Clopidogrel Inhibition of Stent, Graft, and Vascular Thrombogenesis With Antithrombotic Enhancement by Aspirin in Nonhuman Primates

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Background—A recent study showed that clopidogrel reduces thrombo-occlusive complications in patients with symptomatic atherosclerosis more effectively than aspirin.

Methods and Results—The effects of clopidogrel and aspirin have been compared, singly and in combination, for measurements of $^{111}$In-labeled platelets and $^{125}$I-labeled fibrin deposition in baboon models of arterial thrombosis and related to platelet aggregation and expression of activation epitopes induced by ADP, collagen, and thrombin receptor agonist peptide (TRAP) and to template bleeding times (BTs). Low-dose oral clopidogrel ($0.2$ mg $\cdot$ kg$^{-1}$ $\cdot$ d$^{-1}$) produced cumulative (1) intermediate decreases in $^{111}$In-platelet and $^{125}$I-fibrin deposition for segments of prosthetic vascular graft, deployed endovascular metallic stents, and endarterectomized aorta ($P<0.009$ in all cases); (2) elimination of ADP-induced platelet aggregation ($P<0.001$); (3) modest inhibition of collagen-induced platelet aggregation ($P<0.01$); (4) no reduction in TRAP-induced platelet aggregation; and (5) minimal prolongation of BTs ($P=0.03$). High-dose oral clopidogrel ($\geq 2$ mg/kg) produced the same effects within 3 hours. The effects of clopidogrel dissipated over 5 to 6 days. Aspirin $10$ mg $\cdot$ kg$^{-1}$ $\cdot$ d$^{-1}$ alone did not decrease $^{111}$In-platelet and $^{125}$I-fibrin deposition on segments of vascular graft but detectably decreased $^{111}$In-platelet and $^{125}$I-fibrin accumulation on stents ($P<0.01$), minimally inhibited ADP- and collagen-induced platelet aggregation ($P<0.05$ in both cases), and minimally prolonged BTs ($P=0.004$). Within 3 hours of aspirin administration, the antithrombotic effects of acute high-dose or chronic low-dose clopidogrel were substantially enhanced, and BTs were modestly prolonged without inhibiting platelet aggregation induced by TRAP ($P<0.001$ in all cases compared with clopidogrel alone).

Conclusions—Clopidogrel produces irreversible, dose-dependent, intermediate reduction in thrombosis that is substantially enhanced by the addition of aspirin. The effects of combining aspirin and clopidogrel need to be evaluated in patients at risk of vascular thrombosis. (Circulation. 1998;98:2461-2469.)

Key Words: clopidogrel ▪ thrombus ▪ stents ▪ aspirin

Heart attacks, strokes, and peripheral arterial occlusion are generally caused by thrombo-occlusive episodes in stenotic atherosclerotic arteries.1 Ruptured atherosclerotic plaques initiate complex interactions among damaged atheromatous vascular structures and highly reactive platelets and coagulation proteins, resulting in the formation of tissue factor–dependent vascular thrombosis. Tissue factor–initiated thrombotic occlusion also complicates interventional procedures used in the management of patients with symptomatic atherosclerotic vascular disease, including thrombolytic reperfusion for acute coronary thrombosis, angioplasty, various types of atherectomy, deployment of endovascular stents, endarterectomy, and implantation of small-caliber vascular grafts.2,3

Large-scale, randomized, placebo-controlled trials have established that daily oral aspirin therapy decreases the relative risk of vaso-occlusive episodes in patients with symptomatic atherosclerotic disease by $20\%$ to $25\%$.1,4 The benefits of aspirin are attributable to its irreversible interruption of thromboxane A$_2$ (TXA$_2$) generation by platelets.5 Controlled clinical trials have also established that oral ticlopidine therapy decreases the relative risk of vaso-occlusive events in symptomatic atherosclerotic patients by $30\%$ to $35\%$.1,4,6-9 Ticlopidine, a thienopyridine requiring hepatic modification in vivo to exhibit antiplatelet effects, selectively inhibits ADP-dependent platelet aggregation that is cumulative over 8 to 10 days.10-12 Recent clinical studies demonstrate that adding aspirin to ticlopidine therapy markedly reduces thrombo-occlusive events associated with the deployment of coronary stents.13-16 Unfortunately, ticlopidine therapy has a number of troublesome adverse effects, including reversible neutropenia, diarrhea, and idiosyncratic cutaneous rashes.
Clopidogrel inhibits thrombogenesis

