CLINICAL INVESTIGATIONS

Platelet Activation and Aggregation during Cardiopulmonary Bypass

Christine S. Rinder, M.D.,* John Bohnert, M.D.,† Henry M. Rinder, M.D.,‡
Jane Mitchell,§ Kenneth Ault, M.D.,¶ Robert Hillman, M.D.**

Increases in plasma concentrations of platelet granule products such as platelet factor 4 and β-thromboglobulin during cardiopulmonary bypass suggest that platelets are activated during extracorporeal circulation. Subsequent circulation of these activated platelets may be responsible for the ubiquitous platelet dysfunction associated with cardiopulmonary bypass. Using flow cytometry and a monoclonal antibody directed against an α-granule membrane protein, granule membrane protein 140 (GMP-140), which is expressed on the platelet surface membrane after activation, we directly measured the percentage of circulating activated platelets in 41 patients before, during, and after cardiopulmonary bypass. In addition, we compared the GMP-140 expression with platelet aggregation in response to adenosine diphosphate (ADP). Cardiopulmonary bypass produced a significant increase in the percentage of GMP-140-positive platelets persisting in the circulation; the percentage peaked at a mean of 29% (range 10–58%) before separation from extracorporeal circulation. A significant percentage of these activated platelets continued to circulate in the early postoperative period. Simultaneous measurement of platelet aggregation in response to ADP demonstrated an aggregation defect that had a time course distinct from platelet activation and whose magnitude did not correlate with the degree of platelet activation in individual patients. We conclude that cardiopulmonary bypass causes a complex constellation of platelet defects, which include α-granule release, prolonged circulation of activated, "spent" platelets, and impaired platelet aggregation. (Key words: Blood, coagulation: platelet activation; platelet aggregation; platelet glycoproteins. Measurement technique: flow cytometry. Surgery, cardiac: cardiopulmonary bypass.)

CARDIOPULMONARY BYPASS (CPB) produces changes in platelet structure and function that may contribute to a

---

* Assistant Professor of Anesthesiology, Department of Anesthesiology, Yale University School of Medicine, New Haven, Connecticut.

† Resident, University of Utah School of Medicine, Salt Lake City, Utah.

‡ Fellow, Department of Hematology, Yale University School of Medicine, New Haven, Connecticut.

§ Research Assistant, Maine Cytometry Research Institute, South Portland, Maine.

¶ Director, Maine Cytometry Research Institute, South Portland, Maine.

** Chief of Medicine, Maine Medical Center, Portland, Maine.

bleeding diathesis postoperatively. After CPB, platelets are unable to aggregate normally in response to weak agonists such as adenosine diphosphate (ADP) and epinephrine and show a decreased response to lower doses of the strong agonist collagen.[1–3] Electron microscopic studies of platelets from CPB patients show shape change[4] and loss of granule contents[5] consistent with platelet activation. This is supported by reports of increased serum concentrations of platelet factor 4, β-thromboglobulin, and thromboxane B_2 during bypass.[4,6] A decrease in releasable ADP from platelets during CPB[7] suggests that one source of platelet dysfunction associated with CPB may be the persistent circulation of activated degranulated platelets. Recent evidence suggests, however, that activated platelets are cleared preferentially from the circulation.[8] If this is the case, activated platelets produced during bypass would have a short half-life and should contribute only to postoperative thrombocytopenia and not to the qualitative aggregation defect that persists postoperatively.

It is now possible to measure the expression of platelet activation antigens on the platelet surface using monoclonal antibodies and flow cytometry.[9] Platelet α-granule release is associated with fusion of the granule membrane with the surface membrane. A 140-kD protein, granule membrane protein 140 (GMP-140), is found exclusively on the inner surface of the α granule in the unstimulated platelet and with activation can be measured as a stable activation antigen on the platelet surface.[10,11] GMP-140 therefore serves as a marker to identify platelets in the circulation that have undergone α-granule release, i.e., platelets that are are activated.

