Effects of Clopidogrel and Aspirin in Combination Versus Aspirin Alone on Platelet Activation and Major Receptor Expression in Patients After Recent Ischemic Stroke

For the Plavix Use for Treatment of Stroke (PLUTO-Stroke) Trial

Victor L. Serebruany, MD, PhD; Alex I. Malinin, MD; Wendy Ziai, MD; Alex N. Pokov, MD; Deepak L. Bhatt, MD; Mark J. Alberts, MD; Dan F. Hanley, MD

Background and Purpose—Clopidogrel is widely used in patients after recent ischemic stroke; however, its ability to yield additional antiplatelet protection on top of aspirin has never been explored in a controlled study. To determine whether clopidogrel with aspirin (C/ASA) will produce more potent platelet inhibition than aspirin alone (ASA) in patients after ischemic stroke, we conducted the Plavix Use for Treatment of Stroke trial.

Methods—Seventy patients after ischemic stroke were randomly assigned to C/ASA or ASA groups. Platelet studies included aggregometry; cartridge-based analyzers; expression of PECAM-1, P-selectin, GP IIb/IIIa (antigen and activity), vitronectin receptor, and formation of platelet-leukocyte microparticles by flow cytometry. Platelet tests were performed at baseline and after 30 days after randomization.

Results—There were no deaths, hospitalizations, or serious adverse events. There were no differences in the baseline platelet characteristics between C/ASA and ASA groups, or significant changes in platelet parameters in the ASA group, except diminished collagen-induced aggregation (P=0.001). In contrast, therapy with C+ASA resulted in a significant inhibition of platelet activity assessed by ADP (P=0.00001) and collagen-induced (P=0.02) aggregation; closure time prolongation (P=0.03), and reduction of platelet activation units with Ulegra (P=0.00001); expression of PECAM-1 (P=0.01), and GP IIb/IIIa activity with PAC-1 (P=0.02) when compared with ASA group. Therapy with C+ASA also resulted in the reduced formation of platelet–leukocyte microparticles (P=0.02).

Conclusion—Treatment with C+ASA for 1 month provides significantly greater inhibition of platelet activity than ASA alone in patients after recent ischemic stroke in the frame of the small randomized trial. (Stroke. 2005;36:2289-2292.)

Key Words: aspirin ■ clinical trial ■ clopidogrel ■ platelets ■ stroke

Clopidogrel selectively and irreversibly binds to the P2Y12 ADP receptor on the platelet surface and reduces the combined incidence of stroke, myocardial ischemia, or vascular death.1 It is currently the drug of choice in the prophylaxis of stent thrombosis and is widely used in postischemic stroke management. However, the ability of clopidogrel to inhibit platelets on top of aspirin has never been explored in a controlled study. The primary hypothesis of the Plavix Use for Treatment of Stroke (PLUTO-Stroke) trial was to determine, in a randomized fashion, the antiplatelet properties of clopidogrel with aspirin when compared with those of aspirin alone in patients after ischemic stroke.
Inclusion Criteria
- Ischemic stroke within the last three months
- Age 21 or over
- Signed informed consent

PLUTO-stroke algorithm.

Assessment of platelet function at baseline and one month after randomization

PLUTO-stroke algorithm.

Assessed using a Chronolog Lumi-Aggregometer (model 560-Ca) with the AggroLink software package. Aggregation was expressed as the maximal percent change in light transmittance from baseline using platelet-poor plasma as a reference. Curves were analyzed according to international standards.2

Platelet Function Analyzers

PFA-100
Using the Dade Behring platelet function analyzer (PF-100) instrument, the blood–citrate mixture is aspirated under a constant negative pressure and contacts an ADP and collagen-coated membrane. The blood then passes through an aperture that induces high shear and simulates primary hemostasis after injury to a small blood vessel under flow conditions. The time to aperture occlusion (the closure time) is recorded in seconds and is inversely related to the degree of shear-induced platelet reactivity.3

Ultegra
The Ultegra analyzer (Accumetrics, Inc) is a turbidometric-based optical detection system, which measures platelet-induced aggregation as an increase in light transmittance. Fibrinogen-coated micro-particles are used in the Ultegra cartridge to bind to available platelet receptors. The Ultegra Analyzer is designed to measure this agglutination as an increase in light transmittance. Ultegra RPFA assay results are reported as platelet activation units (PAU).4