Clopidogrel is a ticlopidine-like thienopyridine that is severalfold more potent and is free of the adverse effects plaguing ticlopidine therapy. Like ticlopidine, clopidogrel is devoid of direct antiplatelet effects and must undergo hepatic metabolic modification to exhibit selective inhibition of ADP-induced platelet aggregation. Clopidogrel acts by irreversibly inactivating platelet ADP receptor–initiated signaling in a dose-dependent manner. In a recently reported large-scale, randomized, controlled clinical trial, clopidogrel was shown to be significantly more effective than and at least as safe as aspirin in decreasing arterial thrombo-occlusive episodes in patients with symptomatic atherosclerotic disease. Clinical trials reporting that ticlopidine plus aspirin markedly reduces thrombotic occlusion of coronary stents suggest that adding aspirin to clopidogrel therapy may enhance its antithrombotic efficacy.

Clopidogrel and aspirin each inhibit platelet recruitment by interrupting ADP- and TxA2-mediated platelet activation, respectively. Presumably, the relative contributions of ADP- and TxA2-dependent platelet recruitment varies with different clinical thrombotic processes. Accordingly, the present study was designed to measure the relative antithrombotic effects of administering clopidogrel and aspirin, singly and in combination, for 3 different thrombosis models in baboons, ie, graft, stent, and vascular thrombosis models. The relative antihemostatic and antithrombotic effects of oral clopidogrel, clopidogrel with aspirin, clopidogrel with heparin, or the combination of clopidogrel, aspirin, and heparin were determined.

Methods

Baboon Model of Thrombosis and Hemostasis

Twenty-four normal juvenile male baboons weighing 9 to 11.5 kg and bearing chronic exteriorized arteriovenous (AV) femoral shunts were used in these studies. Before experimentation, all animals were observed to be disease-free for at least 3 months. All procedures were approved by the Institutional Animal Care and Use Committee (Emory University) in compliance with National Institutes of Health guidelines (Guide for the Care and Use of Laboratory Animals, 1985), Public Health Service policy, the Animal Welfare Act, and related university policies. In these models, quantitative, reproducible nonocclusive thrombi were formed on deep and endovascular metallic stents, segments of vascular graft, and segments of endarterectomized baboon aorta interposed in the AV shunts under physiological flow conditions for 60 minutes without systemic anticoagulation. The forming thrombus incorporated circulating prelabeled autologous 111In-labeled platelets and homologous 125I-labeled fibrinogen. These chronic AV shunts, per se, do not detectably activate platelets or coagulation.

Thrombogenic Devices

Nonocclusive thrombi were formed over 60 minutes in the exteriorized AV shunts of awake animals by deployment of metallic endovascular stents, interposing 2-cm-long, 4-mm-ID thrombogenic segments of uncrimped Dacron vascular grafts or incorporating segments of endarterectomized homologous aorta in established AV shunts while blood flow was controlled at 100 mL/min.

Stent thrombosis was produced by deployment of stainless steel endovascular stents (3.5 mm) in the exteriorized chronic AV shunt. The stainless steel stents, a gift from Johnson & Johnson Interventional Systems, Warren, NJ, were deployed over a balloon that was then advanced through the stent and inflated to 2 atm to allow stent expansion. The balloon was then gently withdrawn while the stent was held in place. After deployment, the balloon was removed from the stent and deployed into the AV shunt. The stent was then immediately withdrawn from the AV shunt, and the balloon was inserted into a 3.3-mm-ID, 20-cm-long segment of silicone rubber tubing (Technical Products Inc). The balloon was inflated 3 times to a pressure of 10 atm to achieve maximal apposition of the stent struts with the tubing wall. The shunt tubing was then filled with sterile saline to remove potentially confounding air bubbles from the surface of the stent and to facilitate interpositioning of stent-containing segments in the exteriorized chronic AV femoral shunt.

Graft thrombosis was produced by segments of Dacron vascular grafts (Bioknit, C.R. Bard, Inc) rendered impervious to blood leakage by external wrapping in Parafilm (American Can Co) and 5.3-mm-ID “heat-shrinkable” Teflon tubing. Connections were constructed to ensure that the devices were isodiametric and suitable for incorporation into the AV shunts.

