Analysing responses to aspirin and clopidogrel by measuring platelet thrombus formation under arterial flow conditions

Kazuya Hosokawa1,2; Tomoko Ohnishi1; Hisayo Sameshima1; Naoki Miura3; Takashi Ito3; Takehiko Koide3; Ikuro Maruyama3

1Research Institute, Fujimori Kogyo Co., Ltd., Yokohama, Kanagawa, Japan; 2Department of Veterinary Medicine, Faculty of Agriculture, Kagoshima University, Kagoshima, Japan; 3Department of System Biology in Thromboregulation, Graduate School of Medical and Dental Sciences, Kagoshima University, Kagoshima, Japan

Summary
High residual platelet aggregability and circulating platelet-monocyte aggregates in patients administered aspirin and clopidogrel are associated with ischaemic vascular events. To determine the relevance of these factors with residual thrombogenicity, we measured platelet thrombus formation using a microchip-based flow-chamber system in cardiac patients receiving aspirin and/or clopidogrel, and evaluated its correlation with agonist-inducible platelet aggregation and platelet-monocyte aggregates. Platelet thrombus formation was analysed by measuring flow pressure changes due to the occlusion of micro-capillaries and was quantified by calculating AUC10 (area under the flow pressure curve). The growth and stability of platelet thrombi that formed inside microchips at shear rates of 1000, 1500, and 2000 s⁻¹ were markedly reduced in patients receiving aspirin and/or thienopyridine compared to healthy controls (n=33). AUC10 values of aspirin therapy patients (n=20) were significantly lower and higher than those of healthy controls and dual antiplatelet therapy patients (n=19), respectively, and showed relatively good correlations with collagen-induced platelet aggregation and platelet-monocyte aggregates at 1000 and 1500 s⁻¹ (r > 0.59, p < 0.01). In contrast, AUC10 in dual antiplatelet therapy patients was significantly correlated with ADP-induced platelet aggregation at all examined shear rates (r > 0.59, p < 0.01), but did not correlate with collagen-induced aggregation.

Aspirin monotherapy patients with high residual platelet thrombogenicity also exhibited significant elevations in both collagen-induced platelet aggregation and platelet-monocyte aggregates. Our results, although preliminary, suggest that residual platelet thrombogenicity in aspirin-treated patients is associated with either collagen-induced platelet aggregation or circulating platelet-monocyte aggregates, but it is predominantly dependent on ADP-induced platelet aggregation in patients receiving dual antiplatelet therapy.

Keywords
Antiplatelet therapy, aspirin, clopidogrel, platelet thrombus, blood flow

Introduction
Aspirin and thienopyridines (clopidogrel and ticlopidine), which inhibit thromboxane A2 (TXA2) synthesis and the P2Y12 receptor, respectively, are the most widely used antiplatelet agents for the secondary prevention of coronary heart disease. These drugs are frequently administered in combination (dual antiplatelet therapy) for long-term treatment after percutaneous coronary intervention (PCI) and significantly improve patient outcomes (1, 2). However, despite the well-demonstrated efficacy in several trials, considerable numbers of patients receiving aspirin and thienopyridines experience recurrent vascular events, even with dual antiplatelet therapy. In addition, several studies have shown that low sensitivity to aspirin and clopidogrel, as determined by platelet function tests, is associated with poor therapeutic outcomes (3–7).

For monitoring antiplatelet treatment efficacy and identifying low responsiveness to therapy, several assay systems have been developed in the clinical setting, such as the VerifyNow™ (Accumetrics, San Diego, CA, USA) and Multiplate™ (Dynabyte Medical, Munich, Germany) systems, which measure platelet aggregation and aggregation in response to an exogenous agonist. The PFA-100 test is also capable of quantifying platelet aggregate formation on the surface of collagen coated with a specific agonist under arterial shear conditions. Although these systems are less labor intensive than light transmission aggregometry (LTA) and permit the use of whole blood, the choice of platelet agonist strongly affects the observed platelet activity and inhibitory effects of the antiplatelet agents. In addition, as the ability to identify patients with low responsiveness to antiplatelet agents varies depending on the test used (8–10), consensus methodologies to assess antiplatelet treatment efficacy have not yet been established.

