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Cilostazol Inhibits Platelet-Monocytic THP1 Cell Aggregation, Platelet CD4OL, and P-selectin Expression

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Background: Platelet activation is critical during inflammation, thrombosis, and tumor progression and metastasis. This activation leads to platelet-monocyte aggregation, platelet P-selectin, and CD40 ligand (CD40L) expression. Platelets can increase acute myelogenous leukemia (AML) blast proliferation. Cilostazol is widely used for arteriosclerosis obliterans and is known to inhibit platelet function. We hypothesized that cilostazol preconditioning impairs subsequent AML blast proliferation through a reduction in platelet activation. We aim to determine the effects of cilostazol on platelet activation as measured by platelet-monocyte aggregation, platelet P-selectin, and CD40L expression. **Methods:** Platelet-rich plasma samples from healthy volunteers were treated with cilostazol. THP1 is a human AML cell line that resembles human monocytes, which was used as a model to measure platelet-monocyte aggregation. The blood samples were stimulated by adenosine diphosphate (8 μ M) for evaluation of platelet-monocytic THP1 cell aggregation and P-selectin expression and were stimulated by thrombin (0.1 U/mL) for detecting CD40L expression. All samples were stained with fluorochrome-conjugated antibodies and were analyzed by flow cytometry. **Results:** Pretreatment with cilostazol significantly suppressed platelet-monocytic THP1 cell aggregation, P-selectin, and CD40L expression in a concentration-dependent manner at concentrations of 10^1, 10^2, and 10^1 mM, respectively. **Conclusions:** Our data showed that cilostazol can downregulate not only cellular interactions between platelets and monocytic THP1 cells but also platelet P-selectin and CD40L expression.

Key words: THP1 cell, CD40L, P-selectin, cilostazol, platelet

INTRODUCTION

The activation of platelets plays an important role in thrombosis and inflammation[1,2] and is essential in tumor progression and metastasis[3,4]. Platelet activation enhances levels of platelet-monocyte aggregation[5,6], P-selectin[7], and platelet CD40 ligand (CD40L) surface expression[8], which was known to modulate tumor invasion and metastasis[4,9-11]. Platelet P-selectin is present in the α-granules of platelets and translocates rapidly to the cell surface after platelet activation[12]. Platelet P-selectin may bind to monocytes and form platelet-monocyte aggregates, mostly via binding of platelet P-selectin to P-selectin glycoprotein ligand-1, which is constitutively expressed on the monocyte surface[13]. CD40L was not detectable on unstimulated platelets, but activation of platelets by thrombin results in the expression of CD40L[14]. Elevated levels of CD40L in circulation could provide additional diagnostic markers and therapeutic targets for cancer patients[8,15].

Platelet functions were also important in acute myelogenous leukemia (AML)[10,16]. Platelets can interact with primary AML cells and may contribute to AML cell proliferation, regulation of AML cell apoptosis, and susceptibility to intensive chemotherapy[16-19]. Thus, platelets may have a role in AML after the development of the disease and during treatment[16-18]. In addition, interfering with platelet activation may be valuable in the management of AML blast proliferation.

Cilostazol is a selective inhibitor of phosphodiesterase 3[20] that has been used in treating patients with arteriosclerosis obliterans and intermittent claudication[21]. It also has

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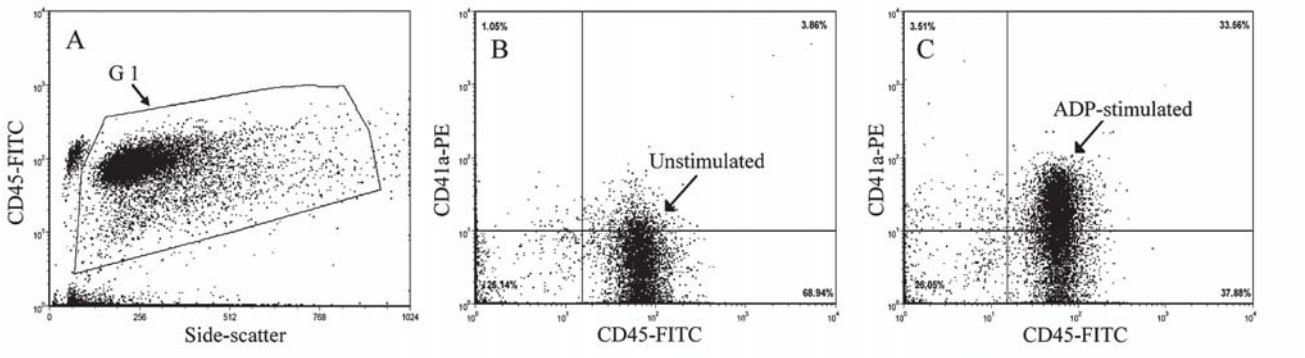