Endarterectomy thrombosis was produced by endarterectomizing fresh baboon aorta (5- to 6-mm ID) obtained from other donor animals, flushed with saline, and divided into 4-cm lengths. Branches were ligated, and specimens were stored in normal saline. For endarterectomies, the aortic segments were inverted and the intima and inner media were removed for a distance of 1 cm in the central portion of the vessel by sharp dissection. After completion of the endarterectomy, each segment was returned to its normal configuration and cannulated with 1-cm lengths of heat-shrinkable Teflon tubing (Small Parts, Inc). The aortic segments were encased with heat-shrinkable Teflon tubing and each end was carefully sealed by heating, but direct heat to tissues was avoided. The resultant configuration maintained stable geometry, with a smooth transition from vessel to tubing.

Clopidogrel Dosing

Clopidogrel was administered orally at 7 doses spanning more than 2 orders of magnitude, ie, 0.1, 0.2, 0.5, 2, 5, 10, and 20 mg·kg−1·d−1. Antithrombotic and antihemostatic effects of high-dose clopidogrel for stent and graft thrombosis were obtained 3 hours after dosing with 2 to 20 mg/kg. Because the effects of low-dose clopidogrel were cumulative, doses of 0.1, 0.2, and 0.5 mg/kg clopidogrel were administered daily for 6 days to determine full dose-response effects (see below). Thrombus formation was measured in the following sequence on different days during the subsequent 2 weeks, assessing the effects of (1) clopidogrel 0.2 mg·kg−1·d−1 alone, (2) clopidogrel 0.2 mg·kg−1·d−1 with heparin 100 IU/kg bolus and 100 IU/kg infused over 1 hour, (3) clopidogrel 0.2 mg·kg−1·d−1 plus aspirin 10 mg/kg administered 2 hours previously, and (4) clopidogrel 0.2 mg·kg−1·d−1 in combination with aspirin 10 mg/kg and heparin 100 IU/kg infused over 1 hour. Treatments were discontinued for 1 week to permit the antiplatelet effects of clopidogrel and the enhancing effects of aspirin to dissipate before other studies were begun.

