Anesthesiology
1999; 91:362–8

Platelet-activated Clotting Time Does Not Measure Platelet Reactivity during Cardiac Surgery

Linda Shore-Lesserson, M.D., Tameshwar Ammar, M.D.,† Marietta DePerio, R.N.,‡ Frances Vela-Cantos, R.N.,§ Cherie Fisher, M.D.,‖ Kaya Sarier, M.D.#*

Background: **Platelet dysfunction is a major contributor to bleeding after cardiopulmonary bypass (CPB), yet it remains difficult to diagnose. A point-of-care monitor, the platelet-activated clotting time (PACT), measures accelerated shortening of the kaolin-activated clotting time by addition of platelet activating factor. The authors sought to evaluate the clinical utility of the PACT by conducting serial measurements of PACT during cardiac surgery and correlating postoperative measurements with blood loss.**

Methods: **In 50 cardiac surgical patients, blood was sampled at 10 time points to measure PACT. Simultaneously, platelet reactivity was measured by the thrombin receptor agonist peptide-induced expression of P-selectin, using flow cytometry. These tests were temporally analyzed. PACT values, P-selectin expression, and other coagulation tests were analyzed for correlation with postoperative chest tube drainage.**

Results: **PACT and P-selectin expression were maximally reduced after protamine administration. Changes in PACT did not correlate with changes in P-selectin expression at any time interval. Total 8-h chest tube drainage did not correlate with any coagulation test at any time point except with P-selectin expression after protamine administration ($r = -0.4$; $P = 0.03$).**

Conclusions: **The platelet dysfunction associated with CPB may be a result of depressed platelet reactivity, as shown by thrombin receptor activating peptide–induced P-selectin expression. Changes in PACT did not correlate with blood loss or with changes in P-selectin expression suggesting that PACT is not a specific measure of platelet reactivity. (Key words: Hemostasis; platelet activating factor; P-selectin; point-of-care.)**

A POINT-OF-CARE assay for measuring platelet function has eluded clinicians caring for patients undergoing cardiopulmonary bypass (CPB). The absence of a specific bedside test of platelet function has made it difficult to construct efficient and rational transfusion algorithms and has encouraged further reliance on empiric platelet transfusions to treat bleeding after cardiac surgery. This is especially true when platelet count is reduced and the function of the remaining platelet complement is unknown.

The platelet-activated clotting time (PACT), or Hemostatus® (Medtronic, Parker, CO), is a point-of-care test that has been evaluated in cardiac surgical patients for its predictive value for blood loss. Improvements in the PACT after desmopressin therapy and platelet transfusion suggest improved platelet function.[1] Correlations with chest tube drainage (CTD) have been documented by some investigators but not by others,[2–4] and thus the clinical utility of this test has been questioned. The assay measures the platelet activating factor (PAF)–mediated shortening of a whole blood kaolin-activated clotting time. This PAF-mediated procoagulant activity may be a marker that can be followed as a measure of platelet function during CPB.

The study objective was to measure the temporal course of PACT during cardiac surgery and to evaluate its correlation with blood loss postoperatively. Concurrently the temporal course of platelet reactivity, as measured by the agonist-induced platelet expression of P-selectin,[5] was also assayed. These and other coagulation tests were correlated with postoperative mediastinal tube drainage.

* Assistant Professor of Anesthesiology, The Mount Sinai Medical Center, New York, New York.

† Attending Anesthesiologist, Good Samaritan Hospital, East Islip, New York.

‡ Research Nurse in Anesthesiology, The Mount Sinai Medical Center, New York, New York.

§ Research Nurse in Anesthesiology, The Mount Sinai Medical Center, New York, New York.

‖ Attending Anesthesiologist, Mount Sinai Hospital, Miami, Florida.

Clinical Assistant Professor in Anesthesiology, The Mount Sinai Medical Center, New York, New York.

