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 prothrombotic 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 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 αIIbβ3 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.

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 αIIbβ3, which mediates platelet aggregation.6 Transformation of integrin αIIbβ3 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 vitro 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

This article is accompanied by an Editorial View. Please see:

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.

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 further. 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 μg/kg/min until 8 h after the start 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·kg⁻¹·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. Pentyl was administered for surgical pain relief at the rate of 2.5 μg·kg⁻¹·h⁻¹ during surgery and 0.4 μg·kg⁻¹·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
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 protocols and reagents (BD Biosciences, Franklin Lakes, NJ). To evaluate the total amount of p38 MAPK, ERK1/2, and Akt, whole blood was stained with p38 MAPK (Cell Signal-
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% CO2 after collagen stimulation. Washed platelets were generated from platelet-rich plasma by washing twice with citrate wash buffer (128 mm NaCl, 7.5 mm Na2HPO4, 4.3 mm NaH2PO4, 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 (157 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) to adjust platelet counts to 200,000/µl. Peripheral blood mononuclear cells were isolated from whole blood by density gradient centrifugation (NycoPrep blood mononuclear cells were isolated from whole blood by density gradient centrifugation (NycoPrep; 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), SB20474 (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 cocultured with peripheral blood mononuclear cells for 2 h at 37°C with 5% CO2 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.](image1)

![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.](image2)
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.

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**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 < 0.05 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.**
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 and P-selectin on platelets was significantly lower in the Mil(+) group compared with 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 compared with the Mil(-) group, indicating that heterotypic blood cell interactions were attenuated by milrinone infusion.

**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) were 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 \(\pm SD, n = 17\)-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.

**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 \(\pm SD, n = 17\)-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 baseline (0 h) in each group. \(\text{Tissue factor (MFI)}\)

**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 \(\pm SD, n = 17\)-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.
Blood loss during 24 h, ml 651
Transfusion, ml 360
Duration of tourniquet inflation, min 126
Duration of surgery, min 181
SBP/HR at 24 h after start of surgery, mmHg, beats/min 120
Sex, M/F 6/11 5/12 0.71
INR 1.01
SBP/HR at 8 h after start of surgery, mmHg, beats/min 122
Platelets, 10^3/mm^3 229
Height, cm 155
Fibrinogen, mg/dl 268
Weight, kg 65
PDE3 INHIBITOR AND ORTHOPEDIC SURGERY

Pression on platelets in response to ADP and collagen
leukocyte aggregates, and both PAC-1 and P-selectin ex-

ments. These samples from healthy volunteers in
in vitro

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 exper-
iments. 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 stimu-
lation (2 µg/ml) was diminished by incubation with
milrinone (table 4; P < 0.05). Furthermore, milrinone
inhibited monocyte tissue factor expression after coin-
cubation 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–medi-
at intracellular signaling attenuation in platelet inhibi-
tion by pretreatment with each intracellular protein
kinase inhibitor. With regard to the synthesis of platelet-
leukocyte aggregates and P-selectin expression on plate-
lets, 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). Further-
more, tissue factor expression on monocytes after coin-
cubation 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 1. Preoperative Patient Characteristics

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

Data are presented as mean ± SD (n = 17/group).
APTT = activated thromboplin time; INR = international normalized ratio;
milrinone(−) = group not treated with milrinone; milrinone(+) = milrinone-
treated group; PT = prothrombin time.

Table 2. Perioperative Management

<table>
<thead>
<tr>
<th></th>
<th>Milrinone(−)</th>
<th>Milrinone(+)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>SBP/HR after induction of anesthesia, mmHg, beats/min</td>
<td>118 ± 17/71 ± 14</td>
<td>120 ± 15/73 ± 13</td>
<td>0.71/0.82</td>
</tr>
<tr>
<td>SBP/HR at 2.5 h after start of surgery, mmHg, beats/min</td>
<td>124 ± 19/75 ± 13</td>
<td>119 ± 18/77 ± 11</td>
<td>0.45/0.64</td>
</tr>
<tr>
<td>SBP/HR at 8 h after start of surgery, mmHg, beats/min</td>
<td>122 ± 19/76 ± 13</td>
<td>116 ± 18/78 ± 15</td>
<td>0.39/0.70</td>
</tr>
<tr>
<td>SBP/HR at 24 h after start of surgery, mmHg, beats/min</td>
<td>120 ± 18/74 ± 12</td>
<td>114 ± 18/76 ± 11</td>
<td>0.39/0.62</td>
</tr>
<tr>
<td>Duration of surgery, min</td>
<td>181 ± 46</td>
<td>184 ± 50</td>
<td>0.84</td>
</tr>
<tr>
<td>Duration of anesthesia, min</td>
<td>271 ± 53</td>
<td>267 ± 33</td>
<td>0.76</td>
</tr>
<tr>
<td>Duration of tourniquet inflation, min</td>
<td>126 ± 16</td>
<td>124 ± 17</td>
<td>0.67</td>
</tr>
<tr>
<td>Fluid infusion, ml</td>
<td>2.623 ± 597</td>
<td>2.454 ± 732</td>
<td>0.43</td>
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<tr>
<td>Transfusion, ml</td>
<td>360 ± 256</td>
<td>380 ± 242</td>
<td>0.80</td>
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<tr>
<td>Blood loss during surgery, ml</td>
<td>270 ± 191</td>
<td>280 ± 162</td>
<td>0.86</td>
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<tr>
<td>Postoperative hemoglobin after 24 h, g/dl</td>
<td>11.6 ± 2.0</td>
<td>11.7 ± 2.1</td>
<td>0.88</td>
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<td>Blood loss during 24 h, ml</td>
<td>651 ± 130</td>
<td>673 ± 147</td>
<td>0.61</td>
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</table>