Fig. 1 Flow cytometric analysis of platelet-monocytic THP1 cell aggregation. (A) Monocytic THP1 cells ("G1" area) were identified in the side-scatter properties (granularity; x-axis) versus monocyte-specific monoclonal antibody (CD45-FITC; y-axis). (B and C) Representative results of the binding of unstimulated and adenosine diphosphate (ADP)-stimulated platelets to THP1 cells. A right upper quadrant plot of CD45-FITC (x-axis) against CD41a-PE (y-axis) was then used to determine the monocytic THP1 cells positive for platelets. The results are revealed as percentage (%) which was defined as platelet-coupled THP1 cells in the THP1 population.

a potent inhibitory effect on platelet aggregation both in vitro and in vivo[22,23]. Cilostazol might have a dispersing effect on platelet aggregates, similar to prostaglandin I2[22]; however, the effect of cilostazol on platelet activation is not well understood. We hypothesized that cilostazol preconditioning would reduce platelet activation and in vitro heterotypic aggregation between platelets and promyelomonocytes, which may impair subsequent AML blast proliferation. We tested the effects of cilostazol on platelet-monocyte aggregation, platelet CD4OL, and P-selectin expression.

METHODS

Blood Collection

This study was approved by the institutional review board of this hospital, and informed consent was obtained from all volunteers before enrollment. Whole blood for this in vitro study was obtained from healthy men (ages 27-33 years) who had not taken any medication for at least 15 days. We used the described protocol to study the effects of cilostazol on platelet activation[24]. Briefly, blood was collected from an antecubital vein with an 18-gauge needle without a tourniquet, using a two-syringe technique. The first sample of 2 mL was discarded to avoid tissue contamination, and the second sample was used in the experiments. All samples were anticoagulated with a 1:9 volume of 3.8% sodium citrate solution and then immediately processed for stimulation procedures and flow cytometric analyses. The anticoagulated blood samples were centrifuged at 100 g for 10 min to obtain platelet-rich plasma. One sample of the blood was diluted with Hank's balanced salt solution to serve as a control. The other portions were preincubated with desired concentrations of cilostazol (10^{-2} to 10^{2} mM) at 37 ^{o}C for 1 min.

Reagents and Flow Cytometry

The following reagents were used: cilostazol (Otsuka, Tokyo, Japan) was dissolved in dimethyl sulfoxide (DMSO). Adenosine diphosphate (ADP, Sigma-Aldrich, St Louis, MO, USA) and thrombin (Chrono-log, Havertown, PA, USA) were used for platelet stimulation. Anti-CD41a antibody (clone HIP8, Becton Dickinson, San Jose, CA, USA), a platelet-specific monoclonal antibody (mAb) conjugated with PE or FITC, recognizes the platelet GPIIb/IIIa complex independent of activation. Anti-CD62P-phycoerythrin (PE) antibody (clone AK4, Becton Dickinson, San Jose, CA, USA), a mAb directed against P-selectin expressed on platelet surface and anti-CD45-FITC (clone HI30, Becton Dickinson, San Jose, CA, USA), a mAb against the leukocyte common antigen. Anti-CD40L-fluorescein isothiocyanate (FITC) antibody (clone TRAP1, Becton Dickinson, San Jose, CA, USA) directs against CD40L expressed on the platelet surface. We used negative IgG1-FITC and IgG1-PE antibodies (clone MOPC-21, Becton Dickinson, San Jose, CA, USA) for testing nonspecific binding and establishing background fluorescence. Paraformaldehyde was obtained from Sigma-

