Alexander Wahba

THE INFLUENCE OF CARDIOPULMONARY BYPASS ON PLATELET FUNCTION AND BLOOD COAGULATION

Determinants and Clinical Consequences

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Blut ist ein ganz besondrer Saft.

Faust I
Johann Wolfgang von Goethe
(1749 – 1832)

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Abbreviations

CPB	cardiopulmonary bypass
IVBT	in vitro bleeding tests
GP	glycoprotein
PFA	platelet function analyser
PACT	platelet-activated clotting time
ACT	activated clotting time
ADP	adenosine diphosphate
ATP	adenosine triphosphate
PBS/BSA	phosphate buffered saline/ bovine serum albumin
FITC	fluorescein isothiocyanate
PE	phycoerythrin
LDH	lactic dehydrogenase
AST	aspartate aminotransferase
ALT	alanine aminotransferase
PT	prothrombin time
aPTT	activated partial thromboplastin time
TT	thrombin time
AT	antithrombin
t-PA	tissue plasminogen activator
TAT	thrombin antithrombin
F_{1+2}	prothrombin fragment 1+2
PF_4	platelet factor 4
ANOVA	analysis of variance
β-TG	β-thromboglobulin
TRAP-6	thrombin receptor agonist peptide-6

List of Papers

Paper I
Cardiopulmonary bypass leads to a preferential loss of activated platelets: a flow cytometric assay of platelet surface antigens. A. Wahba, G. Black, M. Koksch, G. Rothe, J. Preuner, G. Schmitz, D.E. Birnbaum. Europ. J. Cardiothorac. Surg. 10, 1996, 768-773.

Paper II
Aprotinin has no effect on platelet activation and adhesion during cardiopulmonary bypass. A. Wahba, G. Black, M. Koksch, G. Rothe, J. Preuner, G. Schmitz, D.E. Birnbaum. Thrombosis and Haemostasis 75, 1996, 844-8.

Paper III
The blood saving potential of vortex versus roller pump with and without aprotinin. A. Wahba, A. Philipp, M.F. Bauer, M. Kaiser, H. Aebert, D.E. Birnbaum. Perfusion 10, 1995, 333-341.

Paper IV
Effects of Extracorporeal Circulation and Heparin on the Phenotype of Platelet Surface Antigens Following Heart Surgery. Alexander Wahba, Gregor Rothe, Heiko Lodes, Stefan Barlage, Gerd Schmitz, Dietrich E. Birnbaum.Thromb Res 97, 2000, 379-386.

Paper V
Predictors of blood loss following coronary artery bypass grafting. A. Wahba, G. Rothe, H. Lodes, S. Barlage, G. Schmitz, D.E, Birnbaum. J. Cardiothorac. Vasc. Anesth. 11,1997, 824-827.

Paper VI
Are in Vitro Platelet Function Tests Useful to Estimate the Blood Loss Following Open Heart Surgery ? A. Wahba, S. Sandner, D.E. Birnbaum. Thorac. Cardiovasc. Surg. 46, 1998, 228-231.

Introduction

The key to the astonishing progress of open heart surgery during the last 5 decades has been cardiopulmonary bypass (CPB) by an extracorporeal circulation (1). The concept is simple: the patients blood returning to the right atrium through the superior and inferior caval vein is removed from the body by the use of one or several cannula and diverted into a reservoir. All the blood is pumped back via an arterial line after it has been oxygenated and the carbon dioxide removed.
Ever since its invention, the deleterious effects of extracorporeal circulation on the blood and its constituents have been recognised. In contrast to the pathways of the blood within the body, the extracorporeal circuit is not lined with endothelial cells. The passage of the patient's blood through the circuit affects nearly all physiologic processes within the body (2). Today it is widely believed that activation of the blood cells and plasma protein systems, which are delineated in Table 1, are causally connected to the whole body inflammatory reaction, described by Kirklin et al (3). The resulting injury to the body and its organs is variable in its extent, but some degree of impaired function of kidneys, lungs, and the brain is usually apparent (4).

Table 1: Cells and Plasmatic Systems Activated by Cardiopulmonary Bypass. Modified from Edmunds et al. (5)

cells	plasmatic systems
platelets	contact
endothelial cells	coagulation
neutrophils	fibrinolytic
monocytes	complement
lymphocytes	

Activation of the clotting cascade via the intrinsic system (Figure 1) is initiated by the contact of blood with the foreign surface of the extracorporeal circuit. Activated monocytes adhere to the perfusion circuit and express tissue factor which probably further activates coagulation (6). In addition, high levels of tissue factor within the surgical wound lead to activation of the extrinsic system of coagulation (Figure 1) (7). These reactions would ultimately lead to clotting of the patients blood within the extracorporeal circuit by fibrin formation and platelet activation. Therefore the use of heparin as an anticoagulant during CPB is a condition sine qua non. However, heparin is far from being an ideal anticoagulant. Heparin

does not inhibit the coagulation cascade completely (Figure 1). At least F XII, F XI, and prekallikrein are activated, and high-molecular-weight kininogen is cleared (8). Thus markers of fibrin formation can be detected in most patients during and early after CPB, and fibrin emboli can occur (9). In addition to the activation of the clotting cascade, it has been shown that CPB leads to significant fibrinolysis (10).

Immediately after the initiation of CPB, there is a rapid increase in the circulating levels of tissue-plasminogen activator (11). Tissue- plasminogen activator, which is stored and released by endothelial cells, catalyses the fibrinolytic system leading to a lysis of blood clots. The fibrinolytic cascade is depicted in Figure 2.

