

Phosphodiesterase 3 Inhibition Reduces Platelet Activation and Monocyte Tissue Factor Expression in Knee Arthroplasty Patients

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Background: **Tissue damage during surgery activates platelets and provokes a prothrombic state. The current study attempted to determine the impact of phosphodiesterase 3 inhibitors on platelet activation, platelet–leukocyte aggregate formation, and monocyte tissue factor expression during and after total knee arthroplasty.**

Methods: **Thirty-four patients undergoing scheduled total knee arthroplasty were randomly assigned to receive either the phosphodiesterase 3 inhibitor milrinone or the same amount of saline perioperatively. The effects of milrinone on platelet and leukocyte function *in vitro* were then assessed in healthy volunteers.**

Results: **Perioperative infusion of milrinone significantly attenuated platelet activation; phosphorylation of intraplatelet p38 mitogen-activated protein kinase, extracellular signal–regulated kinase 1/2, and Akt; and platelet–leukocyte aggregation. Furthermore, perioperative tissue factor expression on monocytes and fibrin monomer complex production were reduced by milrinone infusion in patients undergoing total knee arthroplasty. *In vitro* studies using adenosine diphosphate– and collagen–stimulated blood samples from healthy volunteers confirmed the antiplatelet effects and reduced monocyte tissue factor expression by milrinone. These studies further showed that platelet aggregation and integrin $\alpha_{IIb}\beta_3$ activation were modified by intraplatelet phosphatidylinositol 3-kinase/Akt and mitogen-activated protein kinase/extracellular signal–regulated kinase pathways, and that P-selectin expression on platelets and platelet–leukocyte aggregation were modulated by intraplatelet p38 mitogen-activated protein kinase pathway.**

Conclusion: **Continuous milrinone infusion has the potential to reduce platelet activation and monocyte tissue factor expression during the perioperative period in total knee arthroplasty. These events may be mediated in part by the ability of milrinone to reduce activation of intraplatelet mitogen-activated protein kinases and phosphatidylinositol 3-kinase. The clinical impact of phosphodiesterase 3 inhibition on perioperative hemostasis remains to be elucidated.**

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SURGICAL trauma and inflammation enhances platelet activity at the site of vascular damage and provokes the release of subendothelial adhesive proteins, such as collagen and von Willebrand factor, or soluble agonists, such as adenosine diphosphate and thrombin, subsequently inducing platelet aggregation and increasing blood coagulability.[1–4] We have previously reported that the activities, as well the interactions, of platelets, leukocytes, and the endothelium are enhanced, particularly in venous blood from the lower half of the body, during the perioperative period of total knee arthroplasty.[5]

Circulating platelets are continuously exposed to adhesive and activating molecules in the perioperative inflammatory state. Most of these factors bind to specific platelet receptors and stimulate intraplatelet signaling molecules to promote platelet adhesion, aggregation, and secretion. Although different agonists induce platelet activation *via* different signaling pathways, these signals converge to common signaling events such as calcium mobilization and activation of the ligand binding function of integrin $\alpha_{IIb}\beta_3$, which mediates platelet aggregation.[6] Transformation of integrin $\alpha_{IIb}\beta_3$ into a competent receptor for fibrinogen on the platelet surface is detected by flow cytometry using an activation-dependent antibody such as PAC-1. These conformational changes on the platelet surface membrane are therefore closely linked to platelet aggregation, because circulating platelets do not bind fibrinogen or stick to each other without being activated.

Similarly, P-selectin is a component of the α-granule membrane of resting platelets that is expressed on the surface membrane with platelet activation and degranulation. However, circulating platelet–leukocyte aggregates are currently considered a more sensitive and reliable marker of platelet activation *in vivo* than platelet surface P-selectin, which is the classic marker of platelet activation.[7] Degranulated platelets can initially aggregate with monocytes and neutrophils on the leukocyte surface membrane *via* platelet surface P-selectin at glycoprotein ligand 1 (CD162).[8] Furthermore, activated platelets induce expression of tissue factor on monocytes *via* cross-talk between P-selectin/P-selectin glycoprotein ligand 1 and CD40/CD40 ligand.[8,9]

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Mitogen-activated protein kinases (MAPKs) (*e.g.*, p38 MAPK, extracellular signal–regulated kinase [ERK], c-Jun amino-terminal kinase) and phosphatidylinositol 3-kinase (PI3K) control cellular responses to proliferative and chemotactic stimuli, such as growth factors and hormones. The MAPK and PI3K/Akt pathways have recently become the focus of research into multiple platelet signaling pathways. However, these intraplatelet pathways have mostly been studied in conventional models of agonist-induced platelet aggregation, and perioperative changes in these pathways have not been elucidated.[10]

Phosphodiesterase 3 (PDE3), which plays a pivotal role in the regulation of intracellular cyclic adenosine monophosphate levels, can be found in cardiac muscle, vascular muscle, platelets, and leukocytes. PDE3 inhibitors, widely used as inodilators (*i.e.*, positive inotropes and arteriovenous dilators) in clinical situations, are also recognized for their antiplatelet activity. Cilostazol has been approved in the United States for the treatment of intermittent claudication[11] and in Japan for the prevention of stroke recurrence.[12] Milrinone is approved for clinical use as an inodilator, and *in vitro* studies have reported that its antiplatelet effects are as potent as those of cilostazol.[13,14] However, the antiplatelet effects of milrinone during the perioperative period need to be elucidated further.[15,16] Therefore, the current study aimed to examine the impact of milrinone infusion on platelet activity, platelet–leukocyte aggregation, and subsequent monocyte tissue factor production, as well as fibrin monomer formation, during the first 24 h after anesthesia induction in patients undergoing total knee arthroplasty, and to clarify the underlying intraplatelet MAPK and PI3K/Akt pathways *in vitro*.

Materials and Methods

In Vivo *Clinical Study*

The Review Board for Human Experiments at Kyoto Prefectural University of Medicine, Kyoto, Japan, approved this prospective, randomized, double-blind study. Written informed consent was obtained from all patients and volunteers. Sample size was calculated from a pilot study on the primary outcome (perioperative increase in platelet–monocyte aggregation). A sample size estimate indicated that 16 patients/group would provide 80% power for detecting differences of 1 SD at an α level of 0.05 for the primary outcome. We studied 34 patients diagnosed with osteoarthritis and undergoing unilateral total knee arthroplasty. All had American Society of Anesthesiologists physical status I or II and were aged 60–80 yr. We excluded patients with conditions known or suspected to independently increase platelet activity, specifically, those with venous thrombosis, sepsis, acute infection, pregnancy, acute coronary syndromes, heparin-induced thrombocytopenic purpura, transient ischemic attacks, severe hypertension (> 160/95 mmHg), recent cardiopulmonary bypass, or multiple sclerosis. We also excluded patients who had preoperative hepatic or renal dysfunction or severe cardiac or respiratory disease. All patients discontinued aspirin 1 week before surgery and other nonsteroidal antiinflammatory medication at least 48 h preoperatively. Intermittent pneumatic compression boots were applied on both feet postoperatively. Postoperative fondaparinux sodium was administered more than 24 h after the end of surgery according to the recommendation of the pharmaceutical company (GlaxoSmithKline, Tokyo, Japan). The same surgical team performed all surgical procedures using the same techniques and prosthesis.