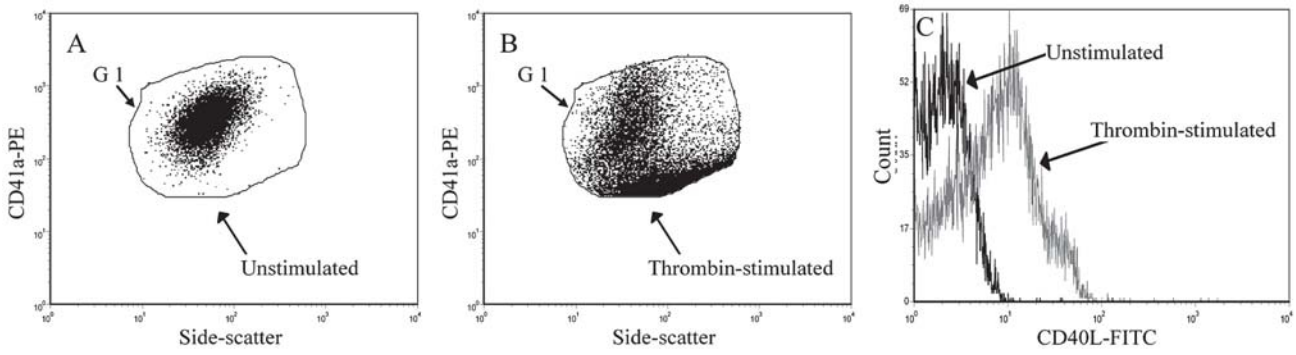

Fig. 2 Flow cytometric analysis of platelet CD40L expression in platelet-rich plasma. (A and B) Single platelet events are identified by their characteristic side-scatter properties (granularity; x-axis) and positive labeling with a platelet-specific monoclonal antibody (CD41a-PE; y-axis). Dot plot of gating platelets (G1) by unstimulated and thrombin-stimulated fluorescence of the events within the "G 1" were analyzed. (C) Histogram plots overlaid by unstimulated and thrombin-stimulated fluorescence (CD40L-FITC, x-axis) of the events within the "G 1" were then further analyzed.

Aldrich (St Louis, MO, USA). A FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) was equipped with a 488 nm argon ion laser, a standard two-color filter configuration, and CELLQuest cell analysis software (Becton Dickinson, San Jose, CA, USA).

Cell Culture

The human monocytic THP1 cell line, a human promyelomonocytic cell line, was obtained from the American Type Culture Collection (Manassas, VA, USA) and was grown in RPMI 1640 medium with 2 mM L-glutamine, 4.5 g/L glucose, 10 mmol/L HEPES balanced salt solution, 1.0 mmol/L sodium pyruvate, 10% fetal bovine serum, and 1% antibiotic-antimycotic mixture. The cell density was maintained between 5×10^4 and 8×10^5 viable cells/mL, and the medium was refreshed every 2-3 days.

Detection of Platelet-Monocytic THP1 Cell Aggregation (Fig. 1)

After preincubation with desired concentrations of cilostazol in platelet-rich plasma, platelet stimulation with and without ADP was used at a final concentration of 8 μM at room temperature for 5 min. Equal volumes of platelet-rich plasma and suspensions of monocytic THP1 cells were mixed and incubated at 37 °C for 5 min. Samples were stained with saturating concentrations of anti-CD41a-PE and anti-CD45-FITC antibodies at room temperature for 20 min. After fixation with 1% paraformaldehyde at 4 °C for 30 min, they were immediately processed for flow cytometry. Platelet-monocytic THP1 cell aggregation was measured as positive for CD41a-PE and CD45-FITC (THP1 cells). The two-color analysis enabled discrimination of platelet-coupled and platelet-free THP1 cells. The results are shown as percentages (%), which are defined as platelet-coupled THP1 cells in the THP1 population. For each sample, 10,000 THP1 cells were measured.

Detection of Platelet P-selectin Expression

After preincubation with desired concentrations of cilostazol in platelet-rich plasma, platelet stimulation with and without ADP was used at a final concentration of 8 μM at room temperature for 5 min. Samples were stained with a saturating concentration of anti-CD41a-FITC and anti-CD62P-PE mAb at room temperature in the dark for 20 min. After fixation with 1 % paraformaldehyde at 4 °C for 30 min, samples were immediately processed for flow cytometry. With flow cytometry, individual platelets were identified by side scatter and anti-CD41a-FITC. P-selectin expression on the surface of platelets was defined as that positive for anti-CD62P-PE. Results are expressed as mean fluorescence intensity (MFI) of P-selectin expression, which was defined as P-selectin expressed on the surface membrane of platelets. For each sample, 10,000 platelets were collected.

Detection of Platelet CD40L Expression (Fig. 2)

After preincubation with desired concentrations of cilostazol in platelet-rich plasma, platelet stimulation with and without thrombin was used at a final concentration of 0.1 U/mL at 37 °C for 1 min. Thereafter, blood samples were mixed with saturating concentrations of anti-CD41a-PE and anti-CD40L-FITC mAb at room temperature in the dark for 20 min. Both samples were then fixed with 1% paraformaldehyde and were maintained at 4 °C for 30 min.

