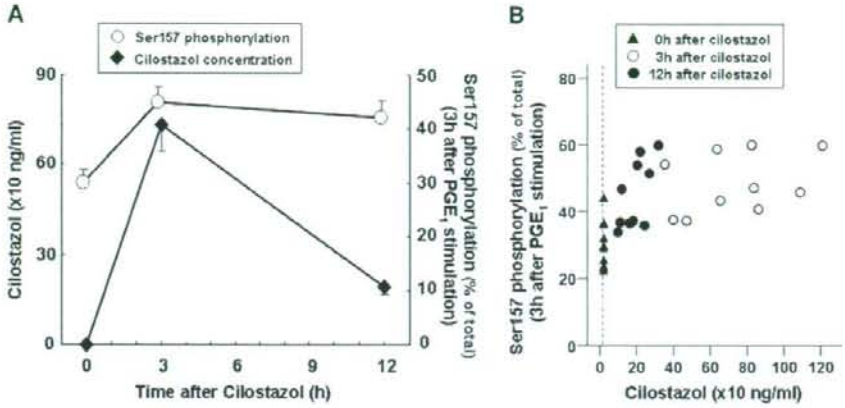

Fig 6. Relationship of plasma cilostazol concentrations with vasodilator-stimulated phosphoprotein (VASP)-P157 levels under prostaglandin E1 (PGE1) treatment. (A) The mean plasma concentration of cilostazol and the mean VASP-P157 levels under PGE1 treatment for 3 h over time are shown. The data are mean ± SE (n=10). (B) Individual plots of cilostazol plasma concentrations and VASP-P157 levels under PGE1 treatment for 3 h are shown.

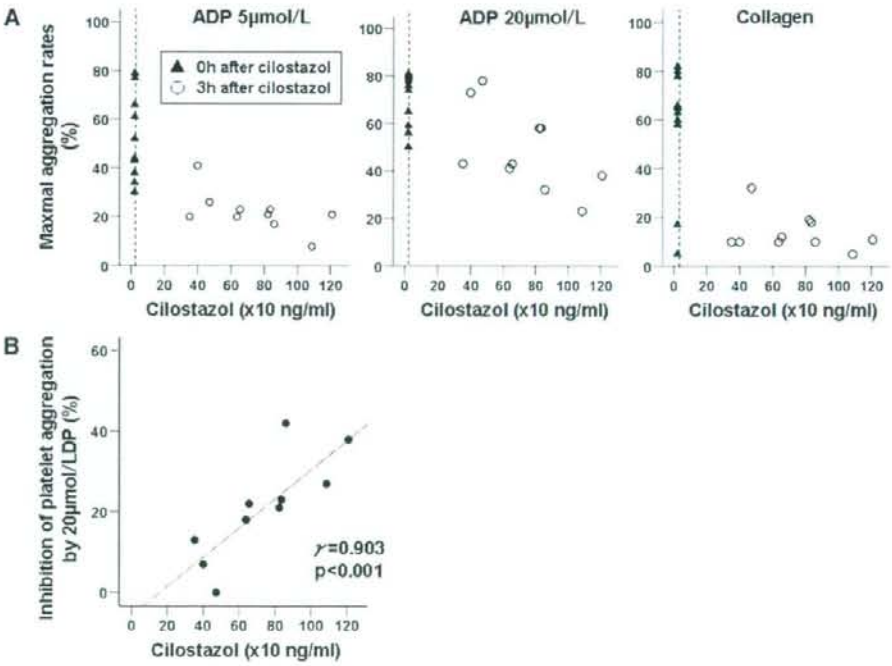

Fig 7. Relationship of plasma cilostazol concentrations with platelet aggregation under prostaglandin E1 (PGE1) treatment. (A) Individual plots of cilostazol plasma concentrations and the 5 μmol/L ADP, 20 μmol/L ADP and 1 μg/ml collagen-induced maximal aggregation rates under PGE1 treatment are shown. (B) Correlation of plasma concentrations of cilostazol with the rate of inhibition of platelet aggregation induced by 20 μmol/L ADP is shown. This correlation was assessed by Spearman's rank test.

tive values using a standard, which was VASP-P239 and VASP-P157/239 at 3 h after 100 mg cilostazol intake treated with 8 nmol/L PGE1 for 3 h, the correlations were not obtained.

Similarly, cilostazol intake apparently inhibited the MAR of platelets treated with 8 nmol/L PGE1 for 1 h (Figs 4,5) at 3 h after cilostazol intake, as assessed by longitudinal analysis; there was a significant inverse correlation between the plasma concentration of cilostazol and the 5 μmol/L and 20 μmol/L ADP-induced MARs of platelets treated with

8 nmol/L PGE_1 for 1 h (p=0.038 and p=0.004, respectively) (data not shown). On the other hand, we did not detect a correlation of cilostazol plasma concentrations with the 1 μg/ml collagen-induced MAR of platelets under PGE_1 treatment (p=0.255) (data not shown). Individual plots of cilostazol levels and MARs are shown in Fig 7A.

We used Spearman's rank test to analyze the correlation between cilostazol plasma concentrations and the rates of IPA (%) under PGE_1 treatment induced by 20 μmol/L ADP; we found a strong correlation between them (r=0.903, p<0.001) (Fig 7B). On the other hand, we did not detect a correlation of plasma cilostazol level with 5 μmol/L ADP-induced IPA (%) (r=0.413, p=0.235) and 1 μg/ml collagen-induced IPA (%) (r=0.503, p=0.138) (data not shown), probably because of inappropriately weak stimulation where aggregation was strongly suppressed even at low concentrations of cilostazol (Fig 7A).

Thus, VASP phosphorylation levels and platelet aggregation under PGE_1 treatment could reflect the levels of cilostazol in the plasma.

