PLATELET COMPOSITION AND FUNCTION IN PATIENTS UNDERGOING CARDIOPULMONARY BYPASS FOR HEART SURGERY

Giampiera Bertolino, Alessandro Locatelli,* Patrizia Noris, Marco Maurelli,* Piero Ceriana,* Giuliano Mazzini,° Pierangelo Spedini, Simona Belletti, Carlo L. Balduini

Department of Internal Medicine (Internal Medicine and Medical Oncology), University of Pavia, IRCCS Policlinico S. Matteo, *Department of Anesthesia and Intensive Care, IRCCS S. Matteo, and °Centro di Studio per l'Istochimica, Department of Animal Biology, Pavia, Italy

ABSTRACT

Background. Previous studies showed severe biochemical and functional damage to platelets in patients undergoing cardiopulmonary bypass for cardiac surgery, and suggested that this derived from the proteolytic action of plasmin on the platelet surface.

Methods. A double-blind study was carried out to compare platelet function and composition in patients randomized to receive the protease inhibitor aprotinin or placebo during reoperation for valvular prosthesis replacement or coronary artery bypass grafting.

Results. Flow cytometry with specific monoclonal antibodies and polyacrylamide gel electrophoresis did not show any significant proteolysis of platelet glycoprotein Ib and IIb-IIIa either in the placebo or the aprotinin group. Functional studies were consistent with these results, since ristocetin-induced platelet agglutination was unchanged and platelet aggregation and ATP release induced by collagen and ADP were only slightly reduced by cardiopulmonary bypass. These mild defects in platelet function were partially prevented by aprotinin infusion.

Conclusions. On the basis of our data and those from literature, we suggest that platelets may be affected very little or severely damaged during cardiopulmonary bypass for cardiac surgery, probably depending on some aspects of the technical procedure which remain to be identified. Aprotinin infusion significantly protects platelets in the latter condition, while its role is obviously slight in the former.

_____

Key words: heart surgery, cardiopulmonary by-pass, platelets

Blood loss is a frequent complication of cardiac surgery and cardiopulmunary bypass (CPB); this problem is particularly severe when patients undergo reoperation.[1] A functional platelet defect is generally considered the main cause affecting the hemostatic system, although both coagulation and fibrinolysis were found to be activated during CPB.[2] The exact mechanism(s) responsible for platelet dysfunction is still the object of debate. Several studies have reported depletion of some functional platelet membrane glycoproteins (GP),[2-6] and it has been suggested that this abnormality derived from the proteolytic activity of plasmin. In fact, this enzyme is able to modify platelet membrane composition *in vitro* and to induce both activation and inhibition of platelet function.[7,8]

Aprotinin, a nonspecific serine protease inhibitor active against plasmin, has recently been shown to decrease the blood loss associated with CPB when administered at high doses intraoperatively.[9] It is possible therefore that it reduces bleeding through inhibition of plas-

min-induced platelet damage. Several studies were designed to test this hypothesis, but conflicting results were obtained.[4,6,10] A placebo-controlled, double-blind trial of aprotinin administration during CPB for cardiac surgery was set up to investigate this topic further.

Surprisingly, our results showed that platelet composition and function were affected very little by CPB in both the placebo and the aprotinin group, and that there was therefore little place for a protective effect of aprotinin on platelets.

Materials and Methods

Patients

Twenty patients undergoing elective reoperation for valve replacement or coronary artery bypass grafting gave informed consent for entry into this placebo-controlled, double-blind study approved by the local Ethics Committee. Ten patients (5 valve prosthesis replacements and 5 coronary bypass) received aprotinin and 10 (5 valve prosthesis replacements and 5 coronary bypass) placebo. The two groups were similar with respect to age, sex and preoperative cardiac function.

All patients were operated on by the same team of surgeons and anesthetists. The preoperative antiaggregant/anticoagulant drug regimen was stopped 10 days before the operation and replaced with subcutaneous heparin in order to prolong the activated partial thromboplastin time up to 2-2.5 times normal values. Anesthesia was always induced and maintained with the same balanced technique (fentanyl, diazepam, isoflurane and pancuronium), and myocardial preservation was achieved with a crystalloid cardioplegic solution administered as a single bolus of 10-15 mL/kg into the aortic root plus topical hypothermia with slushed ice. CPB equipment included a Gambro roller pump with membrane oxygenator (Monolith, Sorin, Italy), arterial line filter (ABF 40, Sorin, Italy) and cardiotomy reservoir (CRF 28, Sorin, Italy). The circuit was always primed with lactated Ringer's solution plus 0.5 g/kg of 20% mannitol plus 40 g of albumin, and CPB was conducted at a blood flow of 2.4 L/min/m^2 while maintaining moderate systemic hypothermia (32°C nasopharyngeal temperature).

