Shear-Induced Platelet Aggregation in Cerebral Ischemia

Shinichiro Uchiyama, MD; Masako Yamazaki, MD; Shoichi Maruyama, MD; Makoto Handa, MD; Yasuo Ikeda, MD; Mayumi Fukuyama; Ichiro Itagaki

Platelet activation has an important role in arterial thrombosis and can be induced not only by physiological agonists but also by physical shear stress. Deposition of platelet aggregates in the arterial circulation occurs preferentially at sites of bifurcation, sharp bend, and stenosis, where flow separation is likely to occur. In vitro experiments have shown that fluid shear stress may promote platelet aggregation by direct activation or by enhancing the response to chemical stimuli. This mechanism may be of great importance for thrombogenesis in patients with ischemic stroke or transient ischemic attacks (TIA), who develop thrombi in the arteries of the neck or the brain. Spontaneous platelet aggregation or rheological platelet aggregation induced in vitro by shearing forces alone has occasionally been found in patients with various atherothrombotic diseases.

In the present study we measured shear-induced platelet aggregation (SIPA) in patients with cerebral ischemia with a newly developed apparatus and studied the correlation of SIPA with other hemostatic parameters as well as the influence of antiplatelet agents.

Subjects and Methods

The subjects were 63 patients with cerebral infarction (41 men and 22 women; mean age, 65 years range, 30 to 87 years) and 12 patients with TIA (7 men and 5 women; mean age, 65 years [range, 49 to 83 years]), who were studied within 3 weeks of the onset of illness and had not been given any antiplatelet agents, anticoagulants, or fibrinolytic agents. In addition, we studied 14 healthy nonsmoking volunteers (6 men and 8 women; mean age, 31 years [range, 27 to 42 years]) and 12 nonsmoking patients with various neurological disorders except for stroke and without hypertension, diabetes mellitus, or hyperlipidemia (7 men and 5 women; mean age, 60 years [range, 48 to 75 years]).

Cerebral infarction was classified as atherothrombotic, cardioembolic, or lacunar based on the clinical manifestations and the findings of brain computed tomography, brain magnetic resonance imaging, cerebral angiography, electrocardiography, and echocardiography, according to the Classification of Cerebrovascular Diseases III of the National Institute of Neurological Disorders and Stroke. Atherothrombotic infarction was diagnosed when there was evidence of arterial stenotic plaque or occlusion at one or more sites in the extracranial and major intracranial arteries along with the absence of potential cardiac sources of embolism. A diagnosis of cardioembolic infarction was made on the basis of evidence of a potential cardiac source of embolism, a rapid onset of symptoms, evidence of multiple brain or systemic infarcts, and the absence of atherosclerotic lesions in the large arteries. Lacunar infarction was defined as small, deep subcortical infarcts (<1.5 cm in diameter) in the territory of the perforating arteries in patients without large arterial lesions or potential cardiac sources of embolism. Patients who did not meet the diagnostic criteria for atherothrombotic, cardioembolic, or lacunar infarction were classified as "other".
the criteria for any of the above three clinical categories were excluded from this study. Patients with primary coagulation disorders including antiphospholipid antibody syndrome, hereditary deficiencies of coagulation inhibitors, abnormalities of fibrinolysis, thrombocytosis, and polycythemia were also excluded.9

Fig 1 shows a diagram of the apparatus used to measure SIPA.16 Our method for the continuous monitoring of SIPA was based on a turbidimetric technique that uses a thermostatted cone-plate streaming chamber made of polymethylacrylate. The light beam from a helium-neon laser was passed through a sample of platelet-rich plasma in the chamber to the photodetector, and sequential changes in the light transmittance resulting from platelet aggregation due to shear stress caused by rotation of the cone were displayed with a computer system. The cone-plate chamber was composed of a rotating opaque cone with a diameter of 30 mm and an angle of 1° and a detachable concave cell with a transparent cylindrical wall and an opaque base plate. The distance from the apex of the cone to the base plate was adjusted to 0.04 mm by means of a micrometer screw in conjunction with a rotation unit. The cone was rotated by means of a rotor, and a constant shear stress was obtained throughout the entire streaming sample without producing turbulent flow because of a very small angle between the cone and plate.11 A rotation rate of up to 1800 rpm (the corresponding shear rate was 10 800 per second) was applied to the samples. Two optical fibers were fixed to either end of the cylindrical wall of the cell. The first fiber was used to carry the incident light (wavelength, 633 nm) from a helium-neon laser light source to the cell. The second optical fiber was connected to a silicone photodiode sensor, and the photodiode output current was converted to a voltage signal by an optical power meter. The change in the logarithmic value of the transmitted light intensity was proportional to the change in the platelet count.