Randomization. Patients were allocated to treatment according to a two-way randomization process. Randomization was computer generated using a randomization scheme from the Randomization Web site,# and codes were maintained in sequentially numbered envelopes. The envelopes were opened after informed consent was obtained. Patients assigned to the milrinone group [Mil(+) group] were given an infusion of intravenous milrinone (Astellas Pharmaceutical Co. Ltd., Tokyo, Japan) at a dose of 50 μg/kg infusion for the first 10 min after anesthetic induction, with a subsequent continuous infusion of 0.5–0.75 $\mu g \cdot kg^{-1} \cdot min^{-1}$ until 8 h after the start of surgery (5–6 h after the end of surgery). The remaining patients were given an identical volume of nutrient-free standard saline solution [Mil(−) group]. Both solutions were covered with opaque foil to prevent physicians from determining group assignment.

Protocol. General anesthesia was induced with intravenous propofol (2 mg/kg), and tracheal intubation was performed after administering intravenous vecuronium (0.15 mg/kg). Anesthesia was maintained using an inhalation of 1–1.5% sevoflurane and 66% nitrous oxide in oxygen. An intravenous infusion of 0.025 $mg \cdot kg^{-1} \cdot h^{-1}$ vecuronium was adjusted to maintain one or two twitches in response to supramaximal stimulation of the ulnar nerve at the wrist. Mechanical ventilation was adjusted to maintain end-tidal partial pressure of carbon dioxide between 35 and 40 mmHg. The ambient operating room temperature was maintained at approximately 23°C. Patients were laid on a circulating water-warming mattress maintained at 37°C and covered with a sheet during surgery. Fentanyl was administered for surgical pain relief at the rate of 2.5 $\mu g \cdot kg^{-1} \cdot h^{-1}$ during surgery and 0.4 $\mu g \cdot kg^{-1} \cdot h^{-1}$ after surgery until postoperative day 2. A tourniquet was applied to the thigh of all patients from the beginning of surgery for approximately 120 min. Blood samples were collected from the radial artery at (1) baseline (after induction of anesthesia), (2) 2.5 h (after tourniquet deflation and around the end of surgery), (3) 8 h (5–6 h after the end

http://www.randomization.com/. Accessed October 12, 2008.

of surgery), and (4) 24 h after the start of surgery. Blood was withdrawn into sterile 3.8% sodium citrate Vacutainers (Becton Dickinson, San Jose, CA). Blood pressure and heart rate were recorded at 5-min intervals during surgery.

Measures of Platelet Activation.

Impedance Aggregometry. Impedance aggregometry was performed using a Chrono-log aggregometer (Chrono-log, Havertown, PA). Anticoagulated whole blood was diluted with an equal volume of saline and incubated for 10 min. Aggregation in response to collagen (2 μg/ml) was determined as the change in impedance units over 6 min.

Platelet–Leukocyte Aggregate Formation, and PAC-1 and P-selectin Expression on Platelets. To determine platelet–leukocyte aggregates, whole blood was stained with CD14-FITC and CD41a-PE (BD Pharmingen, San Diego, CA). To determine PAC-1 and P-selectin (CD62P) expression on platelets or platelet–leukocyte aggregates, whole blood was stained with PAC-1-FITC, CD62P-PE, and CD61-PerCP or CD14-PerCP (BD Pharmingen). Samples in polypropylene tubes were incubated for 20 min without any stimulant, in the dark, at room temperature (20°–25°C), fixed by adding 100 μl Optilyse B (Beckman Coulter, Fullerton, CA), and lysed in 1 ml distilled water. The samples were stored at 4°C until flow cytometric analysis using a standard four-color filter configuration (FACSCalibur with CellQuest software; Becton Dickinson, Franklin Lakes, NJ) on the day of collection. Platelets were selected by gating CD61+ events on a two-parameter dot plot displaying side scatter *versus* CD61-PerCP (FL3). A total of 30,000 CD61+ events were acquired. Monocytes were selected by gating CD14-bright+ events on a two-parameter dot plot displaying side scatter *versus* CD14-FITC (FL1) or -PerCP (FL3), whereas neutrophils were selected by gating CD14-dim+ and high side light scatter populations in whole blood. Back-gating onto forward scatter *versus* side scatter plots was performed to verify the morphology of these cells. A total of 3,000 CD14+ events were acquired. Nonspecific fluorescence was determined using irrelevant isotypic control antibodies. Geometric mean fluorescence intensity values were used for statistical analyses. Platelet–monocyte aggregates were also presented as percentages of platelet-conjugated monocytes in the total monocyte population.

Intraplatelet p38 MAPK, ERK1/2, and Akt Phosphorylation. To analyze the phosphorylation status of p38 MAPK, ERK 1/2, and Akt within platelets, whole blood was stained with p38 MAPK-FITC (pTpY180/182) (BD Pharmingen), ERK1/2-PE (pT202/pY204) (BD Pharmingen), CD61-PerCP, and CD62P-APC (BD Pharmingen) or with Akt-PE (pT308) (BD Pharmingen), CD61-PerCP, and CD62P-APC according to the commercially available protocol and reagents (BD PhosFlow; BD Pharmingen). Further, to evaluate the total amount of p38 MAPK, ERK1/2, and Akt, whole blood was stained with p38 MAPK (Cell Signaling Technology, Tokyo, Japan), ERK1/2 (Cell Signaling Technology), and Akt (Cell Signaling Technology), respectively, with CD61-PerCP and CD62P-APC. It was then further stained with secondary antibody: Alexa Fluor® 488 F(ab′)2 fragment of goat anti-rabbit immunoglobulin G (H+L) (Invitrogen, Tokyo, Japan) or PE F(ab′)2 fragment of donkey anti-mouse immunoglobulin G (H+L) (eBioscience, San Diego, CA). Platelets were selected by gating CD61+ or CD62P+ events. A total of 30,000 CD61+ or 10,000 CD62P+ events were acquired. Nonspecific fluorescence was determined using irrelevant isotypic control antibodies. Geometric mean fluorescence intensity values were used for statistical analyses.

Monocyte Tissue Factor Expression. Whole blood (100 μl) was stained with mouse anti-human monoclonal antibodies CD142-PE and CD14-PerCP (BD Pharmingen) to determine leukocyte surface tissue factor (CD142) expression. The subsequent procedures are the same described previously.

Fibrin Monomer. Plasma soluble fibrin monomer complex concentration was measured by latex immunoturbidimetric assay (Mitsubishi BCL, Tokyo, Japan).

In Vitro *Studies*

Protocol. We studied healthy volunteers (all males; aged 25–35 yr) who had not received any medication for more than 2 weeks. Blood samples were collected without stasis from the antecubital vein with a 20-gauge needle. Whole blood samples were anticoagulated with sterile 3.8% sodium citrate Vacutainers. To clarify the underlying mechanism of the antiplatelet effect of PDE3 inhibitors, we mainly investigated the impact of milrinone (a gift from Astellas Pharma Inc., Tokyo, Japan) on platelet function and intraplatelet signaling *in vitro*. Blood samples were divided into groups that received 0 μM (positive control) and 1 μM of milrinone coincubation. Milrinone was dissolved in 0.5% lactic acid and then diluted with saline. Equivalent volumes of lactic acid and saline were added to control samples. The dose of milrinone was assumed to be a therapeutic blood concentration (milrinone < 1.5 μM).[17] Samples were incubated for 10 min at room temperature and then stimulated with either 10 μM adenosine diphosphate (ADP) or 2 μg/ml collagen. The negative control groups were incubated without ADP or collagen.