The cellular system participating in the process of blood clotting is the blood platelets. It has been shown repeatedly that alteration of platelet function is responsible for some of the effects of CPB on the human body (12).

The net clinical effect of CPB on coagulation, fibrinolysis, and blood platelets is an impairment of blood clotting in some patients if not all (13). Bleeding is responsible for a considerable part of the morbidity associated with open heart surgery. Roughly 3-5 % of patients need more than 10 units of blood transfusions to compensate for abnormal blood loss (13). 3-14 % of patients require reoperation for bleeding (14). Surgeons and intensivists involved in the postoperative care of these patients are well aware of the fact that the disposition for bleeding varies greatly. Many variables may influence the individual patients disposition for bleeding.

The aim of this study is to investigate some of the mechanisms involved in impairment of blood coagulation during CPB with particular reference to evaluation of platelet function. In addition, methods to predict the amount of the blood loss will be explored.

Study Objectives

Paper I
In this prospective study the effects of CPB on platelet surface antigens associated with platelet activation, aggregation, and adhesion and the platelet volume were measured. In 20 patients, the expression of the GPIIb-IIIa, Ib, 53, and P-selectin were measured using flow cytometry in platelet rich plasma before and after in vitro stimulation with adenosine diphosphate.

Paper II
Aprotinin, a serin protease inhibitor, is known to reduce blood loss following CPB by limiting hyperfibrinolysis. Its influence on circulating platelets is uncertain. In this prospective trial we investigated activation, adhesion, and aggregation receptors on the platelet surface in 20 patients who underwent elective coronary artery bypass grafting. These patients were randomly assigned to receive either a high dose of aprotinin or placebo. Flow cytometry was performed to determine platelet activation (P - selectin, GP 53), adhesive (GPIb), and aggregatory (GPIIb-IIIa) receptors on circulating platelets, before, during, and after CPB.

Paper III
In this study the potential of centrifugal blood pumps for saving blood was investigated. 120 patients scheduled for elective coronary artery bypass grafting were entered into a prospective randomised trial comparing different perfusion strategies. In group I standard roller pumps were used. Centrifugal blood pumps were used in group II In group III roller pump were employed and aprotinin was given. The rationale was to test whether the potential blood saving effect of centrifugal pumps was comparable to the effect of using aprotinin as a supplement to a standard perfusion circuit. In addition the effect of different pumps on platelet function was investigated.

Paper IV
In this prospective study, the time dependent effects of extracorporeal circulation and heparin-mediated effects on platelet surface antigens in vitro were investigated using whole blood flow cytometry.
Blood samples were drawn prior to and following CPB in 89 patients. The response of surface antigen expression GPIIb-IIIa, P-selectin, and GPIb was measured.

Paper V
The predictive value of parameters possibly associated with blood loss following CPB was investigated in a prospective study.

The data of 89 patients scheduled for elective heart surgery with CPB were included and blood samples drawn before and after surgery. Chest tube drainage was measured hourly until removal of drains.
Activation of coagulation and fibrinolysis, routine clotting tests and expression of platelet surface antigens, using flow cytometry, were analysed.

Paper VI
The suitability of two commercially available in vitro bleeding tests (IVBT), the PFA-100® and the Hepcon® HMS to predict blood loss following operations with CPB was compared to conventional coagulation studies.
In 40 patients a blood sample was taken before and after CPB to measure platelet count, prothrombin time, aPTT, d-dimers, fibrinogen, and PFA-100® and Hepcon® HMS data. The postoperative blood loss was recorded hourly until removal of drains.

Material and Methods

All studies were approved by the institutional review board. Informed consent was obtained from all patients. Patients were referred to the University Hospital of Regensburg, Germany for elective coronary artery bypass grafting. Patients subjected to emergency procedures were excluded. Patients receiving platelet-active medication for less than ten days before the operation, patients on intravenous heparin and patients with unstable angina were excluded from the study. None of the patients investigated had a history suggestive of a haemostatic disorder. Details of operations, anaesthesia and postoperative regimen were similar in all studies. Details are outlined below.

Anaesthesia and Surgical Procedures

All patients had anaesthesia induced with fentanyl, etomidate, and pancuronium, maintained with oxygen, isoflurane and fentanyl. An arterial line was placed into the left femoral or radial artery. A central venous line and a Swan-Ganz catheter (Baxter Deutschland, Unterschleißheim, Germany) were placed into the internal jugular vein. Heparin (Liquemin N, Hoffman La Roche, Germany, 375 IU/kg) was used as anticoagulant before cannulation. The kaolin activated clotting time (ACT) was maintained over 480 seconds during CPB by additional doses of heparin, if required. CPB was performed at a minimal temperature of 31°C with a nonpulsatile flow ranging from 2.4 to 2.6 l/min/m^2 with a roller pump (Stöckert Instrumente, Munich, Germany). In paper III a centrifugal pump was used with a BP 80 pump head (Biomedicus, Medtronik, Bad Homburg, Germany) in group II. The extracorporeal circuit was primed with 1,500 ml of Ringer's solution and 100 ml of mannitol 10%. 7,500 IU of heparin were added to the pump prime. A membrane oxygenator (Maxima II, Medtronik, Bad Homburg, Germany) was used in all cases. Heparin was completely neutralised after discontinuation of CPB with protamin sulphate (Protamin, Hoffman La Roche, Germany). Additional protamin sulphate was given, if the ACT was above 120 s. Surgical haemostasis was achieved using a standardised protocol. Blood remaining in the extracorporeal circuit was returned to the patient in the early postoperative period. The cardiotomy reservoir was used to collect mediastinal blood loss. The chest tubes were connected to the reservoir before closure of the chest. Chest tube drainage was recorded hourly until removal of drains on the morning following surgery. Mediastinal shed blood was not reinfused. Transfusion of packed red cells was considered below an haemoglobin value of 9 g/dl, particularly in bleeding patients. Fresh frozen plasma and platelet transfusions were given, if the blood loss via the mediastinal drains was more than 200 ml for 3 consecutive hours.