We used 1E3, which is a murine monoclonal antibody specific for GMP-140, and flow cytometry to detect the presence of circulating activated platelets in whole blood of patients undergoing CPB. If activated platelets continued to circulate in significant numbers, we could then explore the contribution of these circulating activated platelets to the platelet dysfunction produced by CPB. Unlike the products of α granule release, such as β-thromboglobulin, which provide an index of total activation summed over time, this technique permits measurement of the fraction of circulating degranulated (activated) platelets at any given point in time. By measuring

the percentage of activated platelets in a sample of 10,000 individually analyzed platelets, this technique is not affected by hemodilution, unlike measurement of serum concentrations of β-thromboglobulin, platelet factor 4, and thromboxane B2. Therefore, we also could estimate the rate of clearance of activated platelets after termination of CPB and its relation to postoperative bleeding. In addition, by simultaneously measuring platelet aggregation and the percentage of circulating activated platelets at different time points, we were able to investigate whether platelet activation and the platelet aggregation defect produced on CPB evolve in parallel. Both platelet activation and the aggregation response to ADP also were compared to chest-tube drainage to determine their contributions to postoperative blood loss.

Materials and Methods

All patient studies were approved by the Maine Medical Center institutional review board. After informed consent was obtained, 41 consecutive patients undergoing elective procedures requiring CPB were studied. Thirty-six of these were undergoing coronary artery bypass grafting and 5 aortic valve replacements. Patients undergoing repeat coronary artery bypass surgery were excluded. All patients had a preoperative template bleeding time within normal limits (less than 8 min).

Patients were anticoagulated with heparin to an activated clotting time greater than 400 s and cooled to 26° C during CPB using a membrane oxygenator at comparable flow rates. Whole-blood samples were drawn from the radial artery catheter at the following time points: immediately before incision; 5 min postanticoagulation; 5, 15, 30, and 60 min after the start of bypass; 5 min after protamine administration had titrated to an activated clotting time within 10 s of baseline; 2–4 h after termination of bypass; and 18 h after bypass. Preliminary work had demonstrated no difference in platelet activation or aggregation in samples drawn 5 min after anticoagulation with heparin or immediately before CPB, so the latter time point was not included in the current study.

A 50-μl aliquot of whole blood was placed immediately in 2% paraformaldehyde in phosphate-buffered saline for baseline studies; a second aliquot was stimulated with 10 μM ADP for five min and then fixed in 2% phosphate-buffered saline. Fixed samples were stored at 4° C for 1 h and then prepared as described below. Whole blood samples were analyzed also for hematocrit, hemoglobin, and platelet count by an automated blood cell counter (Coulter Electronics, Hialeah, FL), and platelet counts less than $100 \times 10^9/l$ were confirmed by manual counting. Blood loss was assessed by measuring chest tube drainage in the first 24 h after bypass. The number of packed red blood cell units and platelet transfusions received in the same time period was recorded.

Monoclonal Antibodies

1E3, a murine monoclonal antibody of the immunoglobulin G1 (IgG1) subclass, recognizes a 140-kD glycoprotein (GP) that becomes associated with the platelet surface during α granule secretion. This GP has been designated GMP-140,[8] or platelet-activation-dependent granule external membrane (PADGEM)[12] protein. The 1E3 monoclonal antibody was biotinylated in the standard fashion.[9] The monoclonal antibodies anti-GPIb and anti-GPIIb/IIIa (AMAC, Westbrook, ME) were conjugated to fluorescein isothiocyanate (FITC) and used as platelet markers in whole-blood samples as previously described.[13] Preliminary studies demonstrated that during CPB, no platelets were produced that completely lacked either GPIb or GPIIb/IIIa. Because of its greater number of receptors on the platelet surface, the majority of patient studies used GPIIb/IIIa as the primary platelet marker.

Flow Cytometric Analysis

After incubating for 1 h at 4° C, fixed samples were washed and incubated with a saturating concentration of biotinylated 1E3 and FITC-anti-GPIb or FITC-anti-GPIIb/IIIa, as described previously.[9] The sample was then washed, and 20 μl phycoerythrin (PE)-conjugated avidin (Becton Dickinson Immunocytometry Systems, San Jose, CA) was added for an additional 20 min. Samples were washed again in Tyrode's buffer and then analyzed in the FACScan flow cytometer (Becton Dickinson Immunocytometry Systems). The instrument was set to measure forward light scatter (FSC) as an index of particle size and to measure FITC and PE fluorescence for platelet markers and GMP-140 expression, respectively. Platelets were identified in whole-blood samples by triggering the cytometer on size and FITC (GPIIb/IIIa or GPIb) fluorescence.[9] Analysis of 10,000 platelets was performed, and the percentage of activated platelets was determined by the proportion of platelets that expressed PE fluorescence greater than background (fig. 1). Analysis of platelet aggregation on the FACScan was performed by identifying the upper limit of FSC for single platelets based on platelets drawn from normal volunteers into EDTA anticoagulant, as described previously.[14] ADP-stimulated (10 μM) patient samples were then analyzed for forward scatter versus FITC (GPIb or GPIIb/IIIa) fluorescence, and the percentage of platelets exceeding the limit for single platelets was recorded as platelet aggregates (fig. 2). We have demonstrated previously that flow cytometric measurement of platelet aggregates correlates well with standard platelet aggregation measurements.[14]