Measurements of Thrombus Formation

Autologous platelets were labeled with 1 mCi 111In[111In]indium oxine (111In) as previously described and reinfused at least 1 hour before the thrombogenic devices were interposed. 111In-labeled baboon fibrinogen (5 μCi), purified and labeled as described previously, was injected intravenously 10 minutes before thrombogenic devices were introduced. Images of the segments containing vascular graft, deployed stents, or endarterectomized aorta were acquired separately with a General Electric 400T MaxiCamera, stored, and analyzed with a Medical Data Systems A1 image processing system (Medtronic) interfaced with the camera, by routines already described. The total numbers of deposited platelets in regions of interest were calculated by dividing the deposited platelet activity (counts/min) by the circulating blood activity (counts·min−1·mL−1) and multiplying by the circulating platelet count (platelets/mL). Fibrin was determined after completion of the experiments by removal of the thrombogenic segments for counting 111In-fibrin radioactivity 30 days later when the 111In activity had decayed. Total fibrin accumulation was calculated by dividing the deposited 111In activity (counts/min) by the clottable fibrinogen activity (counts·min−1·mL−1) and multiplying by the plasma fibrinogen level (mg/mL).
Aggregation (AC50). The appearance of activated platelets in the
expression as the agonist concentration that induced half-maximal
spanning the range of responsiveness. The results were plotted and
optical densities of PPP and PRP. ADP (Sigma Chemical Co),
E. Plow, Cleveland, Ohio), and the secretory granule membrane,
P-selectin, a gift from Biogen Inc, Cambridge, Mass.
Plateau levels of thrombotic accumulation are achieved by
shunts flowing at 100 mL/min in nonanticoagulated baboons.
metallic stents (bottom) interposed in exteriorized chronic AV
ments of Dacron vascular graft (top) or deployed endovascular
Vascular Thrombosis
Platelet deposition in formation of graft and stent
Figure 1. Platelet deposition in formation of graft and stent
thrombus. Autologous 111In-platelets accumulate rapidly on seg-
ments of Dacron vascular graft (top) or deployed endovascular
metallic stents (bottom) interposed in exteriorized chronic AV
shunts flowing at 100 mL/min in nonanticoagulated baboons.
Plateau levels of thrombotic accumulation are achieved by ~60
minutes. For graft thrombosis, platelet deposition is not signifi-
cantly reduced by heparin (100 IU/kg bolus and 100 IU/kg
infused during 60 minutes) or aspirin (10 mg/kg PO 2 hours
before study). Although heparin fails to decrease stent thrombo-
sis, aspirin therapy produces intermediate reduction in platelet
deposition.
Laboratory Studies
Platelet counts, erythrocyte counts, and total leukocyte counts were
performed on whole blood collected in 2 mg/mL disodium EDTA
with a Serono Baker model 9000 whole-blood analyzer. Blood
samples for testing platelet hemostatic function were collected in
citrate (3.2% for platelet aggregation studies and 3.8% for flow
cytometric determination of P-selectin and ligand-induced binding
site [LIBS] expression).
Platelet aggregation was determined within 1 hour of drawing
blood with a Chrono-Log aggregometer by recording the increase in
light transmission through a stirred suspension of platelet-rich
plasma (PRP) maintained at 37°C. PRP and platelet-poor plasma
(PPP) were prepared by differential centrifugation, as previously
described. The platelet count in the PRP was adjusted to 300 ×
10^3/µL. Percent aggregation was calculated linearly between the
optical densities of PPP and PRP. ADP (Sigma Chemical Co),
collagen (Nycomed Arzenmittel), and thrombin receptor agonist
peptide (TRAP1–6) (Peninsula Laboratories) were added at doses
spanning the range of responsiveness. The results were plotted and
expressed as the agonist concentration that induced half-maximal
aggregation (AC50). The appearance of activated platelets in the
peripheral blood was evaluated by flow cytometry using fluoresce-
inated monoclonal antibodies against neoantigens expressed on
membrane surfaces of activated platelets, including conformationally
altered integrin αIIbβ3 (or glycoprotein IIb/IIIa), LIBS (a gift from Dr
E. Plow, Cleveland, Ohio), and the secretory granule membrane,
P-selectin, a gift from Biogen Inc, Cambridge, Mass.
Template bleeding time (BT) measurements were performed at
baseline and at 60 minutes. BT testing was carried out on the shaved
volar surface of the forearm as previously described in nonhuman
primates.
Statistical Analysis of Data
Data were presented as mean±SD. Student’s t test for paired or
unpaired data was used when data were normally distributed.
Otherwise, Mann-Whitney nonparametric analysis was used. Facto-
trial ANOVA and ANCOVA were used. A value of P≤0.05 was
considered to be the estimate of statistical significance.
Results
Baboon Models of Graft, Stent, and
Vascular Thrombosis
Segments of Dacron vascular graft interposed in exteriorized
AV shunts flowing at 100 mL/min induced rapid platelet
deposition that reached plateau levels by 60 minutes (Figure 1,
top). Neither aspirin 10 mg/kg PO 2 hours previously nor
heparin 100 IU/kg IV bolus and 100 IU/kg infused over 60
minutes significantly reduced thrombus formation (Figure 1,
bottom). Deployed metallic endovascular stents in AV shunts
flowing at 100 mL/min also produced thrombus that reached
plateau values by ~60 minutes (Figure 1, bottom). Whereas
heparin had no effect on stent thrombosis (P>0.2), oral
aspirin 10 mg/kg modestly decreased platelet accumulation on
stents, ie, 111In-platelet deposition decreased from 2.56±0.96×10^8 to
1.76±0.57×10^8 platelets (P<0.01; Figure 1, bottom).
Control endarterectomized aortic segments produced sub-
stantial thrombus by 60 minutes, ie, platelet deposition
averaged 1.6±0.39×10^9 platelets/cm, and mean fibrin accu-
mulation was 1.1±0.3 mg/cm.
Baseline platelet concentrations averaged 285±60×10^3/µL,
and control mean plasma fibrinogen concentration was
2.95±0.27 mg/mL. Baseline template BT averaged 3.2±0.8
minutes. During baseline thrombus formation on thrombo-
genic devices, platelets remaining in the circulation did not
change aggregatory responsiveness or express activation

