% for LJ-P4, 5% for anti-LIBS1, and 7% for PAC1. These data demonstrate that under the conditions employed, platelets maintain their initial binding levels of the specific mAbs.

We also assessed the possibility that either heparin or dextran administered periprocedurally to patients undergoing PTCA might interfere with binding of mAbs to platelets. The binding of mAbs to platelets in peripheral blood samples collected before and after the administration of heparin and dextran was unchanged. Moreover, in vitro additions of heparin and dextran to a level comparable to fivefold the estimated in vivo plasma peak levels showed no effect on binding of mAbs to...
The effects of flow rate on the FAFC analysis of platelets were also evaluated in preliminary studies with whole-blood samples by recording fluorescence intensity and forward light scatter at increasing flow rates (Figure 2). At flow rates exceeding 2,000 cells per second, the number of platelets identified by the positive membrane immunofluorescence and their typical light-scatter profiles decreased with reciprocal increases in the number of cells with a light-scatter profile of red blood cells and white blood cells exhibiting similar fluorescence levels. This effect indicated coincidental detection of platelets, red blood cells, and white blood cells at higher flow rates. Similar observations have been made by Shattil et al, who reported that about 5% of the total platelet population may be associated with leukocytes by using an anti-GPIIb mAb to identify platelets. However, since in these experiments flow rates of 10,000 cells per second were used, flow-coincidence artifact cannot be excluded.

Detection of Activated Platelets During PTCA

To examine whether platelet activation is directly detectable, five patients undergoing PTCA were analyzed by whole-blood FAFC and mAbs specific for activation-dependent platelet membrane epitopes. Before angioplasty, platelets bound minimal amounts of PAC1, S12, 9F9, and anti-LIBS1, similar to those found in platelets of control patients undergoing catheterization without PTCA and normal volunteers (Table 1). Moreover, no increased binding of these antibodies was observed with platelets in blood obtained by peripheral venipuncture (data not shown), indicating that the heparin-coated catheter used in this study did not induce detectable platelet activation. Similarly, plasma levels of platelet-secreted proteins (βTG and PF4) and FPA were comparable among specimens of blood obtained via catheter from the coronary sinus before PTCA and peripheral venipuncture (data not shown). Before PTCA, basal plasma levels of βTG or FPA were detectably elevated in three patients; a concomitant increase in plasma βTG and FPA was observed in one subject only (patient 3; Table 2). Although bolus heparin transiently mobilizes PF4 from its endothelial binding sites into the circulation over about 5 minutes, the sustained elevation in plasma levels of PF4 ranging from 19.4 to 72.0 ng/ml found in all five patients probably reflects platelet activation as opposed to the effect of heparin.

After angioplasty, activated platelets were identified in the coronary sinus blood obtained from four of the five patients, as evidenced by a mean increase in binding of PAC1 (2.6-fold) and anti-LIBS1 (2.7-fold). PTCA was associated with lesser increases in 9F9 binding (1.5-fold to twofold); in one patient increased binding with S12 was detected. Baseline and peak levels of the mean platelet membrane immunofluorescence intensity obtained with the different FITC-conjugated mAbs by one-color flow cytometric analysis are shown for each patient in Table 1. Evaluation of the GPIIb-IIIa complex using mAb LJ-P4 that binds to both resting and activated platelets revealed a concomitant mean increase of approximately 12% in the five patients undergoing PTCA. This increase in LJ-P4 binding may reflect a translocation of GPIIb-IIIa molecules from the α-granule membrane to the platelet surface on activation.

Mean platelet membrane immunofluorescence intensity of binding of the different activation-specific mAbs to platelets in whole blood, plasma levels of βTG and PF4, and their course in relation to coronary angiography and angioplasty are depicted in Figure 3. No correlation was found between the expression of activation-dependent platelet epitopes and platelet secretion, as determined by βTG and PF4 release, as shown in Tables 1 and 2 and Figure 3. For example, an approximately 10-fold increase in binding of S12 observed after PTCA in several blood specimens over a time period of 20 minutes was associated with a minor and only transient increase in plasma βTG (Figure 3A). In another patient, even at peak levels of plasma βTG, no S12-positive platelets were detectable in coronary sinus blood samples after PTCA (Figure 3B and Table 1). This discrepancy in results was not obviously attributable to concurrently administered medications capable of modifying platelet function, although drug effects could not be excluded.