Flow cytometry (FCM) analysis is also capable of evaluating circulating activated platelets in vivo, and platelet-monocyte aggregate formation and platelet CD62P (P-selectin) expression are re-
ported to be sensitive markers of activated platelets, which have been reported to be associated with ischaemic vascular events (11, 12).

A recent study has demonstrated that shear stress in the coronary arteries of patients receiving aspirin and clopidogrel activates platelets and monocytes, and increases platelet-monocyte aggregates by an agonist-independent pathway (13). These findings suggest that measuring agonist-inducible platelet activation may not be sufficient to accurately assess the risk of ischaemic vascular events. However, it is unclear whether and how agonist-dependent and -independent platelet activation pathways are related to residual thrombogenicity in patients receiving antiplatelet therapy. We speculate that the measurement of platelet thrombus formation (PTF), which involves platelet adhesion and aggregation, granule secretion, and thrombus growth, under conditions that mimic arterial flow should more directly reflect therapeutic responses to aspirin and clopidogrel than platelet aggregation induced by exogenous agonists (14).

Here, we investigated inter-individual variability of PTF using an automated microchip-based flow-chamber system in cardiac patients administered either aspirin monotherapy or dual antiplatelet therapy, and analysed the association between residual PTF and agonist-induced platelet aggregation and two FCM parameters, platelet-monocyte aggregates (PMA) and P-selectin (CD62P), in whole-blood samples.

Materials and methods

For platelet aggregation assays, ADP, collagen, arachidonic acid (AA) reagents, and blood collection tubes containing hirudin (final concentration, 25 µg/ml) were purchased from Dynabyte Medical (Munich, Germany). Blood collection tubes containing EDTA-2K, which were used for determining hematocrit and platelet counts, and collection tubes containing 3.13% sodium citrate, which were used for determining clotting parameters and performing flow cytometric analyses, were purchased from Terumo (Tokyo, Japan).

FITC-conjugated anti-CD41 antibody, FITC-conjugated anti-CD62P antibody, PE-conjugated anti-CD42b antibody, PE-conjugated anti-CD14 antibody, FITC-conjugated IgG1, and PE-conjugated IgG2a were purchased from Beckman Coulter (New York, NY, USA).

Patient population and study protocol

The study protocol was approved by the ethics committee of Kinki University (Osaka, Japan). After obtaining written informed consent, blood samples were collected from 42 consecutive patients (males; 38; females; 4; mean age, 58.2±7.9 years) receiving aspirin and/or thienopyridine for the treatment of heart disease and who were recruited from March 2010 to April 2011. All patients had been receiving antiplatelet therapy and had not experienced myocardial infarction and/or percutaneous coronary intervention for >3 months prior to the collection of blood samples. Exclusion

Table 1: Patient characteristics.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Healthy controls (n=33)</th>
<th>Aspirin monotherapy group (n=20)</th>
<th>Dual antiplatelet therapy group (n=19)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male (n)</td>
<td>19</td>
<td>16</td>
<td>19</td>
</tr>
<tr>
<td>Female (n)</td>
<td>14</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Age (years)</td>
<td>36.5 ± 11.6</td>
<td>58.3 ± 7.9</td>
<td>56.6 ± 7.7</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>21.8 ± 3.0</td>
<td>25.6 ± 3.2</td>
<td>25.3 ± 3.0</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>41.9 ± 4.0</td>
<td>43.6 ± 3.1</td>
<td>42.8 ± 2.9</td>
</tr>
<tr>
<td>Platelet count (10⁹/µl)</td>
<td>25.8 ± 4.5</td>
<td>23.6 ± 4.8</td>
<td>22.3 ± 4.9</td>
</tr>
<tr>
<td>APTT (s)</td>
<td>27.6 ± 1.37</td>
<td>27.1 ± 1.43</td>
<td>26.9 ± 1.64</td>
</tr>
<tr>
<td>PT (s)</td>
<td>11.08 ± 0.54</td>
<td>10.78 ± 0.52</td>
<td>10.95 ± 0.42</td>
</tr>
<tr>
<td>Statin (n)</td>
<td>-</td>
<td>9</td>
<td>15</td>
</tr>
<tr>
<td>ACE/ARB-inhibitor (n)</td>
<td>-</td>
<td>5</td>
<td>8</td>
</tr>
<tr>
<td>Beta-blockers (n)</td>
<td>-</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>NSAIDs</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Previous MI (n)</td>
<td>-</td>
<td>10</td>
<td>8</td>
</tr>
<tr>
<td>Previous PCI (n)</td>
<td>-</td>
<td>5</td>
<td>15</td>
</tr>
</tbody>
</table>