The percentages of activated and aggregated platelets for baseline and ADP-stimulated samples were plotted over time; all patient values for each time point were pooled and subjected to analysis of variance. Baseline and ADP-stimulated values were compared with chest-tube
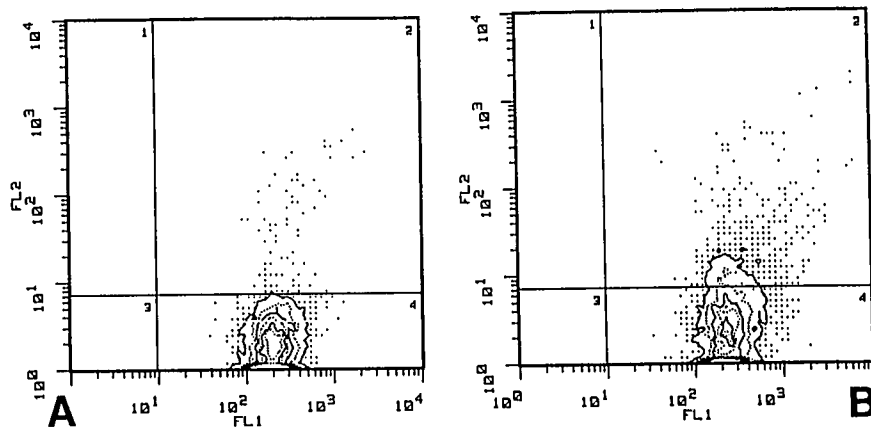
FIG. 1. Contour graphs of platelets in whole blood by flow cytometry. *A:* Whole blood drawn from a patient prior to the start of CPB. Events to the right of the vertical line represent platelets by virtue of their binding the monoclonal antibody to GPIIb/IIIa. Platelets above the horizontal line also express the activation-specific glycoprotein GMP-140. In *A*, 4% of the platelets are activated. *B:* The platelets in the same patient at the end of CPB, with 27% of platelets now expressing GMP-140. Note that all of the scales are logarithmic.

drainage by a two-tailed t test. The sample size did not permit multivariate analysis.

Results

The percentage of GMP-140-positive, *i.e.*, activated, platelets in whole blood increased significantly during CPB (Figure 3). The baseline sample (7%) and the postanticoagulation sample taken after sternotomy and right atrial cannulation (6%) were not significantly different. However, by 5 min after the initiation of CPB, there was a significant increase in platelet activation that progressed with duration of CPB. Just before termination of CPB, the percentage of activated platelets peaked at a mean of 29% (range 10–58%). Peak levels of platelet activation did not, however, correlate with CPB duration, which averaged 83 min (r = 0.4). Protamine administration did not immediately change the level of platelet activation. By 2–4 h after CPB, the percentage of activated platelets had decreased (19%) but remained significantly higher than baseline; at 18 h it approached the baseline value (11%). The platelet count, even when corrected for dilution based on the hematocrit, decreased significantly as

early as 10 min after the start of CPB, increased slightly at the end of bypass, and then stabilized at a lower value for the remainder of the study period (fig. 4). Aortic valve replacement patients (n = 5) were analyzed separately and found not to differ significantly from coronary artery bypass grafting patients (n = 36) in terms of peak activation levels, change in platelet count, or chest-tube drainage in the postoperative period.