Optical Aggregometry. Whole blood was centrifuged at 130*g* for 20 min at 22°C. The upper layer was collected as platelet-rich plasma. This layer was diluted with platelet-poor plasma to adjust platelet counts to 200,000/μl. Aliquots of platelet rich plasma (500 μl) were placed in a siliconized glass tube, maintained at 37°C, and stirred at a speed of 1,000/min to simulate intravascular shear. Platelet aggregation was measured for 5 min using an aggregometer with the turbidimetric method (CAF-110; JASCO, Tokyo, Japan). The baseline optical density point was defined as 0% for the platelet-

rich plasma and 100% for the platelet-poor plasma after maximal platelet activation.

Flow Cytometry. The experimental procedure was the same as that described in the *in vivo* clinical study. Further, to determine whether inhibition of monocyte tissue factor expression by milrinone is directly affected by monocytes or through platelet–monocyte interaction, various blood samples (whole blood, peripheral blood mononuclear cells only, and washed platelets plus peripheral blood mononuclear cells [cell number ratio = 50:1]) were incubated for 2 h at 37°C with 5% CO_2 after collagen stimulation. Washed platelets were generated from platelet-rich plasma by washing twice with citrate wash buffer (128 mM NaCl, 7.5 mM Na_2HPO_4, 4.3 mM NaH_2PO_4, 4.8 mM trisodium citrate, 2.4 mM citric acid, 0.28 μM prostaglandin E1, and 0.35% bovine serum albumin, pH 6.5) and resuspending into modified HEPES–Tyrode buffer (137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$ $6H_2O$, 12 mM $NaHCO_3$, 0.4 mM Na_2HPO_4, 10 mM HEPES, 5.5 mM glucose, and 0.35% bovine serum albumin, pH 7.4) to adjust platelet counts to 200,000/μl. Peripheral blood mononuclear cells were isolated from whole blood by density gradient centrifugation (NycoPrep 1077; AXIS-SHIELD, Oslo, Norway), with subsequent density gradient centrifugation (ρ = 1.063 g/ml) using iodixanol (Optiprep; AXIS-SHIELD) for the complete removal of platelets. Peripheral blood mononuclear cell viability (> 97%) was assessed by trypan blue exclusion, and the percentage of monocytes with adherent platelets (< 5%) was detected by the procedure described in the *in vivo* clinical study.

Intracellular Pathway Inhibition *In Vitro*. Blood samples were separately preincubated for 10 min with dimethyl sulfoxide (positive control), LY294002 (20 μM, PI3K inhibitor), SB202190 (20 μM, p38 MAPK inhibitor), SB203580 (20 μM, p38 MAPK inhibitor), SB202474 (20 μM, inactive analogue of SB203580 and SB202190), U0126 (20 μM, MAPK kinase [MEK] 1/2 inhibitor), or U0124 (20 μM, inactive analogue of U0126) (Calbiochem, San Diego, CA). The effects of these pathway inhibitors on platelet aggregation, platelet–leukocyte aggregation, platelet surface expression of PAC-1 and P-selectin, and monocyte expression of tissue factor in response to ADP and collagen were investigated by the methods described previously. Only in the experiments for monocyte tissue factor expression, washed platelets were pretreated with a different kinase inhibitor for 10-min incubation, and supernatant was eliminated after centrifugation. Platelets were then coincubated with peripheral blood mononuclear cells for 2 h at 37°C with 5% CO_2 after collagen stimulation.

Statistical Analysis

The effects of PDE3 inhibition in the perioperative period shown in figures 1–6 were analyzed using general

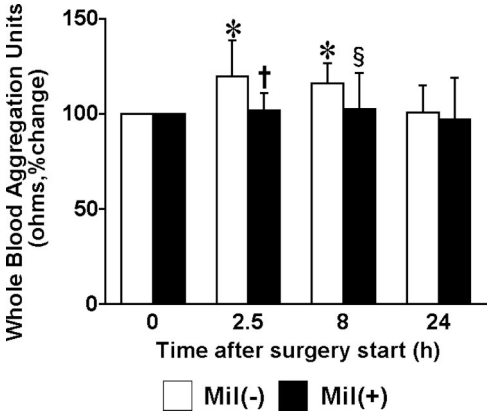

Fig. 1. Perioperative changes in aggregation units in response to collagen (2 μg/ml). Acceleration of perioperative whole blood aggregation was significantly lower in the milrinone-treated group [Mil(+)] compared with the group not treated with milrinone [Mil(−)] at 2.5 and 8 h after the commencement of surgery. Data are shown as percent change from preoperative (0 h) values (mean ± SD, n = 17/group). *Open bars* = Mil(−) group; *solid bars* = Mil(+) group. * P < 0.01 compared with baseline (0 h) in each group. † P < 0.01 compared with Mil(−) at each time point. § P < 0.05 compared with Mil(−) at each time point.

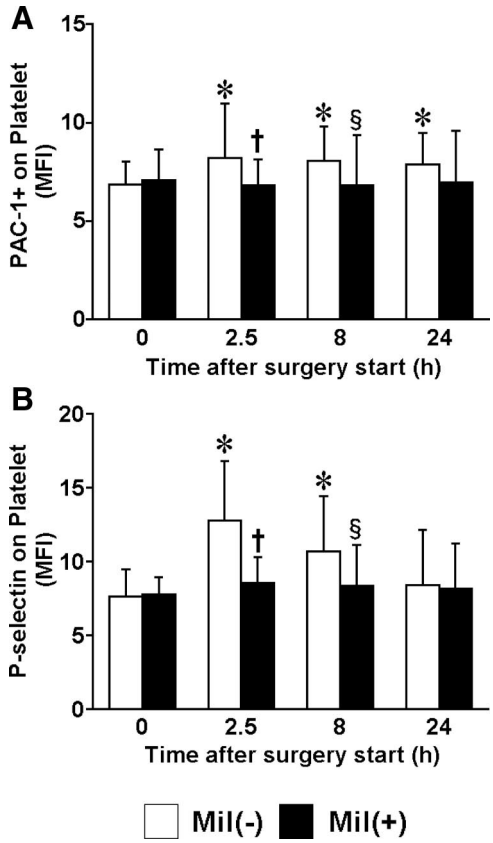

Fig. 2. Perioperative changes in PAC-1 and P-selectin expression on platelets. Perioperative PAC-1 (*A*) and P-selectin (*B*) expression on platelets was attenuated in the milrinone-treated group [Mil(+)] compared with the group not treated with milrinone [Mil(−)]. Data are shown as geometric mean fluorescence intensity (MFI) values (mean ± SD, n = 17/group). *Open bars* = Mil(−) group; *solid bars* = Mil(+) group. * P < 0.01 compared with baseline (0 h) in each group. † P < 0.01 compared with Mil(−) at each time point. § P < 0.05 compared with Mil(−) at each time point.