APTT, activated partial thromboplastin time; BMI, body mass index; MI, myocardial infarction; NSAIDs, non-steroidal anti-inflammatory drugs; PCI, percutaneous coronary intervention; PT, prothrombin time.
criteria were age (<18 or >70 years), severe liver or renal dysfunction, infectious disease, and intake of anticoagulant agents.

Three patients were excluded from the study due to the intake of an anticoagulant agent. Ultimately, 20 patients receiving aspirin monotherapy (100 mg/day aspirin; 16 males and 4 females; mean age, 58.3 ± 7.9 years), and 19 patients receiving dual antiplatelet therapy with aspirin (100 mg/day) plus a thienopyridine (75 mg/day clopidogrel, 17 males; or 100 mg/day ticlopidine, 2 males; mean age, 56.6 ± 7.7 years) were enrolled in the study.

To allow comparative estimates of antiplatelet treatment efficacies, we also measured PTF for blood samples collected from 33 healthy volunteers (19 males and 14 females; mean age, 36.5 ± 11.6 years) who had not taken any medications in the preceding two weeks that might have affected platelet function or coagulation.

**Analysis of PTF**

PTF assays were performed using an automated microchip flow-chamber system, as described previously (15). Characteristics of the PTF assay, including the practical requirements and coefficient of variation (CV) values, are summarised in Supplemental Tables 1 and 2 (available online at www.thrombosis-online.com). Briefly, whole blood (350 μl) anticoagulated by hirudin (25 μg/ml) was perfused into the collagen-coated microchip at flow rates of 12, 18, and 24 μl/minute (min), corresponding to initial wall shear rates of 1000, 1500, and 2000 s⁻¹, respectively (15). The PTF process inside the microchip was analysed by monitoring flow pressure. AUC₁₀ (area under the flow pressure curve for 10 min) was calculated to evaluate platelet thrombogenicity, as reported previously (15).

**Whole blood platelet aggregometry (WPA)**

Whole blood platelet aggregation was evaluated for each hirudin-anticoagulated blood sample 1 hour after the blood draw using a Multiplate™ analyser (Dynabyte Medical). Briefly, 300 μl saline and 300 μl blood sample were pipetted into a single-use cuvette. After a 3-min incubation at 37°C, one of the following agonists (final concentration) was added: ADP (6.5 μM), collagen (3.2 μg/ml), or AA (0.5 mM). Platelet adhesion and aggregation were then monitored for 6 min. The impedance change caused by the adhesion of

![Figure 1: Platelet thrombus formation (PTF) process in blood samples of healthy individuals and patients receiving antiplatelet therapy as evaluated by flow pressure waveforms. A microchip-based PTF assay was used to generate flow pressure waveforms at shear rates of 1000, 1500, and 2000 s⁻¹ from blood samples of 33 healthy individuals (A), 20 patients receiving aspirin monotherapy (B), and 19 patients receiving dual antiplatelet therapy (C).](image-url)
Platelets onto the sensor surface was plotted against time and the area under the aggregation curve was used to measure the aggregation response.

**Analysis of PMA and CD62P by flow cytometry (FCM)**

PMA and CD62P-positive platelet cells were measured as previously described (16). All of the following operations were performed at room temperature in the dark, and the results of both assays are expressed as the percentage of positive cells.