Received from the Departments of Cardiothoracic Anesthesiology and Cardiac Surgery, The Mount Sinai Medical Center, New York, New York. Submitted for publication April 22, 1998. Accepted for publication March 1, 1999. Supported by a grant from Medtronic Hemotec, Inc., Parker, Colorado. Data were presented in part at the Society of Cardiovascular Anesthesiologists' Annual Meeting, Baltimore, Maryland, May 13, 1997.

Address reprint requests to Dr. Shore-Lesserson: One Gustave L. Levy Place, Box 1010, New York, New York 10029. Address electronic mail to: linda_shore@SMTPLINK.mssm.edu

Materials and Methods

The protocol was approved by our Institutional Review Board, and all patients gave informed consent to participate. Adult patients were recruited if they were scheduled to undergo a cardiac surgical procedure requiring CPB. Patients with preoperative renal failure were excluded from study, but patients receiving preoperative aspirin therapy were included. In all patients, CPB was instituted after administration of bovine lung heparin 300 U/kg and demonstration of ACT greater than 400 s. Perfusion was maintained using non-heparin coated systems, membrane oxygenators, and moderate hypothermia (25–28°C core temperature). Heparin was antagonized with 1 mg protamine per 100 U total heparin administered, and all of the residual volume of the CPB circuit was transfused to the patient at the completion of CPB. Patients undergoing repeat operations or complex combined procedures received ε-aminocaproic acid 150 mg/kg, followed by 15 $mg \cdot kg^{-1} \cdot h^{-1}$ infusion.

After the removal of 10 ml of blood to reduce dead space volume, blood was withdrawn from the arterial catheter or the extracorporeal circuit at the following time points: (1) baseline (before sternotomy), (2) after sternotomy, (3) after heparin (pre-CPB), (4) after 30 min on CPB (CPB30), (5) after 60 min on CPB (CPB60), (6) after 90 min on CPB (CPB90), (7) off CPB (before protamine), (8) after protamine, (9) at intensive care unit (ICU) arrival (n = 29), and (10) 18–24 h postoperatively.

After study of the first 20 patients, ICU arrival was added as a time interval for blood sampling.

Platelet-activated Clotting Time: Materials

The PACT cartridge was prewarmed to 37°C in the Hepcon® (Medtronic Hemotec, Parker, CO) machine for 15–30 min. Three milliliters of whole blood in a precalibrated syringe was mounted on the instrument, which automatically dispensed 0.35 ml of blood into each of the six channels of the cartridge. Each channel of the cartridge contains an equivalent amount of kaolin activator and calcium chloride. Channels 1 and 2 contain no PAF, and their average is defined as the "control" ACT. Channels 3, 4, 5, and 6 contain 1.25, 6.25, 12.5, and 150 nM of PAF, respectively. There were four cartridges with varying heparin concentrations. The cartridge selected was the one that would yield a "control" ACT of 400–1,500 s, for this increases the sensitivity of the ACT-based test. For the purpose of the current study, only two types of cartridges were used. Blood samples from patients in the nonheparinized state were studied using a cartridge containing 3 U/ml of heparin per channel. Samples from patients who had already received heparin for CPB were studied using a cartridge with no additional heparin.

The clot ratio for each PAF concentration was mathematically calculated from the following formula:

$$1 - (\text{ACT [activated channel]}/\text{ACT "control"}).$$

Clot ratio can be expressed as a percentage of maximal platelet function using the mean clot ratio values obtained from channel 6 in a normal reference population (Package insert, HemoSTATUS® [Medtronic Hemotec). The clot ratio for the reference population was derived from 22 donors (87 samples) and was 0.51 ± 0.06. Division of clot ratio by 0.51 yields the patient's clot ratio normalized to that of the reference population.