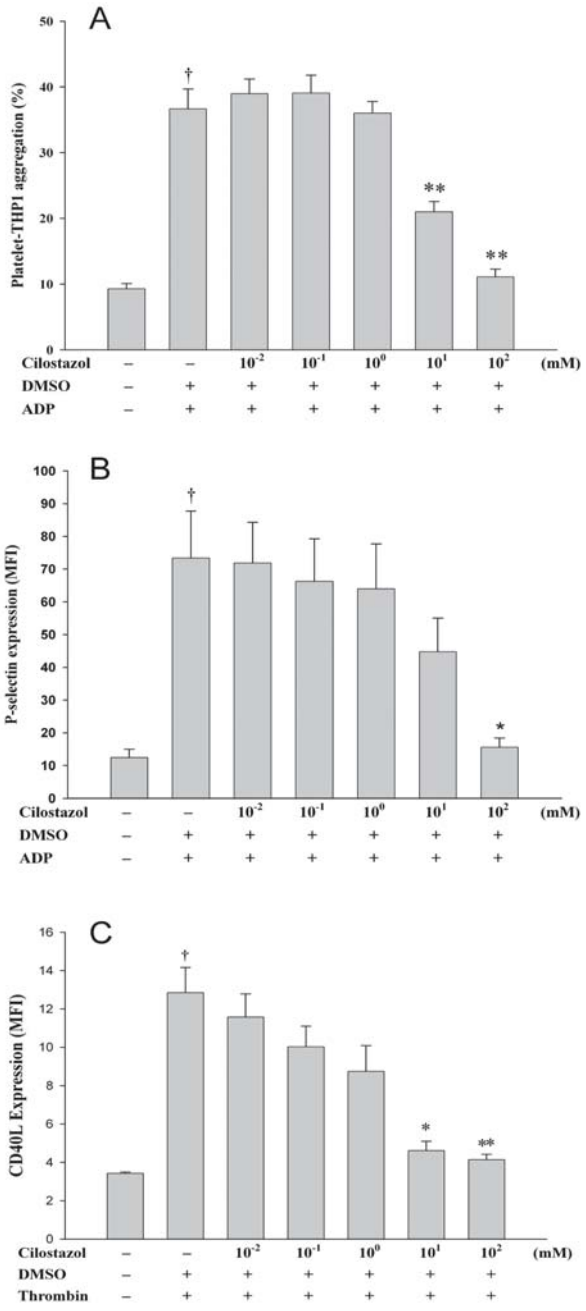

Fig. 3 The inhibitory effect of cilostazol on platelet-monocytic THP1 cell aggregation (A), platelet P-selectin (B) and CD40L (C) expression, compared with the vehicle control [+dimethyl sulfoxide (DMSO)], measured by flowcytometry. Various doses of cilostazol were co-incubated with the platelet-rich plasma and stimulated with ADP (8 μ M) or thrombin (0.1 U/mL). MFI = mean fluorescence intensity. Values are the mean ± standard error. †$P < 0.01$ (vs. the unstimulated platelets); *$P < 0.05$, **$P < 0.001$ (vs. isotype control platelets).

After fixation, blood samples were immediately processed for flow cytometry. Under flow cytometry, individual platelets were identified by side scatter and anti-CD41a-PE using a logarithmic scaled dot plot. Results are also expressed as MFI.

Statistical Analysis

All data are presented as mean ± standard error. Differences between the experimental groups were evaluated using a one-way ANOVA analysis of variance. If the overall P value was < 0.05, pairwise comparisons were carried out using the Fisher's protected least significant difference test.

The authors had full access to the data and take responsibility for its integrity. All authors have read and agree to the manuscript as written.

RESULTS

The MFI of percentage of platelet-monocytic THP1 aggregation, P-selectin, and CD40L expression decreased in a concentration-dependent manner in platelets pretreated with cilostazol (Fig. 3). The percentage of aggregation of platelet-monocytic THP1 cells at concentrations of 10^1 and 10^2 mM were 21.02 ± 1.55 and 11.09 ± 1.19, respectively, which were 42.7% (P < 0.001) and 69.8% (P < 0.001) lower than the vehicle control group (+ dimethyl sulfoxide) correspondingly (n = 6, Fig. 3A). Expression of P-selectin was downregulated by cilostazol 79% at concentrations of 102 mM (P = 0.019, n = 8, Fig. 3B).

Furthermore, cilostazol reduced CD40L expression in a concentration-dependent manner (n = 6). Pretreatment with cilostazol significantly suppressed CD40L expression to 64.1% (P = 0.001) and 67.8% (P < 0.001) at concentrations of 10^1 and 10^2 mM, respectively (Fig. 3C). These data also show that a lower concentration (101 mM) of cilostazol is required to downregulate platelet-THP1 cell aggregation and platelet CD40L expression than the concentration (10^2 mM) needed to inhibit platelet P-selectin expression.