**PMA**

Fifty microliter aliquots of whole blood were incubated with either 10 μl FITC-conjugated anti-CD41 antibody and PE-conjugated anti-CD14 antibody or isotype-matched controls for 15 min. The samples were then mixed with 250 μl OptiLyseC (Beckman Coulter) and further incubated for 15 min. Before starting the FCM measurements, samples were diluted by adding 250 μl phosphate-buffered saline (PBS). PMA were defined as double positive for CD41 and CD14, and at least 500 monocytes were evaluated for each sample. All samples were analysed using an EPICS-XL flow cytometer (Beckman Coulter).

**CD62P**

Fifty-microliter aliquots of whole blood were incubated either with 10 μl FITC-conjugated anti-CD62P and PE-conjugated anti-CD42b or with isotype-matched controls for 15 min. The samples were then mixed with 250 μl OptiLyseC (Beckman Coulter) and further incubated for 15 min. Samples were diluted by adding 250 μl PBS and CD62P/CD42b double-positive platelet cells were then quantified using an EPICS-XL flow cytometer (Beckman Coulter). For each blood sample, a minimum of 10,000 platelets was evaluated.

**Statistical analysis**

All data analyses were performed using JMP IN® software (SAS Institute, Inc., Tokyo, Japan). Statistically significant differences be-

![Figure 2: Evaluation of platelet thrombus formation (PTF), whole blood platelet aggregometry (WPA), and flow cytometry (FCM) measurement values in healthy controls and patients receiving anti-platelet therapy.](image-url)

(A) PTF 1000 s⁻¹ 1500 s⁻¹ 2000 s⁻¹

(B) WPA

WPA-AA  WPA-COL  WPA-ADP

(C) CD62P

(D) PMA

Figure 2: Evaluation of platelet thrombus formation (PTF), whole blood platelet aggregometry (WPA), and flow cytometry (FCM) measurement values in healthy controls and patients receiving anti-platelet therapy. A) AUC₁₀ at the shear rates of 1000 (○), 1500 (△), and 2000 s⁻¹ (●); B) WPA-arachidonic acid (AA) (△), WPA-collagen (COL) (○), and WPA- adenosine diphosphate (ADP) (●); and C & D) CD62P (○) and platelet-monocyte aggregates (PMA) (●) in healthy individuals (control; black), patients receiving aspirin monotherapy (aspirin; blue), and patients receiving dual antiplatelet therapy (dual; green).
between AUC_{10}, WPA, and FCM measurements in each treatment group were determined using the Wilcoxon signed-rank test. Pairwise correlations between PTF (AUC_{10}), WPA, and FCM measurements in each group were analysed using Spearman's rank correlation coefficients (r). A p-value of <0.05 was considered statistically significant.

**Results**

Characteristics of the aspirin monotherapy and dual antiplatelet therapy patient groups are shown in ▶ Table 1. No significant differences in the standard measures of clotting parameters (PT and APTT) or blood cells (haematocrit and platelet counts) were detected between the two patient groups.

**Evaluation of PTF**

**Aspirin monotherapy patients**

Aspirin treatment moderately prolonged the onset of the flow pressure increase caused by the occlusion of capillaries with thrombi, and efficiently lowered the sustained pressure waveform peak, which is a measure of platelet thrombi firmness and stability (▶ Figure 1B).

At shear rates of 1000, 1500, and 2000 s^{-1}, the mean AUC_{10} values in aspirin monotherapy patients were significantly lower than those of healthy individuals (▶ Figure 2A). However, despite the clear efficacy of aspirin on inhibiting PTF, the observed flow pressure waveforms displayed a wide range of inter-individual variation, and patients with high residual PTF were observed at all three shear rates.

**Dual antiplatelet therapy patients**

Further suppression of the flow pressure waveforms was observed in whole-blood samples of nearly all patients receiving dual antiplatelet therapy, although steady increases of the waveforms were observed for two patients at shear rates of 1500 and 2000 s^{-1} (▶ Figure 1C). The waveforms of blood samples from patients in this group exhibited a characteristic parabolic pattern due to decreased flow pressure in the late phase of the assay (5–10 min), suggesting that the stability and sustainability of platelet thrombi were drastically reduced. Microscopic observation of the PTF process demonstrated that platelet thrombi had markedly lowered firmness and stability in response to dual antiplatelet therapy, and easily collapsed and broke apart with increased blood flow, which gradually decreased in the late phase of the assay (see Suppl. Movie 1, available online at www.thrombosis-online.com).

