Changes in platelet Bax levels contribute to impaired platelet response to thrombin after cardiopulmonary bypass: prospective observational clinical and laboratory investigations

M. Murase¹,†, Y. Nakayama³,†, D. I. Sessler², N. Mukai¹, S. Ogawa¹ and Y. Nakajima³,*

¹Department of Anaesthesiology and Critical Care, Kyoto Prefectural University of Medicine, Kyoto 602-8566, Japan, ²Department of Outcomes Research, Anesthesiology Institute, Cleveland Clinic, OH 44195, USA and ³Department of Anesthesiology and Critical Care, Kansai Medical University, Osaka 573-1191, Japan

*Corresponding author. E-mail: nakajima@koto.kpu-m.ac.jp; nakajiya@hirakata.kmu.ac.jp
†These authors contributed equally to this study.

Abstract

Background. Anucleate platelets can undergo apoptosis in response to various stimuli, as do nucleated cells. Cardiopulmonary bypass (CPB) causes platelet dysfunction and can also activate platelet apoptotic pathways. We therefore evaluated time-dependent changes in blood platelet Bax (a pro-apoptotic molecule) levels and platelet dysfunction after cardiac surgery.

Methods. We assessed blood samples obtained from subjects having on-pump or off-pump coronary artery bypass graft surgery (n = 20 each). We also evaluated the in vitro effects of platelet Bax increase in eight healthy volunteers.

Results. Thrombin-induced platelet calcium mobilisation and platelet-surface glycoprotein Ib (GPIb) expression were lowest at weaning from CPB and did not recover on postoperative day one. On-pump surgery increased platelet expression of Bax, especially the oligomerised form, along with translocation of Bax from the cytosol to mitochondria and platelet-surface tumour necrosis factor-alpha (TNF-α)–converting enzyme (TACE) expression. In contrast, mitochondrial cytochrome c expression was reduced. While similar in direction, the magnitude of the observed changes was smaller in patients having off-pump surgery. In vitro, a cell-permeable Bax peptide increased platelet Bax expression to the same extent seen during bypass and produced similar platelet changes. These apoptotic-like changes were largely reversed by Bcl-xL pre-administration, and were completely reversed by combined application of inhibitors that stabilise outer mitochondrial membrane permeability and TACE.

Conclusions. CPB increases platelet Bax expression, which contributes to reduced platelet-surface GPIb expression and thrombin-induced platelet calcium changes. These changes in platelet apoptotic signalling might contribute to platelet dysfunction after CPB.

Clinical trial registration. UMIN Clinical Trials Registry (number UMIN000006033).

Key words: anaesthesia; apoptosis; blood platelet disorders; intracellular signaling peptides and proteins; platelet membrane glycoproteins
Cardiopulmonary bypass (CPB) serves as a temporary substitute for a patient’s heart and lungs during conventional open-heart surgery. CPB produces dilutional haemostatic defects because of priming-induced dilution. Furthermore, consumption coagulopathy might deplete coagulation factors and platelets because procoagulant pathways are activated. However, bypass-related haemostatic defects are disproportionate to the degree of decrease in coagulation factors, with the exception of fibrinogen, and platelets, possibly because of a qualitative platelet defect that is manifested as decreased aggregation in response to platelet agonists.\(^2\ 3\)

Alterations in platelet apoptotic signalling might induce CPB-induced disturbances.\(^4\) Platelets contain apoptotic regulators, including members of the Bcl-2 protein family, and downstream effectors such as caspases. Consequently, even anucleate platelets can undergo apoptosis.\(^5\) Among the Bcl-2 family proteins, the pro-apoptotic molecules Bax and Bak accelerate the opening of mitochondrial voltage-dependent anion channels, while the anti-apoptotic proteins Bcl-XL and Bcl-2 close these same channels.\(^6\) Clearance of damaged and dead platelets requires the activation of endogenous metalloproteinases, while disintegrin and metalloprotease 17 (ADAM17, also referred to as tumour necrosis factor-\(\alpha\) (TNF-\(\alpha\))-converting enzyme (TACE)) mediate GP Ib shedding.\(^7\)

