Therapeutic Benefit of Low-Dose Clopidogrel in Patients Undergoing Carotid Surgery Is Linked to Variability in the Platelet Adenosine Diphosphate Response and Patients’ Weight

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Background and Purpose—We have previously shown that a single 75-mg tablet of clopidogrel, taken before carotid endarterectomy, significantly reduces postoperative embolization, a marker of thromboembolic stroke. This study explores the antiplatelet effect of this submaximal dose.

Methods—Fifty-six patients on long-term aspirin (150 mg) were randomized to 75 mg clopidogrel or placebo before carotid endarterectomy. Blood samples were taken pre- and postdrug administration and at the end of surgery to measure platelet activation and adenosine diphosphate (ADP) response by flow cytometry and aggregometry.

Results—Surgery produced a significant rise in platelet activation in vivo as evidenced by a rise in the percentage of monocyte–platelet aggregates in patients given placebo, but this was not seen in patients receiving clopidogrel. Before surgery, clopidogrel produced a significant reduction in the platelet response to ADP; for example, with 10⁻⁶M ADP, 77.32±2.3% bound fibrinogen in placebo group compared with 67.16±3.1% after clopidogrel (P=0.01). This was accentuated after surgery when the percentage of platelets binding fibrinogen in response to ADP was 76.53±2.2% in patients given placebo and 62.84±3.3% in the clopidogrel group (P=0.002). Similar differences were seen over a range of ADP concentrations and by aggregometry. Platelet responsiveness before treatment was highly variable and was positively correlated with the inhibitory effect of clopidogrel; patients with the highest baseline response to ADP showed the greatest response to clopidogrel. A negative correlation was seen between the effect of clopidogrel and patients’ weight (r=0.57; P=0.002).

Conclusions—These results explain how a single 75-mg dose of clopidogrel produces a significant clinical impact on embolization. (Stroke. 2007;38:2464-2469.)

Key Words: carotid endarterectomy ■ clopidogrel ■ platelets

Carotid artery disease can be effectively treated by carotid endarterectomy (CEA), but postoperative carotid thrombosis remains a significant problem in 2% to 3% of patients.1 Postoperative carotid thrombosis is preceded by increasing embolization, which can be detected by transcranial Doppler, and there is increasingly convincing evidence that the postoperative transcranial Doppler signals represent platelet microemboli.2 We have previously shown that the rate of platelet embolization is related to the inherent responsiveness of the patients’ platelets to adenosine diphosphate (ADP).3 Those patients who developed high numbers of emboli in the first 1 to 2 hours postoperatively, and were therefore, by definition, at higher risk from stroke,4-7 had platelets that showed a strong aggregatory response to ADP preoperatively despite the use of a standard 150-mg dose of aspirin in all the patients.3 This observation suggests a potential role for dual antiplatelet therapy to reduce the risk of stroke after carotid surgery, and specifically the inclusion of a platelet ADP receptor antagonist.

Clopidogrel is a second-generation thienopyridine derivative that binds irreversibly to the platelet P2Y₁₂ receptor inhibiting ADP-induced platelet activation.8 Dosing at 75 mg clopidogrel per day produces an incremental effect on reducing ADP-mediated platelet activation in human volunteers, reaching a maximum after 7 days. Alternatively, a 300- or 600-mg loading dose of clopidogrel has been shown to produce a rapid and pronounced inhibitory effect.9 This has been replicated in a clinical setting in patients undergoing coronary stenting,10-12 and several studies have now demonstrated the clinical efficacy of combined clopidogrel and aspirin therapy in the prevention of subacute thrombosis in patients undergoing intracoronary interventions.10-14 How-
ever, to date, the potential for clopidogrel therapy in carotid artery disease has received relatively little attention. Unlike patients undergoing coronary artery stenting, in which the percutaneous approach allows a maximal loading dose of clopidogrel (300 or 600 mg) with minimal risk of bleeding during the procedure,9,10 in patients undergoing open surgery, the risk of bleeding is greater. We consequently conducted a randomized, controlled trial of low-dose clopidogrel plus aspirin in patients undergoing CEA. Patients given a single 75-mg tablet of clopidogrel the night before surgery showed a significant 10-fold reduction in postoperative embolization compared with placebo with no increased risk of bleeding.15 This low dose of clopidogrel was selected from previous studies in healthy volunteers16 and in preliminary studies in patients as a dose with minimal risk of causing significant surgical bleeding.