Platelet-rich plasma was separated by centrifugation (at 100g for 15 minutes) of venous blood collected with 1/10 volume of 3.8% trisodium citrate. Then aggregation was induced by applying shear stress at 108 dynes/cm² for 5 minutes by means of the above-mentioned apparatus, and the extent of SIPA was expressed as the percent maximum change in light transmittance during the 5-minute period.

SIPA was determined before and on day 7 after oral administration of either of the following drugs was started: 81 mg aspirin (one baby's Bufferin tablet, Lion Corporation) or 200 mg ticlopidine (two 100-mg tablets of Panadine, Daichi Pharmaceutical Co). Aspirin was administered to 3 patients with chronic cerebral infarction and 2 with TIA, and ticlopidine was administered to 4 patients with chronic cerebral infarction and 2 with TIA, among those entered into this study.

Platelet aggregation was also determined in response to 2 µmol/L ADP (Niko Bioscience), 0.42 mmol/L arachidonic acid (AA) (Nakarai Kagaku), and 0.2 µmol/L platelet-activating factor (PAF) (Funakoshi Yakuhin) according to the method described previously.12 In addition, the β-thromboglobulin (β-TG) level was determined in plasma separated by centrifugation at 1500g for 30 minutes at 4°C from 2.5 mL of venous blood collected into an ice-cold Diatube-H (Diagnostica Stago) containing 0.3 mL of anticoagulants including sodium citrate, citrate, theophylline, adenosine, and dipyridamole. The plasma sample was subjected to enzyme-linked immunosorbent assay using an Asserrachrom β-TG kit (Diagnostica Stago).

Determination of plasma von Willebrand factor (vWF) antigen levels and analysis of vWF multimers were performed with stored frozen plasma samples that were separated by centrifugation from venous blood that was collected in the first 26 consecutive subjects (5 with atherothrombotic stroke, 12 with lacunar stroke, 4 with TIA, and 5 patient control subjects) together with a 1/9 volume of 50 mmol/L EDTA, 3.2% trisodium citrate, 10 mmol/L leupeptin, and 60 mmol/L N-ethylmaleimide. Plasma levels of vWF antigen were determined by a sandwich enzyme-linked immunosorbent assay. vWF multimers were analyzed by sodium dodecyl sulfate-1% agarose gel electrophoresis.13

One-way ANOVA was used to compare results among the stroke groups, TIA patients, and control subjects. Pearson's correlation coefficients were used to analyze the relations between SIPA and the hemostatic parameters. Student's dependent t test was used to compare the results after treatment with those obtained before treatment, and P<.05 was judged to indicate statistical significance.

**Results**

SIPA was determined in 26 control subjects and 75 patients with cerebral ischemia. SIPA was determined at both 12 and 108 dynes/cm² in all subjects. However, the platelet aggregation induced by low shear stress (12 dynes/cm²) proved to be unstable and quite variable, since it was greatly affected by the time elapsed after blood collection. Therefore, only platelet aggregation induced by high shear stress (108 dynes/cm²) was analyzed in this study. SIPA at 108 dynes/cm² was determined on different days in 8 normal volunteers, and there was no significant difference between the first and second determinations (the mean±1 SD of the first and second determinations was 48.6±9.7% and 49.9±6.8%, respectively; P=.54). There was no significant correlation between SIPA and the age of either the control
subjects or the patients with cerebral ischemia, and there were no significant differences between men and women. There was also no significant correlation between SIPA and the duration after onset in the patients with cerebral ischemia. SIPA was increased in the 21 patients with atherothrombotic stroke and the 12 TIA patients compared with normal or patient control subjects, but it was not increased in the 11 patients with cardioembolic stroke or the 31 patients with lacunar stroke (Table 1). In addition, SIPA was significantly higher in the patients with atherothrombotic stroke or TIA than in the patients with cardioembolic stroke or lacunar stroke. There was no significant correlation between SIPA and platelet count or platelet aggregation in response to ADP, AA, or PAF, as well as no correlation between fibrinogen or β-TG levels and SIPA (Table 2).