Perioperative changes in platelet apoptotic signalling remain unclear. Flow cytometry techniques using whole-blood specimens fixed immediately after collection can quantify cell-surface antigen expression and thus monitor platelet signalling changes in the perioperative period.\(^8\)

The aim of this study was to evaluate time-dependent changes in platelet Bax function and platelet response to thrombin during and after cardiac surgery. We tested the primary hypothesis that increased platelet Bax levels after CPB contribute to reduced perioperative platelet-surface GP Ib expression and impaired platelet response to thrombin because of increases in outer mitochondrial membrane permeability and TACE activity. Second, we investigated if changes in platelet Bax, and related pathways, were less pronounced during and after off-pump cardiac procedures than during and after on-pump procedures.

**Methods**

The Review Board for Human Experiments, Kyoto Prefectural University of Medicine, approved this prospective clinical observational clinical study and laboratory study. Written informed consent was obtained from all participants.

**Clinical study**

The patient sample size used in this study was based on a pilot study that showed that CPB increased platelet Bax expression in terms of geometric mean fluorescence intensity (MFI) from 8.5 (1.5) arbitrary units (au) to 14 (2.9) au, and decreased platelet-surface GP Ib expression from 720 (43) au to 440 (110) au (\(n\)=10).\(^2\)

Based on these data, we calculated that 18 subjects per group would provide 80% power for detecting differences in fluorescence intensity by one standard deviation (SD) at a \(\alpha\) level of 0.05 using analysis of variance (ANOVA) with repeated measures between factors as the primary outcome. Because we anticipated that some subjects would be eliminated from the analysis by virtue of receiving perioperative platelet transfusions, we enrolled a total of 22 subjects in each group. The sample size was determined using PASS 14 (NCSS LLC, Kaysville, UT, USA).

We excluded patients with conditions believed to modulate platelet activity, including venous thrombosis, sepsis, acute infection, pregnancy, heparin-induced thrombocytopenia, transient ischaemic attacks, severe hypertension (>160/95 mm Hg), recent CPB, multiple sclerosis, preoperative hepatic or renal dysfunction, and severe respiratory disease. We also excluded patients who could not safely discontinue antiplatelet drugs, other than aspirin, before surgery, or who took any nonsteroidal anti-inflammatory medication within 48 h before surgery.

We enrolled a total of 44 subjects undergoing on-pump or off-pump coronary artery bypass graft (CABG) surgery via median sternotomy between July 2011 and October 2015. Subjects were aged 20–80 yr and had an ASA physical status of III or IV. The same surgical team performed all operations. We excluded four subjects who received platelet transfusions in the perioperative period after enrolment.

General anaesthesia was induced using i.v. midazolam, fentanyl and vecuronium, and maintained with 1–1.5% sevoflurane in oxygen/air throughout on-pump and off-pump CABG surgery. Sevoflurane was administered through a heart-lung machine during CPB. Fentanyl was continued intraoperatively at 4 \(\mu\)g kg\(^{-1}\) h\(^{-1}\) and through the second postoperative day (POD) at 0.5 \(\mu\)g kg\(^{-1}\) h\(^{-1}\). Subjects undergoing CPB were given porcine heparin (300 IU kg\(^{-1}\)) and additional boluses of 50 IU kg\(^{-1}\) as necessary to maintain an activated clotting time of at least 480 s during bypass.

The CPB circuit included a roller pump (Maquet Inc., Wayne, NJ, USA), cardiotomy reservoir, membrane oxygenator (Terumo, Tokyo, Japan), venous drainage cannula, and a 40-mm arterial line filter with arterial return to the ascending aorta. The circuit was primed with a mixture of Ringer’s lactate (800 ml), 20% (wt/vol) mannitol (250 ml), and 8.4% (wt/vol) sodium bicarbonate (150 ml).