The magnitude of the clinical effect of the low 75-mg dose of clopidogrel appeared disproportionate with the existing data on the antiplatelet effect of the drug. Therefore, to explore how this low dose could significantly reduce postoperative embolization after CEA, we investigated the platelet response to ADP in a subgroup of the patients using both flow cytometry and aggregometry to assess the degree of inhibition of the platelet response in the patients both pre- and postdrug treatment and at the end of surgery.

Materials and Methods

Study Design

Fifty-six consecutive patients undergoing CEA at the Leicester Royal Infirmary were recruited with informed consent. All patients enrolled were on long-term aspirin therapy, standardized to 150 mg aspirin for 4 weeks before surgery. The methodology has been described previously15 and was a double-blind randomized control trial with patients receiving either a single 75-mg dose of clopidogrel or placebo 12 hours before surgery in addition to their aspirin. Patients were excluded from the trial if they had refused informed consent, reported aspirin intolerance, had not complied with aspirin administration, or were already taking other antiplatelet or anti-thrombotic therapy. The study was approved by the Leicestershire Ethics Committee.

Blood Collection and Testing

Blood samples were collected for measurement of platelet reactivity at 3 time points: (1) before administration of the trial drug, 12 hours before surgery; (2) 12 hours after drug ingestion, immediately before surgery; and (3) at the end of the operation, after restoration of blood flow through the endarterectomized vessel. All blood samples were collected into Vacutainer tubes (Becton Dickinson) with precautions taken to minimize artificial activation of the platelets. The first 3 mL of blood was taken into 0.184 mol/L EDTA and used for a full blood count. Subsequent samples were collected into 0.105 mol/L sodium citrate for analysis of platelet function.

Surgical Procedure

A standardized CEA was performed on all patients with the operation carried out under a normocarbic, normotensive general anesthetic. After administration of unfractionated heparin (5000 IU), all patients underwent endarterectomy using routine shunting, tacking sutures to the distal intimal step, and closure of the arteriotomy with a Hemashield Finesse patch (Boston Scientific Ltd). Completion angiography was performed before restoration of flow, and patients were monitored during and for 3 hours after the procedure with transcranial Doppler as described previously.15

Whole Blood Flow Cytometry

The platelet response to ADP was analyzed in whole blood by flow cytometry.16,17 Blood was processed within 10 minutes of collection and all antibodies were used at optimum concentrations previously determined by titration. Five microliters of citrated whole blood was added to 50 μL of HEPES buffered saline containing fluorescein isothiocyanate conjugated-antifibrinogen antibody (Dako Ltd) together with ADP (final concentrations of 10–6, 10–7, 10–8 M11). These samples were incubated for 20 minutes at room temperature and then diluted 100 times in formyl salmine (0.2% formalin in 0.9% NaCl). Flow cytometric analysis was carried out within 2 hours in an XL-MCL flow cytometer (Beckman Coulter Ltd). Negative controls were set to 2% using samples incubated with the antifibrinogen antibody plus 1.6×10–3 M1 EDTA, which prevents the binding of fibrinogen to GPIIb-IIIa. The results were recorded as the percentage of platelets positive for bound fibrinogen antibody.

The percentage of monocytes in the circulation with bound platelets (monocyte–platelet aggregates [MPAs]) was measured as a sensitive marker of platelet activation occurring in vivo.16 For this, 5 μL of whole blood was added to 35 μL of HEPES buffered saline containing nonspecific mouse IgG (MOP31C, Sigma; to block nonspecific Ig binding). CD45-RPE-Cy5 antibody (Dako; to label leukocytes), and CD42b-RPE antibody (BD Pharmingen; to label platelets bound to monocytes). The samples were incubated for 20 minutes at room temperature, diluted 100 times in 0.5% formyl saline, and analyzed in the flow cytometer within 2 hours. Monocytes were identified by their forward and side scatter characteristics and the MPAs were identified as particles positive for both CD45 and CD42b. A R-phycocerythrin–conjugated isotype control was used to set the baseline for CD42b-positive events at 2%.

Platelet Aggregometry

Born aggregometry was carried out using citrated blood within 1 hour of being taken. Platelet-rich plasma was stimulated with ADP (0.5, 1, 2, and 4×10–7 M1) or arachidonic acid (2.5×10–6 M1). In all cases, platelet aggregation was measured over 10 minutes in PAP-4C aggregometer (BioData Corp) and the results recorded as the percentage of maximal aggregation compared with autologous platelet poor plasma.

Statistical Analysis

All data were normally distributed, as tested by D’Agostino and Pearson omnibus normality test, and analyzed by the Student t test (either paired for comparison of subjects at different time points or unpaired when comparing the 2 groups) and presented graphically as mean±SEM. When more than one concentration of platelet agonist was used, the 2-way analysis of variance test was used. Correlations were performed using linear regression. A value of P≤0.05 was considered statistically significant.