Analysis of Platelet Function

Flowctometry

Blood was collected in commercially available vials (Sarstedt, Nümbrecht, Germany) containing ethylenediaminetetraacetic acid anticoagulant, for measurement of haemoglobin concentration, haematocrit value, platelet count, and platelet volume, using a Technicon H3 hematology analyzer (Bayer Diagnostics, Tarrytown, USA). Another blood sample collected in trisodium citrate was drawn for flow cytometry. In papers I, II, and III platelet function was assessed by using flow cytometry in platelet rich plasma. To obtain further information on platelet haemostatic function, the response of the surface antigens to adenosine diphosphate (ADP) in vitro was performed in all samples.

Blood samples were collected in trisodium citrate and processed immediately. Platelet rich plasma was prepared by centrifugation at 150 g for 15 minutes. The platelet rich plasma was diluted in phosphate buffered saline (Biochrom, Berlin, Germany) containing 1 mg/ml bovine serum albumin (PBS/BSA) and the platelet count was adjusted to approximately 20,000 platelets/µl. The dilution is necessary to ensure analysis of individual platelets. 20 µl of the platelet suspension was incubated at room temperature for 10 minutes with 5 µl of ADP (110 µmol/l) (DiaAdin, DiaMed, Cressier sur Morat, Switzerland) or PBS/BSA. Thereafter all samples were incubated for 5 minutes at room temperature with one of the following fluorescein isothiocyanate (FITC)-labelled monoclonal antibodies: anti-glycoprotein (GP)IIb/IIIa (cluster of differentiation (CD) 41a, P2), anti-GPIb (CD 42b, SZ2), anti-GP53 (CD 63, CLB-Gran/12), and anti-P-selectin (CD 62P, CLB-Thromb/6), all from Immunotech (Marseille, France). Samples were diluted with 300 µl of PBS/BSA and analysed in a Becton Dickinson FACScan flow cytometer (Becton Dickinson, San José, CA, USA). A Lysis II software was used for measurements. 10,000 platelets were acquired in list mode at a flow rate of 12 µl/minute, 500 - 1,000 particles/second.

In papers IV and V the in vitro response of platelets to stimulation was analysed by flow cytometry in a dual colour whole blood assay. Platelets were identified by their high and constitutive expression of glycoprotein (GP) IIb/IIIa (CD 41, integrin $\alpha_{IIb}\beta_3$). This method allows analysis in diluted whole blood independent of variations in platelet concentration and avoids artefactual stimulation during centrifugation. Platelet activation is quantified based on the increased surface expression of P-selectin (CD 62) following degranulation of α-granules (15) and at the same time the decreased surface expression of GPIb, the receptor for von Willebrand factor (vWF), following internalization into the open canalicular system (16). The simultaneous surface recruitment of GPIIb-IIIa, the platelet receptor for

fibrinogen (17), serves as a further independent indicator of platelet activation (18). To obtain further information on platelet haemostatic function, the response of the surface antigens to submaximal stimulation with ADP or thrombin receptor agonist peptide-6 (TRAP-6) in vitro was performed in all samples. In addition the in vitro heparin response on platelet surface antigen expression in the preoperative sample was tested by in vitro incubation with heparin.
In vitro stimulation with ADP or TRAP-6 causes an increased expression of GPIIb-IIIa and P-selectin and a decrease in GPIb expression. A blood sample collected in trisodium citrate was processed immediately to perform measurements of platelet surface antigen expression, using whole blood flow cytometry. 100 µl of the sample were diluted in 900 µl of phosphate buffered saline (Biochrom, Berlin, Germany) containing 1 mg/ml bovine serum albumin (Sigma, Deisenhofen, Germany) (PBS/BSA). 20 µl of this solution were incubated for 10 minutes at room temperature with PBS/BSA or 15 µmol/l of ADP. Thereafter a saturating concentration of Phycoerythrin (PE) - labelled anti – GPIIb-IIIa (CD41, P2) and one of the following FITC - labelled antibodies: anti – GPIb (CD 42b, SZ2), anti - P-selectin (CD 62, CLB - thromb/6) were added and incubated for 5 minutes (all antibodies from Immunotech, Marseille, France) at room temperature. Then 1 ml of cold PBS/BSA was added and all samples were analysed with an Ortho Cytoron Absolute flow cytometer (Ortho Diagnostic Systems, Neckargmünd, Germany). The PE fluorescence of the brightly expressed antigen GPIIb-IIIa was used as a threshold for the detection of platelets in the diluted whole blood. The analysis of the PE fluorescence of GPIIb-IIIa in the dual combination, with the only dimly expressed antigen P-selectin and the brighter GPIb on the FITC channel, allowed the control of the critical compensation of FITC overlap into the PE channel. 10,000 platelets were acquired in list mode at a low sample flow rate (0.25 µl/sec) and sheath flow rate (100 µl/sec). For data analysis platelets were identified automatically, using the AutoGate function of the Immunocount II software (Otho Diagnostic Systems, Neckargmünd, Germany), followed by calculation of mean FITC and PE fluorescence. The calibration of the instrument was controlled for each set of experiments using fluorescent standard beads (Polysciences, Eppelheim, Germany), which are similar to the stained platelets in light scatter and fluorescence intensities.