The aggregation response of platelets to 10 μM ADP at different time points during CPB is shown in figure 5. Aggregation decreased significantly 10 min after the start of CPB and decreased further by the end of CPB. It reached a nadir 1–4 h after termination of CPB and then returned to a value not significantly different from baseline by 18 h after surgery. The decrease in ADP-induced platelet aggregation at the end of CPB did not correlate (r = −0.06) with the degree of platelet activation at the same time point in individual patients (fig. 6). Similarly, at its nadir 2–4 h after CPB, the decrease in platelet aggregation did not correlate with platelet activation at the same time point. Ten patients were receiving sodium nitroprusside for control of postoperative hypertension at the 2–4-h postoperative time point. When these patients
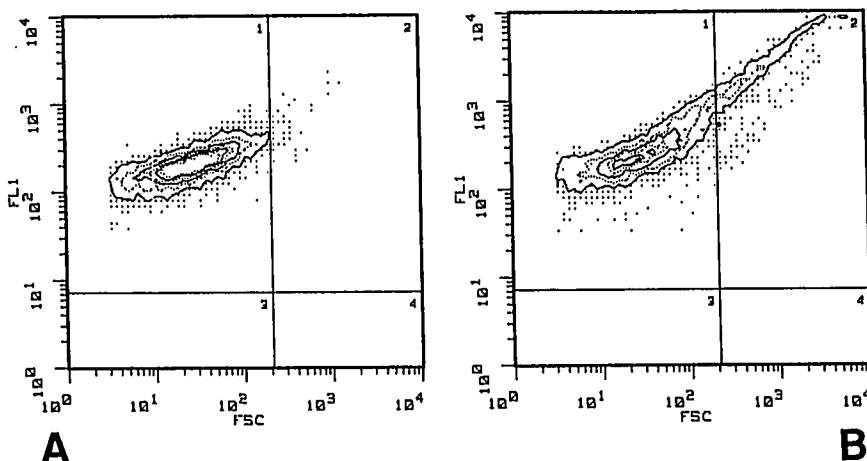

FIG. 2. These contour graphs show forward scatter (FSC), a measure of size, in the x-axis and GPIIb/IIIa binding in the y-axis. *A:* This graph, a sample of unstimulated whole blood, defines the upper limit of the size of single platelets, denoted by the vertical line. *B:* A sample of whole blood stimulated with ADP; the events to the right of the vertical line represent aggregated platelets, comprising 35% of the total. Note that all of the scales are logarithmic.
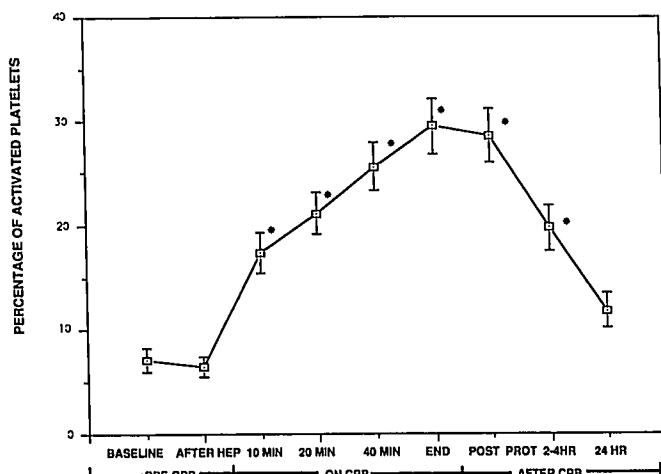
FIG. 3. Percentage of circulating activated platelets in whole blood at each of the various time points before, during, and after CPB. Data are means ± SEM. HEP = heparin; PROT = protamine. Samples are compared to baseline by ANOVA (*$P < 0.05$).

FIG. 5. Percentage of aggregated platelets in response to 10 μM ADP at the various time points. Shown are the means ± SEM. Samples are compared to baseline by ANOVA (*$P < 0.05$).

were excluded from the analysis (to eliminate any additive antiaggregatory effects of the nitroprusside[15]), there still was no correlation between activation and aggregation.