Discussion

We have demonstrated that the antiplatelet effect of cilostazol can be monitored by analyzing intracellular VASP phosphorylation levels and platelet aggregation induced by collagen and ADP under PGE_1 treatment. Although a previous report showed an antiplatelet effect of cilostazol intake using the optical aggregometer and ADP or collagen as stimuli,[21] another report showed that cilostazol intake did not affect ADP- or collagen-induced platelet aggregation.[22] In the present study, we were also unable to detect an antiplatelet effect of cilostazol by optical aggregometer using the same agonists without PGE_1 treatment.

VASP is an abundant substrate of A-kinase and G-kinase in platelets. Cilostazol is a phosphodiesterase 3 inhibitor and inhibits the degradation of cAMP.[1,2] Theoretically, phosphodiesterase 3 inhibitor could also inhibit the degradation of cGMP. Because cilostazol can affect the cAMP and cGMP levels in platelets, we used analysis of phosphorylated VASP levels to evaluate cilostazol's effect on platelets. However, we could not detect VASP phosphorylation without PGE_1 treatment even after cilostazol intake. Thus, it might be difficult to detect the effect of cilostazol intake not only by platelet aggregation but also by VASP phosphorylation, probably because of low concentrations of cAMP and cGMP in isolated platelets. However, in situations where cAMP concentrations increase, cilostazol might achieve antiplatelet effects. Because the expected function of cilostazol is to maintain increased cAMP levels in platelets, but not to produce cyclic nucleotides, it is understandable that we were unable to detect an antiplatelet effect by analyzing levels of VASP phosphorylation or platelet aggregation without PGE_1 treatment, even after cilostazol intake.

In the present study, a low concentration of PGE_1 (8 nmol/L) induced VASP phosphorylation at Ser157 and Ser239 to a certain extent, both of which were not fully phosphorylated. Under these conditions, we observed apparent enhancement of the phosphorylation of each residue by cilostazol. The level of VASP phosphorylated at both the Ser157 and Ser239 residues exhibited the most striking change following cilostazol intake, because VASP-P157/239 was not detected even after treatment with PGE_1 without cilostazol intake (Figs 2A,D). Therefore, to evaluate the level of VASP phosphorylation, measuring the levels of VASP-P157/239 might be the best way of monitoring the effect of cilostazol.

Although we could not detect a cilostazol effect on ADP- or collagen-induced platelet aggregation without PGE_1 treatment, we could clearly detect its effect on both agonist-induced aggregations under PGE_1 treatment. Furthermore, the 20 μmol/L ADP-induced MARs of platelets treated with PGE_1 inversely correlated with the plasma concentration of cilostazol, although the inverse-correlation between collagen-induced aggregation and the plasma concentration of cilostazol was not significant, probably because the agonist concentrations were inappropriately weak, as these aggregations were strongly inhibited in the presence of even low concentrations of cilostazol. Thus, the 20 μmol/L ADP-induced platelet aggregation under PGE_1 treatment could reflect the level of cilostazol in the plasma. Based on these findings, 20 μmol/L ADP stimulation might be a better choice for monitoring the effect of cilostazol on platelet aggregation under PGE_1 treatment.

Although analysis of the levels of VASP phosphorylation by the Western blotting method would more directly reflect the concentration of cAMP and activity of cAMP-dependent protein kinase, it takes more time than the analysis of aggregation, because a much complicated process is involved. Given that we could clearly detect the effect of cilostazol in the evaluation of 20 μmol/L ADP-induced platelet aggregation under ex vivo PGE_1 treatment, it would be the more feasible method for use in the clinical setting.

Evaluation of a drug's efficacy sometimes provides insight into its functional mechanism; this is the case for antiplatelet drugs. In this study, under PGE_1 treatment, we detected apparent enhancement of VASP phosphorylation and antiplatelet effect on the aggregation induced by ADP and collagen. Although PGE_1 is a synthetic compound, which reduces platelet reactivity through the prostacyclin receptor,[14] adenosine and prostacyclin are conceivable intrinsic agonists that increase cAMP.[13] Therefore, the function of cilostazol in pathophysiological conditions would be to enhance and prolong the effects of these cAMP-producing factors in platelets, which could at least partially contribute to their cardiovascular protective effects.

PGE_1 increases cAMP via its *Gas*-coupled receptor. On the other hand, it has been demonstrated that increased cAMP subsequently induces the endothelial type of nitric oxide synthase, thereby increasing cGMP in platelets.[27] Because not only A-kinase, but also G-kinase, has been demonstrated to phosphorylate VASP at serines 157 and 237,[16] the effect of cilostazol, which theoretically inhibits degradation of both cAMP and cGMP,[28] might be partly mediated by inhibition of cGMP degradation. The half-lives of prostacyclin and nitric oxide are 6–7 min[29] and several seconds,[30] respectively. The reason why cilostazol did not achieve antiplatelet effects without PGE_1 treatment might be loss of cyclic nucleotide producing factors, because it took at least 20 min to prepare PRP after blood sampling.

It was recently reported that some people are poor responders to antiplatelet drugs such as aspirin[31,32] and clopidogrel,[33,34] and that cardiovascular risk is elevated in such patients. As for cilostazol, it remains to be seen whether poor responders exist, because its efficacy has not been evaluated extensively because of the lack of suitable monitoring methods. Given that the cilostazol-effect monitoring methods have been established by this study, it should now be possible, and important, to evaluate the antiplatelet effects of cilostazol in a number of patients groups. Values

of any parameter that reflect a patient's clinical status can acquire even greater importance when they are linked to clinical outcomes. Therefore, further examination, especially through a prospective clinical study, is required in order to determine which value might be best for the evaluation of the cilostazol-effect in the prevention of cardiovascular events.

Acknowledgments

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Circ J 2008; **72:** 420–426

Action of Aspirin on Whole Blood-Aggregation Evaluated by the Screen Filtration Pressure Method

Arata Tabuchi, MD; Ryoji Taniguchi, MD*; Kanako Takahashi, MT*; Hirokazu Kondo, MD*; Mitsunori Kawato, MD*; Takeshi Morimoto, MD, MPH**; Takeshi Kimura, MD*; Toru Kita, MD*; Hisanori Horiuchi, MD*

Background There are few monitoring systems widely used in clinical practice for evaluating the effectiveness of aspirin therapy, so in the present study aspirin's antiplatelet effects we investigated with a whole blood aggregometer using a screen filtration pressure (SFP) method.