There was no significant correlation between SIPA and plasma vWF antigen levels in the 26 patients for whom both were determined simultaneously (Table 2), whereas SIPA was likely to be increased in the patients with dense bands of larger vWF multimers (Fig 2). Larger multimers were defined as bands above the 10th band from the bottom, indicating molecular weights of >13 to 14 × 10^6. SIPA indicates shear-induced platelet aggregation.

248 ± 77 arbitrary units) than in 12 patients with lacunar stroke (147 ± 67 U) (P < 0.001) or in 5 patient control subjects (114 ± 43 U) (P < 0.01). There was no significant difference in the level of larger vWF multimers between 4 TIA patients (156 ± 91 U) and patients with atherothrombotic or lacunar stroke or patient control subjects.

The effects of two antiplatelet agents on SIPA were also studied in the patients with cerebral ischemia. Oral aspirin (81 mg/d) did not affect SIPA, whereas oral ticlopidine (200 mg/d) clearly inhibited it (Fig 4).

Discussion

Recent evidence has suggested that SIPA is an important mechanism of thrombogenesis at sites of arterial bifurcation or stenosis. Fluid shear stress in arteries and arterioles stenosed by atherosclerosis or spasm may exceed the normal average level of 20 dynes/cm². In vitro platelet aggregation begins to occur when a fluid shear stress of at least 30 to 60 dynes/cm² is applied for 30 seconds. Regarding the molecular mechanism of SIPA, it is known that the interaction of fibrinogen with glycoprotein (GP) IIb/IIIa is required at low shear stresses, while interaction of vWF with both GP Ib and GP IIb/IIIa is required at high shear stresses. Platelet aggregation induced by a high shear stress of 108 dynes/cm² was increased in our patients with atherothrombotic stroke and TIA but not in those with cardioembolic or lacunar stroke. These differences in SIPA between subtypes of stroke may reflect differences in the pathogenesis of the underlying condition. Atherothrombotic stroke was associated with increased SIPA, whereas SIPA did not increase in patients with lacunar stroke.

Table 1. Shear-Induced Platelet Aggregation in Subtypes of Cerebral Ischemia

<table>
<thead>
<tr>
<th>Subgroup</th>
<th>n</th>
<th>SIPA, % (Mean±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atherothrombotic stroke</td>
<td>21</td>
<td>57.9±14.4</td>
</tr>
<tr>
<td>Cardioembolic stroke</td>
<td>11</td>
<td>48.3±11.8</td>
</tr>
<tr>
<td>Lacunar stroke</td>
<td>31</td>
<td>49.2±8.1</td>
</tr>
<tr>
<td>Transient ischemic attack</td>
<td>12</td>
<td>57.5±8.6</td>
</tr>
<tr>
<td>Patient control</td>
<td>12</td>
<td>46.3±10.3</td>
</tr>
<tr>
<td>Normal control</td>
<td>14</td>
<td>44.9±2.7</td>
</tr>
</tbody>
</table>

*P<.05, **P<.01, ***P<.001 (ANOVA).

Table 2. Correlation Between Shear-Induced Platelet Aggregation and Other Hemostatic Parameters in Patients With Cerebral Ischemia

<table>
<thead>
<tr>
<th>Hemostatic Parameters</th>
<th>n</th>
<th>Range</th>
<th>Mean±SD</th>
<th>Correlation Coefficient</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platelet count, x10⁴/mm³</td>
<td>66</td>
<td>11.4-54.6</td>
<td>31.9±10.4</td>
<td>.228</td>
<td>.097</td>
</tr>
<tr>
<td>Platelet aggregation, %</td>
<td></td>
<td>5.8-266</td>
<td>49±21</td>
<td>.203</td>
<td>.242</td>
</tr>
<tr>
<td>Induced by ADP</td>
<td>45</td>
<td>2-86</td>
<td>69±14</td>
<td>.239</td>
<td>.168</td>
</tr>
<tr>
<td>Induced by AA</td>
<td>45</td>
<td>3-85</td>
<td>42±26</td>
<td>.170</td>
<td>.330</td>
</tr>
<tr>
<td>Induced by PAF</td>
<td>45</td>
<td>127-726</td>
<td>335±159</td>
<td>.121</td>
<td>.533</td>
</tr>
<tr>
<td>Fibrinogen, mg/dL</td>
<td>29</td>
<td>14-360</td>
<td>65±79</td>
<td>.112</td>
<td>.578</td>
</tr>
<tr>
<td>β-Thromboglobulin, ng/mL</td>
<td>27</td>
<td>0.06-2.18</td>
<td>1.32±0.49</td>
<td>.079</td>
<td>.707</td>
</tr>
</tbody>
</table>