Flow Cytometry: Materials

Murine monoclonal IgG1 antibodies to platelet GPIIIa (CD61) and P-selectin (CD62) conjugated with fluoroisothiocyanate (FITC) and phycoerythrin-streptavidin (PE), respectively, were obtained from Becton Dickinson Immunocytometric Systems (San Jose, CA). Anti-CD61 was supplied as a 12.5 μg/ml solution and anti-CD62 as a 1.5 μg/ml solution. 10 mM *N*-[2-hydroxyethyl] piperazine-*N'*-[2-ethanesulfonic acid] (HEPES; Sigma Chemical, St. Louis, MO), pH 7.40, in 0.15 M NaCl was the isotonic buffer used. A thrombin receptor activating peptide (TRAP), SFLLRN,[6] was prepared as a 2 mM solution in PBS (0.15 M NaCl, 0.01 M sodium phosphate buffer, pH 7.2).

Flow Cytometry: Platelet Analysis

Surface expression of P-selectin in response to TRAP activation was measured using flow cytometry on samples obtained at the 10 time points described previously, according to the following protocol.

Blood was placed into glass tubes containing 1/9th volume of 3.8% buffered sodium citrate and prepared as described by Shattil *et al.*[7] Blood samples were prepared immediately after sampling and were handled with care so as to ensure that platelet activation did not occur. Briefly, 40-μl aliquots of blood were placed into polypropylene tubes, and 10 μl TRAP or buffer was added to each tube to produce final TRAP concentrations of 0.0, 1.0, 1.5, or 2.0 μM. The samples were then incubated at 22°C for 5 min and then reacted for 15 min at 22°C with saturating concentrations of anti-CD61 (10 μl) and anti-CD62 (10 μl). Samples were then diluted with HEPES

buffer (500 μl) and fixed with 50 μl of paraformaldehyde fixative solution (final concentration of 1% paraformaldehyde [Coulter, Miami, FL]).

Flow cytometric analyses were performed using the Epics-Profile II Coulter flow cytometer (Coulter, Hialeah, FL). The instrument is equipped with a 5-W argon laser, generating 400 mW at 488 nm. Daily calibration was performed using FITC and PE microspheres (Flow Cytometry Standards, Research Triangle Park, NC). Platelets were identified by their characteristic light scattering properties and their ability to bind anti-GPIIIa (CD61) antibody. Five thousand platelets were analyzed per sample. For the purpose of gating, a platelet was considered positive for P-selectin (CD62) when its fluorescence exceeded the upper limit that encompassed 99% of the platelets incubated without antibody. Fluorescence data were recorded as the percentage of platelets expressing P-selectin.

The potential effect of thrombocytopenia on TRAP-induced P-selectin expression was determined by evaluating blood samples from 10 patients. TRAP-induced P-selectin expression was determined, as described previously, on whole blood, samples of whole blood diluted with an equal volume of platelet-poor plasma, or whole blood diluted with an equal volume of Plasma-Lyte A® (Baxter Healthcare, Deerfield, IL).

Statistical Analysis

Clot ratio and P-selectin expression were analyzed using repeated measures analysis of variance (ANOVA) with *post hoc* Tukey's test. P-selectin expression was quantified by subtracting the baseline level of platelet activation (% expression at TRAP = 0 μM) from the activated level (% expression at TRAP = 2 μM).

Linear regression was used to test for significance of correlations between coagulation tests and 8-h CTD as well as P-selectin expression and 8-h CTD postoperatively. Both the postprotamine and ICU arrival time points were analyzed. Categorical data were analyzed using chi-square analysis or Fisher exact test. Continuous data are reported as the mean ± SD. Data not conforming to normal distribution are reported as median (range). All statistical analyses were two-tailed, and $P \leq 0.05$ was considered significant.

Results

Fifty patients were enrolled in the study and all completed the protocol. There were no perioperative deaths. The surgery performed included 26 coronary artery bypass grafting (CABG) procedures, 7 aortic valve replacements, 6 mitral valve replacements, 6 combined procedures (valve replacement plus CABG, double valve replacement, or valve replacement plus left ventricular aneurysmectomy), and 5 thoracic aortic aneurysm resections. Thirty-seven men and 13 women with a mean age of 66 ± 12 yr were studied. Twenty-one patients were taking aspirin preoperatively; eight were receiving intravenous heparin preoperatively.