Methods and Results Thirty-five healthy male volunteers took 100 mg/day aspirin for 14 days. Whole-blood aggregation was analyzed at baseline and on days 7 and 14, using collagen and adenosine diphosphate as the stimuli, and compared with the platelet-rich plasma (PRP) aggregation measured by optical aggregometer. The platelet-aggregation threshold index (PATI) for both methods, which was defined as the putative agonist-concentration giving half-maximal aggregation, and the PRP-maximal aggregation rate were analyzed. The maximal aggregation rate induced by 1.6 mg/L collagen decreased from 85.5% (80.8–92.8) [median (interquartile range)] at baseline to 51.5% (39–63.8) on day 14 ($p<0.0001$). The PRP-PATI and whole-blood PATI for collagen increased from 0.32 (0.28–0.70) to 1.82 mg/L (1.25–2.89) ($p<0.0001$) and from 0.28 (0.22–0.3) to 1.06 mg/L (1.01–1.29) ($p<0.0001$) respectively.

Conclusions The whole-blood PATI and PRP-PATI for collagen, as well as the maximal PRP aggregation rate, clearly distinguish platelet aggregability before and after aspirin intake. However, whole-blood analysis by the SFP-method is easier to perform, and is a promising method of monitoring aspirin's effects. (*Circ J* 2008; **72:** 420–426)

Key Words: Aggregation; Aspirin; Platelet-aggregation threshold index (PATI); Screen filtration pressure method; Whole blood-aggregation

Aspirin is an irreversible inhibitor of cyclooxygenase-1, which is the rate-limiting enzyme for producing thromboxane A_2 in platelets.[1] Aspirin is the antiplatelet drug used most widely and its effect on prevention of cardiovascular events has been established for high-risk patients.[2–4] However, the efficacy of aspirin is rarely monitored in clinical practice because valid and comprehensive monitoring systems have not yet been established.

Platelet aggregation has conventionally been examined by the optical aggregometer method, in which a change in the light transmission of platelet-rich plasma (PRP) is monitored under agonist stimulation at certain concentrations.[5] Requirement of centrifugation for the preparation of PRP is one of the hindrances to its routine use. In addition, the PRP method measures the aggregability of platelets alone, whereas in vivo other blood cells also regulate thrombus formation by modulating platelet aggregation; for example, leukocytes produce platelet activating factor[6] and erythrocytes bind prostacyclin to inhibit its activity.[7] Therefore, if whole-blood aggregation is properly measured, it might reflect a more physiological phenomenon. Several systems for measuring whole-blood aggregation, such as the PFA-100® (Dade-Behring, Deerfield, IL, USA), Verify-Now® (Accumetrics, San Diego, CA, USA) and ROTEM analyzer® (Pentapharm, Munich, Germany), have been developed and are under investigation for establishment as a clinical test.

The screen filtration pressure (SFP) method was established in the 1960s to analyze whole-blood aggregation by measuring the absorbing pressure of agonist-stimulated whole blood through a microsieve.[8,9] Although the principle of this method is simple and understandable, it has not been widely used, until recently, when a semi-automatic aggregometer that uses this method was developed and has come into use in the clinical and research fields.[10–12] Using this aggregometer, we have been able to measure whole-blood aggregability quickly and reproducibly.

In the current study, 35 healthy male volunteers took aspirin for 14–21 days and the whole-blood and PRP aggregations were monitored by whole-blood aggregometer and optical aggregometer, respectively.

Methods

Study Population and Procedures

This study was approved by the Ethics Committee, Faculty of Medicine, Kyoto University. Thirty-five healthy male physicians aged 26–48 years (35±5.5 years old) voluntarily participated after giving informed consent. Each subject took 100 mg enteric-absorbed aspirin® (Bayer, Leverkusen, Germany) after breakfast for 14 days. Fasting

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*Departments of Geriatric Medicine, *Cardiovascular Medicine and **Medical Education, Graduate School of Medicine, Kyoto University, Kyoto, Japan*

Mailing address: Hisanori Horiuchi, MD, Department of Cardiovascular Medicine, Graduate School of Medicine, Kyoto University, Kyoto 606-8507, Japan. E-mail: horiuchi@kuhp.kyoto-u.ac.jp

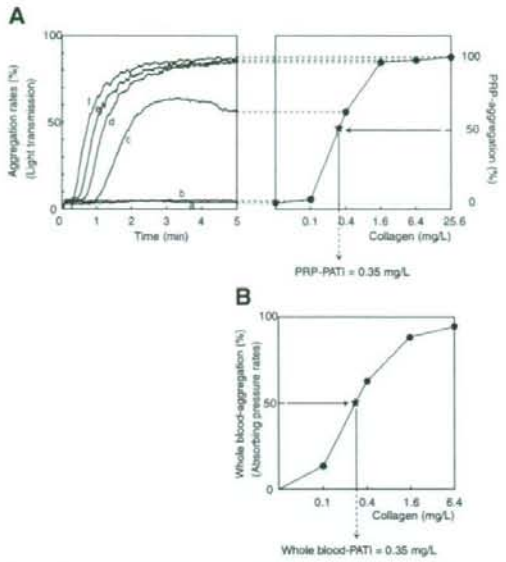

Fig 1. Definition of platelet-aggregation threshold index (PATI). Definitions and methods of calculation of platelet-rich plasma (PRP) (A) and whole-blood (B) PATI are shown schematically. (A, Left) Light transmission curves obtained with 0 (a), 0.1 (b), 0.4 (c), 1.6 (d), 6.4 (e) and 25.6 mg/L (f) of collagen. (B) 0% and 100% absorbing pressure rates represent –6 mmHg and –130 mmHg, respectively.

blood samples were collected in the morning before aspirin intake. Measurements were made at baseline and on days 7 and 14. Nine of the subjects took 300 mg/day aspirin for 7 days following the 14-day period of 100 mg/day aspirin intake and their fasting blood samples were analyzed on day 21.