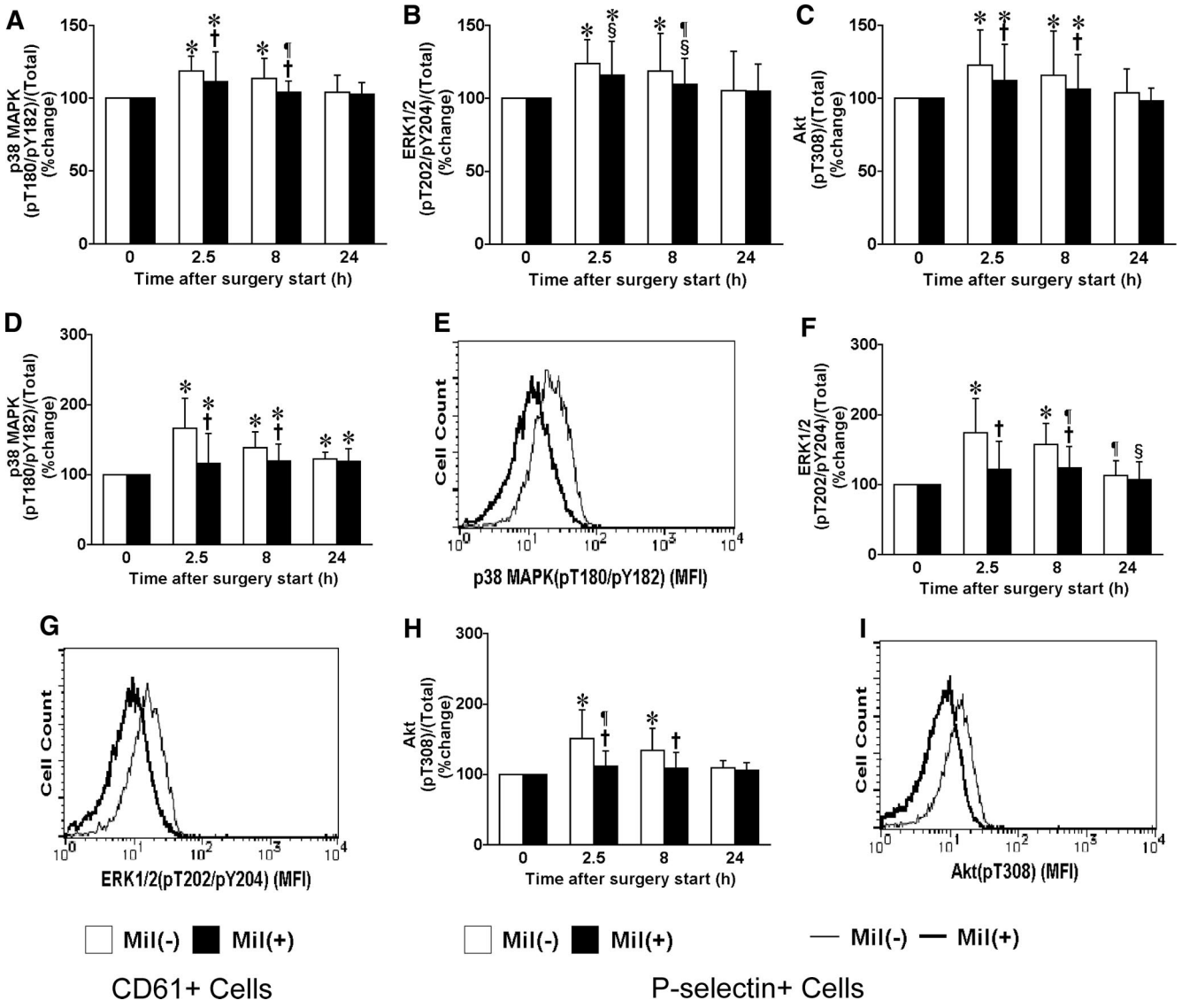

Fig. 3. Perioperative changes in the geometric mean fluorescence intensity ratio of phosphorylated and total platelet p38 mitogen-activated protein kinase (MAPK), extracellular signal–regulated kinase (ERK) 1/2, and Akt. Perioperative increase in phosphorylation of intraplatelet p38 MAPK (*A*), ERK1/2 (*B*), and Akt (*C*) was attenuated in the milrinone-treated group [Mil(+)] compared with the group not treated with milrinone [Mil(−)]. These differences between the groups were exaggerated in activated (P-selectin positive) platelets (*D*, *F*, and *H*). Data are shown as geometric mean fluorescence intensity values (mean ± SD, n = 17/group). *Open bars* = Mil(−) group; *solid bars* = Mil(+) group. * $P < 0.01$ compared with baseline (0 h) in each group. ¶ $P < 0.05$ compared with baseline (0 h) in each group. † $P < 0.01$ compared with Mil(−) at each time point. § $P < 0.05$ compared with Mil(−) at each time point. Representative flow cytometric histograms of P-selectin positive platelets at 2.5 h after the commencement of surgery in the two groups are shown on the *right* (*E*, *G*, and *I*). *Thin line* = Mil(−) group; *bold line* = Mil(+) group.

linear regression modeling for two-way analysis of variance with repeated measures (one between factor and one within factor), followed by Tukey multiple comparison testing. Other continuous variables were analyzed with one-way analysis of variance (one between factor). The chi-square test was used to compare categorical variables. Analyses were performed using SuperANOVA (Abacus Concepts, Inc., Berkeley, CA) and Statcel2 (Oms-publishing, Saitama, Japan). Values are expressed as mean ± SD or as percent change from baseline value. *P* values less than 0.05 were considered significant.

Results

Perioperative Milrinone Infusion Attenuates Platelet Activity and Subsequent Tissue Factor Production and Coagulability in Patients Undergoing Total Knee Arthroplasty

In the current clinical trial, morphometric and demographic characteristics were similar in the two groups, as were preoperative hemostatic data (table 1). Furthermore, anesthetic and surgical management and clinical factors, including perioperative blood loss and transfusion, did not differ significantly between the groups.

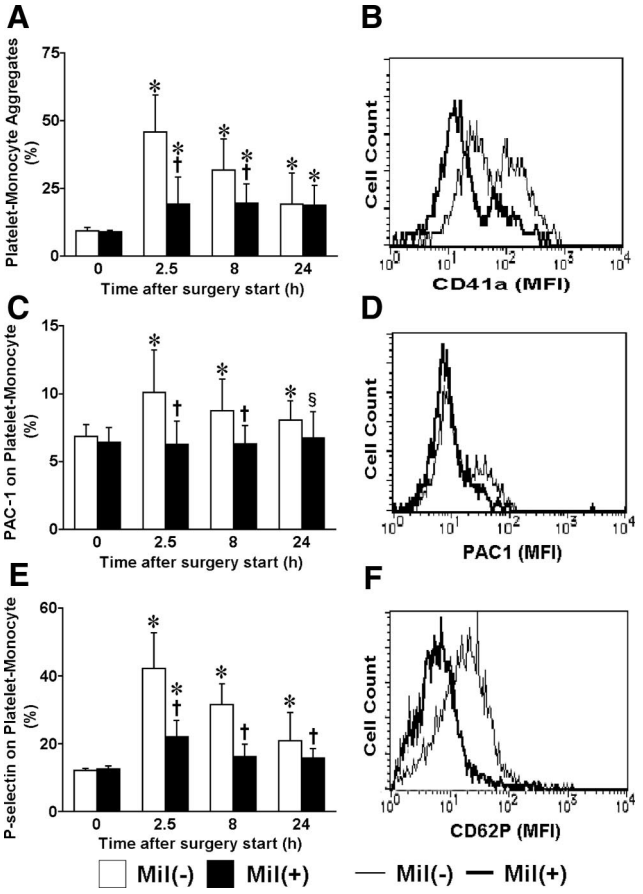

Fig. 4. Perioperative changes in platelet–monocyte aggregates, and PAC-1 and P-selectin expression on platelet–monocyte aggregates. Perioperative platelet–monocyte aggregation (*A*), PAC-1 expression (*C*), and P-selectin expression (*E*) on platelet–monocyte aggregates (activated platelet-monocyte aggregates) was attenuated in the milrinone-treated group [Mil(+)] compared with the group not treated with milrinone [Mil(−)]. The *bar charts on the left* present data as percentages of CD41a (platelet surface antigen), PAC-1, and P-selectin conjugated monocytes in the total monocyte population, respectively (mean ± SD, n = 17/group). Open bars: Mil(−) group; solid bars: Mil(+) group. * $P < 0.01$ compared with baseline (0 h) in each group. † $P < 0.01$ compared with Mil(−) at each time point. § $P < 0.05$ compared with Mil(−) at each time point. Representative flow cytometric histograms of mean fluorescence intensity (MFI) values at 2.5 h after the commencement of surgery in the two groups are shown on the *right* (*B*, *D*, and *F*). *Thin line* = Mil(−) group; *bold line* = Mil(+) group.