PFA-100® and the platelet-activated clotting time (PACT) (Paper VI)

The PFA-100® and the platelet-activated clotting time (PACT) in the Hepcon® HMS were used as platelet function test in paper VI.
The PFA-100® (DADE Diagnostika, Unterschleißheim, Germany) is based on the test proposed by Kratzer and Born (19). A citrated whole blood

sample is introduced into a disposable test cartridge. The blood is aspirated through a glass capillary into a cup. There it comes into contact with an ADP or epinephrine coated membrane with a central aperture (150 µm diameter). During aspiration of blood, platelets adhere to the aperture. During the course of the measurement a stable platelet plug forms that eventually occludes the aperture. The time from beginning until occlusion of the aperture is defined as the ”closure time”. This closure time was shown to be indicative of the platelet function in the sample (20).
The PACT in whole blood is measured with the Hepcon® HMS (Medtronic, Düsseldorf, Germany). The test was performed in duplicate. Platelet procoagulant activity was measured by determining the kaolin-activated clotting time (ACT) with platelet activating factor (PFA) added to the cartridge. The PACT values were expressed as the percent maximum response (21).

Additional Methods Used in Individual Papers

Paper I

Blood samples were taken just prior to heparinization while purse string sutures were applied to the aorta (A), before cannulation and after heparin was allowed to recirculate (B), at one hour of CPB (C), and one hour after discontinuation of CPB (D). A serum sample was taken for total protein value, creatinine. The platelet count was corrected for haemodilution according to the method described by Harker et al. (12).

Paper II

Patients were randomly allocated to one of two groups. In group APR, aprotinin (Trasylol, Bayer AG, Leverkusen, Germany) was given: 2×10^6 IU with induction of anaesthesia, 2×10^6 IU were added to the pump prime, and 5×10^5 IU per hour were infused continuously during CPB. Group CON served as the control. Blood samples were taken just prior to heparinization (A), at one hour of CPB (B), and one hour after completion of CPB (C) from the arterial line. Another blood sample was taken prior to heparinization (A), one hour after discontinuation of CPB (C), and six hours following discontinuation of CPB (D) for measurement of the specific degradation product of fibrinogen, d-dimers, using a latex immunoassay.

Paper III

In group I, a conventional standard roller pump (Stöckert Instrumente, Munich, Germany) was used. This group served as a control. In group II,

CPB was conducted with a centrifugal blood pump with a BP 80 pump head (Biomedicus, Medtronik, Bad Homburg, Germany). Group III individuals were placed on CPB using a roller pump (Stöckert Instrumente, Munich, Germany) and aprotinin (Trasylol, Bayer AG, Leverkusen, Germany) was given: 2 x 10^6 I.E. with induction of anaesthesia, 2 x 10^6 I.E. were added to the pump prime, and 5 x 10^5 I.E. per hour were infused continuously during CPB. The conduction of CPB was otherwise identical in all groups. A blood sample for serum levels of creatinine, free haemoglobin, full blood count, total protein, lactic dehydrogenase (LDH), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) was taken during and after the operation. In a subset of 10 individuals per group, further blood samples were taken for investigation of thrombocyte function using flow cytometry as described above.

Paper V

Blood samples were drawn from all patients on the day prior to surgery by venipuncture in the right antecubital fossa and immediately after arrival of the patient in the intensive care unit following surgery from an indwelling arterial line. Besides all samples described in the general section, another sample was collected in trisodium citrate (Sarstedt, Nümbrecht, Germany) for measurement of prothrombin time (PT), activated partial thromboplastin time (aPTT), thrombin time (TT) and antithrombin (AT), using a chromogenic assay. Fibrinogen was measured using the Clauss method. The specific degradation products of fibrinogen, d-dimers, were measured quantitatively using a latex immunoassay. Enzyme linked immunosorbent assays were used to measure tissue plasminogen activator (t-PA), thrombin antithrombin complex (TAT) and prothrombin fragment 1+2 (F_{1+2}). Chromogenic substrates were employed to determine α_2-antiplasmin. Another sample was collected in citrate-theophylline-adenosine-dipyridamole-tubes (Diatube H vials, Boehringer Mannheim, Mannheim, Germany) and stored on ice for measurement of platelet factor 4 (PF_4), using an enzyme linked immunsorbent assay.

Paper VI

Blood samples for evaluation were taken during anaesthesia before CPB was instituted and at the end of surgery. A full blood count was performed and the prothrombin time, the activated partial thromboplastin time (aPTT), d-dimers, and fibrinogen were determined. One sample each was taken to measure the closing time in the PFA-100® and the platelet-activated clotting time (PACT) in the Hepcon® HMS.

Statistical Analysis

SPSS software for Windows® with an advanced statistics module was used on a personal computer for statistical analysis.

The student's t-test for paired data was used to test for statistical significance between groups of normally distributed data. If more than two groups were compared, Bonferroni correction was used (paper I). The U test according to Wilcoxon, Mann and Whitney was used as a nonparametric test. One way analysis of variance (ANOVA) with repeated measures was performed for all variables that were measured at three or more different time points. The student's t-test for paired data was used as a post hoc test. The level of significance was adjusted according to the Bonferroni correction. The correlation of the preoperative and postoperative variables was tested according to Pearson. Spearman's rank correlation was used for non-parametric data (paper V). In paper V further analysis was performed with multiple regressions using a linear regression model with blood loss as the dependent variable. Independent variables were included stepwise into the regression equation using score statistics at 0.05 level for entry. The correlation coefficient (r^2 value) was recorded. The student's t-test and the Wilcoxon-Mann-Whitney test were used to test for significant differences between subgroups that were formed for further analysis as indicated in the results section. Multiple logistic regression was performed to determine the predictive value of different parameters for blood loss between ”bleeders” and ”non-bleeders” as defined in the results section.