Measurement of PRP- and Whole-Blood Aggregation

After placement of a tourniquet, blood was collected from an arm vein using a 21G needle and placed in a glass tube containing a final concentration of 0.313% sodium citrate. The effect of the tourniquet on both forms of aggregation was negligible when blood collection was performed smoothly (data not shown), as described before[10] PRP was prepared by centrifugation of blood at 200 G at 25°C for 10 min and platelet-poor plasma was prepared by centrifugation at 2,000 G at 25°C for 10 min. Aggregability immediately after blood collection was weaker, but stabilized at a later time[10,13] Because the aggregability of the PRP and whole blood was stable during the 60–120 min after blood collection (data not shown), we analyzed aggregability during that period.

Horse tendon collagen (Horm®) was purchased from Nycomed (Munich, Germany) and adenosine diphosphate (ADP) from Sigma (St Louis, MO, USA). PRP aggregation was measured by the addition of 20 μl of collagen (final concentrations; 0, 0.1, 0.4, 1.6, 6.4, and 25.6 mg/L) and ADP (0, 1, 3, 9, 27, and 100 μmol/L) to 180 μl of PRP while constantly stirring at 37°C using a light transmission aggregometer, MCM HEMA TRACER 212® (MC Medical, Tokyo, Japan). The degree of light transmission of the PRP was defined as showing an aggregation rate of 0% and that of the platelet-poor plasma as 100%. The platelet-aggregation threshold index (PATI) was defined as the putative agonist-concentration giving 50% aggregation based on the light transmission rate at 5 min after stimulation (Fig 1A). The light transmission rate at 5 min during stimulation by an excessively high concentration of 25.6 mg/L of collagen or 100 μmol/L of ADP was defined as 100% of PRP aggregation and the rate without agonist stimulation as 0%. In most cases, the aggregation rates at 5 min obtained with 25.6 mg/L of collagen and 100 μmol/L of ADP were similar to those obtained with 6.4 mg/L of collagen and 27 μmol/L of ADP, respectively (data not shown), indicating that PRP aggregation was saturated at 6.4 mg/L of collagen and 27 μmol/L of ADP.

Whole-blood aggregation was measured with a whole-blood aggregometer using the SFP method (WBA-Neo®; ISK, Tokyo, Japan). The reaction was started by the addition of 20 μl of agonist solution to 180 μl of whole blood while constantly stirring at 37°C. The final concentrations of collagen were 0.1, 0.4, 1.6, and 6.4 mg/L, and those of ADP were 1, 3, 9, and 27 μmol/L. At 5 min after stimulation, the absorbing pressure of aggregated whole blood was measured through a microsieve with 30×30 μm windows, and a negative pressure of –130 mmHg was defined as 100% of aggregation and –6 mmHg as 0%, the latter deviation from 0 mmHg being designated because of the viscosity of unstimulated whole blood[10] As shown in Fig 1B, the putative agonist-concentration giving 50% aggregation was calculated and defined as the PATI[10]

Whole-blood aggregation was measured in triplicate and PRP-aggregation in duplicate, and the average value of these measurements was analyzed.

Laboratory Testing

General serum values were measured by the SRL Laboratory (Tokyo, Japan).

Table 1 Clinical and Biochemical Characteristics

	Baseline	*Day 7*	*Day 14*
Age, years	*35±5.5*		
Body mass index, kg/m²	*23.7±2.5*	*NT*	*NT*
RBC, ×10¹²/L	*4.98±0.35*	*4.94±0.36*	*4.93±0.37*
Hemoglobin, ×10² g/L	*1.54±0.87*	*1.53±0.11*	*1.52±0.11*
WBC, ×10⁹/L	*5.90±1.00*	*5.89±1.07*	*5.86±1.27*
Platelets, ×10¹¹/L	*2.32±0.48*	*2.35±0.39*	*2.41±0.43*
Fibrinogen, g/L	*2.29±0.47*	*NT*	*NT*
PT (INR)	*1.05±0.07*	*NT*	*0.95±0.30**
aPTT, s	*35.8±5.0*	*NT*	*33.2±11.5*
Fasting glucose, g/L	*1.00±0.11*	*NT*	*NT*
AST, IU/L	*23±8*	*NT*	*NT*
ALT, IU/L	*30±25*	*NT*	*NT*
Total cholesterol, g/L	*2.01±0.26*	*NT*	*1.98±0.29*
HDL-cholesterol, g/L	*0.58±0.13*	*NT*	*0.57±0.15*
LDL-cholesterol, g/L	*1.19±0.21*	*NT*	*1.06±0.42*
Triglyceride, g/L	*1.19±0.53*	*NT*	*1.26±0.92*
BUN, mg/L	*134±28*	*NT*	*NT*
Creatinine, mg/L	*8.7±0.9*	*NT*	*NT*
Na, mmol/L	*141±1.9*	*NT*	*NT*
K, mmol/L	*4.5±0.6*	*NT*	*NT*
hs-CRP μg/L	*719±1,420*	*721±1,690*	*700±1,330*

*Data are means±SD; males=35. *p<0.05 compared with baseline by Wilcoxon's ranked test.*
NT, not tested; RBC, erythrocyte; WBC, leukocyte; PT (INR), prothrombin time (international normalized ratio); aPTT, activated partial thromboplastin time; AST, aspartate transaminase; ALT, alanine transaminase; HDL, high-density lipoprotein; LDL, low-density lipoprotein; BUN, blood urea nitrogen; hs-CRP, high-sensitivity C-reactive protein.