The mean duration of CPB was 105±21 and 125±16 minutes for the subjects in the placebo and in the aprotinin group, respectively. Anticoagulation was achieved by administration of 300 IU/kg heparin before the start of CPB; additional heparin was administered during the procedure according to a fixed protocol: 100 IU/kg every 30 min for the entire duration of CPB for both groups of patients. Heparin reversal was achieved with protamine sulphate (1 mg/100 IU of heparin) after cessation of CPB.

The trial drug, aprotinin (Trasylol, kindly supplied by Bayer, Italy), and placebo were provided in identical case packs; 2,000,000 KIU aprotinin were given at the start of anesthesia, followed by a constant infusion of 500,000 KIU per hour. An additional 2,000,000 KIU were added to the priming of the CPB. The perioperative course was always devoid of complications and blood products were never transfused.

Blood sampling and laboratory assays

Blood samples were obtained through a central venous catheter at three different time points: a) after induction of anesthesia, before hypothermia and administration of heparin and aprotinin (or placebo); b) during CPB at the end of rewarming; c) after the neutralization of heparin by protamine.

Blood was anticoagulated with 3.8% trisodium citrate (9 parts of blood and one part of anticoagulant) for platelet functional studies, and with EDTA (10 mM, final concentration) for flow cytometry and platelet electrophoresis; samples for whole blood counting were collected into 10 mM EDTA and analyzed with a Coulter S-Plus cell counter.

Platelet rich (PRP) and platelet poor plasma (PPP) were obtained by centrifuging the blood at 200 g for 10 min and at 4,500 g for 20 min, respectively, at room temperature.

Platelet aggregation and ATP release were studied in PRP (adjusted to 150×10^9 platelets/L with PPP) in a Lumi aggregometer (Chronolog Corporation, Havertown, PA), respectively, by the method of Born[11] and by the luciferase-

luciferin method, as previously described.[12] Collagen (Mascia Brunelli, Milan, Italy), ADP and ristocetin (Sigma Chemical Co., St Louis, MI) were the stimulating agents.

Monoclonal antibody (MoAb) binding to platelets was studied by flow cytometry. Paraformaldehyde was added to PRP (1% final concentration) and after 5 min incubation at room temperature, platelets were washed and resuspended in phosphate buffer saline (PBS) containing 3.0 mM EDTA and 5% bovine serum albumin (BSA) (Sigma). Ten μL of the working solutions of SZ1 (anti-GPIb/IX) and P2 (anti-GPIIb-IIIa) (Immunotech SA, Luminy, France) were added to 100 μL of the samples adjusted to 50×10^9 platelets/L and incubated for 30 min at room temperature. Platelets were then washed and resuspended in 100 μL of PBS containing 0.3 mM EDTA and 5% BSA. One hundred μL of fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Sigma Chemical Company, St. Louis, MI, USA), at a dilution of 1/50, was added. Following 30-min incubation at room temperature in the dark, platelets were washed, resuspended in 200 μL of PBS containing 0.3 mM EDTA and 0.1% BSA, and analyzed by a FACStar flow cytometer (Becton-Dickinson Immunocytometry System, Mountain View, CA), with argon laser excitation at 4888 (100 mW operating output). Correlated dual para-meter analysis of side scatter (log) and green fluorescence (log) was performed on about 10,000 platelets per sample. Mean platelet fluorescence intensity in arbitrary units was taken into account.

Platelet sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to Laemmli,[13] as previously described.[12] High molecular weight standards (Bio-Rad, Richmond, CA) were simultaneously electrophoresed. Each sample contained 100 mg of protein, as measured by the method of Lowry.[14] Gels were stained with PAS and Coomassie Blue, respectively, for glycoprotein and molecular weight marker detection, and scanned with a densitometer using a yellow-green filter. On the glycoprotein profile, the platelet content of GPIb and IIb-IIIa was expressed by the area below the peak of each single glycoprotein that was identified on the basis of the molecular weight. Platelet glycoprotein content during and after CPB was expressed as % of pre-CPB values.

The statistical significance of the differences was determined by the Scheffè test.