Summary of Results

Paper I
Heparinization resulted in a significant increase of GP 53 and P-selectin and a significant decrease of GPIb expression. During and following cardiopulmonary bypass GPIIb-IIIa and GPIb were significantly decreased. Similarly, the expression of the activation markers was reduced significantly. The mean platelet volume decreased significantly from 8.5 $\pm$ 0.7 fl at baseline to 7.9 $\pm$ 0.8 fl at the end of the study period ($p < 0.017$).

Paper II
Aprotinin had neither a significant effect on platelet activation nor on adhesive and aggregatory receptors. Plasma levels of d-dimers were measured before and after CPB to assess fibrinolytic activity. d-dimers following CPB (0.6 $\pm$ 0.5mg/l vs. 1.4 $\pm$ 0.9 mg/l) and chest tube drainage (363 $\pm$ 119 ml vs. 858 $\pm$ 333 ml, $p<0.05$) were significantly less in the aprotinin group.

Paper III
There was no significant difference between group I and II with respect to free haemoglobin, lactic dehydrogenase, serum bilirubin, platelet surface GPIIb-IIIa and P-selectin, chest tube drainage, use of blood products, length of stay in intensive care, time on ventilator, and postoperative mortality. Aprotinin reduced chest tube drainage (840 $\pm$ 335 ml vs. 513 $\pm$ 409 ml) and use of blood products significantly.

Paper IV
A significant correlation between the duration of extracorporeal circulation and the postoperative response of GPIIb-IIIa, GPIb, and P - selectin was found. Postoperative P-selectin and GPIb expression stimulated with ADP correlated to blood loss. Heparin in vitro significantly reduced GPIb expression (278.6 ± 47.5 versus 259.1 ± 36.8).
Heparin as well as the duration of extracorporeal circulation independently correlated with phenotypic changes of platelets following extracorporeal circulation.

Paper V
A significant correlation was found between blood loss and variables of coagulation (activated partial thromboplastin time, fibrinogen, prothrombin fragment 1+2, d-dimers, and thrombin antithrombin complex) and platelet count. In addition a correlation between blood loss and platelet count, GPIb and P-selectin expression on platelets was found. Intraoperative variables had also an influence on bloos loss (use of the internal thoracic artery, cross

clamp time). In a multiple regression model, GPIb expression on platelets, platelet count, use of the internal thoracic artery and d-dimers were significantly associated with blood loss. Logistic regression analysis revealed that postoperative values of GPIb and d-dimers predicted an increased blood loss with a positive predictive value of 73 % and a negative predictive value of 91 %.

Paper VI
A significant correlation was found between total blood loss (250-1750 ml) and the preoperative PFA-100® (r=0.41, p=0.022), the preoperative platelet count (r=0.42, p=0.007), the preoperative D-dimer concentration in the plasma (r=0.41, p=0.01), and duration of CPB (r=0.35, p=0.044). There was no significant correlation between blood loss and the Hepcon® HMS system. There appears to be a great variability in individual results.

Discussion

Flow Cytometry – Measurement of Platelet Function

A great number of laboratory tests have been designed to test platelet function. Many of them have been used to investigate the platelet function defect of CPB. As early as 1980 several of these tests were available to Harker et al.: platelet aggregometry, plasma levels of β-TG, PF_4, platelet content of ADP and ATP, and morphometric analysis of platelet granule number (12). Some of these, i.e. plasma contents of β-TG and PF_4, are only indirect markers of platelet function and may simply indicate that degranulated platelets have left the circulation, or could be due to artefacts during measurement (22, 23). Aggregometry has been used extensively to investigate platelet function (12, 24-26). However aggregometry is semiquantitative and has standardization problems (27).

We used single colour flow cytometry in platelet rich plasma from peripheral blood samples before, during, and after CPB in papers I, II, and III. A dual colour assay was used in papers IV and V. The advantage of flow cytometry over other methods is that it allows for analysis of surface antigens on individual platelets, independent of platelet-to-platelet interaction and platelet concentration (28). Within the flow cytometer, platelets are hydrodynamically focussed to a serial cell flow that crosses a laser beam. At the time of intercept with the laser beam, each cell generates forward light scatter, dependent on cell size, and right angle light scatter, dependent on cell granularity. Thus different cell populations are characterised, providing a typical two-dimensional plot shape. Additional information is gained by fluorescent light emission impulses from laser light excitation of prestained cells. This is achieved by allowing platelet surface epitopes to react with monoclonal antibodies that were conjugated with fluorochrome dyes. We chose to mark surface antigens that are known to be involved in platelet aggregation, such as GPIIb-IIIa, or platelet activation, such as P-selectin or GP 53. Flow cytometry was shown to be advantageous over other ex vivo methods and is particularly suited to investigate platelet function (28).

In papers I, II, and III the assay was performed in platelet rich plasma to simplify the experimental protocol. The authors are aware of the fact that the preparation of platelet rich plasma may lead to platelet activation (29). Provided a standardised method is used, this should result in a constant error, i.e. the expression of platelet surface markers may be artificially increased. All samples were carefully drawn and processed immediately, according to the protocol described in the methods section. Attention was paid to consistently minimise platelet activation during preparation of the blood sample.

Other scientists fixed platelets after blood samples were drawn to eliminate ex vivo platelet activation (30, 31). However, we chose to stimulate platelets in vitro with ADP to obtain further information on platelet haemostatic function as suggested by Michelson (28). Thus platelets were not fixed.
Fixation of samples following stimulation and prior to measurement, as suggested by Kestin et al. (22), was unnecessary since all our samples were measured immediately after their preparation.