Detection of activated circulating platelets after PTCA, as documented by FAFC analysis, was restricted to the blood specimens obtained from the coronary sinus. In peripheral venous blood samples that were obtained and analyzed in parallel, no activated circulating platelets could be identified.

Subpopulations of Activated Platelets and Platelet-Derived Microparticles

To quantify the relative proportion of activated platelets, for each antibody an analysis marker within the fluorescence profile was set at baseline level (before PTCA) to define a 2% and 10% platelet subpopulation with the brightest membrane immunofluorescence (Figure 4). This procedure permitted the analysis of a distinctly activated platelet subpopulation for increases in relative number and fluorescence intensity. In addition, a separate proportion of GPIIb-positive microparticles before and after PTCA was quantified.

In the five patients undergoing PTCA, the relative proportion of platelets positive for PAC1 and anti-LIBS1 increased from 2.0±0.3% and 2.0±0.5% at baseline to 18.2±14.2% and 28.0±14.4%, respectively (p<0.01 in both cases). A lesser increase (2.0±0.2% at baseline to 6.7±2.5%) was observed in the relative proportion of platelets that had expressed the epitope in platelet-bound fibrinogen recognized by 9F9 (Figures 5A and 5B). Compared with the mean fluorescence levels obtained with the different mAbs for the total platelet population, the analysis of a 2% and 10% subpopulation with the highest fluorescence levels at baseline did not improve sensitivity for detecting activated platelets but increased the resolution of platelet activation results. This is illustrated for the individual patients by the data depicted in Figure 6A, demonstrating quantitative functional changes in membrane GP epitopes of activated platelet subpopulations after PTCA. The improved resolution of activation obtained by analysis of platelet subpopulations was also evident when baseline and peak levels of fluorescence intensity from all five patients undergoing PTCA were averaged (Figure 6B). For example, by comparing pre-PTCA and...
Membrane Immunofluorescence

FIGURE 4. Flow cytometric quantification of activated-platelet subpopulations in patients undergoing percutaneous transluminal coronary angioplasty (PTCA). For each monoclonal antibody, a gate within the fluorescence profile at baseline level (---) was set to define a 2% (left panels) and 10% (right panels) platelet subpopulation as indicated. Increase in platelet number with high fluorescence intensity was detected in blood obtained during PTCA (- - - -). Representative histograms are shown for immunofluorescence of fluorescein isothiocyanate (FITC)-PAC1, FITC-9F9, and FITC-anti-LIBS1. Histograms representing the binding of S12 to platelets are taken from patient No. 2 (Table 1 and Figure 3A).

post-PTCA data, the amount of PAC1 binding to the activated GPIIb-IIIa complex increased 2.6-fold in the total platelet population and 3.4-fold in the platelet subpopulation with the brightest fluorescence at baseline. Corresponding mean rates for binding of anti-LIBS1 were 2.7 and 4.5, and 1.8 and 2.3 for binding of 9F9.

The mean proportion of platelet-derived microparticles was 16±4% of the total platelet population at baseline (before PTCA). Angioplasty caused a minor increase in the relative proportion of microparticles, i.e., 20±6% (p>0.05). These changes were not associated with a detectable change in platelet concentration in whole blood.
FIGURE 5. Effect of percutaneous transluminal coronary angioplasty (PTCA) on relative proportion of activated-platelet subpopulations. Binding of fluorescein isothiocyanate (FITC)-PAC1, FITC-9F9, and FITC-anti-LIBS1 was evaluated by one-color flow cytometric analysis in five patients undergoing PTCA. As illustrated in Figures 1 and 4, a 2% platelet subpopulation with the brightest membrane fluorescence intensity at baseline was analyzed separately and quantified for relative increase in platelet number after PTCA. Panel A: Data depict the relative proportion of activated platelets in each individual patient before and during/after PTCA. Panel B: Corresponding bar graph of mean values ±SD of the relative proportion of activated-platelet subpopulations before and after PTCA is shown. Probability values for increased binding of PAC1, anti-LIBS1, and 9F9 were <0.01, <0.01, and <0.03, respectively.