DISCUSSION

We found that cilostazol attenuated platelets conjugated with monocytic THP1 cells in a concentration-dependent manner. THP1 cells were isolated from a patient with AML. Normal platelets during in vitro culture caused a dose-dependent increase in AML blast proliferation[19]. This is caused both by direct adhesion and through platelet release of soluble mediators, including platelet-derived

growth factor, platelet factor 4[16,19]. Cilostazol blocks the adhesion of platelets, and monocytic THP1 cells may have a role in AML blast proliferation. We also showed that cilostazol attenuated ADP-induced platelet P-selectin expression in a concentration-dependent manner. Platelet-monocyte aggregation results from platelet P-selectin binding to monocytes[13,25]. Cilostazol downregulates P-selection expression and may partially inhibit cilostazol on platelet-monocytic THP1 cell aggregation. There is an important cascade between platelets and leukocytes; cilostazol may block this cascade by acting on platelet P-selectin, through P-selectin glycoprotein ligand-1, to inhibit the crosstalk between platelets and leukocytes[26,27]. Furthermore, platelet P-selectin binds to leukocytes, especially monocytes, which have been reported to be potential markers of excessive proinflammation in sepsis[28], cardiopulmonary bypass[29], thrombosis[30], and unstable angina[31]. Therefore, cilostazol may also modulate the proinflammatory response.

How cilostazol inhibits P-selection expression appears to be suppressed by phosphodiesterase inhibition[22]. This is because phosphodiesterase inhibition increases intracellular cAMP levels, leading to the activation of protein kinase A. Protein kinase A activation induces a decrease in intracellular Ca^{2+}, which may cause suppression of platelet α-granule release and P-selectin expression[22]. Thus, any agent increasing cAMP levels could mimic the effect of cilostazol on the suppression of P-selectin. Nomura et al[32] observed that the P-selectin levels were high in patients with diabetes mellitus but that daily oral medication with cilostazol for four weeks significantly decreased this parameter. Thus, cilostazol may be clinically useful for diseases with increased platelet P-selectin expression. Furthermore, we demonstrated that platelet CD40L expression decreased after cilostazol treatment.

Platelets are a major source of CD40L[33] and play important roles in cancer and leukemia[8,34-37]. Therefore, the downregulation of CD40L expression by cilostazol may have a potential role in clinical settings, in particular through the activation of platelets and in conditions associated with neoplasm. Given the similar mechanism of P-selectin expression decreased by cilostazol, CD40L expression also decreased by way of increased intracellular cAMP content[38].

This study also showed that a lower concentration of cilostazol is required to downregulate platelet CD40L expression and platelet-THP1 cell aggregation than platelet P-selectin expression. Measurement of platelet CD40L expression and platelet-THP1 cell aggregation may be a more sensitive indicator of in vitro platelet activation than P-selectin expression in response to a platelet inhibitor such as cilostazol.

Ligation of platelet CD40L with CD40 induces α-granule release, which leads to P-selectin expression. CD40 signals through protein kinase cascades, including protein kinase C (PKC). PKC signaling also occurs in platelets, and the CD40L-CD40 interaction with P-selectin expression could be due to platelet CD40 signaling via protein kinase C[33]. CD40L-mediated platelet P-selectin expression can also induce platelet-leukocyte aggregation[33]. Therefore, cilostazol may play a role in modulating platelet activation either directly or indirectly to inhibit platelet-monocyte aggregation and the expression of P-selectin and CD40L.

The peak concentration-time profile after cilostazol 100 mg was 1200 ng/ml (~3.23 mM) in plasma[39], which was within the inhibitory range (10^1 to 10^2 mM). On the other hand, information on acute overdosage with cilostazol in humans is limited[39]. Even in small animals, the oral LD50 of cilostazol is > 5.0 g/kg in mice and rats and > 2.0 g/kg in dogs[39], which was far higher than the daily required human dose. Therefore, the concentration used in the present study might be relatively safe and be clinically relevant doses. However, extrapolation of the data to clinical situations is still speculative.

In conclusion, the effects of cilostazol on the interaction of platelets and monocytic THP1 cells as well as the CD40L and P-selectin expression have been investigated. Our data showed that cilostazol downregulates platelet-monocytic THP1 cell aggregation, platelet CD40L, and P-selectin expression. Our findings provide new insights into the pleiotropic effects of cilostazol and implicated cilostazol in preventing platelet-monocyte aggregation, platelet CD40L, and P-selectin-mediated AML blast proliferation. However, it has not yet been characterized in detail whether platelet interactions with AML blasts are important for carcinogenesis or treatment responsiveness of malignancies. Therefore, the clinical implication of cilostazol in patients with AML remains to be further investigated.

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