Results

Table 1 summarizes the results of *ex vivo* platelet studies in the placebo and aprotinin groups. The values observed just before the end

	placebo		aprotinin	
	CPB	post-CPB	CPB	post-CPB
Platelet count				
actual	59.7±10.2**	63.3±14.1**	69.2±11.4**	62.3±11.6**
corrected	91.8±17.2	85.4±17.2*	97.4±19.5	82.9±13.2**
Aggregation by				
collagen (20 mg/mL)	76.8±11.3**	80.5±18.6**	91.1±15.4°	89.9±23.0
ADP (20 mM)	79.8±14.4*	65.7±21.4**	89.1±21.0*	79.3±15.9**
ristocetin (1 mg/mL)	82.1±24.9	103.1±23.9	90.7±49.0	86.5±29.7
ATP release by				
collagen (20 mg/mL)	67.3±21.7*	68.8±25.7*	93.2±38.5°	104.1±36.0°
ADP (20 mM)	71.8±26.1*	65.8±20**	82.7±27.2	87.7±28.4
GP content				
Ib	99.4±21.9	103.2±23.0	100.7±20.8	97.6±23.2
IIb-IIIa	101.1±8.1	93.7±11.7	103.2±13.3	101.2±15.8
MoAb binding				
anti-GPIb	93.5±15.0	92.0±13.4	94.7±12.7	93.1±10.5
anti-GPIIb-IIIa	94.5±4.6	121.5±24.7*	90.7±11.9	116.0±8.9*

Table 1. Effect of CPB on platelet function and composition. Values (mean ± SD) are expressed as % of pre-CPB.

* p < 0.05; ** p < 0.01 with respect to pre-CPB value
° p < 0.05; °° p < 0.01 with respect to placebo

of CPB and after heparin neutralization by protamine are expressed as % of the values observed in the same patient before CPB.

The actual platelet count was greatly and significantly reduced by CPB in both the placebo and the aprotinin group; however, when platelet counts were corrected for the hemodilution caused by the priming solution and intravenous infusions, a significant reduction was observed only after heparin neutralization by protamine at the end of CPB. Aprotinin treatment did not show any significant protective effect on platelet counts.

Concerning *in vitro* platelet function, CPB reduced both aggregation and ATP release induced by collagen and ADP in the placebo group, but the extent of reduction never exceeded 35% of pre-CPB values. In the aprotinin group, platelet aggregation and ATP release after collagen stimulation were significantly better than in placebo patients, while platelet response to ADP was not significantly different. Ristocetin-induced platelet agglutination was unchanged at all sampling times.

SDS-PAGE studies showed that the GPIb and IIb-IIIa content of platelets was not modified by CPB. Flow cytometry results demonstrated that surface GP Ib was unchanged during and after the surgical procedure; on the other hand, the binding of a MoAb reacting with GPIIb-IIIa was normal during CPB, but significantly increased after the neutralization of heparin by protamine. No significant differences between the aprotinin and the placebo group were observed with respect to SDS-PAGE and flow cytometry.

Discussion

Our study was designed to verify the hypothesis that aprotinin infusion during CPB protects the platelet surface from the proteolytic action of plasmin and preserves platelet function. To this end we randomized patients undergoing elective open heart reoperation to receive intraoperatively aprotinin or placebo, and we studied platelet function and composition at different times during the surgical procedure.

Our results do not allow any conclusions about the ability of aprotinin to preserve platelet membrane GP since we did not observe direct or indirect signs of membrane proteolysis in either the placebo or the aprotinin group. SDS-PAGE and flow cytometry with anti-GPIb and anti-IIb-IIIa MoAbs showed that the total content and surface expression of these glycoproteins were not reduced by the CPB procedure; on the other hand, the only significant modification we observed was an up-regulation of surface GPIIb-IIIa after heparin neutralization at the end of CPB. This phenomenon is not surprising since thrombin formation occurs after heparin neutralization,[10,15,16] and it is well known that thrombin activates platelets and induces exposure of the α-granule GPIIb-IIIa complex on the cell surface. Furthermore, the small, but statistically significant, decrease of the corrected platelet count after protamine administration may be explained by thrombin formation.

The results of the functional studies are consistent with the evidence that the GP content of the platelet membrane was not reduced during CPB. Ristocetin-induced platelet agglutination was unchanged at all sampling times, suggesting normal GPIb content, and collagen- or ADP-induced platelet aggregation was only slightly reduced, confirming that large-scale GPIIb-IIIa proteolysis did not occur. The mild defects of ATP release and platelet aggregation we observed may be explained by the transient platelet activation known to occur during CPB.[2] Aprotinin infusion partially prevented this mild platelet damage, thus suggesting that it inhibited platelet activation.