Perioperative systolic blood pressure values in the milrinone group tended to be lower than those in the control group, although significant differences were not observed between the two groups (table 2). We planned to exclude patients who developed persistent severe hypotension (systolic blood pressure < 70 mmHg for 10 min) or persistent tachycardia (heart rate > 140 beats/min for 10 min); however, no patients met these exclusion criteria.

The increase in perioperative platelet aggregability assessed by whole blood impedance aggregometry was suppressed in the Mil(+) group compared with the Mil(−) group (fig. 1; $P < 0.05$). The expression of PAC-1

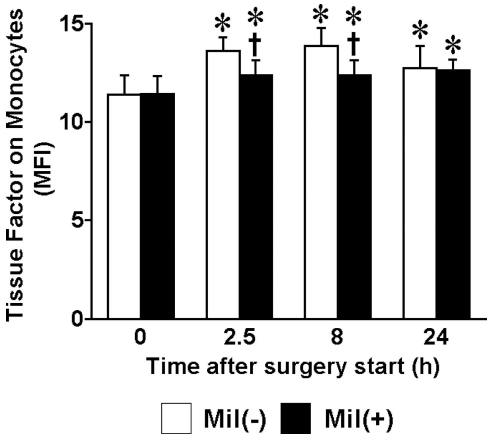

Fig. 5. Perioperative changes in tissue factor expression on monocytes. Perioperative increase in this antigen, which reflects the prothrombic state, was attenuated in the milrinone-treated group [Mil(+)] compared with the group not treated with milrinone [Mil(−)] (mean ± SD, n = 17/group). *Open bars* = Mil(−) group; *solid bars* = Mil(+) group. * $P < 0.01$ compared with baseline (0 h) in each group. † $P < 0.01$ compared with Mil(−) at each time point. MFI = mean fluorescence intensity.

and P-selectin on platelets was significantly lower in the Mil(+) group compared with the Mil(−) group during the perioperative period (fig. 2; $P < 0.05$). A perioperative increase in phosphorylation status of intraplatelet p38 MAPK, ERK1/2, and Akt was prevented in the Mil(+) group when compared with the Mil(−) group. These differences were enhanced in the activated (P-selectin–positive) platelets (fig. 3; $P < 0.05$). The amount of platelet–monocyte aggregation and expression of PAC-1 and P-selectin on platelet–monocyte aggregates (activated platelet–monocyte aggregates) were significantly lower in the Mil(+) group when compared with the Mil(−) group, indicating that heterotypic blood cell interactions were attenuated by milrinone infusion

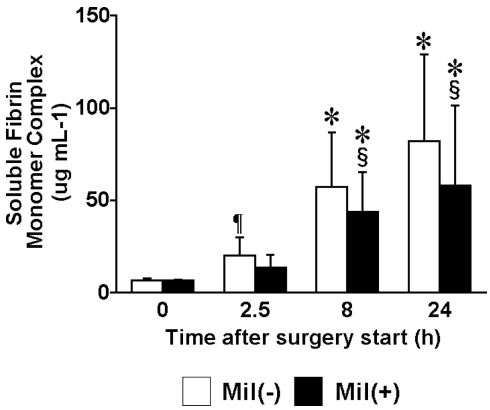

Fig. 6. Perioperative changes in fibrin monomer complex concentrations. Perioperative increase in this product, which reflects the prothrombic state, was attenuated in the milrinone-treated group [Mil(+)] compared with the group not treated with milrinone [Mil(−)] (mean ± SD, n = 17/group). *Open bars* = Mil(−) group; *solid bars* = Mil(+) group. * $P < 0.01$ compared with baseline (0 h) in each group. ¶ $P < 0.05$ compared with baseline (0 h) in each group. † $P < 0.01$ compared with Mil(−) at each time point. § $P < 0.05$ compared with Mil(−) at each time point.

Table 1. Preoperative Patient Characteristics

	Milrinone(−)	Milrinone(+)	*P* Value
Age, yr	71 ± 8	72 ± 8	0.73
Weight, kg	65 ± 12	64 ± 11	0.87
Height, cm	155 ± 10	156 ± 9	0.88
Sex, M/F	6/11	5/12	0.71
Hemoglobin, g/dl	12.0 ± 2.0	12.2 ± 1.7	0.77
Leukocytes, $\times 10^3/mm^3$	6.5 ± 1.9	6.3 ± 1.9	0.72
Platelets, $\times 10^3/mm^3$	229 ± 77	237 ± 74	0.71
PT, s	11.4 ± 0.5	11.3 ± 0.7	0.64
INR	1.01 ± 0.05	0.99 ± 0.08	0.43
APTT, s	34.1 ± 4.8	34.8 ± 5.0	0.65
D-dimer, μg/ml	1.6 ± 0.6	1.8 ± 1.2	0.61
Fibrinogen, mg/dl	268 ± 35	261 ± 38	0.57

Data are presented as mean ± SD (n = 17/group).

APTT = activated thromboplastin time; INR = international normalized ratio; milrinone(−) = group not treated with milrinone; milrinone(+) = milrinone-treated group; PT = prothrombin time.

perioperatively (fig. 4; $P < 0.05$). The perioperative changes in platelet–neutrophil conjugates were similar to those observed for platelet–monocyte conjugates (data not shown). The changes in these platelet activation markers were markedly clear at 2.5 h after the commencement of surgery and were reduced by perioperative milrinone infusion. The perioperative increases in tissue factor expression on monocytes, as well as the generation of fibrin monomer complexes, which reflect increases in blood coagulability, were reduced by perioperative milrinone infusion (figs. 5 and 6; $P < 0.05$).

In Vitro *Platelet Activity, Monocyte Tissue Factor Expression, and Intraplatelet Signaling Are Attenuated by Preincubation with Milrinone*

To validate the clinical findings, we measured blood samples from healthy volunteers in *in vitro* experiments. These *in vitro* studies showed that milrinone decreased platelet aggregation in response to ADP and collagen (table 3; $P < 0.05$). The increase in platelet–leukocyte aggregates, and both PAC-1 and P-selectin expression on platelets in response to ADP and collagen was attenuated by preincubation with milrinone (table 3; $P < 0.05$). Milrinone attenuated the phosphorylation of Akt, p38 MAPK, and ERK1/2 in platelets, without altering the total amounts of these molecules (table 3; $P < 0.05$). Tissue factor expression on monocytes 2 h after whole blood incubation following collagen stimulation (2 μg/ml) was diminished by incubation with milrinone (table 4; $P < 0.05$). Furthermore, milrinone inhibited monocyte tissue factor expression after coincubation with both washed platelets and peripheral blood mononuclear cells, but it did not do so when incubated with peripheral blood mononuclear cells alone (table 4; $P < 0.05$).