Chest tube drainage was recorded in all patients for the first 24 h after surgery. Two patients required reexploration for excessive bleeding, and one of the two was found to have arterial bleeding; his data was eliminated from the study. Six patients received platelet transfusions; 3 of these 6 also received cryoprecipitate, and 2 received both cryoprecipitate and fresh frozen plasma. All transfusions were at the discretion of the cardiothoracic surgeon for perceived excessive bleeding and were analyzed separately; they were not found to differ significantly from
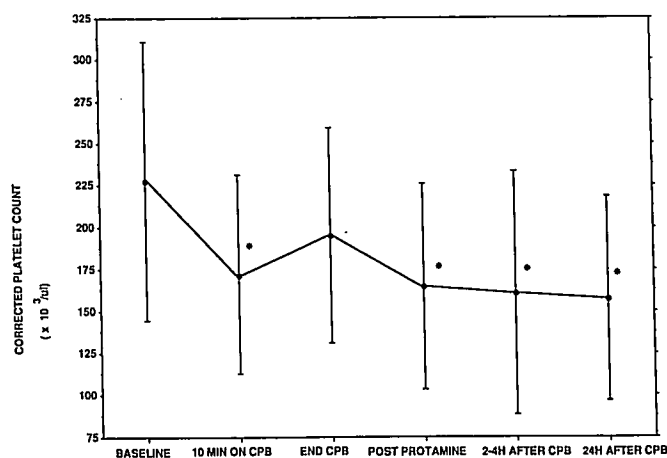
the other study patients in terms of activation or aggregation either before or after their transfusion. Three patients received aspirin and one received a nonsteroidal antiinflammatory drug the week before their surgery. All had a normal preoperative template bleeding time and showed comparable platelet activation and aggregation impairments during CPB when analyzed separately. Chesttube drainage averaged 1200 ml; values exceeded 2000 ml in only 2 of 40 patients. There was no correlation between chest tube drainage and 1) the peak percentage of circulating activated platelets (r = −0.2), 2) the nadir

FIG. 4. Platelet count corrected for hemodilution based on the change in hematocrit. Data are means ± SD. Samples are compared to baseline by ANOVA (*$P < 0.05$).
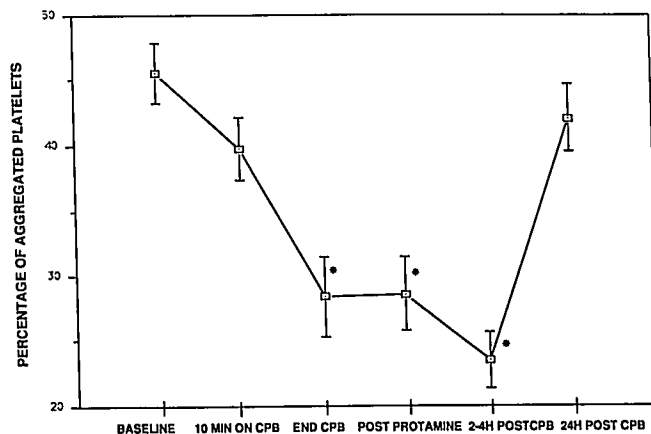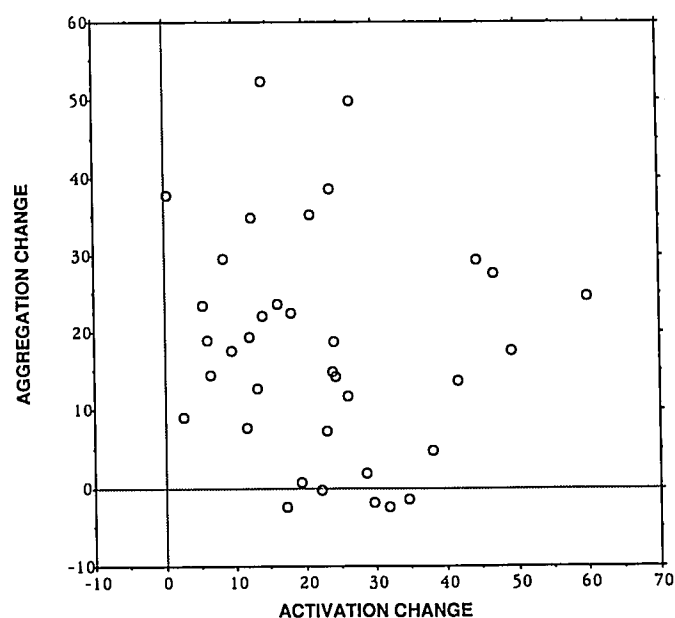

FIG. 6. Correlation between change in activation and change in aggregation (absolute value) between baseline and the end of CPB. No correlation was observed at r = 0.03.

of the aggregation defect (r = 0.03), or 3) the platelet count (r = −0.07).