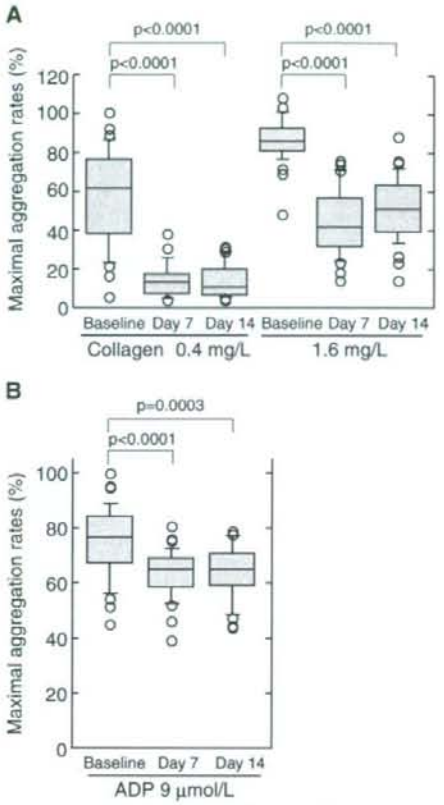

Fig 2. Effects of aspirin intake on maximal aggregation rates of platelet-rich plasma. Maximal aggregation rates induced with 0.4 and 1.6 mg/L of collagen (A) and 9 μmol/L of adenosine diphosphate (ADP) (B) were measured at baseline, and on days 7 and 14 of 100 mg/day aspirin intake as described in the text (n=35). The box–whisker plots show the median, 25th and 75th percentiles (boxes), and the 10th and 90th percentiles (whiskers).

Statistical Analysis

Statistical analysis was performed using Excel® (Microsoft, Redmond, WA, USA) and Statview® (SAS Institute, Cary, NC, USA). Aggregation rates and PATI values are presented as median (interquartile range) and other data as means ± SD. Wilcoxon's signed-rank test was used for statistical analysis. Correlations between valuables were analyzed using Spearman's correlation; $p<0.05$ was considered significant. Receiver-operator-characteristics (ROC) analysis for the detection of aspirin intake was performed using the values obtained from both PRP- and whole-blood aggregation measurements. In some figures, data are shown using box and whisker plots in which the boxes show median and interquartile ranges, and bars represent the 10th and 90th percentiles, with the median representing the 18th value among 35 subjects. The lower boundary of the box represents the 10th value and the upper one shows the 26th value. The lower bar represents the 4th value and the upper bar shows the 32nd value. Upper and lower open circles represent the 1st to 3rd and the 33rd to 35th values respectively.

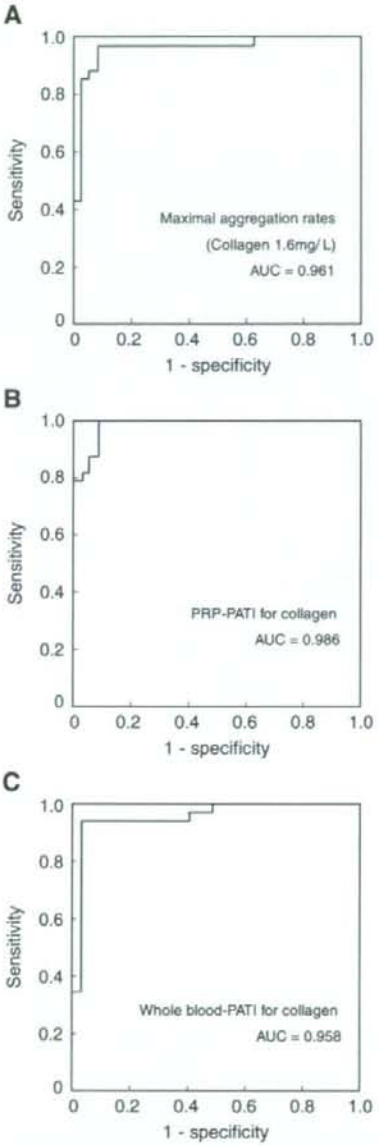

Fig 3. Receiver-operator-characteristics (ROC) curve analysis. ROC curves of maximal aggregation rates with 1.6 mg/L of collagen stimulation (A), PRP-PATI for collagen (B) and whole-blood PATI for collagen (C) in detecting the intake of aspirin. AUC, area under the curve. See Fig 1 for other abbreviations.

Results

Baseline Characteristics

None of the 35 volunteers took drugs that would affect platelet aggregation for 7 days before the study began or at any time during the study. Laboratory tests for the group as a whole were normal (Table 1), and none of the individual subjects had values that lay outside the normal range. None

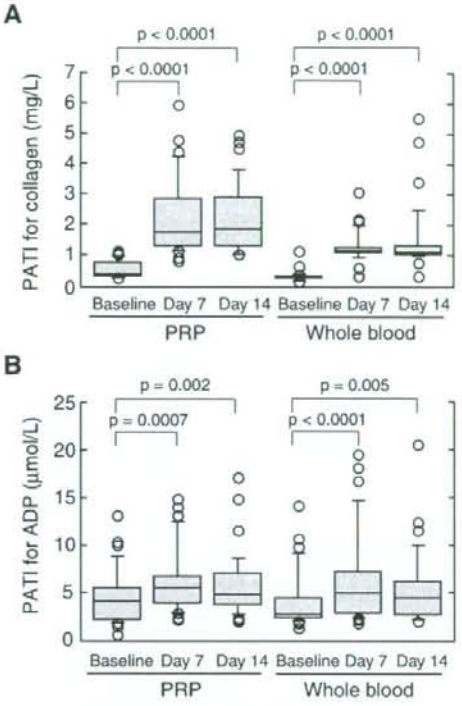

Fig 4. Effects of aspirin intake on the PRP-PATI and whole-blood PATI for collagen (A) and ADP (B). All values were analyzed at baseline and on days 7 and day of 100 mg/day aspirin intake as described in the text (n=35). The box–whisker plots show the median, the 25th and 75th percentiles (boxes), and the 10th and 90th percentiles (whiskers). See Figs 1 and 2 for abbreviations.