Role of p38 MAP, ERK1/2, and Akt Phosphorylation in Platelet Function

Finally, we assessed the role of PDE3 inhibitor–mediated intracellular signaling attenuation in platelet inhibition by pretreatment with each intracellular protein kinase inhibitor. With regard to the synthesis of platelet–leukocyte aggregates and P-selectin expression on platelets, significant differences were observed, particularly among SB202474, SB202190, and SB203580 (table 5; $P < 0.05$). Significant differences in platelet aggregation were observed between the positive control (dimethyl sulfoxide pretreatment) and LY294002 and between U0124 and U0126 (tables 6 and 7; $P < 0.05$). There were also significant differences in platelet PAC-1 expression between positive controls and LY294002, and between U0124 and U0126 (tables 6 and 7; $P < 0.05$). Furthermore, tissue factor expression on monocytes after coincubation of washed platelets and peripheral blood mononuclear cells was prevented by SB202190 and SB203580 (table 5; $P < 0.05$). Inhibition of intraplatelet p38 MAPK pathway thus reduced P-selectin expression, platelet–monocyte conjugate formation, and monocyte tissue factor expression, but not platelet aggregation and PAC-1 expression. Inhibition of the intraplatelet MEK/ERK pathway and PI3K/Akt pathway inhibited platelet aggregation and PAC-1 expression, but not P-selectin

Table 2. Perioperative Management

	Milrinone(−)	Milrinone(+)	*P* Value
SBP/HR after induction of anesthesia, mmHg, beats/min	118 ± 17/71 ± 14	120 ± 15/73 ± 13	0.71/0.82
SBP/HR at 2.5 h after start of surgery, mmHg, beats/min	124 ± 19/75 ± 13	119 ± 18/77 ± 11	0.45/0.64
SBP/HR at 8 h after start of surgery, mmHg, beats/min	122 ± 19/76 ± 13	116 ± 18/78 ± 15	0.39/0.70
SBP/HR at 24 h after start of surgery, mmHg, beats/min	120 ± 18/74 ± 12	114 ± 18/76 ± 11	0.39/0.62
Duration of surgery, min	181 ± 46	184 ± 50	0.84
Duration of anesthesia, min	271 ± 53	267 ± 33	0.76
Duration of tourniquet inflation, min	126 ± 16	124 ± 17	0.67
Fluid infusion, ml	2,623 ± 597	2,454 ± 732	0.43
Transfusion, ml	360 ± 256	380 ± 242	0.80
Blood loss during surgery, ml	270 ± 191	280 ± 162	0.86
Postoperative hemoglobin after 24 h, g/dl	11.6 ± 2.0	11.7 ± 2.1	0.88
Blood loss during 24 h, ml	651 ± 130	673 ± 147	0.61

Data are presented as mean ± SD (n = 17/group).

HR = heart rate; milrinone(−) = group not treated with milrinone; milrinone(+) = milrinone-treated group; SBP = systolic blood pressure.

Table 3. *In Vitro* Changes in Platelet Aggregation, Platelet Activity Markers, and Intraplatelet MAPKs and Akt after Pretreatment with Milrinone

	NC	PC	Mil 1
Platelet aggregation units, % change			
ADP	—	100 ± 0	82.8 ± 4.9*
Collagen	—	100 ± 0	70.6 ± 11.9*
Platelet–monocyte aggregates, %			
ADP	8.5 ± 3.9	45.7 ± 5.4	29.3 ± 8.8*
Collagen	8.4 ± 3.3	61.2 ± 7.0	43.2 ± 4.9*
PAC-1 on platelet, MFI			
ADP	7.4 ± 2.0	11.1 ± 1.3	8.9 ± 1.7†
Collagen	7.5 ± 1.7	15.8 ± 2.0	12.1 ± 1.4†
P-selectin on platelets, MFI			
ADP	8.1 ± 1.6	35.6 ± 3.7	31.2 ± 4.3†
Collagen	8.1 ± 1.6	52.6 ± 4.8	45.0 ± 4.7†
p38 MAPK (pT180/pY182)/(total), % change			
ADP	100 ± 0	125.2 ± 8.6	111.9 ± 10.6†
Collagen	100 ± 0	182.5 ± 10.4	161.3 ± 10.2*
ERK1/2 (pT202/pY204)/(total), % change			
ADP	100 ± 0	132.1 ± 9.6	115.8 ± 10.6†
Collagen	100 ± 0	154.7 ± 7.5	133.0 ± 15.4*
Akt (pT308)/(total), % change			
ADP	100 ± 0	131.5 ± 9.4	112.2 ± 10.2*
Collagen	100 ± 0	152.5 ± 11.6	134.1 ± 12.0*

Blood samples were incubated with milrinone (Mil 1, 1 μM) or the same amount of vehicle for 10 min and then stimulated with adenosine diphosphate (ADP, 10 μM) or collagen (2 μg/ml). Platelet aggregation was measured using platelet-rich plasma samples by optical aggregometry, and the data are shown as percent change from positive control (PC) values. Flow cytometry analysis was performed using whole blood samples. Platelet-monocyte aggregates are shown as percent of platelet-conjugated monocytes in the total monocyte population. PAC-1 and P-selectin expression on platelets are shown as geometric mean fluorescence intensity (MFI) values. The geometric MFI ratio of phosphorylated and total platelet Akt, p38 mitogen-activated protein kinase (MAPK), and extracellular signal–regulated kinase (ERK) 1/2 are shown as percent change from negative control (NC) values. Data are presented as mean ± SD (n = 5/group).

* $P < 0.01$ and † $P < 0.05$ compared with each PC.

Table 4. *In Vitro* Changes in Tissue Factor Expression on Mononuclear Cell in Response to Collagen after Pretreatment with Milrinone

	Collagen(−)		Collagen(+)	
	Control	Mil 1	Control	Mil 1
Whole blood, MFI	12.0 ± 0.7	11.9 ± 0.7	17.2 ± 1.2	15.2 ± 1.5*
Mononuclear cells, MFI	13.0 ± 0.8	12.9 ± 1.9	12.1 ± 0.9	11.8 ± 0.7
Mononuclear cells + washed platelets, MFI	13.0 ± 0.9	12.5 ± 1.7	18.2 ± 3.3	13.9 ± 1.8*

Various blood samples (whole blood, peripheral blood mononuclear cells only, and washed platelets + peripheral blood mononuclear cells [cell number ratio = 50:1]) were incubated for 2 h at 37°C with 5% CO_2 after collagen (2 μg/ml) stimulation. Tissue factor expressions on monocyte are shown as geometric mean fluorescence intensity (MFI) values. Data are presented as mean ± SD (n = 6/group).

* $P < 0.01$ compared with each control.

Mil 1 = milrinone, 1 μM.

expression, platelet–monocyte conjugate formation, and monocyte tissue factor expression.