Total time spent on CPB was 135 ± 25 min with a mean cross-clamp period lasting 92 ± 21 min. The mean total heparin dose was 36.9 ± 10 K units, and the protamine dose was 420 ± 99 mg.

Platelet-activated Clotting Time

Analysis of variance (ANOVA) demonstrated a significant effect of channel ($P < 0.0001$) and of time ($P < 0.0001$) on the PACT clot ratio. Clot ratio was significantly reduced from baseline at the postheparin, CPB30, CPB60, CPB90, off CPB, postprotamine, and ICU arrival time points ($P < 0.01$), in channels 4, 5, and 6.

P-Selectin

P-selectin data were available from 38 of the 50 patients at various time points. ANOVA demonstrated a significant effect of TRAP concentration ($P < 0.0001$) and of time ($P < 0.0001$) on P-selectin expression measured by flow cytometry. P-selectin expression was significantly reduced from baseline at the CPB90, postprotamine, and day 1 postoperative time points for all TRAP concentrations (fig. 1). Changes in PACT did not correlate with changes in P-selectin expression at any time interval.

PACT as a Function of "Control ACT"

The "control" ACT was not constant in each patient blood sample because this value varies with the patient's intrinsic coagulation, antithrombin activity, and the total heparin concentration in the cartridge (cartridge plus plasma level). Clot ratio showed a significant correlation with the "control." Table 1 shows the "control" ACT, clot ratio in channel 6, and their correlation at various time intervals.

Blood Loss and Transfusions

Eight-hour CTD was 585 (35–3680) ml and 24-h CTD was 935 (92–4645) ml. Seventeen patients (34%) received transfusion of packed erythrocytes, five (10%)

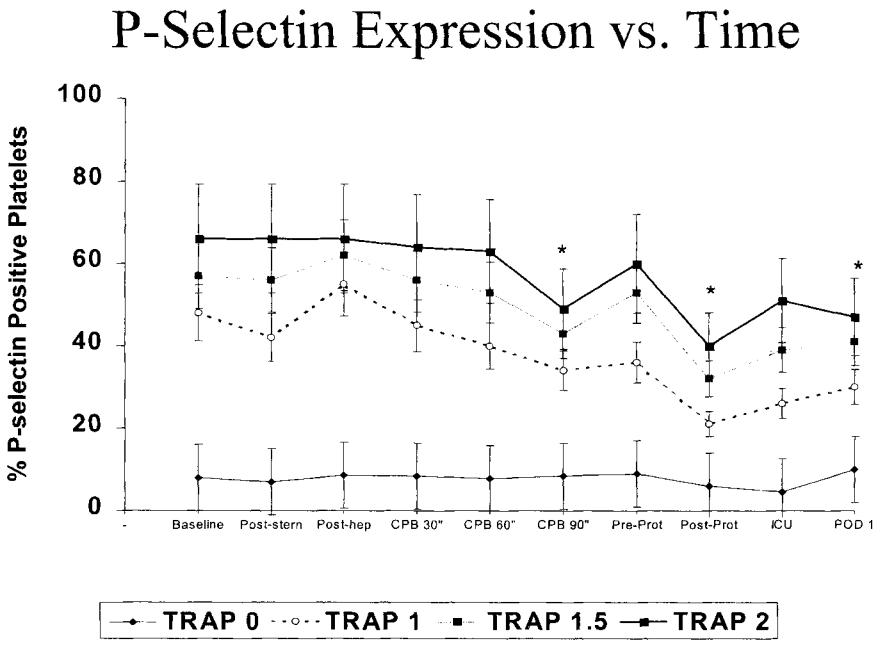

Fig. 1. P-selectin expression over time as measured using fluorescence flow cytometry at the 10 time points during the perioperative period. The graded response is demonstrated by the increase in the percent of platelets expressing P-selectin in response to increased levels of thrombin receptor agonist peptide (TRAP). stern = sternotomy, hep = heparin, CPB = cardiopulmonary bypass, prot = protamine, ICU = intensive care unit, POD = postoperative day. *P < 0.01 difference from baseline for TRAP = 1, 1.5, and 2 μM. There were no significant differences in P-selectin expression over time for TRAP = 0 μM.