Discussion

The present study shows that PTCA-induced injury at sites of coronary artery atherosclerotic stenosis activates circulating platelets, as detected by flow cytometric analysis, despite aspirin pretreatment. The panel of mAbs directed to distinct activation-specific platelet epitopes permits the definition of platelet subpopulations exhibiting activation markers in blood emerging from vessels undergoing PTCA. PTCA is a useful clinical model to investigate platelet activation in humans, since this vascular insult is localized, well characterized experimentally, and occurs prospectively.

An important prerequisite for this study was the availability of the heparin-coated coronary sinus catheters. These catheters permitted collection of blood specimens at close proximity to the site of mechanical vascular injury. The relevance of this vascular access for collection of representative blood samples is demonstrated by the fact that no activated platelets were detectable in blood specimens obtained throughout the study by peripheral venipuncture. This striking difference in results may be due to at least two facts. First, once activated, platelets may be immediately removed from the circulation and therefore not be detectable. Second, direct demonstration of activated platelets by FAFC requires the presence of at least 0.8% activated platelets in a whole-blood sample.24,66 Thus, subpopulations of activated platelets that continue to circulate after expression of activation-dependent epitopes would not be detectable in peripheral blood samples because of dilution within the systemic circulation. The heparin-coated surface of the coronary sinus catheter was shown to prevent catheter-induced activation of the hemostatic apparatus. Thus, no significant binding of activation-dependent mAbs was found in blood samples obtained either before angioplasty or throughout angiography without PTCA. Since these patients underwent the same invasive diagnostic procedures, which included coronary angiography and permitted collection of serial blood samples over a period of approximately 45 minutes, they served as appropriate controls in the study design. This finding corroborates the results of βTG and PF4 plasma levels that, although elevated, remained unchanged in the control patients (Table 2). Therefore, any change in platelet activation markers detected in patients undergoing PTCA may be attributable to this coronary intervention procedure.

The present study demonstrates that expression of activation-dependent platelet membrane epitopes is detectable only in a portion of the total platelet population, which prompted the performance of separate analyses of these platelet subpopulations for increases in particle number and fluorescence intensity. The relative proportion of platelets positive for one or more activation-specific mAbs increased up to 20-fold after PTCA, as compared with baseline before angioplasty (Figure 5A). Analysis of platelet subpopulations for binding of FITC-PAC1, FITC-9F9, or FITC-anti-LIBS1 also permitted the quantification of functional changes in platelet membrane epitopes after PTCA. Compared with FAFC analysis of the total platelet population, this procedure resulted in an improved resolution of platelet activation (Figures 6A and 6B).

The activation of platelets following PTCA was accompanied by a detectable increase in the formation of platelet-derived microparticles, although patients un-
deteriorating cardiopulmonary bypass exhibit greater relative increases in platelet-derived microparticles. This difference in results may largely reflect the difference in the magnitude of the stimulus for platelet activation rather than a difference in the mechanism of platelet activation by the cardiopulmonary bypass apparatus.

The mAbs employed in the present study are directed to distinct activation-dependent epitopes on the platelet surface and could, therefore, reflect different aspects of the platelet activation process. The affinity of the antibody for its platelet epitope is another variable that will affect the amount of binding. After PTCA, binding of FITC-PAC1, FITC-9F9, and FITC-anti-LIBS1 increased concordantly in four of the five patients, as evidenced by an increase in mean platelet membrane immunofluorescence (Table 1 and Figures 3 and 6). The increase in binding of these mAbs to the platelet surface indicates expression of the activated GPIIb-IIIa complex, receptor occupancy by ligands, and expression of ligand-induced binding sites on GPIIIa.