CPB under cardiac arrest was performed under near-normothermic conditions (>35 °C), with a flow rate adjusted to the calculated cardiac index of 2.4 litre min\(^{-1}\) m\(^{-2}\). Ultrafiltration was only used when haemococoncentration was required. The myocardium was protected by blood cardioplegia repeated at 30-min intervals.

Subjects having off-pump coronary bypass surgery were given >150 IU kg\(^{-1}\) heparin after graft harvesting to maintain an activated clotting time of >300 s. A mechanical stabilizer (Octopus; Medtronic, Minneapolis, MN, USA) and a heart positioner (Starfish or Urchin, Medtronic) were used to control the beating heart.

Heparin was neutralized using protamine sulphate (~1 mg of protamine per 100 IU of heparin given) within 10 min after weaning from CPB, in on-pump, and after completion of bypass graft anastomosis in off-pump cases.

Blood specimens were collected in sterile 3.8% sodium citrate tubes from subjects just after the induction of anaesthesia (Pre), during CPB (During, 1 h after CPB initiation), just after
weaning from CPB (Pt, before protamine administration), six h after weaning from CPB (Pt6), and 24 h after anaesthesia induction (POD 1) in off-pump surgery.

Platelet calcium measurements
To measure platelet cytosolic free calcium mobilisation, anticoagulated whole blood was diluted 1:10 in modified HEPES-Tyrode's buffer (consisting of 137 mM NaCl, 2.7 mM KCl, 1 mM MgCl2·6H2O, 12 mM NaHCO3, 0.4 mM Na2HPO4, 10 mM HEPES, 5.5 mM glucose, and 0.35% bovine serum albumin, pH 7.4) and incubated at 37 °C for 15 min with 5 µM Fluo-3/AM, followed by CD41-PE labelling. Gly-Pro-Arg-Pro was added at a final concentration of 2.5 mM to prevent thrombin-induced fibrin clot formation and platelet aggregation without inhibiting thrombin-induced platelet activation.10

Flow cytometric analysis of platelet-surface protein expression
To determine platelet-surface GPIb and TACE expression, whole blood was stained with an allophycocyanin labelled anti-GPIb antibody (BD Biosciences, San Jose, CA, USA) or a PE-labelled anti-TACE antibody (R&D Systems, Minneapolis, MN, USA) respectively. Samples were incubated without any stimulant in the dark at ambient temperature (20–25 °C), and then fixed by adding OptiLyse B (Beckman Coulter, Fullerton, CA, USA).3

Flow cytometric analysis of platelet signalling proteins
Commercially available reagents (BD cytofix/cytoperm kit, BD Biosciences) and a standard intracellular staining protocol were used to evaluate intra-platelet Bcl-2 family protein expression.11 12 Antibodies included anti-Bax (6A7; Sigma-Aldrich, Tokyo, Japan), anti-Bax-PE (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and anti-Bcl-xL-Alexa 488 (Cell Signalling Technology, Danvers, MA, USA). Activated mitochondrial Bax can be distinguished from cytosolic and loosely attached, inactive Bax by a conformational change in the N-terminus, which exposes the formerly buried 6A7 epitope comprising amino acids 13–19. Intra-mitochondrial cytochrome c expression was determined according to the manufacturer’s recommendations (Calbiochem, San Diego, CA, USA).13 Briefly, cells were first treated with digitonin to selectively permeabilise the plasma membrane and allow release of cytosolic cytochrome c. Apoptotic cells release cytochrome c into the cytoplasm from the mitochondria but healthy cells do not. The cells were then fixed to prevent further mitochondrial damage and loss of any unreleased cytochrome c during the subsequent steps.