Mechanisms of Abnormal Bleeding After CPB

Haemodilution

Simple haemodilution during CPB in itself leads to a reduction of coagulation factors by approximately 50% (13). However, the resulting levels are usually high enough to maintain the coagulation process (12). Thus it is not surprising that most studies were unable to find a correlation between the level of soluble coagulation factors and blood loss (32).

Platelet Dysfunction

Impaired platelet function is an important cause of abnormal bleeding. Platelet activation and subsequent impairment of function starts already with the administration of heparin.
High dose heparin was shown to prolong the bleeding time and other parameters of platelet function before CPB was started (33). In addition, we found an in vivo effect of heparin on platelet surface antigen expression, namely GPIb (paper I). This was confirmed by others, using flow cytometry (22) or platelet aggregometry (34, 35). In vitro experiments (paper IV) confirmed these findings: an effect of heparin on the expression of GPIb but not GPIIb-IIIa and P-selectin was shown. Moreover the in vitro response correlated significantly with the ratio of pre- and postoperative GPIb expression. This indicates that heparin may reduce GPIb expression on circulating platelets.

Despite the use of high doses of heparin sufficient thrombin is generated to induce a marked increase in circulating TAT complexes after CPB starts (36, 37). In paper V, we describe an increase in circulating TAT complexes and F_{1+2} reflecting a significant activation of the coagulation cascade. The activation of coagulation and the resulting thrombin formation leads to platelet activation early during CPB (38).
Other agonists, such as activated complement, plasmin, platelet-activating factor, serotonin, epinephrin, as well, contribute to platelet activation (33,

38). This activation results in a change of shape of circulating platelets with formation of pseudopods, especially at the beginning of CPB (23). Activated platelets adhere to the surface of the artificial circuit and thus leave the circulation (39). This and blood haemodilution are the major causes of thrombocytopenia following CPB. Thrombocytopenia, however, contributes to abnormal bleeding, since platelet count is inversely correlated to blood loss (paper V).

The functional status of platelets remaining within the circulation may be described by their expression of platelet surface antigens. In this respect, we found a significant decrease in GPIIb-IIIa on the surface of circulating platelets during and after CPB. Therefore, we conclude that the fibrinogen receptor plays a role in the platelet function defect of CPB. Most studies performed in this field are in accordance with our results (31, 40).
Also GPIb was significantly reduced during CPB with and without in vitro stimulation with ADP. Several authors obtained similar results using different methods, including whole blood cytometry (25, 31, 41, 42). Some studies found no effect of CPB on GPIb (22, 43). This lead to a continued debate on whether the functional defect is intrinsic or extrinsic to the platelet (22). Likewise show studies on platelet activation (P-selectin and GP53) inconsistent results. Some report activation of platelets by CPB (31, 44), just as we did (paper I), others found no significant change before and during CPB (22, 30, 40, 42). Reports on increased numbers of platelet-leukocyte aggregates during CPB support our results, because the formation of these aggregates is mediated via P-selectin on the platelet surface (45). It appears that the effect of CPB on platelet surface antigens is time dependent (paper IV). We found a direct correlation of the duration of CPB with platelet adhesion receptor phenotype in vitro and the difference of pre- and postoperative platelet count. Moreover the degree of platelet activation (expression of GPIb and P-selectin) by in vitro stimulation with ADP after CPB correlated significantly with postoperative blood loss.

The results described in the preceding paragraph and the observation of a continued reduction of mean platelet volume (MPV) during CPB (paper I), support the following hypothesis:
Circulating platelets are activated by heparin in vivo before CPB. During the initial phases of CPB some platelets are lost from the circulation by adherence to artificial surfaces (30, 38). After the release of the aortic cross-clamp a significant blood activation takes place with increasing thrombin/antithrombin III complex levels and other markers of clotting and fibrinolysis (37). The administration of protamin reverses the inactivation of thrombin by heparin. This leads to further consumption of circulating platelets. The surface expression of GP53 and P-selectin of in vitro

stimulated platelets is therefore lowest shortly after CPB (paper I). It appears that mainly large and thus more active platelets disappear, particularly towards the end of CPB. Less activated and smaller platelets remain within the circulation. This mechanism may contribute to the platelet function defect of CPB. Our hypothesis is also supported by findings of Huang et al., who reported a progressive decrease of ristocetin-induced platelet agglutination during and after CPB (41).

Hyperfibrinolysis

Only recently has hyperfibrinolysis been recognised to be an important contributor to the development of abnormal bleeding following open heart surgery (46, 47). Again heparin seems to play a role before CPB by stimulating release of plasmin from cell surfaces (48). It is of note that plasmin and d-dimer interact with platelets and thus possibly contribute to platelet dysfunction (49).

In paper V we describe our data on hyperfibrinolysis confirming activation of the fibrinolytic system (d-dimers, t-PA, and α_2-antiplasmin). We found a positive correlation between indicators of hyperfibrinolysis and blood loss, confirming the importance of the described mechanisms. In addition d-dimers are a useful in predicting the amount of blood loss.

Other Factors

Several other factors associated with CPB may contribute to abnormal blood loss. Obviously administration of drugs acting on platelets or fibrinolytics have an influence, but their effect is not necessarily exclusive to the use of CPB. Further discussion will thus be omitted.

Skin temperature and hypothermic CPB were shown to be an important determinant of bleeding (50, 51). This is probably mediated by the effect of temperature on platelet function (52).