Discussion

Persistent circulation of activated platelets and abnormalities in platelet aggregation have been postulated as a cause of excessive bleeding after CPB.[5] Other investigators have shown that CPB is associated with platelet activation based on increases in serum platelet factor 4, thromboxane B_2, and urinary β-thromboglobulin.[4,6] These proteins accumulate in the serum and urine after platelet activation by both surgical stimulation and CPB and thus neither provide information about the timing of activation during the procedure nor indicate whether these activated platelets remain in the circulation. The current study used a monoclonal antibody to GMP-140, an α-granule marker that has been shown to be found exclusively on the surface of platelets that have undergone α-granule release.[10,11] It serves, therefore, as a specific marker for platelet activation. Recent studies have suggested that GMP-140 expression is correlated also with increased platelet clearance,[8] indicating that under normal circumstances, activated platelets do not remain in the circulation but rather are actively cleared. Using flow cytometry, we were able to directly measure platelet activation in whole-blood samples at frequent time points during and after CPB to detect more precisely the time course of platelet activation and the development of the aggregation defect.

This study clearly demonstrates that during CPB, platelets undergo progressive activation characterized by α-granule release and that the activation affects an average of nearly one third of circulating platelets and as much as 50% of the platelets in selected patients at the termination of CPB. Surgical stimulation before heparin administration, including graft and internal mammary harvesting and sternotomy, did not increase the number of activated platelets remaining in the circulation. After CPB, activated platelets continued to circulate in numbers significantly higher than at baseline, though lower than during CPB. These "spent" platelets may be incapable of contributing their α granule contents, such as thrombospondin, to the platelet aggregate, thereby potentially rendering it more vulnerable to disaggregation by proteases. The percentage of circulating activated platelets at the end of CPB did not correlate with CPB duration, preoperative drug exposure, or platelet transfusion.

Using the technique of ADP stimulation of platelets to measure ability to aggregate, it also was possible to demonstrate the development of an aggregation defect during CPB. This has been reported previously by a number of investigators using standard aggregometry. In our study, however, the timing of the peak aggregation defect did not match that of the peak percentage of platelet activa-

tion. Moreover, there was no correlation between the degree of activation and the magnitude of the aggregation abnormality, suggesting that additional factors other than platelet activation operate during CPB and result in a primary defect in platelet aggregation. This aggregation defect had reversed by 18 h after surgery, at the same time as the number of activated platelets had declined, reflecting either some degree of functional platelet "recovery," or the influx of new platelets from the bone marrow and splenic pools. Neither the peak percentage of activated platelets nor the aggregation defect predicted postoperative bleeding; however, the number of patients with major postoperative bleeding was small. A larger study with a longer average duration of CPB might reveal synergistic action of both the primary aggregation defect and the level of α-granule-depleted platelets, correlating with major bleeding diatheses.

Likely candidates for the pathophysiology of the aggregation defect independent of activation include destruction of platelet surface adhesion receptors. Plasmin is capable *in vitro* of platelet activation and cleavage of both the von Willebrand binding site, GPIb, and the fibrinogen binding site, GPIIb/IIIa.[16,17] The success of the antiplasmin agent aprotinin in reducing post-CPB blood loss[18,19] therefore may result from its ability to preserve both GPIb-dependent platelet adhesion and GPIIb/IIIa-dependent platelet–platelet interaction. In addition, if aprotinin inhibits plasmin-induced platelet activation *in vivo*, then more platelets would be capable of contributing their granule contents to the formation of a stable, protease-resistant clot. It would be of interest to determine in the *in vivo* clinical setting which of these mechanisms is the predominant beneficial effect of aprotinin.

In summary, we have shown that CPB (but not surgery) produced a significant increase in the level of circulating activated platelets, such that by the end of CPB, an average of 29% of platelets remaining in the circulating had undergone α-granule release. By 2–4 h after termination of CPB, the percentage of activated platelets had decreased but remained significantly greater than normal baseline values, demonstrating either that these activated platelets are cleared slowly relative to other clinical settings or that their persistent activation occurs after separation from bypass. Platelets also developed an aggregation defect that was temporally and qualitatively distinct from platelet activation. Neither measurement alone correlated with chest tube drainage during the first 24 h. This study has determined that CPB produces multiple distinct functional platelet defects and provides a critical foundation for further clinical investigation.