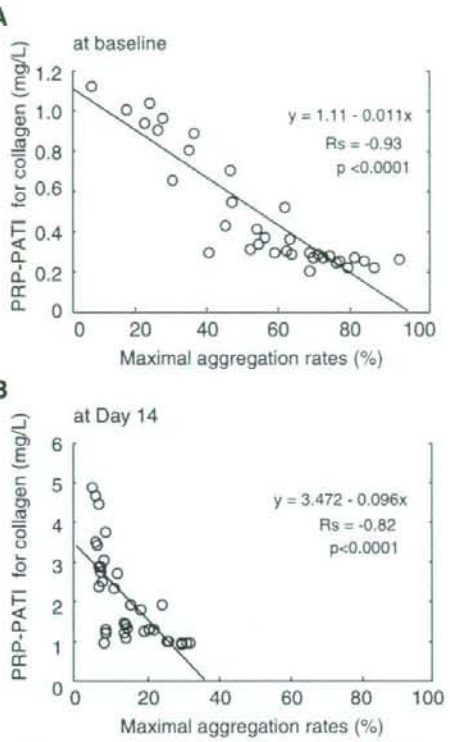

Fig 5. Correlation of the PRP-PATI for collagen and the maximal aggregation rate induced with 0.4 mg/L of collagen. The PRP-PATI and the maximal aggregation rate at baseline (A) and on day 14 (B) for individuals are plotted. Rs, Spearman rank correlation coefficient. See Fig 1 for abbreviations.

of the factors shown in Table 1, including platelet and other blood-cell counts, correlated with either collagen- or ADP-induced PRP- aggregability or whole-blood aggregability at baseline or on day 14 (data not shown). None of the laboratory tests subjected to serial testing showed a significant change before and after aspirin intake, except prothrombin time (PT), but the PT values at both baseline and on day 14 were within the normal range (Table 1).

Effect of Aspirin on the Maximal Rate of PRP Aggregation

The maximal aggregation rates for collagen were decreased by aspirin in all 35 subjects. The rates induced by 0.4 mg/L of collagen were 62.0% (38.3–76.6) [median (interquartile range)] at baseline, 13.0% (7.6–17.5) on day 7 and 11.0% (6.5–19.6) on day 14 (Fig 2A), the latter 2 values significantly different from baseline ($p<0.0001$). The rates induced by 1.6 mg/L collagen also decreased significantly from 85.5% (80.8–92.8) at baseline to 41.5% (31.9–56.4) on day 7 ($p<0.0001$) and 50.5% (39.0–63.8) on day 14 ($p<0.0001$) (Fig 2A). The rates induced by 1.6 mg/L of collagen were distributed evenly and the rates in 29 subjects (10–90th percentile) were in the range of 76.5–108.5% at baseline and 33–69.5% on day 14 (Fig 2A). The area under the ROC curves analyzing the rates at baseline and on day 14 for those induced by collagen at 0.4 mg/L (data not shown) and 1.6 mg/L (Fig 3A) was 0.947 and 0.961, respectively. The maximal aggregation rates with 0.4–1.6 mg/L of collagen appeared useful for evaluating aspirin's effect on inhibition of platelet aggregation, although the ranges of these rates was distributed rather widely (Fig 2A).

We also analyzed the maximal aggregation rate induced by ADP. As shown in Fig 2B, aspirin significantly decreased the rate induced by 9 μmol/L of ADP from 76.5% (67.3–84.3) at baseline to 65.0% (58.1–69.0) on day 7 ($p<0.0001$) and to 65.0% (58.9–70.8) on day 14 ($p=0.0003$) (Fig 2B). However, the decreases were small and the rates were widely distributed and extensively overlapped.

Effect of Aspirin on PRP-PATI for Collagen and ADP

The PRP-PATI for collagen was increased by aspirin in all 35 subjects: 0.32 mg/L (0.28–0.70) at baseline, 1.74 mg/L (1.27–2.84) on day 7, and 1.82 mg/L (1.25–2.89) on day 14 (Fig 4A). Aspirin intake significantly increased the PRP-PATI for collagen on both day 7 ($p<0.0001$) and day 14 ($p<0.0001$), compared with baseline. PRP-PATI values for collagen were distributed evenly and the values in 29 subjects (10–90th percentile) were in the range of 0.23–0.97 mg/L at baseline and 1.00–3.80 mg/L on day 14 (Fig 4A). The PRP-PATI for collagen negatively well-correlated with the maximal aggregation rate induced by 0.4 mg/L collagen at base-

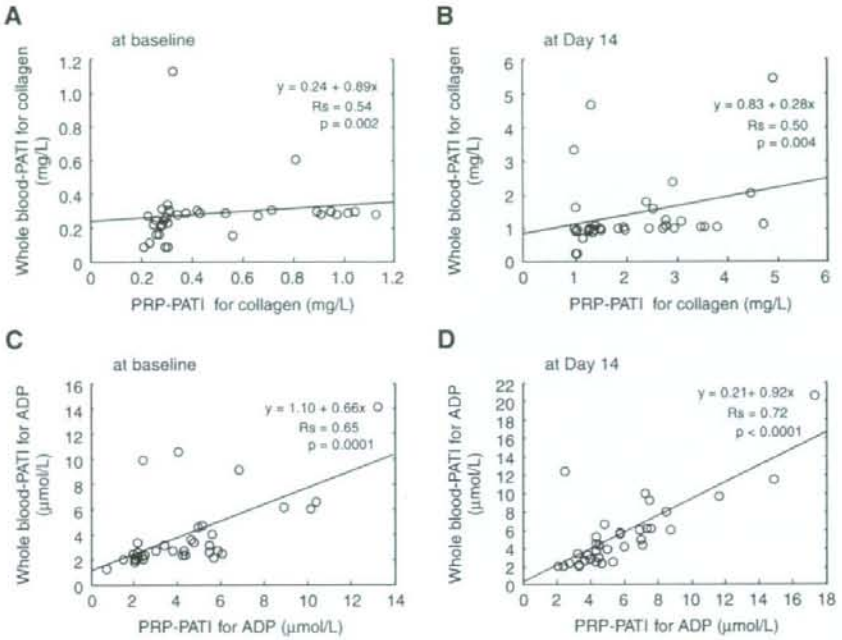

Fig 6. Correlation between the whole-blood and PRP-PATI values. Collagen stimulation at baseline (A) and on day 14 (B). ADP stimulation at baseline (C) and on day 14 (D). See Figs 1 and 2 for abbreviations.

line (Rs=–0.93; p<0.0001) (Fig 5A) and on day 14 (Rs=–0.82; p<0.0001) (Fig 5B). Similar negative correlations were observed with 1.6 mg/L collagen at both baseline (Rs=–0.39; p=0.02) and on day 14 (Rs=–0.72; p<0.0001) (data not shown). The area under the ROC curve analyzing the PRP-PATI for collagen at baseline and on day 14 was 0.986 (Fig 3B). Thus, the PRP-PATI for collagen clearly distinguished aggregability under aspirin intake from that at baseline.