Discussion

In the current study, we demonstrated that platelet activity, phosphorylation of intraplatelet p38 MAPK, ERK1/2, and Akt, and heterotypic blood cell interactions were enhanced in patients undergoing total knee arthroplasty. Furthermore, we showed that perioperative milrinone infusion attenuated these events, and this may have consequently reduced monocyte tissue factor and soluble fibrin monomer complex production. *In vitro* studies using blood samples from healthy volunteers also demonstrated that milrinone at clinically relevant concentrations diminished stimulant-induced acceleration of platelet activity, platelet–leukocyte interactions, and monocyte tissue factor expression by reducing the activity of p38 MAPK, MEK/ERK, and PI3K/Akt signaling pathways in platelets as downstream signals of PDE3 inhibition. Therefore, these intraplatelet signaling pathways may play individual roles in platelet activation and in inducing monocyte tissue factor production.

Orthopedic surgery, particularly arthroplasty of the lower extremities, is known to promote platelet activation and heterotypic blood cell interactions. Tourniquet application augments these responses as well as surgical inflammation during total knee arthroplasty.[5] Vascular endothelial injury,[18] pain,[19] and ischemia–reperfusion injury[20] from tourniquet application are probably the main mechanisms for exaggeration of blood cell interactions and blood coagulability during this type of surgery. Intraoperative milrinone infusion, with target blood concentrations between 1.0 and 1.5 μM, reduces the platelet markers relevant to activation status in the perioperative period.[17] This finding is consistent with our previous report that milrinone inhibits platelet aggregation and calcium release by thrombin (< 1.5 μM).[21] Kikura *et al.*,[16] however, reported that perioperative PDE3 inhibition (with milrinone or amrinone) did not cause deterioration of platelet function or hemostasis during cardiopulmonary bypass surgery. The discrepancy between the results of these studies may be explained by differences in platelet condition. Platelet hypofunction is observed after cardiopulmonary bypass, whereas platelet hyperactivity occurs in total knee arthroplasty.

Perioperative blood loss and total transfusion amounts did not change significantly with perioperative milrinone infusion. Despite the lack of strong evidence supporting a link between bleeding time and clinical bleeding

Table 5. *In Vitro* Changes in Platelet Aggregation, Platelet–Monocyte Aggregates, Platelet PAC-1 and P-selectin Expression, and Monocyte Tissue Factor Expression by p38 MAPK Inhibitors

	NC	PC	SB202474	SB202190	SB203580
Platelet aggregation units, % change					
ADP	—	100 ± 0	53.3 ± 5.1	49.0 ± 10.8	49.0 ± 10.8
Collagen	—	100 ± 0	81.9 ± 2.8	76.4 ± 10.5	71.3 ± 10.7
Platelet–monocyte aggregates, %					
ADP	8.7 ± 0.8	51.1 ± 3.2	49.0 ± 2.6	20.5 ± 2.9*	21.9 ± 2.4*
Collagen	8.8 ± 0.3	71.9 ± 2.7	68.8 ± 3.4	39.6 ± 3.3*	36.4 ± 3.6*
PAC-1 on platelets, MFI					
ADP	7.3 ± 1.8	10.4 ± 1.8	10.5 ± 1.6	11.7 ± 1.9	11.5 ± 1.7
Collagen	7.5 ± 1.1	14.3 ± 1.5	14.6 ± 2.0	13.5 ± 2.0	14.3 ± 1.8
P-selectin on platelets, MFI					
ADP	7.9 ± 1.5	37.1 ± 2.3	39.0 ± 2.6	21.4 ± 2.6*	22.3 ± 3.1*
Collagen	8.1 ± 2.2	53.8 ± 3.9	53.0 ± 2.1	37.6 ± 3.5*	36.7 ± 4.8*
Tissue factor expression on monocytes, MFI					
Collagen	11.7 ± 0.5	18.2 ± 2.8	16.2 ± 1.3	9.4 ± 0.9*	9.2 ± 0.4*

Blood samples were preincubated for 10 min with different kinase inhibitors or the same amount of dimethyl sulfoxide (control). Only in the experiments for monocyte tissue factor expression, washed platelets were pretreated with a different kinase inhibitor for 10-min incubation, and supernatant was eliminated after centrifugation. Platelets were then coincubated with peripheral blood mononuclear cells for 2 h at 37°C with 5% CO_2 after collagen stimulation. Platelet aggregation and flow cytometry protocol is the same as that described in tables 3 and 4. Platelet–monocyte aggregates are shown as percentage of platelet-conjugated monocytes in the total monocyte population. PAC-1 and P-selectin expression on platelet and tissue factor expression on monocyte are shown as geometric mean fluorescence intensity (MFI) values. Data are presented as mean ± SD (n = 5/group).

* $P < 0.01$ compared with each SB202474.

ADP = adenosine diphosphate, 10 μM; collagen = collagen, 2 μg/ml; MAPK = mitogen-activated protein kinase; NC = negative control; PC = positive control; SB202190, SB203580 = p38 mitogen-activated protein kinase inhibitor; SB202474 = inactive analogue of SB203580 and SB202190.

tendency, bleeding time remains a popular method for evaluating primary hemostasis.[22] Researchers have attempted to develop novel antiplatelet drugs that minimally affect bleeding time to primarily reduce clinical bleeding events.[23,24] Among these drugs, PDE3 inhibitors are generally recognized to differ from other antiplatelet drugs (*e.g.*, acetylsalicylic acid, ticlopidine) in that they inhibit platelet aggregability without prolonging bleeding time.[25–27] Previous clinical studies have shown that perioperative PDE3 inhibitor administration does not increase perioperative blood loss and bleeding events, which is consistent with the current study.[15,16,28] The dissociation between bleeding time and platelet inhibition might be explained by the effects of PDE 3 inhibitors on improving endothelial cell function.[29]

Recent studies have shown that a PDE3 inhibitor (cilostazol) decreases lipopolysaccharide-induced p38 MAPK and stress-activated protein kinase/c-Jun NH2-terminal kinase phosphorylation in THP-1 cells.[30] However, there have been few studies investigating the effects of PDE3 inhibitors on intraplatelet MAPK and PI3/Akt signaling. Our *in vivo* and *in vitro* findings indicate that, at clinically relevant concentrations, milrinone can reduce enzyme activity of intraplatelet p38 MAPK, MEK/ERK, and PI3/Akt. Furthermore, these effects are more pronounced in P-selectin–positive platelets. Taken together, these findings suggest that perioperative milrinone infusion reduces the intraplatelet MAPK and PI3/Akt pathways as a downstream signal of PDE3 inhibition, particularly in activated platelets.

Tissue factors are expressed constitutively in the adventitia of blood vessels and play a crucial role in coagulation as an initiator of the extrinsic coagulation cascade. Recent evidence has shown that so-called blood-borne tissue factor, mostly derived from monocytes, is strongly associated with hypercoagulation, thrombus development, and venous thromboembolism.[31,32] In our study, intraoperative PDE3 inhibitors may have inhibited monocyte tissue factor production by attenuating the interaction between monocytes and platelets. The current *in vitro* data indicating that milrinone reduces expression of tissue factor on monocytes in the presence of activated platelets support our *in vivo* results for tissue factor.

The main role of the tissue factor pathway (formally known as the extrinsic pathway) is to generate a thrombin burst. Thrombin activation generates an excess of fibrin monomers that combine with fibrin degeneration products and fibrinogen to form fibrin monomer complexes. Therefore, the concentration of soluble fibrin monomer complexes reflects thrombin generation. The current findings may be explained on the basis that milrinone reduces monocyte tissue factor production and down-regulates the extrinsic coagulation cascade and subsequent formation of soluble fibrin monomer complex.