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

<table>
<thead>
<tr>
<th>Platelet aggregation units, % change</th>
<th>NC</th>
<th>PC</th>
<th>Mil 1</th>
</tr>
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<tbody>
<tr>
<td>ADP</td>
<td></td>
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</tr>
</tbody>
</table>
| Collagen                            | 100 ± 0 | 100 ± 0 | 12.0 ± 0.7
| Collagen                            | 100 ± 0 | 100 ± 0 | 11.9 ± 0.7
| PAC-1 on platelet, MFI              | 7.4 ± 2.0 | 11.1 ± 1.3 | 15.5 ± 1.1 |
| Collagen                            | 7.5 ± 1.7 | 15.8 ± 2.0 | 12.1 ± 1.4 |
| p-selectin on platelets, MFI        | 8.1 ± 1.6 | 35.6 ± 3.7 | 31.2 ± 4.3 |
| p38 MAPK (pT180/pY182)/(total),% change | 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 | 100 ± 0 | 132.1 ± 9.6 | 151.8 ± 10.6 |
| Collagen                            | 100 ± 0 | 154.7 ± 7.5 | 133.0 ± 15.4 |
| Akt (pT308)/(total),% change        | 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 negative 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 percent of platelet-conjugated monocytes in the total monocyte population. 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% CO2 after collagen (2 μg/ml) stimulation. Tissue factor expression on monocyte is shown as geometric mean fluorescence intensity (MFI) values. Data are presented as mean ± SD (n = 6/group).

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. Vascular endothelial injury, pain, and ischemia–reperfusion injury 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. This finding is consistent with our previous report that milrinone inhibits platelet aggregation and calcium release by thrombin ( < 1.5 μM). Kikura et al., 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...
Tissue factor expression on monocytes, MFI

Platelet–monocyte aggregates, %

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

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 cocultured with peripheral blood mononuclear cells for 2 h at 37°C with 5% CO₂ 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.

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. 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 degradation 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. Collagen, located in the matrix of underlying vascular endothelial cells, is exposed to the bloodstream after surgical and tourniquet-induced injury. ADP, which is rich in dense granules and is released with platelet activation, induces

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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, whereas another recent study showed that MAPK activity plays an important role in stimulating secretion of platelet α-granules and dense granules. The current findings are in accord with these studies.

Adenosine diphosphate- and collagen-stimulated platelet aggregation and integrin αIIbβ3 activation were also prevented by MEK inhibitors. Hence, the MEK/ERK pathway contributes to the attenuation of platelet aggregation and integrin αIIbβ3 activation by PDE3 inhibitors. Recent studies have implicated the MEK/ERK pathway in the activation of integrin αIIbβ3 by von Willebrand factor and thrombin. 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.

Platelet aggregation and integrin αIIbβ3 activation induced by ADP and collagen were largely prevented by the P3K inhibitor LY294002 in our study. This indicates that inhibition of P3K/Akt signaling pathways plays a major role in PDE3 inhibitor-mediated reduction of platelet aggregation and integrin αIIbβ3 activation. P3K regulates at least two important platelet responses: integrin αIIbβ3 activation to promote stable platelet aggregation and actin formation assembly to change platelet shape. A recent study showed that platelet protease-activated receptor stimulation causes rapid phosphorylation of Akt, independently of P3K and ADP, whereas P3K 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 αIIbβ3 activation. Therefore, PDE3 inhibitors may suppress Akt phosphorylation to some degree in platelets, independently of P3K 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 repercussion-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.

References


Anesthesiology, V 111, No 6, Dec 2009

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