References

1. Friedenberg WR, Myers WO, Plotka ED, Feathard JN, Kummer DJ, Gatlin PF, Stoiber DL, Ray JF Sautter RD: Platelet dys-

function associated with cardiopulmonary bypass. Ann Thorac Surg 25:298–305, 1978

2. McKenna R, Bachmann F, Whittaker B, Gilson JR, Weinberg M: The hemostatic mechanism after open-heart surgery. J Thorac Cardiovasc Surg 70:298–308, 1975

3. Mammen EF, Koets MH, Washington BC, Wolk LW, Brown JM, Burkick M, Selik NR, Wilson RF: Hemostasis changes during cardiopulmonary bypass surgery. Semin Thromb Hemost 11: 281–292, 1985

4. Zilla P, Fasol R, Groscurth P, Klepetko W, Reichenspurner H, Wolner E: Blood platelets in cardiopulmonary bypass operations. J Thorac Cardiovasc Surg 97:379–88, 1989

5. Harker LA, Malpass TW, Branson HE, Hessel EA, Slicter SJ: Mechanism of abnormal bleeding in patients undergoing cardiopulmonary bypass: acquired transient platelet dysfunction associated with selective α-granule release. Blood 56:824–834, 1980

6. Mezzano D, Aranda E, Urzua J, Lema G, Habash J, Irarrazabal M, Pereira J: Changes in platelet β-thromboglobulin, fibrinogen, albumin, 5-hydroxytryptamine, ATP, and ADP during and after surgery with extracorporeal circulation in man. Am J Hematol 22:133–142, 1986

7. Beurling-Harbury C, Galvan CA: Acquired decrease in platelet secretory ADP associated with increased postoperative bleeding in post-cardiopulmonary bypass patients and in patients with severe valvular heart disease. Blood 52:13–23, 1978

8. Rinder HM, Murphy M, Mitchell J, Stocks J, Hillman R, Ault KA: Progressive platelet activation with storage: evidence for shortened survival of activated platelets following transfusion. Transfusion 31:409–414, 1991

9. Shattil SJ, Cunningham M, Hoxie JA: Detection of activated platelet in whole blood using activation-dependant monoclonal antibodies and flow cytometry. Blood 70:307–315, 1987

10. Johnston GI, Kurosky A, McEver RP: Structural and biosynthetic studies of the granule membrane protein, GMP-140, from human platelets and endothelial cells. J Biol Chem 264:1816–1823, 1989

11. Johnston GI, Cook RG, McEver RP: Cloning of GMP-140, a granule membrane protein of platelets and endothelium: sequence similarity to proteins involved in cell adhesion and inflammation. Cell 56:1033–1044, 1989

12. Larsen E, Celi A, Gilbert G, Furie BC, Erban JK, Bonfanti R, Wagner DD, Furie B:PADGEM protein: a receptor that mediates the interaction of activated platelets with neutrophils and monocytes. Cell 59:305–312, 1989

13. Johnston GI, Pickett EB, McEver RP, George JN: Heterogeneity of platelet secretion in response to thrombin demonstrated by fluorescence flow cytometry. Blood 69:1401–1403, 1987

14. Ault KA, Rinder HM, Mitchell JG, Rinder CS, Lambrew CT, Hillman RS: Correlated measurement of platelet release and aggregation in whole blood. Cytometry 10:448–455, 1989

15. Hines R, Barash PG: Infusion of sodium nitroprusside induces platelet dysfunction in vitro. ANESTHESIOLOGY 70:611–615, 1989

16. Stricker RB, Wong D, Shiu DT, Reyes PT, Shuman MA: Activation of plasminogen by tissue plasminogen activator on normal and thrombasthenic platelets: effects on surface proteins and platelet aggregation. Blood 68:275–280, 1986

17. Adelman B, Michelson AD, Loscalzo J, Greenberg J, Handin RI: Plasmin effect on platelet glycoprotein Ib-von Willebrand factor interactions. Blood 65:32–37, 1985

18. Bidstrup BP, Royston D, Sapsford RN, Taylor KM: Reduction in blood loss and blood use after cardiopulmonary bypass with high dose aprotinin (Trasylol). J Thorac Cardiovasc Surg 97: 364–372, 1989

19. Dietrich W, Spannagl M, Jochum M, Wendt P, Schramm W, Barankay A, Sebening F, Richter JA: Influence of high-dose aprotinin treatment on blood loss and coagulation patterns in patients undergoing myocardial revascularization. ANESTHESIOLOGY 73: 1119–1126, 1990