**Evaluation of WPA**

Multiplate-AUC (area under impedance curve) of WPA induced by AA and collagen (WPA-AA and WPA-COL, respectively) in

<table>
<thead>
<tr>
<th>Group</th>
<th>Parameters AUC_{10} at 1000 s^{-1}</th>
<th>Parameters WPA- and FCM-variables</th>
<th>R-value</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspirin monotherapy group</td>
<td>AUC_{10}</td>
<td>WPA-AA</td>
<td>0.54</td>
<td>0.0147</td>
</tr>
<tr>
<td></td>
<td></td>
<td>WPA-COL</td>
<td>0.63</td>
<td>0.0027</td>
</tr>
<tr>
<td></td>
<td></td>
<td>WPA-ADP</td>
<td>0.51</td>
<td>0.0205</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PMA</td>
<td>0.61</td>
<td>0.0044</td>
</tr>
<tr>
<td></td>
<td>AUC_{10} at 1500 s^{-1}</td>
<td>WPA-AA</td>
<td>0.51</td>
<td>0.0218</td>
</tr>
<tr>
<td></td>
<td></td>
<td>WPA-COL</td>
<td>0.59</td>
<td>0.0064</td>
</tr>
<tr>
<td></td>
<td></td>
<td>WPA-ADP</td>
<td>0.49</td>
<td>0.0279</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PMA</td>
<td>0.60</td>
<td>0.0055</td>
</tr>
<tr>
<td></td>
<td>AUC_{10} at 2000 s^{-1}</td>
<td>WPA-AA</td>
<td>0.48</td>
<td>0.0329</td>
</tr>
<tr>
<td></td>
<td></td>
<td>WPA-COL</td>
<td>0.49</td>
<td>0.0293</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PMA</td>
<td>0.51</td>
<td>0.0212</td>
</tr>
<tr>
<td>Dual antiplatelet therapy group</td>
<td>AUC_{10} at 1000 s^{-1}</td>
<td>WPA-ADP</td>
<td>0.59</td>
<td>0.0082</td>
</tr>
<tr>
<td></td>
<td>AUC_{10} at 1500 s^{-1}</td>
<td>WPA-AA</td>
<td>0.48</td>
<td>0.0373</td>
</tr>
<tr>
<td></td>
<td></td>
<td>WPA-ADP</td>
<td>0.60</td>
<td>0.0062</td>
</tr>
<tr>
<td></td>
<td>AUC_{10} at 2000 s^{-1}</td>
<td>WPA-ADP</td>
<td>0.60</td>
<td>0.0061</td>
</tr>
</tbody>
</table>

*Only pairs showing significant correlation (p<0.05) are included. ^Spearman’s rank correlation coefficients (r). AA, arachidonic acid; ADP, adenosine diphosphate; AUC_{10}, area under the flow pressure curve for 10 min; COL, collagen; FCM, flow cytometry; PMA, platelet-monocyte aggregates; WPA, whole blood platelet aggregometry.*
blood samples from both patient groups were significantly lower than those observed in healthy individuals (both p<0.0001). However, although WPA-AA was efficiently reduced in all aspirin-treated patients, considerable inter-individual variation of WPA-COL was observed in both patient groups. In addition, WPA-ADP was significantly reduced in patients receiving dual antiplatelet therapy compared to healthy controls and patients receiving aspirin monotherapy, although non-reduced values were observed for three patients (►Figure 2B).

**Evaluation of PMA and CD62P**

PMA and CD62P determined flow cytometric analysis were moderately reduced in patients receiving aspirin monotherapy and dual antiplatelet therapy compared with healthy controls. Although PMA values in both patient groups were significantly lower than those of healthy controls (►Figure 2D), CD62P values were only statistically reduced in patients receiving aspirin monotherapy (p<0.05; ►Figure 2C). Both parameters showed considerable variability in all three groups, with high PMA and CD62P being observed in both patient groups.