Figure 1. Detection of activated platelets in vivo as MPAs in patients undergoing CEA. The percentage of MPAs in blood samples from CEA patients treated with 75 mg clopidogrel (●) or placebo (○) (n=27 and n=28, respectively) taken at 3 time points: before drug treatment, after drug/placebo, and at the end of the operation. Data shown as mean±SEM.
Results

Patients
Over a 12-month period, 56 consecutive patients who met the inclusion criteria were randomized to 75 mg clopidogrel (n=27) or placebo (n=29). There were no significant differences in terms of age, male-to-female ratio, weight, atherosclerotic risk factors, presenting complaint, or degree of carotid stenosis between the groups. These patients represent an unselected subgroup of subjects entered into our randomized trial of clopidogrel reported previously, and their demographic and clinical characteristics were comparable.

Arachidonic Acid-Mediated Platelet Aggregation
Platelet aggregation in response to a high dose of arachidonic acid (2.5x10^{-3} M) was tested in all patients before clopidogrel. Aggregation was <20% of the maximum response in all patients, demonstrating that their platelets were effectively inhibited by aspirin, and none could be classified as being “aspirin-resistant” (data not shown).

Platelet Activation In Vivo Detected by Measuring Monocyte–Platelet Aggregates
Before drug treatment, the level of platelet activation in vivo, as evidenced by the presence of MPAs in the circulation, although lower in the placebo-treated group, was not statistically different from that in the clopidogrel-treated group. The levels of MPAs did not change significantly in either group after administration of the trial drug (Figure 1). However, after surgery, the percentage of MPAs in the placebo-treated group increased significantly from 17.9±1.8 to 23.7±2.2% (P=0.0005), whereas in the clopidogrel-treated group, there was a smaller, nonsignificant increase in the percentage of MPAs in the circulation, from 13.9±1.5% preoperatively/postclopidogrel to 16.6±1.7% postoperatively (P=0.06). The level of MPAs in the placebo-treated group was significantly higher postoperatively than in the clopidogrel-treated patients (P=0.013). This indicates that at this dose, clopidogrel, combined with aspirin, effectively prevented the activation of platelets in vivo produced by the surgical procedure.

Platelet Response to Adenosine
Diphosphate—Flow Cytometric Analysis
To explore the antiplatelet effect of the low dose of clopidogrel, the platelet response to ADP was measured by flow cytometry using the binding of fibrinogen to activated GPIIb-IIIa as an index of activation. Figure 2 shows the data for the response to the intermediate concentration of ADP (10^6 M L^{-1}), but the same pattern was seen at all three ADP concentrations (Table 1). Before taking the trial drug, the response to ADP was the same in both groups (P=0.552). After clopidogrel treatment, platelet fibrinogen binding in response to 10^6 M L^{-1} ADP fell significantly from 73.00±3.1% predrug to 67.16±3.1% postdrug (P=0.011). More importantly, at the end of surgery, ADP-mediated platelet fibrinogen binding did not increase in the clopidogrel-treated group (67.16±3.1% preoperatively/postclopidogrel versus 62.84±3.3% at the end of the operation; P=0.142) and the response at the end of the operation was still significantly lower than before drug treatment (P=0.002). By comparison, in the placebo group, the fibrinogen binding in response to ADP did not change significantly at any time point, but was significantly higher than the clopidogrel group both postdrug and at the end of the operation (P=0.011 and 0.002, respectively; Table 1). Similar patterns of response were seen using P-selectin as a marker of platelet activation (data not shown).

Table 1. The Percentage of Platelets Binding Fibrinogen in Response to ADP in Venous Blood Samples From Patients Treated With Clopidogrel (75 mg) or Placebo*

<table>
<thead>
<tr>
<th>Group</th>
<th>Time Point</th>
<th>ADP</th>
<th>P Value (analysis of variance)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1x10^{-7} M</td>
<td>1x10^{-8} M</td>
</tr>
<tr>
<td>Clopidogrel</td>
<td>Predrug</td>
<td>26.19±3.5</td>
<td>73.00±3.1</td>
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<tr>
<td>Placebo</td>
<td></td>
<td>28.62±3.2</td>
<td>74.65±3.0</td>
</tr>
<tr>
<td>Clopidogrel</td>
<td>Postdrug</td>
<td>21.22±2.6</td>
<td>67.16±3.1</td>
</tr>
<tr>
<td>Placebo</td>
<td></td>
<td>28.67±2.7</td>
<td>77.32±2.3</td>
</tr>
<tr>
<td>Clopidogrel</td>
<td>End of operation</td>
<td>19.42±2.7</td>
<td>62.84±3.3</td>
</tr>
<tr>
<td>Placebo</td>
<td></td>
<td>29.28±3.1</td>
<td>76.53±2.2</td>
</tr>
</tbody>
</table>