<table>
<thead>
<tr>
<th>Oral Dose, mg/kg</th>
<th>ADP, µmol/L</th>
<th>Collagen, µg/mL</th>
<th>TRAP, µmol/L</th>
<th>Platelet AC50</th>
<th>Bleeding Time, min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>5.1±2.8</td>
<td>2.2±1.0</td>
<td>38±25</td>
<td>3.1±0.7</td>
<td></td>
</tr>
<tr>
<td>0.1 after 6 d</td>
<td>&gt;100</td>
<td>8.4±4.9</td>
<td>31±24</td>
<td>6.9±5.3</td>
<td></td>
</tr>
<tr>
<td>0.2 after 6 d</td>
<td>&gt;100</td>
<td>7.2±4.0</td>
<td>45±23</td>
<td>7.7±7.4</td>
<td></td>
</tr>
<tr>
<td>0.5 after 6 d</td>
<td>&gt;100</td>
<td>9.5±8.8</td>
<td>43±27</td>
<td>16±11</td>
<td></td>
</tr>
<tr>
<td>2.0 after 3 h</td>
<td>20±13</td>
<td>7.5±4.9</td>
<td>59±36</td>
<td>24±9.5</td>
<td></td>
</tr>
<tr>
<td>20 after 3 h</td>
<td>&gt;100</td>
<td>12±6.0</td>
<td>74±54</td>
<td>&gt;30</td>
<td></td>
</tr>
</tbody>
</table>

(Oral Dose, mg/kg | ADP, µmol/L | Collagen, µg/mL | TRAP, µmol/L | Platelet AC50 | Bleeding Time, min)
epitopes, ie, ex vivo platelet aggregation induced by ADP, collagen, or TRAP was unaltered, and there was no significant flow cytometric expression of P-selectin or LIBS.

**Antithrombotic and Antihemostatic Effects of Clopidogrel**

In vitro, clopidogrel exhibited no direct inhibitory effects on baboon platelet aggregation or LIBS expression induced by ADP, collagen, or TRAP (P > 0.5).

Oral dosing of clopidogrel at 0.1 mg/kg for 6 days abolished ADP-induced platelet aggregation (undetectable aggregation despite the addition of >100 μmol/L ADP; Table 1). This dosing regimen also modestly inhibited platelet aggregation induced by collagen, as shown by the increased concentration of collagen required to produce half-maximal aggregation, ie, from 2.2 ± 1.0 to 8.4 ± 4.9 μg/mL (P < 0.008), and prolonged the BT to 6.9 ± 5.3 minutes (Table 1; P < 0.01). However, neither TRAP-induced platelet aggregation nor thrombus formation on segments of vascular graft or deployed endovascular stents was significantly decreased (P > 0.2 in all cases).

Increasing the dose of clopidogrel to 0.2 mg/kg for 6 days significantly reduced platelet and fibrin accumulation on vascular grafts and stents (Table 2; P < 0.01 in all cases), prolonged the BT to 7.7 ± 7.4 minutes (Tables 1, 2, and 3; P < 0.01 compared with baseline values) without additional inhibition of platelet aggregation and expression of activation epitopes induced by ADP or collagen, and produced no reduction in TRAP-induced platelet activation (Tables 1 and 3).

Within 3 hours, increasing clopidogrel dosing 10-fold to 2 mg/kg significantly decreased platelet deposition (Figure 2; Table 2; P < 0.01 in both cases) but not fibrin accumulation (Table 2; P > 0.1 in both cases) for both vascular grafts and stents. ADP-induced aggregation was markedly inhibited (AC50 20 ± 13 μmol/L; P < 0.0001 compared with baseline), collagen-induced aggregation was inhibited beyond that produced after 6 days of 0.1 mg · kg−1 · d−1 clopidogrel (Table 1), and BT was prolonged to 24 ± 9.5 minutes (P < 0.0001 compared with baseline). TRAP-induced platelet aggregation was not reduced (Table 1).

When the dose of clopidogrel was increased another 10-fold to 20 mg/kg, the accumulation of both platelets and fibrin on segments of vascular graft and stents was significantly decreased within 3 hours of oral administration (Table 2; Figure 2; P < 0.02 in all cases). ADP-induced aggregation was abolished, and BT was increased to >30 minutes. TRAP-induced platelet aggregation was minimally inhibited (Table 1; P < 0.06 compared with baseline), and no additional inhibition of collagen-induced aggregation was produced (Table 1; P > 0.4). The reduction in thrombosis and prolongation of the BT resulting from high-dose clopidogrel gradually dissipated over 6 days, after therapy was discontinued. Thus, high-dose clopidogrel selectively and irreversibly abolished ADP-dependent platelet activation within 3 hours (Tables 1 and 3).