Western blotting
Washed platelet suspensions were prepared as previously described and protein extracts prepared.14 After the protein concentration was adjusted, each sample was separated by 4–12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrothermally transferred to a polyvinylidene difluoride membrane using the iBlot dry blotting system (Life Technologies, Tokyo, Japan).

After incubating blots overnight with a blocking solution to block residual protein-binding sites, blots were incubated with anti-Bax antibody (N-20, Santa Cruz Biotechnology, Dallas, TX, USA) diluted 1:500 in Tris-buffered saline with 0.1% Tween 20 for two h. They were further incubated with secondary HRP-linked donkey anti-rabbit antibody (NA934, GE Healthcare, Little Chalfont, UK) diluted 1:10 000 in Tris-buffered saline with 0.1% Tween 20 for one h, and subsequently exposed to enhanced chemiluminescence reagents for five min. Chemiluminescence was captured by an image acquisition system (Ez Capture II Atto, Tokyo, Japan).

Laboratory study
We studied eight healthy men aged 25–40 yr who denied taking any medications for at least two weeks. Freely flowing blood samples were collected from an antecubital vein with a 20-gauge needle. Whole blood samples were anticoagulated with sterile 3.8% sodium citrate in Vacutainers (1:9 anticoagulant to blood ratio).

Cell-permeable peptide application study
To assess the effect of platelet Bax increases on downstream signalling, washed platelet suspensions were treated with a cell-permeable Bax peptide (Enzo Life Sciences, Tokyo, Japan) and a Bcl-xL peptide (Calbiochem). To increase the cell permeability of these peptides, they were covalently linked to the 10 amino acid TAT transporter.14 Platelets were washed because the effect of cell-permeable peptides is blocked in the presence of plasma proteins. The concentration of cell-permeable peptides was determined according to the manufacturer’s instructions. Thrombin-induced platelet calcium changes and the expression of platelet inactive Bax, platelet-surface GPIb, TACE, and mitochondrial cytochrome c were evaluated after peptide treatment.

Intracellular pathway inhibition in vitro
Washed platelet suspensions were pre-incubated separately for 30 min with dimethyl sulphoxide (positive control), furosemide (blocker of Bax translocation to mitochondria; Sigma, Tokyo, Japan), cyclosporine A (inhibitor of mitochondrial permeability transition pores (mPTP); Sigma), TAPI-1 (TACE inhibitor; Calbiochem), or a combination of these inhibitors. The effects of these pathway inhibitors on the platelet-surface expression of GPIb and Bax in response to Bax cell-permeable peptide were evaluated by flow cytometry, as described above.

Analysis
The effect of time after cardiac surgery was analysed using a general linear regression modelling for two-way ANOVA with repeated measures (one between-factor and one within-factor), followed by Tukey’s multiple comparison testing with Excel add-in software (Statcel 2, OMS-publishing, Saitama, Japan). Other continuous variables were analysed using two-tailed, unpaired Student’s t-tests. Geometric MFI values were used for statistical analyses of flow cytometry data. Values are expressed as means (±) or as percent change from baseline values.

Results
Clinical study
Subject characteristics, preoperative haemostatic data, and perioperative management are presented in Table 1. Off-pump procedures were selected more often in patients with a history of stroke or carotid artery occlusion to reduce the risk of post-CABG stroke.15 Only perioperative blood loss, autologous blood salvage, allogenic transfusion volume, perioperative platelet counts, and
coagulation tests (activated partial thromboplastin time and fibrinogen concentration) at the end of surgery differed significantly between the on-pump and off-pump subjects.

Perioperative platelet function and surface GPIb expression

In subjects who had on-pump CABG surgery, the decrease in thrombin-induced calcium mobilisation and GP1b expression were not observed in platelets from subjects who had off-pump CABG surgery (Fig. 1A, Supplementary data S1A). In contrast, decreases in thrombin-induced calcium mobilisation and GP1b expression were not observed in platelets from subjects who had off-pump CABG surgery (Fig. 1A, Supplementary data S1A).