Our results are at variance with the majority of those reported in the literature. Many authors,[2-6] using different methodological approaches, observed a significant CPB-dependent decrease of GPIb (together with a reduction of ristocetin-induced platelet agglutination) and/or of the GPIIb-IIIa complex (together with a large defect in platelet aggregation). Only Kestin *et al.*[17] concluded that CPB was not responsible for an intrinsic platelet defect, since they did not observe a loss of platelet surface GPIb and GPIIb-IIIa or severe defects in platelet reactivity *in vitro*.

Since it is difficult to hypothesize that these

inconsistencies derive from differences in analytical methods, we suggest that they might be related to the technical variations in the operative procedures or the many uncontrolled variables associated with CPB, including anesthetic or pharmacologic agents, the nature of the priming solution, the degree of hypothermia, the type of oxygenator, etc.

In conclusion, on the basis of our data and those from literature we suggest that platelet composition and function during CPB for heart surgery may be well preserved or severely damaged depending on some unknown technical aspects of the operative procedures; aprotinin infusion significantly protects platelets in the latter condition, while it has a limited role in the former.

References

1. Giordano GF, Giordano GF Jr, River SL, et al. Determinants of homologous blood usage utilizing autologous platelet-rich plasma in cardiac operations. Ann Thorac Surg 1989; 47:897-902.
2. Dechavanne M, French M, Pages J, et al. Significant reduction in the binding of a monoclonal antibody (LYP 18) directed against the IIb/IIIa glycoprotein complex to platelets of patients having undergone extracorporeal circulation. Thromb Haemost 1987; 57:106-9.
3. Woodman RC, Harker LA. Bleeding complications associated with cardiopulmonary bypass. Blood 1990; 76:1680-97.
4. van Oeveren W, Harder MP, Roozendaal KJ, Eijsman L, Wildevuur CRH. Aprotinin protects platelets against the initial effect of cardiopulmonary bypass. J Thorac Cardiovasc Surg 1990; 99:788-97.
5. Rinder CS, Mathew JP, Rinder HM, Bonan J, Ault KA, Smith BR. Modulation of platelet surface adhesion receptors during cardiopulmonary bypass. Anesthesiology 1991; 75:563-70.
6. Huang H, Ding W, Su Z, Zhang W. Mechanism of the preserving effect of aprotinin on platelet function and its use in cardiac surgery. J Thorac Cardiovasc Surg 1993; 106:11-8.
7. Bertolino G, Noris P, Previtali M, et al. Platelet function after in vivo and in vitro treatment with thrombolytic agents. Am J Cardiol 1992; 69:457-61.
8. Grignani G, Zucchella M, Brocchieri A, Saporiti A. Current concepts in coronary thrombolysis. Haematologica 1994; 79:475-82.
9. Westaby S. Aprotinin in perspective. Ann Thorac Surg 1993; 55:1033-41.
10. Orchard MA, Goodchild CS, Prentice CRM, et al. Aprotinin reduces cardiopulmonary bypass-induced blood loss and inhibits fibrinolysis without influencing platelets. Br J Haematol 1993; 85:533-41.
11. Born GVR. Aggregation of blood platelets by adenosine diphosphate and its reversal. Nature 1962; 194:927-9.
12. Balduini CL, Bertolino G, Noris P, et al. Defect of platelet aggregation and adhesion induced by autoantibodies against platelet glycoprotein IIIa. Thromb Haemost 1992; 68:208-13.
13. Laemmli UK. Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature 1970; 227:680-5.
14. Lowry OH, Rosenbrough NJ, Farr AL, Randal RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1970; 193:265-75.
15. Brister SJ, Ofosu FA, Buchanan MR. Thrombin generation during cardiac surgery: is heparin the ideal anticoagulant? Thromb Haemost 1993; 70:259-62.
16. Boisclair MD, Lane DA, Philippou H, Sheikh S, Hunt B. Thrombin production, inactivation and expression during open heart surgery measured by assay for activation fragments including a new ELISA for prothrombin fragments F1+2. Thromb Haemost 1993; 70:253-8.
17. Kestin AS, Valeri CR, Khuri SF, et al. The platelet function defect of cardiopulmonary bypass. Blood 1993; 82:107-17.