received transfusion of fresh frozen plasma, and five (10%) received transfusion of platelets.

On ICU arrival, there was no significant correlation of prothrombin time (fig. 2), activated partial thromboplastin time (fig. 3), platelet count (fig. 4), or PACT (fig. 5) with postoperative 8-h CTD. There was also no significant correlation of P-selectin expression with 8-h CTD (n = 15). At the postprotamine time point (n = 39), there was a significant correlation of P-selectin expression with 8-h CTD (fig. 6), not seen with PACT (fig. 7).

Discussion

The hemostatic defect of CPB is multifactorial in etiology. Harker *et al.*[8] suggest that platelets are activated during extracorporeal circulation leading to α granule release. With platelet activation, α granule membranes fuse with the platelet surface membrane such that P-selectin becomes expressed on the platelet surface. Thus, platelet surface expression of P-selectin reflects the ability of platelets to undergo α granule release in response to a stimulus.

Rinder *et al.*,[9,10] using flow cytometry, have demonstrated that CPB induces *in vivo* platelet P-selectin expression and *in vitro* aggregation defects that are temporally distinct. Kestin *et al.*[11] also studied platelet reactivity using flow cytometric techniques and concluded that *in vitro*, the release reaction is intact but a deficiency of *in vivo* platelet activators exists during CPB. Other authors have reported the selective deple-

Table 1. Correlation of Clot Ratio with "Control" ACT

	n	"Control" ACT Channels 1+2	Clot Ratio Channel 6	r	*P* Value
Baseline		727 ± 235	0.55 ± 0.09	0.66	0.005
Poststernotomy	50	627 ± 303	0.58 ± 0.08	0.52	0.001
60 min CPB	50	492 ± 223	0.38 ± 0.14	0.61	0.0001
90 min CPB	37	446 ± 178	0.38 ± 0.10	0.62	0.001
Postprotamine	50	457 ± 223	0.35 ± 0.15	0.35	0.01
ICU admission	30	608 ± 178	0.46 ± 0.11	0.76	<0.0001
Postoperative day 1	32	693 ± 309	0.57 ± 0.09	0.91	<0.0001

At each time point, clot ratio significantly correlates with the "control" ACT. Each measurement was used in one regression equation.

ACT = activated clotting time; CPB = cardiopulmonary bypass; ICU = intensive care unit.

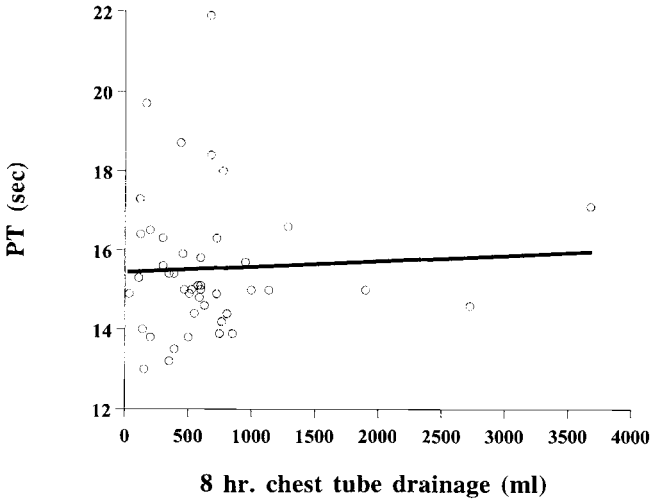

Fig. 2. There was no correlation between prothrombin time (PT) at intensive care unit arrival and 8-h chest tube drainage. R = 0.05; *P* = 0.7.