**Correlations of AUC_{10} with WPA and FCM**

AUC_{10} values for both patient groups showed significant correlation with several measurements of WPA and FCM (►Table 2), although no significant correlation was observed between those of WPA and FCM. In patients receiving aspirin monotherapy, AUC_{10} displayed relatively good correlation with WPA-COL and PMA at shear rates of 1000 and 1500 s^{-1}, respectively (all r>0.59, p<0.01) (►Table 2). In contrast, AUC_{10} in patients administered dual antiplatelet therapy showed significant correlation with WPA-ADP at all examined shear rates (all r>0.59, p<0.01), but was not significantly associated with WPA-COL or PMA, regardless of the shear rate (►Table 2).

**Analysis and identification of patients with high residual PTF**

Receiver-operating characteristic (ROC) curves for AUC_{10} and WPA values were plotted to assess the discriminability of these assays to distinguish between healthy controls and patient administered antiplatelet therapy (►Table 3, Suppl. Fig. 1, available online at www.thrombosis-online.com). Among the examined assays, WPA-AA demonstrated the highest ability to discriminate between healthy individuals and aspirin-treated patients. AUC_{10} values and WPA-COL also showed relatively high values of area under the ROC curves (all, >0.913) in distinguishing between healthy controls and the two patient groups.

For further analyses of patients with high residual platelet thrombogenicity, who represent potential candidates of insensitivity to antiplatelet agents, we identified individuals with elevated AUC_{10} values in both patient groups using tentative cut-off values determined by the ROC curve analysis (►Table 3). In the aspirin monotherapy group, five patients had AUC_{10} values that exceeded any of the cut-off values at shear rates of 1000, and 1500, and 2000 s^{-1}, and also displayed significant elevations in WPA-COL (p<0.01) and PMA (p<0.01), but not in WPA-AA (►Figure

<table>
<thead>
<tr>
<th>Parameters</th>
<th>AUC</th>
<th>Cut-off value</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control and aspirin monotherapy</td>
<td>AUC_{10}</td>
<td>1000 s^{-1}</td>
<td>0.945</td>
<td>151.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1500 s^{-1}</td>
<td>0.969</td>
<td>348.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2000 s^{-1}</td>
<td>0.979</td>
<td>401.4</td>
</tr>
<tr>
<td></td>
<td>WPA-</td>
<td>AA</td>
<td>0.998</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td></td>
<td>COL</td>
<td>0.913</td>
<td>76</td>
</tr>
<tr>
<td></td>
<td>Control and dual antiplatelet</td>
<td>AUC_{10}</td>
<td>1000 s^{-1}</td>
<td>1.000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1500 s^{-1}</td>
<td>0.994</td>
<td>292.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2000 s^{-1}</td>
<td>1.000</td>
<td>330.6</td>
</tr>
<tr>
<td></td>
<td>WPA-</td>
<td>AA</td>
<td>1.000</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td></td>
<td>COL</td>
<td>0.984</td>
<td>76</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ADP</td>
<td>0.961</td>
<td>43</td>
</tr>
<tr>
<td></td>
<td>Aspirin monotherapy and dual antiplatelet</td>
<td>AUC_{10}</td>
<td>1000 s^{-1}</td>
<td>0.876</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1500 s^{-1}</td>
<td>0.832</td>
<td>136.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2000 s^{-1}</td>
<td>0.821</td>
<td>158.1</td>
</tr>
<tr>
<td></td>
<td>WPA-</td>
<td>ADP</td>
<td>0.864</td>
<td>43</td>
</tr>
</tbody>
</table>

AUC; area under the ROC curve; AA, arachidonic acid; ADP, adenosine diphosphate; AUC_{10}, area under the flow pressure curve for 10 min; COL, collagen; WPA, whole blood platelet aggregometry.
Hosokawa et al. Platelet thrombogenicity in response to antiplatelet therapy

3). Similarly, AUC_{10} values in three patients administered dual antiplatelet therapy exceeded the tentative cut-off values determined from the ROC curve between healthy controls and the dual antiplatelet therapy group. The patients who exhibited the first and second highest values for thrombogenicity at 1500 and 2000 s^{-1} displayed elevated WPA-ADP values (Figure 4A and B).