*Data presented as mean±SEM.
response to all 4 doses of ADP showed a similar trend to that seen by flow cytometry. Figure 3 shows the response to $2\times10^{-6}$ M$^{-1}$ ADP. As with the flow cytometric analysis, ADP-mediated aggregation in the clopidogrel-treated group was significantly lower after drug treatment (43.5 ± 2.6% predrug versus 35.9 ± 2.3% postclopidogrel; $P = 0.039$). After surgery, aggregation increased to 44.9 ± 3.2 ($P = 0.025$), but only to a comparable level to that prior to drug treatment. In contrast, administration of placebo had no significant effect on the platelet response, but aggregation was significantly higher in this group after surgery, rising from 42.9 ± 2.1 postplacebo/presurgery to 50.9 ± 3.4 postsurgery ($P = 0.025$). The same pattern was seen for all concentrations of ADP (Table 2).

**Variation in Response to Adenosine Diphosphate and to Clopidogrel**

In line with previous reports in the literature, there was considerable variation in the response to ADP in the patients, whether analyzed by flow cytometry or by aggregometry. For example, in all 56 patients, aggregation in response to $2\times10^{-6}$ M$^{-1}$ ADP before clopidogrel/placebo varied from 12.0% to 75.5% (with a mean of 41.9 ± 1.9%). We, and others, have shown a relationship between the pretreatment ADP response and the inhibitory effect of clopidogrel on the subsequent response to ADP.$^{16,19–21}$ Most previous studies have explored this relationship in patients on maximal doses of clopidogrel, but in the current study, even with the submaximal dose that was found to be therapeutically effective in the context of CEA, preclopidogrel and postclopidogrel ADP responses were positively correlated (Figure 4). This was so for all concentrations of ADP and was observed both by aggregometry and flow cytometry (with the exception of the highest ADP concentration) and whether the data were expressed as the difference between the pre- and postdrug time points (for example, for the aggregatory response to $4\times10^{-6}$ M, ADP, $r = 0.679$; $P = 0.0003$) or as the percentage reduction of predrug response ($r = 0.678$; $P = 0.0003$) as shown in Figure 4. This indicates that not only is the absolute reduction in response to ADP greater in high responders, but the reduction is also disproportionately larger. Consequently, the effect of this lower dose is seen predominantly in those patients with platelets that respond very readily to ADP, whereas for patients with a low ADP response, the effect of the drug is minimal.

**Effect of Body Weight on the Response to Clopidogrel**

There was a significant negative correlation between the patients’ weight and the efficacy of a single dose of 75 mg

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**TABLE 2. Platelet Aggregation Expressed as a Percentage of Maximum Aggregation in Response to ADP in Venous Blood Samples From Patients Treated With Clopidogrel (75 mg) or Placebo**

<table>
<thead>
<tr>
<th>Group</th>
<th>Time Point</th>
<th>ADP</th>
<th>$P$ Value (analysis of variance)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$5\times10^{-7}$ M</td>
<td>$1\times10^{-6}$ M</td>
</tr>
<tr>
<td>Clopidogrel</td>
<td>Predrug</td>
<td>12.97 ± 1.6</td>
<td>27.66 ± 2.3</td>
</tr>
<tr>
<td>Placebo</td>
<td></td>
<td>9.09 ± 1.1</td>
<td>20.77 ± 1.8</td>
</tr>
<tr>
<td>Clopidogregel</td>
<td>Postdrug</td>
<td>8.33 ± 1.0</td>
<td>19.63 ± 1.6</td>
</tr>
<tr>
<td>Placebo</td>
<td></td>
<td>11.45 ± 1.4</td>
<td>24.70 ± 1.9</td>
</tr>
<tr>
<td>Clopidogrel</td>
<td>End of operation</td>
<td>13.35 ± 1.7</td>
<td>27.44 ± 2.7</td>
</tr>
<tr>
<td>Placebo</td>
<td></td>
<td>18.06 ± 1.5</td>
<td>33.62 ± 2.5</td>
</tr>
</tbody>
</table>

*Data are presented as mean ± SEM.
clopidogrel. Figure 5 shows the correlation between the patients’ weight and the reduction in the platelet response. Correlation of patients’ body weight with the reduction in the response to ADP 5×10⁻⁷ M 12 hours after 75 mg clopidogrel.