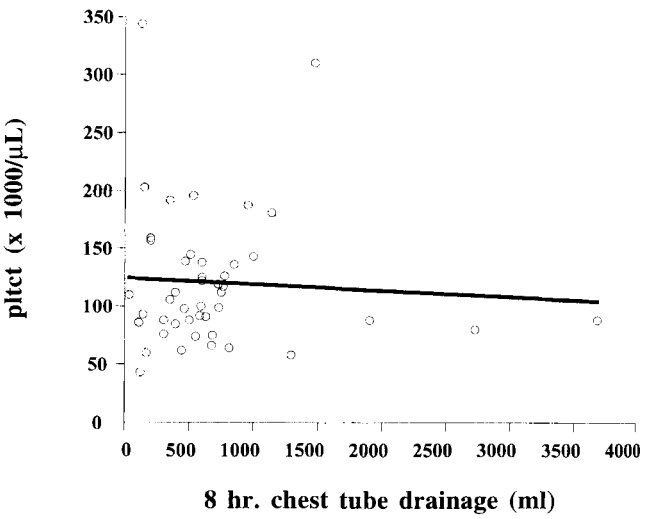

Fig. 4. There was no correlation between platelet count (pltct) at intensive care unit arrival and 8-h chest tube drainage. R = −0.06: *P* = 0.68.

tion or down-regulation of GPIb receptors as a result of CPB, and suggest that preservation of this receptor is one mechanism whereby aprotinin contributes to improved hemostasis.[12,13] The complexity of platelet physiology and numerous etiologies responsible for the platelet defect of CPB make data interpretation increasingly difficult.[14-17]

The measure of P-selectin expression using flow cytometry identifies the portion of activated platelets present in the circulation at a given time and is not affected by hemodilution because the platelet population is specifically identified. PACT is also not affected by moderate thrombocytopenia down to 50,000/μl (Package insert, HemoSTATUS® [Medtronic Hemotec]) and measures the decrease in ACT due to PAF. We sought to evaluate the correlation of PACT with blood loss and with P-selectin expression to determine its ability to measure platelet reactivity and to predict bleeding.

Changes in PACT did not correlate with changes in agonist-induced P-selectin expression. PACT did not correlate with 8-h CTD at either the postprotamine or ICU

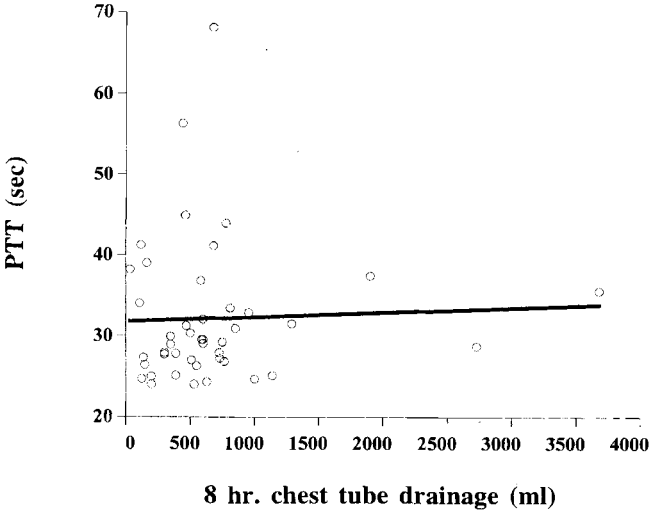

Fig. 3. There was no correlation between partial thromboplastin time (PTT) at intensive care unit arrival and 8-h chest tube drainage. R = −0.04; *P* = 0.78.