Whole Blood Flow Cytometry
The expression of platelet receptors was determined using the following monoclonal antibodies: CD31 (PECAM-1), CD 41 antigen (GP IIb/IIIa, β1), PAC-1 (epitope on the glycoprotein IIb/IIIa complex of activated platelets at or near the platelet fibrinogen receptor), CD51/61 (vitronectin), and CD 62p (P-selectin; PharMingen). Formation of platelet–leukocyte microparticles was assessed by dual labeling with pan-platelet marker (CD151) and then with CD14, the macrophage receptor for endotoxin lipopolysaccharides. The blood–citrate mixture (50μL) was diluted with 450μL Tris-buffered saline. The corresponding antibody was then added (5μL) and incubated at 37°C for 30 min. After incubation, 400μL of 2% buffered paraformaldehyde was added for fixation. The samples were analyzed on a Becton Dickinson FACScan flow cytometer setup to measure fluorescent light scatter as previously described. P-selectin was expressed as percent positive cells, whereas other antigens were expressed as log mean fluorescence intensity.

Statistical Analysis
Comparisons between baseline and 30 days were made using the t test with the Bonferroni correction. Between treatments, comparisons were made at respective time points using t tests. Data were expressed as mean±SD, and P<0.05 was considered significant. Differences between individual flow cytometric histograms were assessed using the Smirnov–Kolmogorov test incorporated in the CELLQuest software.

Results
Eighty-one patients within 1 to 3 months after ischemic stroke were screened, and 70 of them were randomized equally for treatment assignments using a randomization table. All patients completed the trial. No deaths or serious adverse events were reported for either group over the treatment period. Demographic and clinical characteristics are shown in Table 1.

The age was distributed fairly evenly between groups. We observed a slight prevalence of males and blacks in the aspirin group. The distribution of stroke history was similar between 2 treatment arms, whereas patients treated with the
clopidogrel–aspirin combination more often had a family history of stroke or transischemic attack (34% versus 22%). Diabetes mellitus, hyperlipidemia, history of alcohol use, and hypertension were common in both groups. Patients receiving the combined antiplatelet regimen indicated smoking as a risk factor less frequently, and diagnosis of coronary artery disease has been established more often among them as well (51% versus 34%). Stroke localization was similar between groups. The distribution of medications was fairly even between groups except for statins, which were used more often in the combination therapy group.

The platelet data dependent on treatment assignments are presented in Table 2. After 30 days of monotherapy with 81mg aspirin, there was moderate but significant reduction of platelet aggregation induced by collagen ($P=0.001$). No other statistically significant differences, but few trends toward additional platelet inhibition, were observed. Addition of 75mg clopidogrel to 81mg aspirin resulted in a significant potent inhibition of platelet function than monotherapy with aspirin have been proven by conventional aggregometry techniques.

$\text{TABLE 2. Platelet Biomarkers in PLUTO-Stroke}$

<table>
<thead>
<tr>
<th>Conventional aggregometry</th>
<th>C+ ASA (n=35)</th>
<th>ASA, 81 mg (n=35)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>30 Days</td>
</tr>
<tr>
<td>ADP 5μmol/L, (%)</td>
<td>65.9±12.1</td>
<td>26.7±14.8$^*$$\ddagger$</td>
</tr>
<tr>
<td>Collagen 5μmol/L, (%)</td>
<td>79.5±6.6</td>
<td>57.4±19.9$^*$$\ddagger$</td>
</tr>
<tr>
<td>AA 0.7μmol/L, (%)</td>
<td>22.5±28.7</td>
<td>20.9±27.7</td>
</tr>
<tr>
<td>Cartridge-based analyzers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Closure time (sec; PFA-100)</td>
<td>197±38</td>
<td>249±41$^*$$\ddagger$</td>
</tr>
<tr>
<td>PAU (Ultegra)</td>
<td>149.6±20.5</td>
<td>46.6±31.6$^*$$\ddagger$</td>
</tr>
<tr>
<td>Flow cytometry</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD31</td>
<td>63.2±8.9</td>
<td>53.7±13.0$^*$$\ddagger$</td>
</tr>
<tr>
<td>CD41</td>
<td>427±76</td>
<td>359±82$^*$</td>
</tr>
<tr>
<td>PAC-1</td>
<td>7.2±2.4</td>
<td>5.6±2.3$^*$$\ddagger$</td>
</tr>
<tr>
<td>CD51/61</td>
<td>9.1±1.9</td>
<td>8.8±2.7</td>
</tr>
<tr>
<td>CDE2p</td>
<td>9.0±2.9</td>
<td>8.5±2.7</td>
</tr>
<tr>
<td>CD151+14</td>
<td>65.2±13.5</td>
<td>53.7±16.0$^*$$\ddagger$</td>
</tr>
</tbody>
</table>