Discussion

Experimental evidence points to a key role for ADP in thrombus formation and embolization, especially under conditions of high shear,¹⁹ conditions which may pertain in patients undergoing CEA. We have previously shown that high-grade embolization detected by transcranial Doppler after CEA is related to the magnitude of the patients’ platelet ADP response at baseline and occurs despite aspirin therapy. We subsequently showed that a submaximal dose of clopidogrel (75 mg), given with aspirin, before carotid surgery resulted in a significant reduction in postoperative embolization (thus reducing the risk of postoperative embolic stroke) without a real increase in bleeding.¹⁵ The question addressed here is how this low dose of clopidogrel can produce such an effective clinical outcome.

Most previous studies have investigated the efficacy of clopidogrel in patients with coronary artery disease and have focused on the need for a maximal dose achieved either through repeated treatment with 75 mg per day or a loading dose of 300 or 600 mg. In patients undergoing vascular surgery, such treatment runs the risk of hemorrhagic complications. Therefore, a single 75-mg dose was used based on previous studies of bleeding risk in healthy subjects and patients.¹⁵,¹⁶

Firstly, we were able to show that this dose of clopidogrel was sufficient to prevent platelet activation caused by the surgical procedure. Platelets activated in vivo by contact with the surgically damaged carotid artery rapidly bind to leukocytes (predominantly monocytes) in the circulation. The presence of MPAs in the blood is acknowledged to represent an accurate and physiologically relevant indicator of platelet activation occurring in vivo.¹⁸ Carotid surgery in the absence of clopidogrel resulted in a significant rise in MPAs, but this was effectively prevented by the single 75-mg dose of clopidogrel. This could be explained by the effect of clopidogrel in reducing the ADP response, which was higher by the end of surgery in the patients given the placebo compared with the clopidogrel-treated group whether measured by flow cytometry or aggregometry.

It has become increasingly recognized that there is considerable variation in the response of individuals to clopidogrel¹⁶,²⁰–²² resulting in a concept of “clopidogrel resistance,” which is best considered as a relative ineffectiveness of the drug in some subjects rather than a true drug resistance.²³ This variability has been previously reported in patients given maximal doses of clopidogrel,¹⁶,²⁰–²² but we have demonstrated this variability occurs even at low doses of the drug. Before drug treatment, there was wide variation in platelet response to ADP, which is consistent with previous observations in healthy individuals.²⁴,²⁵ There was a significant correlation between the pretreatment response to ADP and the inhibitory effect of clopidogrel. Inhibition of ADP-induced platelet aggregation by clopidogrel was greatest in patients whose platelets showed the highest response to ADP before drug treatment. Conversely, in those patients with a relatively poor baseline ADP response, there was little or no effect of clopidogrel; and in some of the lowest ADP responders, the response to ADP was actually higher in the postclopidogrel sample, most likely a reflection of the known diurnal variation in platelet reactivity.²⁶ These data help to explain how a single, submaximal dose of clopidogrel has a beneficial therapeutic effect in those patients at highest risk of developing postoperative emboli without compromising hemostasis in patients whose ADP response is at the lower end of the response range. Because we have previously shown that it is the patients with the highly ADP-responsive platelets that are at highest risk for developing postoperative emboli,³ it is these patients who require antplatelet therapy. The low dose of drug, therefore, benefits the high ADP responders without putting the low ADP responders at significant risk of bleeding.

Other factors have been proposed as potential causes of the variable response to clopidogrel, including the patient’s body weight.²⁷ This has been shown in animal models²⁸,²⁹ and in a group of patients with coronary artery disease, in which an elevated body mass index was found to be an independent predictor of a poor response to 300 mg clopidogrel.²⁷ We have also shown that the efficacy of single 75-mg dose of clopidogrel was also inversely correlated with the weight of the patient, which suggests that clopidogrel therapy may be improved by tailoring the dose to the patient’s weight.
Overall, these data help to explain how a low, submaximal dose of clopidogrel, combined with aspirin, can prevent postoperative embolization in patients undergoing CEA and provide a rationale for further evaluation of the role of clopidogrel added to aspirin in patients undergoing CEA. The current study is limited in that it was designed to explore the laboratory measure of the antiplatelet effect of low-dose clopidogrel and was therefore not powered to compare these with the clinical end points of embolization or bleeding. However, the data do suggest that larger trials, assessing clinical end points, and tailoring clopidogrel dose to body weight or to baseline platelet reactivity, may be warranted.

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Disclosures
Study drug was supplied by Bristol Myers Squibb.

References

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