Perioperative platelet function and surface GPIb expression

In subjects who had on-pump CABG surgery, the decrease in thrombin-induced platelet calcium mobilisation compared with baseline (MFI: 33 (12) vs 58 (9) compared with baseline, P<0.0001). This was also the case for the decreases in cell surface levels of the thrombin receptor (GP1b) (MFI: 440 (78) vs 700 (830) at the baseline, P<0.0001). Overall, the time course of decreases in calcium mobilisation coincided well with the time course of decreases in cell surface GP1b expression over the 24 h study. These qualitative platelet defects did not recover, even 24 h after induction of anaesthesia (Fig. 1A, Supplementary data S1A). In contrast, decreases in

Table 1 Preoperative characteristics and perioperative management of subjects. Data are presented as means [range] and means (SD) (n=20 per group). FDP, fibrinogen degeneration product; IU, international unit, PT, prothrombin time, INR, international normalized ratio, aPTT, activated partial thromboplastin time, N.S., not significant.

<table>
<thead>
<tr>
<th>Subject characteristics</th>
<th>On-pump</th>
<th>Off-pump</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td>69 [55, 80]</td>
<td>72 [49, 88]</td>
<td>0.25</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>60 (10)</td>
<td>63 (10)</td>
<td>0.44</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>160 (11)</td>
<td>163 (13)</td>
<td>0.45</td>
</tr>
<tr>
<td>Sex (male/female)</td>
<td>11/9</td>
<td>11/9</td>
<td>1</td>
</tr>
<tr>
<td>Haemoglobin (g dL⁻¹)</td>
<td>13.1 (1.8)</td>
<td>12.6 (1.7)</td>
<td>0.33</td>
</tr>
<tr>
<td>FDP (µg mL⁻¹)</td>
<td>2.6 (1.3)</td>
<td>2.5 (1.2)</td>
<td>0.77</td>
</tr>
</tbody>
</table>

Perioperative management

| Duration of surgery (min) | 324 (68) | 313 (58) | 0.58    |
| Duration of anaesthesia (min) | 378 (73) | 368 (71) | 0.64    |
| Duration of cardiopulmonary bypass (min) | 173 (38) | - | <0.001 |
| Total dose of heparin (x 10³, IU) | 241 (4.4) | 17.0 (3.2) | <0.001 |
| Total dose of protamine sulfate (mg) | 225 (47) | 165 (33) | <0.001 |
| Fluid infusion during surgery (ml) | 3100 (640) | 2900 (600) | 0.12    |
| Blood loss during surgery (ml) | 1600 (600) | 980 (300) | 0.005   |
| Blood loss during 24 h (ml) | 1900 (450) | 1300 (400) | <0.001  |

Autologous transfusion by cell salvage (ml)

| Duration of catheterisation during surgery (min) | 750 (290) | 510 (160) | 0.002   |
| Allogenic transfusion during 24 h (ml) | 910 (460) | 440 (420) | 0.002   |
| Red blood cell concentrate during 24 h (ml) | 260 (150) | 140 (140) | 0.009   |
| Fresh frozen plasma during 24 h (ml) | 650 (380) | 300 (330) | 0.003   |

Haemostatic tests (Baseline)

| Platelets (x10³ mm⁻³) | 216 (70) | 226 (76) | N.S.   |
| PT (s)                | 11.4 (1.3) | 11.6 (0.9) | N.S.   |
| INR                   | 1.01 (0.11) | 1.03 (0.08) | N.S.   |
| aPTT (s)              | 34.0 (5.8) | 35.2 (6.6) | N.S.   |
| Fibrinogen (mg dL⁻¹)  | 271 (65) | 293 (75) | N.S.   |