$^*P$ value <0.05 vs baseline.  $^\ddagger P$ value <0.05 between groups.  $^\ddagger$“Aspirin-resistant” patients determined by arachidonic acid-induced aggregation (6 in C+ASA group, and 8 in ASA group).

AA indicates arachidonic acid; PAU, platelet activation units for ADP-cartridge.

Discussion

The PLUTO-Stroke study revealed that addition of clopidogrel to aspirin significantly decreases various platelet activity characteristics after 30 days of treatment in patients after recent (1 to 3 months) ischemic stroke. Importantly, these data are consistent regardless of the method used for assessing platelet function biomarkers. In our study, the additional antiplatelet properties of clopidogrel beyond those afforded by aspirin have been proven by conventional aggregation induced by several agonists, with the platelet analyzers (PFA-100 and Ultegra) and using whole-blood flow cytometry techniques.

Our randomized data support a previous crossover study that the combination of clopidogrel and aspirin provides more potent inhibition of platelet function than monotherapy with each agent. The index data are also concordant with the observation that aspirin inhibits predominantly collagen-induced aggregation, especially in a low daily dose in a poststroke population. We observed less platelet inhibition when platelets were stimulated with ADP compared with collagen in the aspirin poststroke group, which is also supported by a previous report. The most profound platelet inhibition was observed when aggregation was induced by arachidonic acid in the aspirin monotherapy group even at baseline, whereas an additional month of therapy did not change this biomarker. However, we did not observe complete inhibition of arachidonic acid-induced aggregation in poststroke patients, as was reported earlier.

Contrarily, combination antiplatelet therapy has been associated not only with the expected inhibition of ADP-induced aggregation, but also with a significant decrease of aggregability when platelets were stimulated by collagen. Similar trends have been reported in the recent PLUTO-CHF trial, which was designed almost identically with the index study but in a heart failure cohort. It was expected that clopidogrel specifically targeting ADP platelet receptor would inhibit aggregation induced by this particular agonist and that clopidogrel would not affect arachidonic acid-induced aggregation already strongly inhibited by aspirin by...
modulating prostanoid metabolism. On the other hand, significant inhibition of collagen-induced aggregation after 1 month of combination therapy has been never reported in poststroke patients but is supported by the platelet data from the patients undergoing coronary interventions.11 Our data confirm that the aspirin–clopidogrel combination is associated with the additive efficacy to prolong the closure time, suggesting inhibition of platelets under high shear stress conditions using PFA-100 instrument.12 The Ultegra analyzer was even more suitable to detect platelet inhibition after the combination therapy, reducing platelet activation units dramatically (over 3-fold). We found that the aspirin–clopidogrel combination did not effect the expression of P-selectin, despite reported enhanced platelet expression of this activation-dependent alpha-granule constituent in patients after acute ischemic stroke.13

We conclude that treatment with clopidogrel and aspirin for 1 month provides significantly greater inhibition of platelet activity than aspirin alone in patients after recent ischemic stroke in the frame of the small randomized trial. The implication of PLUTO-Stroke for clinical practice is presently uncertain but may be important for future optimization and standardization to protect platelets from excessive activation. However, whether more potent antiplatelet potency of the combination strategy will result in better clinical outcomes remains to be determined in the survival clinical trials.

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References

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