As shown in Fig 4B, aspirin-intake significantly increased the PRP-PATI for ADP from 4.21 μmol/L (2.20–5.53) at baseline to 5.64 μmol/L (3.95–6.79) on day 7 (p=0.0007) and 4.78 μmol/L (3.80–7.13) on day 14 (p=0.002) (Fig 4B). However, the increases were small, and the PATI values were widely distributed and extensively overlapped (Fig 4B).

Effect of Aspirin on PATI of Whole-Blood Aggregation

Whole-blood aggregation was analyzed by semi-automatic aggregometer using the SFP method and the whole-blood PATI for collagen was increased by aspirin in all 35 subjects: 0.28 mg/L (0.22–0.30) at baseline, 1.10 mg/L (1.05–1.22) on day 7, and 1.06 mg/L (1.01–1.29) on day 14 (Fig 4A). Thus, aspirin intake significantly increased the whole-blood PATI for collagen on both day 7 (p<0.0001) and day 14 (p<0.0001), compared with baseline. The whole-blood PATI for collagen in 29 subjects (10–90th percentile) at baseline was in the range of 0.15–0.31 mg/L and 0.93–2.43 mg/L on day 14 (Fig 4A). Importantly these values were distributed in a quite narrower range, as compared with the maximal aggregation rate and the PRP-PATI (Figs 2A,4A), and clearly demonstrated differences in aggregability before and after aspirin intake. The ROC curve analysis revealed that the area under the curve analyzing the whole-blood PATI for collagen at baseline and on day 14 was 0.958 (Fig 3C).

As shown in Fig 4B, aspirin-intake slightly increased the whole-blood PATI for ADP from 2.75 μmol/L (2.35–4.56) at baseline to 4.97 μmol/L (3.03–7.26) on day 7 (p<0.0001), and to 4.45 μmol/L (2.75–6.26) on day 14 (p=0.005) (Fig 4B). However the distribution was wide with extensive overlapping.

Comparison of Whole-Blood PATI and PRP-PATI

As shown in Figs 6A,B, there was a moderate correlation between the PRP-PATI and whole-blood PATI for collagen at both baseline (Rs=0.54; p=0.002) and on day 14 (Rs=0.50; p=0.004), whereas the PRP-PATI and whole-blood PATI for ADP were well correlated at both baseline (Rs=0.65; p=0.0001) (Fig 6C) and on day 14 (Rs=0.72; p<0.0001) (Fig 6D).

Effect of High-Dose Aspirin on Whole-Blood and PRP Aggregations Induced by Collagen

Among the participating 35 subjects, 9 took 300 mg/day aspirin for 7 days following the administration of 100 mg/day of aspirin for 14 days. As shown in Figs 7A,B, the higher dose of aspirin did not increase either the PRP-PATI or whole-blood PATI (p=0.44 and 0.41, respectively). On day 21, the PRP-PATI was 1.96 mg/L (1.36–3.22) and the whole-blood PATI was 1.07 mg/L (1.03–1.08), which did not differ significantly from the values on day 14 [PRP-PATI; 1.82 mg/L (1.35–2.78), whole-blood PATI; 1.05 mg/L (0.99–1.07)]. However, the whole-blood PATI for collagen appeared to be more constant during day 7–21, compared

with the PRP-PATI values for collagen (Figs 7A,B).

Discussion

A semi-automatic whole-blood aggregometer that uses the SFP method has been recently developed, and is considered to be useful in clinical practise. It is a simple and rapid whole-blood assay that requires a small amount of blood (1–2 ml). It has been reported that this whole blood aggregometer can detect the reduction of platelet-aggregability in vitro caused by antiplatelet drugs such as aspirin, cilostazol, dipyridamole and tirofiban[10] To evaluate the utility of this whole-blood aggregometer in clinical practise, we analyzed whole-blood aggregation by the SFP method during daily aspirin intake for 2–3 weeks, and compared the results with those for PRP aggregation, which is the current standard method. As reported previously, the maximal aggregation rate as determined by PRP aggregation showed an excellent ability to distinguish platelet aggregability before and after aspirin intake. In the present study the PRP-PATI for collagen was well correlated with the maximal aggregation rate, and also clearly distinguished platelet aggregability before and after aspirin intake. We also found that the whole-blood PATI for collagen accurately distinguished platelet aggregability before and after aspirin intake. Moreover, the whole-blood-PATI values were distributed in a much narrower range than the PRP-PATI values. Therefore, together with its feasibility, whole-blood aggregation measured with the SFP method is an excellent index for monitoring the efficacy of aspirin therapy.

At present, the maximal aggregation rate measured by optical aggregometer is the standard index for platelet aggregability. However, in the present study the distribution of the individual maximal aggregation rates appeared rather wide (Figs 2A,B), so to try and improve this measurement, we analyzed the PRP-PATI, defined as the putative agonist-concentration giving half-maximal aggregation. In that case, the values for collagen clearly distinguished platelet aggregability before and after aspirin intake (Fig 4A). Also, the area under the ROC curve obtained with the maximal aggregation rate was 0.961 (Fig 3A) and that using the PRP-PATI was 0.986 (Fig 3B). The PRP-PATI for collagen in 90% of cases was below 0.96 mg/L at baseline and in 90% of cases was over 1 mg/L on day 14. Therefore, a PRP-PATI for collagen of approximately 1 mg/L seems a reasonable cut-off value for distinguishing the effects of aspirin intake. A cut-off value between 0.966 and 0.973 mg/L gave 91% sensitivity and 100% specificity, whereas a value between 69.5% and 76.5% of maximal aggregation rate stimulated with 1.6 mg/L of collagen gave 97% sensitivity and 91% specificity. In general, the PATI is a more comprehensible index that has its own significant meaning, whereas the maximal aggregation rate must be interpreted according to the concentration of agonist. The present results suggest that the PRP-PATI may be preferred for monitoring aspirin's effects.