Discussion

We have presented data suggesting that residual PTF, as assessed by a microchip-based flow chamber system, in patients receiving aspirin and clopidogrel is associated with agonist-inducible platelet aggregability and circulating platelet-monocyte aggregates. Although patients receiving either aspirin monotherapy or dual antiplatelet therapy displayed considerable variability in PTF at shear rates of 1000, 1500, and 2000 s^{-1}, those patients showing high residual PTF, who are potential candidates of low responsiveness (also termed “resistance”) to these antiplatelet agents, were successfully identified in both treatment groups. These preliminary findings suggest that residual platelet thrombogenicity in aspirin-treated patients is associated with either collagen-induced platelet aggregation or circulating platelet-monocyte aggregates, but it is predominantly dependent on ADP-induced platelet aggregation in patients receiving dual antiplatelet therapy.

Residual PTF in patients receiving aspirin monotherapy displayed relatively good correlation with WPA-COL and PMA, but not WPA-AA at all three shear rates tested (Table 2). However, as WPA-AA displayed higher sensitivity to aspirin treatment (Table 3), residual platelet thrombogenicity in the presence of aspirin treatment may also be influenced by factor(s) other than the inhibition of platelet cyclooxygenase (COX)-1 activity. Ohmori et al. (17) reported that despite platelet COX-1 inhibition, large inter-individual variability of collagen-induced platelet aggregation exists in aspirin-treated patients, and that elevated collagen-induced aggregation is associated with an increased risk of cardiovascular events. In agreement with these findings, Frelinger et al. (18) demonstrated that cardiovascular events in aspirin-treated patients are associated with the results of COX-1 indirect assays (PFA-100 COL/ADP), but not with platelet activation induced by AA.

Figure 3: Evaluation of values for whole blood platelet aggregometry (WPA) and flow cytometry (FCM) measurements for aspirin monotherapy patients with high residual platelet thrombus formation (PTF). Parameters of area under the flow pressure curve for 10 min (AUC_{10}), WPA measurements, CD62P, and platelet-monocyte aggregates (PMA) of patients below and above any of the three cut-off values, which were determined based on the assay results between healthy individuals and patients receiving aspirin monotherapy, are shown in panels A-D. AA, arachidonic acid; ADP, adenosine diphosphate; COL, collagen.
Our present data also suggest that residual PTF is associated with PMA, which is a sensitive marker of circulating activated platelets in vivo. In addition, a recent study has shown that shear stress in the coronary arteries, even for patients using antiplatelet agents, activates both platelets and monocytes, and increases platelet-monocyte aggregates in an agonist-independent manner (13). In this regard, residual thrombogenicity assessed by our microchip-based flow chamber system should comprehensively reflect the activities of both agonist-dependent and independent platelet activation pathways, which are both associated with ischaemic vascular events (6, 11, 12, 17, 18).

In contrast to aspirin monotherapy, PTF ($AUC_{10}$) in the presence of dual antiplatelet therapy significantly correlated with WPA-ADP, but not WPA-COL, under all examined shear conditions (Table 2), suggesting that the efficacy of thienopyridines for inhibiting PTF is dominant to that of aspirin when administered concurrently. In agreement with our results, several previous clinical studies demonstrated that high residual platelet reactivity to ADP (3, 7, 19–22) and VASP phosphorylation (23) are independent risk factors of ischaemic vascular events in patients receiving dual antiplatelet therapy.

Low-level inhibition of the platelet P2Y$_{12}$ receptor by the poor metabolic activation of clopidogrel due to CYP2C19 polymorphism is associated with an increased risk of vascular events (3, 24, 25). However, individual variability in platelet activation in response to exogenous ADP, as assessed by LTA and the VerifyNow™ system, cannot be fully explained by the CYP2C19*2 allele (26). Thus, the relevance of the inhibitory effect of clopidogrel on PTF involving only endogenous ADP may aide to elucidate clopidogrel resistance.