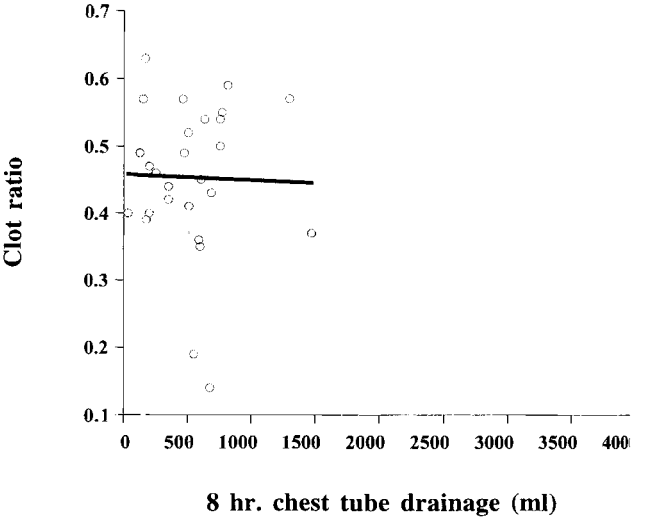

Fig. 5. There was no correlation between platelet activated clot ting time clot ratio at intensive care unit arrival and 8-h ches tube drainage. R = −0.03; *P* = 0.88.

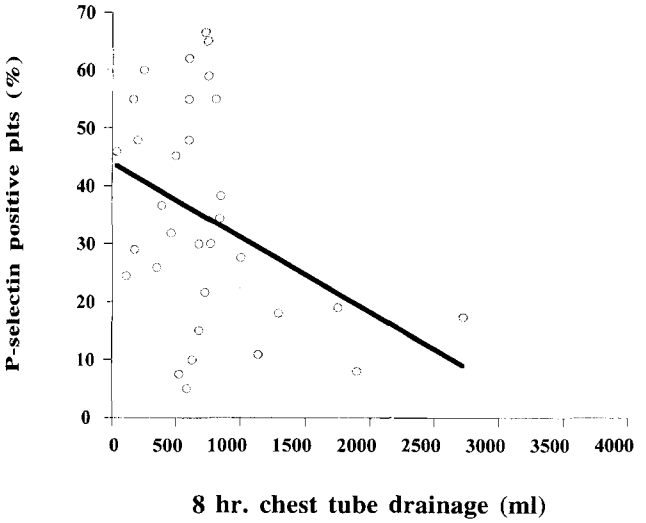

Fig. 6. There was a significant correlation between P-selectin expression at the postprotamine time interval and 8-h chest tube drainage. R = −0.4; *P* = 0.03. plts = platelets.

arrival time points. This is in direct contrast to the correlations reported by Despotis *et al.*,[1] in which 73% of the observed variation in bleeding could be attributed to platelet dysfunction as measured by PACT. There are two potential reasons that subsequent investigators[2,4] have been unable to duplicate Despotis *et al.*'s results. One is the possibility of a greater degree of measurable platelet dysfunction in Despotis *et al.*'s patient population, who had longer durations of CPB, than those of Ereth *et al.*[4] The second and more likely explanation is that Despotis *et al.*'s method of heparin management during CPB differed from those of Ereth *et al.*[4] and from ours. Despotis *et al.*'s maintenance of stable heparin concentrations, rather than a minimum ACT value may have reduced the variability of PACT measurements.

The significant correlation of clot ratio with the "control" ACT, however, indicates that there are at least three inherent problems with the PACT. One is the need for an anticoagulated blood sample (ACT > 400 s) for reduction of the ACT. Another is that if PACT values are to be compared either within or among patients, the level of anticoagulation in the samples (*i.e.*, the "control" ACT) should be consistent. A third is that the test measures many aspects of clot formation that are distinct from "platelet function."

The mechanism whereby PAF shortens ACT may be partly related to α granule release of platelet factor 4, which neutralizes heparin. This is another reason that the heparin level in the test cartridge (cartridge plus patient level) should be constant among samples.