Haemostatic tests (End of surgery)

| Platelets (x10³ mm⁻³) | 106 (37) | 148 (45) | P<0.01  |
| PT (s)                | 13.1 (0.8) | 12.7 (0.6) | N.S.   |
| INR                   | 1.18 (0.07) | 1.14 (0.05) | N.S.   |
| aPTT (s)              | 43.1 (5.4) | 40.8 (6.1) | P<0.05  |
| Fibrinogen (mg dL⁻¹)  | 126 (35) | 171 (47) | P<0.01  |

Haemostatic tests (Postoperative day 1)

| Platelets (x10³ mm⁻³) | 91 (38) | 141 (41) | P<0.01  |
| PT (s)                | 12.2 (1.0) | 12.0 (0.6) | N.S.   |
| INR                   | 1.10 (0.09) | 1.08 (0.06) | N.S.   |
| aPTT (s)              | 38.0 (4.5) | 36.3 (6.0) | N.S.   |
| Fibrinogen (mg dL⁻¹)  | 207 (47) | 231 (55) | N.S.   |

Platelet expression of active Bax, inactive Bax, and Bcl-xl

Throughout the perioperative period, in subjects who had on-pump CABG surgery, platelet expression of both active Bax (i.e. the oligomerised form of Bax) (MFI: 27 (7.8) vs 16 (1.1), P=0.0001) and inactive Bax (MFI: 16 (4.2) vs 9.3 (0.9), P=0.0001) were significantly greater at weaning than at baseline, whereas Bcl-xl levels remained constant (Fig. 1B, Supplementary data S1B). Furthermore, these changes in Bax expression were accompanied by translocation of Bax from the cytosol to the mitochondria (Fig. 2). In contrast, there were no significant changes in either active or inactive Bax, nor was there evidence for Bax mitochondrial translocation, in platelets derived from subjects.
Fig 1 Perioperative changes in thrombin-induced platelet cytosolic calcium, and platelet-surface glycoprotein (GPIb) expression. (A) Thrombin-induced (0.1 U mL$^{-1}$) platelet calcium responses and platelet-surface GPIb expression decreased after weaning from cardiopulmonary bypass (CPB) in subjects undergoing on-pump coronary artery bypass graft (CABG) surgery, but these responses were retained perioperatively in patients undergoing off-pump CABG surgery. Fluo-3 fluorescence changes in the platelets (log FL1 vs time) after thrombin stimulation (0.1 U mL$^{-1}$) were recorded using flow cytometry. Platelets were selected by gating for GPIb$^+$ events on a two-parameter dot plot displaying side scatter vs GPIb-allophycocyanin (FL4). (B) Perioperative changes in intra-platelet Bax, Bcl-xL, mitochondrial cytochrome c, and platelet-surface tumour necrosis factor-alpha (TNF-α)-converting enzyme (TACE). In on-pump CABG subjects, perioperative increases in active Bax and inactive Bax expression were observed while Bcl-xL expression did not change significantly. In off-pump CABG patients, the expression of active Bax and inactive Bax increased only slightly whereas, as in on-pump patients, Bcl-xL expression did not change significantly. Perioperative decrease in mitochondrial cytochrome c content along with increase in platelet-surface TACE expression were observed in on-pump CABG subjects, while no significant changes were observed in off-pump CABG subjects. Platelets were selected by gating GPIb$^+$ events on a two-parameter dot plot displaying side scatter vs GPIb-allophycocyanin (FL4). In all, 30,000 GPIb$^+$ events were acquired. Non-specific fluorescence was determined using irrelevant isotypic control antibodies. Data are shown as geometric MFI values (means (SD), n=20 per group). *$P<0.05$ compared with off-pump CABG at each time point. †$P<0.05$, and §$P<0.01$ compared with preoperative value in each group.
who had off-pump CABG surgery during the perioperative period (Fig. 1B, 2).