AA indicates arachidonic acid; PAF, platelet-activating factor.
ticlopidine (200 mg/d) for 7 days on shear-induced platelet aggregation (SIPA) in patients with cerebral ischemia. Aspirin (81 mg) has been previously shown that this dose was sufficient to suppress both platelet aggregation induced by AA and thromboxane (TX) A2 formation. Failure of inhibition by low-dose aspirin may reflect the lesser importance of TXA2 in the mechanism of SIPA, as was suggested in previous in vitro experiments which showed that neither a cyclooxygenase inhibitor (indomethacin) or a TXA2 synthetase inhibitor (CV-4151) could inhibit SIPA. Moake et al13 also reported that ingestion of a single 600-mg aspirin tablet significantly inhibited SIPA and concluded that aspirin can affect the platelet response to shear forces at doses higher than 300 mg, suggesting a mechanism probably different from that of interference with TXA2 formation.

We found that SIPA was unchanged by treatment with 81 mg of aspirin. We previously demonstrated that this dose was sufficient to suppress both platelet aggregation induced by AA and thromboxane (TX) A2 formation.12 Failure of inhibition by low-dose aspirin may reflect the lesser importance of TXA2 in the mechanism of SIPA, as was suggested in previous in vitro experiments which showed that neither a cyclooxygenase inhibitor (indomethacin) or a TXA2 synthetase inhibitor (CV-4151) could inhibit SIPA. Moake et al13 also reported that the treatment of platelets with aspirin in vivo and in vitro had no inhibitory effect on SIPA. On the other hand, it has been reported that high-dose aspirin inhibits SIPA.24,25 Ratnatunga et al24 reported that ingestion of a single 600-mg aspirin tablet significantly inhibited SIPA and concluded that aspirin can affect the platelet response to shear forces at doses higher than 300 mg, suggesting a mechanism probably different from that of interference with TXA2 formation.

SIPA was inhibited by treatment with 200 mg of ticlopidine. This is the usual dosage in Japan, and we have previously shown that this dose was sufficient to suppress platelet aggregation induced by ADP in the average Japanese patient.12 Ticlopidine is known to be a specific inhibitor of ADP-dependent platelet aggregation,12 and recent studies have suggested that ticlopidine acts by preventing the inhibition of adenylate

![Graph showing effects of oral aspirin (81 mg/d) and ticlopidine (200 mg/d) for 7 days on shear-induced platelet aggregation (SIPA) in patients with cerebral ischemia. Aspirin was administered to 3 patients with chronic cerebral infarction and 2 with transient ischemic attack, and ticlopidine was administered to 4 patients with chronic cerebral infarction and 2 with transient ischemic attack, who were supposedly in a steady state, among those entered into this study.](image-url)
cyclase by ADP through an effect on the ADP receptor–Gi protein complex. Previous studies have demonstrated that ADP released from the dense granules of platelets is capable of inducing the binding of vWF to the GP Ib/IIa complex on the platelet membrane and that SIPA mediated by larger vWF multimers released from platelets or endothelial cells requires the presence of ADP. Based on these studies, it appears that ticlopidine can inhibit vWF-dependent platelet aggregation induced by high shear stresses, which requires ADP as a cofactor, by inhibiting the ADP-dependent receptor mechanism for the binding of vWF to the platelet membrane.

In conclusion, the present study indicates that SIPA is increased in patients with atherothrombotic stroke and TIA, that this increase is correlated with an increase of larger vWF multimers and that it is corrected by ticlopidine but not by low-dose aspirin. SIPA appears to be a useful parameter for evaluating the abnormal rheological properties of platelets and the effects of pharmacological agents. It may be important to investigate correlations between the inhibition of SIPA and the improvement of clinical symptoms in various atherothrombotic diseases in the future.

Acknowledgments
This study was supported in part by a grant-in-aid for scientific research from the Japanese Ministry of Education, Science, and Culture (03670425) and by a research grant for scientific research from the Japanese Ministry of Health and Welfare (62-A-2).

References
Shear-induced platelet aggregation in cerebral ischemia.
S Uchiyama, M Yamazaki, S Maruyama, M Handa, Y Ikeda, M Fukuyama and I Itagaki

Stroke. 1994;25:1547-1551
doi: 10.1161/01.STR.25.8.1547

Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0039-2499. Online ISSN: 1524-4628

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