**TABLE 2. Antithrombotic Effects of Clopidogrel, Heparin, and Aspirin**

<table>
<thead>
<tr>
<th></th>
<th>Graft Thrombosis</th>
<th>Stent Thrombosis</th>
<th>Bleeding</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Platelets x 10^9</td>
<td>Fibrin, mg</td>
<td>Platelets x 10^9</td>
</tr>
<tr>
<td>Baseline</td>
<td>4.36 ± 1.21 (27)</td>
<td>3.06 ± 1.03 (13)</td>
<td>2.56 ± 0.96 (7)</td>
</tr>
<tr>
<td>Three hours after high-dose clopidogrel</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clopidogrel 2.0 mg/kg</td>
<td>2.68 ± 0.64</td>
<td>2.32 ± 0.36</td>
<td>0.66 ± 0.26</td>
</tr>
<tr>
<td>P = 0.03</td>
<td>P = 0.25</td>
<td>P = 0.01</td>
<td>P = 0.07</td>
</tr>
<tr>
<td>Clopidogrel 2.0 mg/kg + aspirin 10 mg/kg</td>
<td>2.66 ± 0.31</td>
<td>2.39 ± 0.66</td>
<td>0.39 ± 0.16</td>
</tr>
<tr>
<td>P = 0.024</td>
<td>P = 0.31</td>
<td>P = 0.006</td>
<td>P = 0.04</td>
</tr>
<tr>
<td>Clopidogrel 20 mg/kg</td>
<td>2.28 ± 0.05</td>
<td>1.83 ± 0.50</td>
<td>0.55 ± 0.23</td>
</tr>
<tr>
<td>P = 0.007</td>
<td>P = 0.07</td>
<td>P = 0.008</td>
<td>P = 0.04</td>
</tr>
<tr>
<td>Clopidogrel 20 mg/kg + aspirin 10 mg/kg</td>
<td>2.05 ± 0.41</td>
<td>1.48 ± 0.48</td>
<td>0.19 ± 0.01</td>
</tr>
<tr>
<td>P = 0.003</td>
<td>P = 0.02</td>
<td>P = 0.003</td>
<td>P = 0.02</td>
</tr>
<tr>
<td>Six days after low-dose clopidogrel</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clopidogrel 0.2 mg/kg</td>
<td>3.16 ± 0.84</td>
<td>1.7 ± 0.42 (6)</td>
<td>1.2 ± 0.77 (8)</td>
</tr>
<tr>
<td>P = 0.03</td>
<td>P = 0.07</td>
<td>P = 0.0094</td>
<td>P = 0.04</td>
</tr>
<tr>
<td>Clopidogrel 0.2 mg · kg−1 · d−1 + aspirin 10 mg/kg</td>
<td>2.07 ± 0.41</td>
<td>1.85 ± 0.28 (6)</td>
<td>0.57 ± 0.6 (7)</td>
</tr>
<tr>
<td>P = 0.0001</td>
<td>P = 0.013</td>
<td>P = 0.0006</td>
<td>P = 0.002</td>
</tr>
<tr>
<td>P = 0.02</td>
<td>P = 0.48</td>
<td>P = 0.1</td>
<td>P = 0.0001</td>
</tr>
<tr>
<td>Clopidogrel 0.2 mg · kg−1 · d−1 + heparin 100 IU/kg</td>
<td>2.45 ± 0.38 (6)</td>
<td>1.22 ± 0.6 (5)</td>
<td>0.64 ± 0.56 (7)</td>
</tr>
<tr>
<td>P = 0.0007</td>
<td>P = 0.002</td>
<td>P = 0.0006</td>
<td>P = 0.004</td>
</tr>
<tr>
<td>P = 0.1</td>
<td>P = 0.15</td>
<td>P = 0.14</td>
<td>P = 0.0002</td>
</tr>
<tr>
<td>Clopidogrel + aspirin + heparin</td>
<td>2.01 ± 1.03 (5)</td>
<td>1.39 ± 0.33 (5)</td>
<td>0.44 ± 0.44 (6)</td>
</tr>
<tr>
<td>P = 0.0003</td>
<td>P = 0.003</td>
<td>P = 0.0004</td>
<td>P = 0.003</td>
</tr>
<tr>
<td>P = 0.09</td>
<td>P = 0.21</td>
<td>P = 0.05</td>
<td>P = 0.001</td>
</tr>
</tbody>
</table>