In our *in vitro* studies, collagen and ADP were used as stimulants of healthy blood to confirm the perioperative accelerated blood cell interactions and prothrombic state occurring after arthroplasty. Collagen is one of the major activators of platelets after injury.[33] Collagen, located in the matrix of underlying vascular endothelial cells, is exposed to the bloodstream after surgical- and tourniquet-induced injury.[18] ADP, which is rich in dense granules and is released with platelet activation, induces

Table 6. *In Vitro* Changes in Platelet Aggregation, Platelet–Monocyte Aggregates, Platelet PAC-1 and P-selectin Expression, and Monocyte Tissue Factor Expression by MEK1/2 Inhibitors

	NC	PC	U0124	U0126
Platelet aggregation units, % change				
ADP	—	100 ± 0	80.2 ± 5.1	50.0 ± 9.2*
Collagen	—	100 ± 0	92.5 ± 4.6	42.7 ± 24.2*
Platelet–monocyte aggregates, %				
ADP	8.7 ± 0.8	51.1 ± 3.2	53.2 ± 3.8	48.9 ± 2.3
Collagen	8.8 ± 0.3	71.9 ± 2.7	70.0 ± 2.3	64.5 ± 3.2
PAC-1 on platelets, MFI				
ADP	7.3 ± 1.8	10.4 ± 1.8	11.6 ± 1.2	7.7 ± 1.2*
Collagen	7.5 ± 1.1	14.3 ± 1.5	15.5 ± 1.4	12.3 ± 1.7*
P-selectin on platelets, MFI				
ADP	7.9 ± 1.5	37.1 ± 2.3	37.5 ± 3.2	36.3 ± 4.0
Collagen	8.1 ± 2.2	53.8 ± 3.9	54.7 ± 3.7	53.7 ± 3.8
Tissue factor expression on monocytes, MFI				
Collagen	11.7 ± 0.5	18.2 ± 2.8	13.5 ± 0.9	13.2 ± 1.2

Experimental protocol was the same as that described in table 5. Data are presented as mean ± SD (n = 5/group).

* $P < 0.01$ compared with each U0124.

ADP = adenosine diphosphate, 10 μM; collagen = collagen, 2 μg/ml; MEK = mitogen-activated protein kinase kinase; MFI = mean fluorescence intensity; NC = negative control; PC = positive control; U0124 = inactive analogue of U0126; U0126 = mitogen-activated protein kinase kinase 1/2 inhibitor.

Table 7. *In Vitro* Changes in Platelet Aggregation, Platelet–Monocyte Aggregates, Platelet PAC-1 and P-selectin Expression, and Monocyte Tissue Factor Expression by PI3K Inhibitor

	NC	PC	LY294002
Platelet aggregation units, % change			
ADP	—	100 ± 0	25.7 ± 6.1*
Collagen	—	100 ± 0	24.4 ± 13.4*
Platelet–monocyte aggregates, %			
ADP	8.7 ± 0.8	51.1 ± 3.2	47.3 ± 2.8
Collagen	8.8 ± 0.3	71.9 ± 2.7	66.0 ± 2.6
PAC-1 on platelets, MFI			
ADP	7.3 ± 1.8	10.4 ± 1.8	7.2 ± 1.3*
Collagen	7.5 ± 1.1	14.3 ± 1.5	7.7 ± 1.8*
P-selectin on platelets, MFI			
ADP	7.9 ± 1.5	37.1 ± 2.3	36.9 ± 3.4
Collagen	8.1 ± 2.2	53.8 ± 3.9	53.3 ± 4.6
Tissue factor expression on monocytes, MFI			
Collagen	11.7 ± 0.5	18.2 ± 2.8	14.1 ± 3.8

Experimental protocol was the same as that described in table 5. Data are presented as mean ± SD (n = 5/group).

* $P < 0.01$ compared with each positive control (PC).

ADP = adenosine diphosphate, 10 μM; collagen = collagen, 2 μg/ml; LY294002 = phosphatidylinositol 3-kinase inhibitor; MFI = mean fluorescence intensity; NC = negative control; PI3K = phosphatidylinositol 3-kinase.

second-wave platelet aggregation. Our *in vitro* findings showed that milrinone reduced platelet–leukocyte aggregation and production of PAC-1 and P-selectin at therapeutic blood concentrations (milrinone < 1.5 μM). These results are compatible with the current *in vivo* findings of perioperative platelet inhibition.

Inhibition of p38 MAPK prevented P-selectin expression by platelets and subsequent formation of platelet–leukocyte aggregates, and as PDE3 inhibitors attenuated p38 MAPK phosphorylation, they may have had such effects. A recent study indicated that p38 MAPK plays a negligible role in calcium mobilization, integrin activation, and aggregation of thrombin-stimulated platelets,[34] whereas another recent study showed that MAPK activity plays an important role in stimulating secretion of platelet α-granules and dense granules.[35] The current findings are in accord with these studies.

Adenosine diphosphate– and collagen–stimulated platelet aggregation and integrin $\alpha_{IIb}\beta_3$ activation were also prevented by MEK inhibitors. Hence, the MEK/ERK pathway contributes to the attenuation of platelet aggregation and integrin $\alpha_{IIb}\beta_3$ activation by PDE3 inhibitors. Recent studies have implicated the MEK/ERK pathway in the activation of integrin $\alpha_{IIb}\beta_3$ by von Willebrand factor and thrombin.[36,37] This activation does not directly depend on ERK activity but rather requires Src/ERK-mediated thromboxane A2 generation. These findings suggest that the antiplatelet effects of PDE3 inhibitors through the intraplatelet MEK/ERK pathway may contribute by the reduction of platelet thromboxane A2 generation. However, platelet activation occurs independently of the MEK/ERK pathway when platelets are exposed to higher concentrations of thrombin and collagen.[10]

Platelet aggregation and integrin $\alpha_{IIb}\beta_3$ activation induced by ADP and collagen were largely prevented by the PI3K inhibitor LY294002 in our study. This indicates that inhibition of PI3K/Akt signaling pathways plays a major role in PDE3 inhibitor–mediated reduction of platelet aggregation and integrin $\alpha_{IIb}\beta_3$ activation. PI3K regulates at least two important platelet responses: integrin $\alpha_{IIb}\beta_3$ activation to promote stable platelet aggregation and actin formation assembly to change platelet shape.[38] A recent study showed that platelet protease-activated receptor stimulation causes rapid phosphorylation of Akt, independently of PI3K and ADP, whereas PI3K and ADP are required for maintaining Akt phosphorylation with continuous stimulation. The investigators also found that activated Akt regulates platelet function by modulating protease-activated receptor-induced platelet aggregation and integrin $\alpha_{IIb}\beta_3$ activation.[39] Therefore, PDE3 inhibitors may suppress Akt phosphorylation to some degree in platelets, independently of PI3K activity.

A limitation of our study is that we did not investigate the hemodynamic mechanisms that underlie the *in vivo* reduction of tissue factor production by milrinone infusion. Endothelial and monocyte tissue factor production is enhanced by ischemia and reperfusion-induced oxygen free radicals, which may be alleviated by increasing peripheral blood flow.[40,41]

In summary, we demonstrated that intraoperative PDE3 inhibition diminished the perioperative increase in hemostasis and subsequent monocyte tissue factor production in patients undergoing total knee arthroplasty. Further studies are needed to delineate the intraplatelet signaling between PDE3 inhibition and downstream p38 MAPK, MEK/ERK, and PI3K/Akt signaling.

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