Unlike anti-LIBS1 and PAC1, detection of an epitope expressed on platelet-bound fibrinogen turned out to be of lower sensitivity, as evidenced by the minor increase of activated platelets positive for binding of 9F9 after PTCA (Figures 5A and 5B). The reason for this finding is unclear at present; however, several possible explanations may be considered. First, the affinity of 9F9 to its epitope appears to be significantly lower than that of PAC1 or anti-LIBS1. Second, 9F9 specifically reacts with fibrinogen bound to its activated receptor on the platelet surface. Since binding of fibrinogen to GPIIb-IIIa is initially reversible, only a proportion of activated platelets may be recognized by 9F9. Conversely, activated platelets that have irreversibly bound fibrinogen may interact with each other at the site of vascular injury to form a platelet aggregate or be removed from the circulation. Finally, it is possible that ligands other than fibrinogen are bound to the functional receptor. In any event, an FAPC assay with 9F9 would fail to detect this fraction of activated platelets.

Increased binding of antibody S12 was obtained in one of five patients only. This antibody is directed to a granule membrane GP, P-selectin (GMP-140), expressed on the platelet membrane surface only after a-granule release. S12, therefore, allows monitoring of platelets that have undergone secretion. The FAPC results obtained with this antibody appear to be inconsistent with the increase in plasma βTG after PTCA (Table 2 and Figure 3A). This discrepancy in results cannot simply be attributed to the variety of medications, although a drug-related effect cannot be excluded. Absent or inconsistent expression of P-selectin has also been observed in patients undergoing cardiopulmonary bypass. A clinical setting in which secretion of platelet-specific proteins has been demonstrated. This discrepancy may result from the fact that activated platelets, which have undergone a-granule release and thereby expressed P-selectin, are only transiently present in the circulation. Alternatively, redistribution or endocytosis of P-selectin may occur, thereby reducing the accessibility to S12 antibodies. If in fact P-selectin is rapidly removed from the platelet surface, then any assays that are dependent on its detection may have insufficient sensitivity. Indeed, it has been suggested that plasma assays for secreted platelet-specific proteins may be more sensitive than are direct flow cytometric analyses.

In contrast to our findings, it has recently been reported that P-selectin-positive platelets were detectable in peripheral venous and coronary sinus blood from patients undergoing angioplasty. These observations suggest that platelets that have undergone secretion and thereby expressed P-selectin on the platelet surface may not be rapidly removed from the circulation. Interestingly, the highest levels of P-selectin ex-
pression after PTCA were observed in those patients who developed acute vascular occlusion and/or myocardial infarction remote from the procedure. The expression after PTCA were observed in those patients who developed acute vascular occlusion and/or myocardial infarction remote from the procedure. The expression after PTCA were observed in those patients who developed acute vascular occlusion and/or myocardial infarction remote from the procedure. The expression after PTCA were observed in those patients who developed acute vascular occlusion and/or myocardial infarction remote from the procedure. The expression after PTCA were observed in those patients who developed acute vascular occlusion and/or myocardial infarction remote from the procedure. The expression after PTCA were observed in those patients who developed acute vascular occlusion and/or myocardial infarction remote from the procedure. The expression after PTCA were observed in those patients who developed acute vascular occlusion and/or myocardial infarction remote from the procedure. The expression after PTCA were observed in those patients who developed acute vascular occlusion and/or myocardial infarction remote from the procedure. The expression after PTCA were observed in those patients who developed acute vascular occlusion and/or myocardial infarction remote from the procedure. The expression after PTCA were observed in those patients who developed acute vascular occlusion and/or myocardial infarction remote from the procedure. The expression after PTCA were observed in those patients who developed acute vascular occlusion and/or myocardial infarction remote from the procedure. The expression after PTCA were observed in those patients who developed acute vascular occlusion and/or myocardial infarction remote from the procedure. The expression after PTCA were observed in those patients who developed acute vascular occlusion and/or myocardial infarction remote from the procedure. The expression after PTCA were observed in those patients who developed acute vascular occlusion and/or myocardial infarction remote from the procedure. The expression after PTCA were observed in those patients who developed acute vascular occlusion and/or myocardial infarction remote from the procedure. The expression after PTCA were observed in those patients who developed acute vascular occlusion and/or myocardial infarction remote from the procedure. The expression after PTCA were observed in those patients who developed acute vascular occlusion and/or myocardial infarction remote from the procedure. The expression after PTCA.


Activation of platelets in blood perfusing angioplasty-damaged coronary arteries. Flow cytometric detection.

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doi: 10.1161/01.ATV.12.12.1475

*Arteriosclerosis, Thrombosis, and Vascular Biology* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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