**Perioperative changes in possible downstream targets of Bax**

In subjects who had on-pump CABG surgery, platelet mitochondrial cytochrome c content declined significantly compared with baseline, especially at weaning from CPB (MFI: 410 (86) vs 660 (87), \(P=0.0001\)), whereas platelet cell surface TACE expression was higher than that at the baseline, especially six h after weaning from CPB (MFI: 26 (6.6) vs 14 (3.5), \(P=0.0002\)). In contrast, similar changes were not observed in platelets from subjects who had off-pump CABG surgery (Fig. 1B, Supplementary data S1B).

**Laboratory study**

**Mechanism of CPB-induced platelet defects**

Platelet exposure to CPB stress triggered Bax generation. We therefore evaluated whether the increase in platelet Bax levels was responsible for the post-bypass platelet defects. Treatment of washed platelets with a cell-permeable Bax peptide (50 \(\mu M\)) increased platelet inactive Bax to the levels observed during bypass in just 30 min. The increased platelet Bax level was associated with reduction in thrombin-induced platelet calcium mobilisation, platelet-surface GP Ib expression, and mitochondrial cytochrome c content, and an increase in platelet-surface TACE expression. These changes were partially reversed by pre-administration of a cell-permeable Bcl-xL peptide (Supplementary data S2).

**Downstream targets of platelet Bax for the decrease in platelet GP Ib expression**

We measured the effects of furosemide, cyclosporine A, and TAPI-1 on cell-permeable Bax peptide-induced mitochondrial cytochrome c expression, platelet-surface TACE expression, and platelet surface GP Ib expression to elucidate the relationship between changes in platelet Bax and mitochondrial cytochrome c levels and platelet-surface TACE and GP Ib expression.

Cell-permeable Bax peptide reduced thrombin-induced platelet calcium mobilisation, surface GP Ib expression, and mitochondrial cytochrome c content. Pre-administration of furosemide or cyclosporine A partially restored thrombin-induced platelet calcium mobilisation, surface GP Ib expression, and mitochondrial cytochrome c content. TAPI-1 pre-treatment also partially prevented reduction in platelet-surface GP Ib expression without affecting cytochrome c status. Combined application of furosemide and cyclosporine A completely prevented reduction in mitochondrial cytochrome c otherwise induced by the cell-permeable Bax peptide. The reduction in platelet calcium mobilisation and GP Ib caused by the cell-permeable Bax peptide were completely reversed only when furosemide, cyclosporine A, and TAPI-1 were combined together (Supplementary data S3).

In summary, administration of the cell-permeable Bax peptide to platelets in vitro introduced comparable platelet apoptotic changes and subsequent platelet defects as caused by...
These changes could largely be reversed by pre-treatment with Bcl-xL peptide, and fully reversed using pre-treatment with a combination of a mitochondrial apoptosis-induced channel blocker (furosemide), an mPTP blocker (cyclosporine A), and a tumour necrosis factor-α (TNF-α)–converting enzyme inhibitor (TAPI-1).

**Discussion**

Platelet dysfunction, a qualitative disorder, contributes to CPB-induced haemostatic defects. Other important causes include thrombocytopenia and alterations in coagulation and fibrinolytic systems. Reduced perioperative agonist-induced platelet aggregation and platelet surface antigen expression contribute to reduced haemostasis in patients undergoing cardiac surgery with cardiopulmonary bypass.

We confirmed a decrease in thrombin-induced platelet calcium mobilisation and GPIb surface expression after CPB that has been previously reported. Our current clinical and laboratory findings extend current understanding by showing that increased platelet Bax, an upstream modulator of the apoptotic pathway, provokes CPB-associated platelet defects by increasing mitochondrial permeability and platelet surface TACE expression (Fig. 3). We further show that this pathway is restricted to on-pump procedures.

A key element of platelet stimulation is the increase in cytosolic free calcium, a common second messenger downstream of most signalling pathways. Increases in cytosolic free calcium precede several activation responses, including shape change, aggregation, secretion, and procoagulant activity. But during and after CPB, thrombin-induced platelet calcium mobilisation was reduced, as was expression of GPIb at the platelet surface.