A recent study using a parallel plate flow chamber system found that the effects of dual antiplatelet therapy on thrombus volume at an arterial shear rate (1500 s$^{-1}$) varied considerably, although the inhibitory effects on thrombus volume measured by confocal videomicroscopy were not proportional to P2Y$_{12}$ inhibition (27). These investigators speculated that the lack of correlation between these factors was because thrombus volume more dominantly reflects initial platelet adhesion and aggregation on a fibrillar col-

Figure 4: Evaluation of values for whole blood platelet aggregometry (WPA) and flow cytometry (FCM) measurements for dual antiplatelet therapy patients with high residual platelet thrombus formation (PTF). Parameters of area under the flow pressure curve for 10 min ($AUC_{10}$), WPA measurements, CD62P, and platelet-monocyte aggregates (PMA) for patients below or above any of the three cut-off values, which were determined based on the assay results between healthy controls and patients receiving dual antiplatelet therapy are shown in panels A-D. AA, arachidonic acid; ADP, adenosine diphosphate; COL, collagen.
What is known about this topic?

- The clinical benefits of aspirin and clopidogrel on reducing cardiac events have been well established in several clinical trials.
- Poor responses to aspirin and clopidogrel, as assessed by platelet aggregation tests, are associated with an increased risk of recurrent vascular events.
- Shear stress in the coronary arteries of patients receiving aspirin and clopidogrel directly activates both platelets and monocytes, and increases platelet-monocyte aggregates by an agonist-independent pathway.

What does this paper add?

- Platelet thrombus formation (PTF), as assessed by a microchip-based flow chamber system, markedly varies among cardiac patients receiving aspirin and clopidogrel.
- Residual PTF in aspirin-treated patients significantly correlates with collagen-induced platelet aggregation and platelet-monocyte aggregates, whereas PTF in patients receiving aspirin and clopidogrel significantly correlates with ADP-induced platelet aggregation.
- Aspirin-treated patients with high residual PTF exhibited markedly elevated values for both collagen-induced platelet aggregation and platelet-monocyte aggregates.

What does this paper add?

- Platelet thrombus formation (PTF), as assessed by a microchip-based flow chamber system, markedly varies among cardiac patients receiving aspirin and clopidogrel.
- Residual PTF in aspirin-treated patients significantly correlates with collagen-induced platelet aggregation and platelet-monocyte aggregates, whereas PTF in patients receiving aspirin and clopidogrel significantly correlates with ADP-induced platelet aggregation.
- Aspirin-treated patients with high residual PTF exhibited markedly elevated values for both collagen-induced platelet aggregation and platelet-monocyte aggregates.

Several limitations of this study warrant mention. First, similar to previous flow chamber-based studies (14, 27), we used whole blood anticoagulated with thrombin inhibitor to analyse the PTF process, although thrombin is a well-known platelet agonist. To more comprehensively simulate the pathologic thrombus formation process, we recently developed a microchip coated with collagen and tissue thromboplastin to evaluate white thrombus formation mediated by both platelet activation and the coagulation system under flow conditions (31, 32). Second, the number of patients was small, and patients taking clopidogrel alone were not examined. Third, the time between the ingestion of antiplatelet drugs and blood sampling was not standardised. Finally, as we only analysed the relevance of residual PTF with agonist-inducible platelet aggregability and the results of flow cytometric analysis, our results should be considered preliminary and further investigation on the clinical relevance of PTF with patient outcomes is necessary to confirm the clinical utility of these combined assays.

Several assay systems are utilised for monitoring antiplatelet therapy in the clinical setting. However, as most systems evaluate platelet activation in response to a supraphysiological exogenous agonist, the results obtained using these systems are strongly affected by the choice of agonist. In addition, the ability to detect low responsiveness to antiplatelet treatment is largely dependent on the assay used (8–10). In this regard, the direct assessment of PTF under arterial shear conditions in combination with conventional platelet function tests, such as LTA, is expected to facilitate the analysis of residual thrombogenicity and optimisation of antiplatelet therapy.

Acknowledgements

The study was supported by the Japan Science and Technology Agency (JST). The authors wish to thank Tomoka Nagasato for her assistance with the flow cytometric measurements.

Conflicts of interest

None declared.

References