Analysis of whole-blood aggregation has the obvious advantage of easy and rapid sample preparation without the need for centrifugation. Recent improvements in the whole blood aggregometer using the SFP method enabled us to consistently examine many samples. As noted, the whole blood-PATI is defined as the putative agonist-concentration that induces half-maximal absorbing pressure[10] and in the present study gave values for collagen that clearly showed an increase during administration of aspirin, compared with

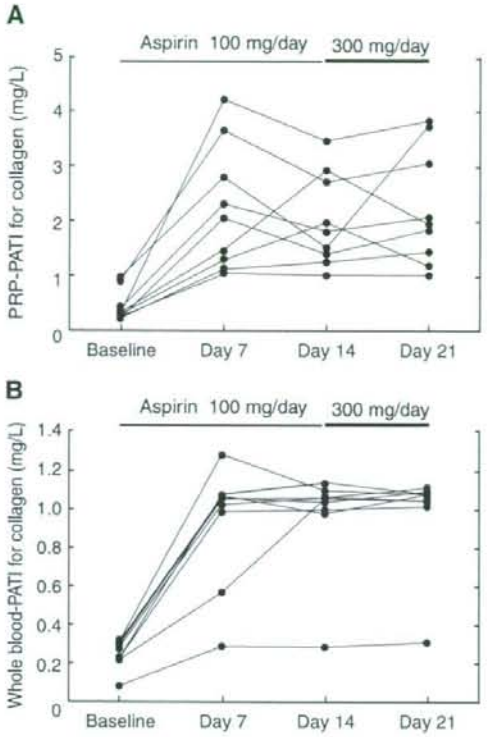

Fig 7. Effects of high-dose aspirin on PRP-PATI (A) and whole-blood PATI (B). Nine subjects took 100 mg/day aspirin for 14 days, followed by 300 mg/day aspirin intake for 7 days. Values were analyzed at baseline and on days 7, 14 and 21. See Fig 1 for abbreviations.

baseline (Fig 4A). Interestingly, the whole-blood PATI values for the 10–90th percentile, especially the 25–75th percentile, were distributed in a narrower range both before and after aspirin intake, and these ranges were also more clearly separated from one another compared with the PRP-PATI values (Fig 4A). The area under the ROC curve obtained with the whole-blood PATI values was 0.958 (Fig 3C). Judging from the distribution (Fig 4A), it seems that approximately 0.7 mg/L is a reasonable cut-off point for the whole-blood PATI for collagen to distinguish the effects of aspirin intake. A cut-off value between 0.61 and 0.74 mg/L gave 94% sensitivity and 97% specificity. These findings suggest that the whole-blood PATI for collagen is an excellent indicator of aspirin's effects as compared with values obtained using the optical aggregometer.

Both the PRP-PATI and whole-blood PATI for ADP also showed significant elevation after aspirin intake, but the increase was relatively small and the distribution of the PATI values was wide and they extensively overlapped before and after aspirin intake (Fig 4B), findings that are consistent with previous investigations[14,15] Therefore, neither of the PATI values for ADP seem particularly useful for evaluating aspirin's effects.

The PRP-PATI is determined from light transmission rates, whereas the whole-blood PATI is based on the absorbing pressures through a microsieve. Therefore, the 2

values are substantially distinct, although both are excellent indicators of aspirin's effect, so it was reasonable to compare them. As shown in Figs 6A,B, there was a moderate correlation between the 2 PATI values for collagen at both baseline and on day 14, whereas the values for ADP were well correlated (Figs 6C,D). One of the reasons for that finding could be that most of the whole-blood PATI values for collagen were distributed in a much narrower range than the PRP-PATI values. The influence of other blood cells on aggregation, which might have the effect of keeping collagen-induced whole-blood aggregability relatively constant both before and after aspirin intake, might also play a role. Further investigation is required to clarify this matter.

The ability of aspirin to inhibit platelet function varies widely among individuals in analyses using the optical aggregometer. It has been reported that platelets with a diminished response to aspirin intake are associated with a higher cardiovascular risk, a phenomenon called as 'aspirin resistance'.[3,16,17] Researchers have not reached a consensus on the definition of aspirin resistance and the prevalence varies between 5% and 60% in the reports.[17] In the present study, aspirin's effects also varied among individuals, and in some subjects blood aggregability after aspirin intake remained within the range of that prior to aspirin intake. These subjects could be aspirin-resistant. If we define the normal range of aggregability before aspirin intake as between the 5th and 95th percentiles, the prevalence of aspirin resistance detected by the maximal aggregation rates, the PRP-PATI and the whole-blood PATI for collagen is 14.2%, 8.6% and 5.7%, respectively.

The ADP receptor inhibitor, clopidogrel, is an essential drug used in coronary stenting. Clopidogrel-resistant patients have also been recognized and the importance of a monitoring system acknowledged.[18,19] We consider that whole-blood analysis by the SFP-method may be a good candidate for that as well.

In conclusion, the PRP-PATI and whole-blood PATI for collagen quantify platelet aggregability before and after aspirin intake in a fashion similar to, or perhaps even superior to, maximal PRP aggregation rates. Furthermore, the whole-blood PATI for collagen may be especially useful for monitoring aspirin's effect on platelets because it is easier to measure without the centrifugation procedure required for preparation of PRP. This is a new finding shown in Japanese subjects; however, whether the results derived from healthy volunteers are directly applicable to actual patients with cardiovascular disease is unknown and deserves further study.

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