The measure of P-selectin expression allowed us to measure the temporal course of platelet reactivity during the perioperative period. Not all patients were studied at all time points due to time constraints in sample acquisition and use of the flow cytometer. Due to the small number of samples at the ICU arrival time point, no conclusions can be drawn regarding the correlation of P-selectin expression and blood loss. The correlation of P-selectin expression at the postprotamine time point (r = −0.4; *P* = 0.03) with CTD suggests that post-CPB bleeding is at least in part related to decreases in platelet reactivity.

Our data (TRAP = 0 μM; fig. 1) do not demonstrate any increase in P-selectin expression as a result of extracorporeal circulation. Rinder *et al.*[10] showed an increase in baseline levels of P-selectin expression during and after CPB in their study of cardiac surgical patients. This discrepancy may be a result of differences in perfusion techniques, pharmacologic agents used during CPB, or methodologic limitations of the current authors' technique, such as the lack of a PE-conjugated IgG antibody control.

The significant reduction in P-selectin expression in response to all doses of TRAP at 90 min on CPB indicates that an intrinsic platelet defect exists. Our data support the contention that hemostasis and platelet abnormalities increase with hypothermia and prolonged duration of CPB.[18] These data are in agreement with those of Ferraris *et al.*[19] however are in contrast to those of Kestin *et al.*,[11] who found intact platelet reactivity *in vitro*. These differences may be attributed to the distinct

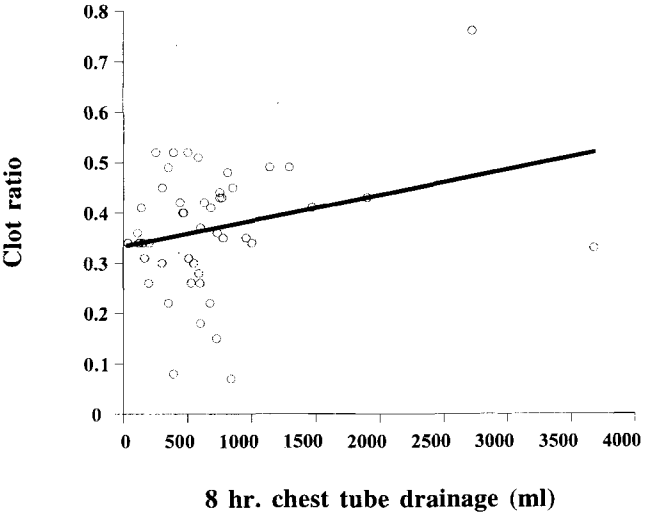

Fig. 7. Lack of significant correlation between platelet activated clotting time clot ratio at the postprotamine time interval and 8-h chest tube drainage. R = 0.27; *P* = 0.06.

platelet agonists used to initiate the release reaction or to differences in patient temperature, or the conduct of CPB. Our data also demonstrate that protamine itself has antiplatelet effects and that these effects are measurable at the doses used to reverse heparin anticoagulation.[20]

An ideal platelet monitor would be one that can assess all of the interrelated facets of platelet function: platelet-endothelial adhesion, receptor integrity, secretion, and aggregation. Currently accepted tests of platelet function such as bleeding time and aggregometry are impractical for the perioperative setting and do not consistently correlate with postoperative bleeding.[21–23] Serum markers of platelet activation can be measured, but these tests are not performed at the "point-of-care." Thromboelastography, which measures the elasticity of the clot due to platelet-fibrin interaction, can be applied as a point-of-care assay, but an abnormal result is not always specific for platelet dysfunction.

Bleeding after CPB may indeed be caused by an intrinsic platelet defect, as suggested by a decrease in TRAP-induced P-selectin expression. Accurate point-of-care measurement of this defect is not available but would be desirable to prescribe prompt and appropriate transfusion therapy to cardiac surgical patients.

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