Coagulation disorders in individual patients may contribute to abnormal bleeding as well. This requires special attention in terms of screening and preoperative as well as postoperative treatment (53).

Prediction of Abnormal Bleeding

As described above, the influence of CPB on coagulation, fibrinolysis and blood platelets is thought to be responsible for the abnormal bleeding seen in a number of patients undergoing heart surgery. It seems logical to assume that the degree of impairment of the systems involved has an influence on the amount of the blood loss following the operation. Thus prediction of the amount of blood loss should be feasible by measuring the values of a predefined set of parameters.

As expected, an activation of the fibrinolytic system (d-dimers, t-PA, and α_2-antiplasmin) was found in a group of 89 patients undergoing operations with CPB. This was significantly correlated to postoperative blood loss (paper V).
Parameters associated with blood platelets, i.e. platelet count and expression of GPIb and P-selectin on the platelet surface also showed a statistically significant correlation. The best correlation with postoperative blood loss of all parameters was found for use of the internal thoracic artery as a bypass graft ($r = 0.433$). This is probably a consequence of the larger wound surface that results from internal thoracic artery harvesting. Routine clotting tests such as aPTT, however, were not significantly correlated to blood loss in our study. In contrast, Gravlee et al. (54) found a statistically significant correlation between mediastinal drainage and routine clotting tests such as aPTT, PT, and TT. Others found no association of clotting factors with abnormal bleeding (32).
Several investigators used aggregometry or hemostatometry to predict blood loss following heart surgery (26, 55). However, the predictive value of these tests was low (26).
According to our working hypothesis, a combination of parameters reflecting different aspects of blood clotting system should have a higher chance of yielding clinically relevant data. In multiple regression analysis the best model for prediction of blood loss was achieved by the combination of postoperative platelet count, platelet surface expression pre- and postoperatively, as well as the use of ITA and d-dimers. When aiming at discriminating”bleeders” from ”non-bleeders”, the combination of postoperative values for d-dimers and GPIb expression yielded the highest predictive value. Bleeders were defined as patients who bled more than mean plus one standard deviation. Therefore the combination of both parameters seems particularly helpful to exclude non-surgical causes in bleeding patients since specificity and the negative predictive value were high. This may aid the identification of individuals who require surgical reexploration.

The usefulness of commercially available tests of platelet function was analysed too (paper VI). The correlation coefficient of blood loss with in vitro bleeding tests was not superior to the results of simple tests of coagulation such as platelet count, d-dimers, or duration of CPB. In fact, the PACT using the Hepcon® HMS was not significantly correlated to blood loss at all. In contrast, Ereth et al. found a significant correlation of blood loss and PACT in a larger group of patients (21). However their PACT result as well was not superior to other tests of coagulation (21).
The reasons for the somewhat disappointing results of these simple in vitro platelet function tests are probably related to the mechanisms of the

haemostatic defect of CPB. Fibrinolysis for example is not directly tested for in the PFA-100® or the Hepcon® HMS. Moreover haematocrit, platelet count, and leukocyte count were shown to influence closure times of the Thrombostat 4000® (56, 57), an in vitro bleeding test similar to the PFA-100®.

The Prevention of Abnormal Bleeding

Obviously the prevention of abnormal blood loss following open heart surgery starts with taking a clinical history on previous episodes of abnormal bleeding and some screening tests. Relative common disorders, such as von Willebrand disease, factor VIII deficiency are often known to the patient. Many others can be detected on routine blood tests, such as thrombocytopenias. Nevertheless represent life threatening bleeding episodes in patients with haemostatic disorders a therapeutic challenge. Prevention or treatment, respectively, consists of transfusion of platelets, transfusion of clotting factors, plasmapheresis, pretreatment with steroids, or use of desmopressin. Non-specific treatment with drugs that have been shown to reduce blood loss, such as aprotinin, aminocarbocyclic acid and other antifibrinolytics may be advisable. Patients on platelet active medication carry an increased risk of abnormal bleeding (58). If possible, these drugs should be stopped prior to surgery.

Prophylactic Drug Treatment

Prophylactic drug treatment is an efficient and well characterised strategy to reduce postoperative blood loss generally and to reduce the incidence of abnormal bleeding in particular. Salzman et al. and Royston et al. were the first to show the efficacy of desmopressin and aprotinin, respectively, in reducing blood loss in cardiac surgery (59) (60). Many others have confirmed their results (41, 42, 61). Probably the most prominent among these prophylactic drugs is aprotinin. The presumed mode of action of this serine protease inhibitor is mediated by its anti-fibrinolytic activity and its influence on platelet function (61-63). The action of aprotinin on hyperfibrinolysis probably through direct inhibition of plasmin is undoubted and well documented (64) (61, 62, 65). Our results (paper II), that show significantly diminished levels of d-dimers in patients treated with aprotinin, are in accordance with this.

The effect of aprotinin on platelets is less clear. A preservation of the platelet surface expression of GPIb or even an increase in GPIb by aprotinin has been claimed by some (38, 41, 63, 66). We were unable to confirm these findings using flow cytometry (paper II).