The interaction between the GPIb/IX/V complex and von Willebrand factor is critical for platelet adhesion, which mediates platelet rolling at both injury sites and inflamed endothelium, and is critical for platelet aggregation under high shear-stress flow conditions. Studies in patients with Bernard–Soulier syndrome, whose platelets have quantitative or qualitative defects in GPIb, reveal a prominent role for GPIb in thrombin-induced platelet activation. Evidence also suggests that the cleavage of protease-activated receptor-1 by thrombin is facilitated by thrombin–GPIb binding. Other studies ascribe platelet aggregation, shape change, secretion, and mitogen-activated protein kinase activation to direct activation of GPIb by thrombin.

Taken together, our observations of reduced perioperative GPIb expression in association with reduced cytosolic calcium response to thrombin, suggests that decreased platelet-surface GPIb expression is a candidate mechanism for CPB-related platelet dysfunction. Our results are consistent with previous reports attributing these phenomena to the apoptotic process in anucleate platelets.

Levels of both the active (oligomerised) and inactive forms of Bax increased during CPB, whereas the level of Bcl-xL remained unchanged. Similar platelet changes were not observed during off-pump cardiac surgery. The platelet increase in the inactive form of Bax along with the decrease in platelet-surface GPIb expression and decreased thrombin-induced platelet calcium changes can be simulated by exposing platelets to a cell-permeable Bax peptide in vitro. Bcl-2 family proteins include anti-apoptotic (e.g, Bcl-2 and Bcl-xL) and pro-apoptotic (e.g, Bax and Bak) members. Balance between these proteins controls the release of mitochondrial...
mechanism sensitive to cyclosporine A. Our results suggest that both mechanisms are active in CPB patients at least until postoperative day one. The decrease in GPIb and changes in cytosolic calcium induced by the cell-permeable Bax peptide were fully reversed by combined treatment with furosemide, cyclosporine A, and TAPI-1. These findings indicate that both mitochondrial status changes and TACE activation can largely explain the observed decreases in GPIb after CPB.

A limitation of our study is that on-pump and off-pump approaches were not randomly assigned. However, it seems unlikely that patient selection would contribute to the observed changes in platelet signalling and GPIb expression and consequent impaired platelet response to thrombin after CPB. Second, although relatively longer CPB time along with the type of CPB circuit and perfusion technique might worsen platelet apoptotic changes, we believe that our data can be applied to more complicated cardiac surgery. Lastly, anti-apoptotic effects of furosemide and cyclosporine A might not be clinically applicable because higher-than-clinical doses were required.

Conclusions

CPB increases intra-platelet Bax expression, which contributes to reduced platelet-surface GPIb expression and thrombin-induced platelet calcium changes. Platelet Bax is critical for reduction in platelet-surface GPIb expression.

Many biochemical and morphological features of apoptotic changes in platelets appear during the conversion to pro-coagulant platelets. However, recent studies show that the intracellular signalling mechanisms underlying platelet pro-coagulant function changes independently of apoptotic cell death signalling. Future studies should explore strategies for controlling platelet apoptotic changes without altering platelet pro-coagulant function, which might help maintain haemostasis in cardiac surgery with CPB.

Supplementary material

Supplementary material is available at British Journal of Anaesthesia online.

Declaration of interest

None declared.

References

8. Kageyama K, Nakajima Y, Shiba Y, Hashimoto S, Mizobe T. Increased platelet, leukocyte, and endothelial cell activity...
are associated with increased coagulability in patients after total knee arthroplasty. J Thromb Haemost 2007; 5: 738–45
10. Michelson AD. Platelet activation by thrombin can be directly measured in whole blood through the use of the peptide GPRP and flow cytometry: methods and clinical applications. Blood Coagul Fibrinolysis 1994; 5: 121–31

Handling editor: Hugh C Hemmings Jr