Other scientists, using several techniques, found no influence of aprotinin on platelet GPIb (67-69). These contradicting results may be a consequence

of methodological problems of studies using radioisotope labelling techniques (41, 63, 67), that require several steps of centrifugation and filtration which possibly influence the expression of platelet surface antigens (22).
GPIIb-IIIa expression was unaffected by aprotinin in our study. This is in accordance with previous studies on GPIIb-IIIa expression (66, 70). In contrast, Lavee et al. claimed that aprotinin protected platelet aggregation during CPB, using electron microscopy (71). However, bubble oxygenators instead of modern membrane oxygenators were used in this study and CPB was conducted at a much lower body temperature (27±4°C). Both factors are known to influence platelet function significantly (50, 72), rendering comparison with our data difficult.
A recent metaanalysis revealed that other antifibrinolytic drugs, such as tranexamic acid and aminocarbocyclic acid, were equally effective in reducing blood loss at much lower costs, compared to aprotinin (73). Being a potent serine protease inhibitor aprotinin has additional effects on other enzyme systems, such as kallikrein (74). This may lead to an attenuation of the whole-body inflammatory response of CPB. The drug may also attenuate neutrophil activation and myocardial damage during aortic cross-clamping (75). However, the clinical significance of these findings in routine cases is by no means proven. In contrast, serious concerns have been raised following reports of increased early occlusion rates of bypass grafts in patients receiving aprotinin (76). However, also this hypothesis has not been proven definitely. Nevertheless, the use of prophylactic drug treatment is usually only recommended in cases with a particular high risk of abnormal bleeding, i.e. patients on platelet active medication (emergency cases) or with surgical risks such as reoperation.

Platelet Anaesthesia

Platelet anaesthesia is an appealing concept that refers to a strategy to preserve platelet during CPB by temporarily inhibiting platelets during the period of extracorporeal perfusion. If the inhibitor is removed immediately after CPB, larger numbers of functionally adequate platelets are available to normalise bleeding time and to reduce postoperative blood losses.
Substances suggested in experimental and clinical studies are prostacyclin or GPII-IIIa inhibitors (77, 78). Due to significant side effects and the insufficient control of drug action, this concept has not yet found its way into routine clinical practice.

Blood Conservation Methods

A substantial number of blood conservation methods have been proposed to reduce bleeding and handle abnormal blood loss. Autologous blood donation, normovolaemic haemodilution before CPB, salvage and transfusion of shed mediastinal blood during and following the operation are appropriate and generally accepted procedures. These methods have proven their efficacy in numerous studies and are routine in many centres (79).

Use of Centrifugal Blood Pumps

High level of shear stress can produce platelet aggregation and promote shedding of microparticles from the platelet plasma membrane (80). Therefore it seems obvious that the use of pumps within the perfusion circuit may contribute to abnormal bleeding. In a search for less traumatic blood pumps, centrifugal pumps where introduced. Indeed centrifugal pumps have been shown to reduce trauma to the blood components in a number of studies performed in vitro and clinically (81-83). Although the advantages of centrifugal pumps in long-term extracorporeal circulation are well established, the blood saving potential of these pumps in routine cardiac surgery is still controversial (82, 84-87).

Our study (paper III) was the first to compare different blood pumps to aprotinin with regard to blood loss and other variables in a prospective randomised trial.

There was no significant difference in postoperative blood loss in our patients, which is in contrast to the findings of Lynch et al. (87). However our findings are in accordance with data published by others (88, 89). The centrifugal pump had little influence on laboratory data that would indicate significant blood trauma, such as LDH and free haemoglobin. These findings are equally supported by published data (86, 89, 90).

In contrast, it is well known that centrifugal pumps are advantageous in long term extracorporeal circulation (extracorporeal lung support, ventricular assist) (82, 84, 85).

In paper III, a comparison of the expression of platelet surface antigens and platelet count between roller pumps and centrifugal blood pumps was done. Platelet count and the expression of platelet surface antigens was not significantly different for the study groups compared to controls. The initial drop in platelet count during and after ECC in all groups is probably caused by haemodilution. These results support the findings of Driessen et al. (90). Our data on platelet surface antigens support the hypothesis that centrifugal blood pumps do not improve platelet function in routine CPB.

Heparin Coated Equipment

Heparin can be attached to certain extracorporeal perfusion materials by ionic or covalent bonds.

Heparin coated equipment reduces complement and granulocyte activation (91-93) and reduces circulating cytokines IL-6 and IL-8 (94). Heparin-coated perfusion circuits attenuate activation of platelets in some studies (95) but not others (93, 96). Different heparin coated systems have been used clinically together with reduced doses of systemic heparin (usually 150 units/kg) and activated clotting times around 280 s in routine bypass surgery (93, 94, 96). Authors reported 4.5 to 24 percent reductions in postoperative blood loss (93, 94, 96). However, concern has been raised about the safety of reducing the systemic heparin dose, which seems to be a prerequisite to actually reduce blood loss when using coated systems. The evidence is empirical and specific for first-time myocardial revascularization operations; extrapolation of this experience to different patients and different protocols may or may not be safe. Nevertheless heparin coated equipment is routinely used in a number of centres and new promising coating techniques are under development.

Conclusions

I. Flow cytometry allows for analysis of surface antigens on individual platelets, independent of platelet-to-platelet interaction and platelet concentration and is particularly suited to investigate platelet function during CPB.

II. Abnormal bleeding following CPB is largely related to heparin, platelets and fibrinolysis. We suggest that a loss of larger and more activated platelets from the circulation contributes substantially to the platelet function defect of cardiopulmonary bypass.

III. Parameters indicating hyperfibrinolysis and platelet dysfunction are useful to predict abnormal bleeding. Commercially available in vitro platelet function tests are significantly correlated to postoperative blood loss but have little clinical value.

IV. Prophylactic drug treatment is an efficient strategy to reduce postoperative blood loss. Aprotinin reduces the fibrinolytic response to CPB, but has no measurable influence on the expression of platelet surface antigens.

V. The use of centrifugal blood pumps for routine heart surgery offers no advantage over conventional roller pumps with respect to platelet function, blood loss, free haemoglobin, or LDH.

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