Numbers in parentheses are numbers of observations. P1 indicates control vs treatment; P2, clopidogrel vs combinations.
The dose-response effects of clopidogrel for platelet accumulation in graft and stent thrombosis are displayed in Figure 3 and documented quantitatively in Table 2. Although clopidogrel has a steep antithrombotic dose-response at 0.2 mg/kg, the dose-response relationship is relatively flat for 10- to 100-fold increased dosing, indicating that the antithrombotic effects of clopidogrel remained intermediate, despite large doses of drug. By contrast, the BT was progressively prolonged to 30 minutes by high-dose clopidogrel.

The overall extent to which clopidogrel reduced thrombus formation was substantially greater for stent thrombosis than for graft thrombosis (Figure 3 and Table 2), implying that ADP-mediated platelet recruitment was quantitatively more important for thrombus forming on stents than for thrombus forming on segments of vascular graft.

The effects of clopidogrel on the formation of thrombus at sites of arterial endarterectomy are shown in Figure 4. Dosing clopidogrel at 0.2 mg · kg⁻¹ · d⁻¹ for 6 days substantially decreased platelet and fibrin accumulation on segments of endarterectomized aorta (P<0.001). Increasing the dose of clopidogrel to 5 mg/kg for 3 days abolished thrombus forming on endarterectomized aorta (Figure 4; P<0.0001).

### Antithrombotic and Antihemostatic Effects of Combining Aspirin and Clopidogrel

Combining aspirin 10 mg/kg and clopidogrel significantly enhanced the reduction in platelet and fibrin deposition produced within 3 hours by high-dose clopidogrel (20 mg/kg), as shown in Figure 5 and Table 2 for stent thrombosis (P<0.01 in both cases) and in Figure 6 and Table 2 for graft thrombosis (P=0.001 in both cases). Although platelet deposition was significantly reduced by 2 mg/kg clopidogrel after 3 hours, fibrin accumulation was not significantly decreased (Table 2; P>0.07), although the BT remained maximally prolonged at >30 minutes.

Single-dose aspirin therapy (10 mg/kg) also enhanced the reduction in platelet and fibrin deposition after 6 days of low-dose clopidogrel (0.2 mg · kg⁻¹ · d⁻¹) for stent and graft

### TABLE 3. Cumulative Effects of Low-Dose Clopidogrel on Platelet Function and Thrombus Formation

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Day 3 (Clopidogrel 0.2 mg · kg⁻¹ · d⁻¹)</th>
<th>Day 6 (Clopidogrel 0.2 mg · kg⁻¹ · d⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Stent thrombosis</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Platelet deposition·10⁹</td>
<td>2.56±0.96 (7)</td>
<td>1.82±1.30 (3)</td>
<td>1.20±0.77 (8)</td>
</tr>
<tr>
<td>p</td>
<td>0.3</td>
<td>0.009</td>
<td></td>
</tr>
<tr>
<td>Fibrin accumulation, mg</td>
<td>0.56±0.27 (6)</td>
<td>0.42±0.08 (3)</td>
<td>0.34±0.21 (8)</td>
</tr>
<tr>
<td>p</td>
<td>0.42</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td><strong>Graft thrombosis</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Platelet deposition·10⁹</td>
<td>4.36±1.21 (27)</td>
<td>3.52±1.42 (3)</td>
<td>3.16±0.84 (6)</td>
</tr>
<tr>
<td>p</td>
<td>0.3</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td>Fibrin accumulation (mg)</td>
<td>3.06±1.03 (13)</td>
<td>2.34±0.23 (3)</td>
<td>1.7±0.42 (6)</td>
</tr>
<tr>
<td>p</td>
<td>0.3</td>
<td>0.0007</td>
<td></td>
</tr>
<tr>
<td>BT, min</td>
<td>3.1±0.7 (14)</td>
<td>4±0.5 (3)</td>
<td>7.7±7.4 (14)</td>
</tr>
<tr>
<td>p</td>
<td>0.05</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td><strong>Platelet aggregation (AC₅₀)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADP, µmol/L</td>
<td>5.1±2.8 (18)</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>p</td>
<td>0.0001</td>
<td>0.0001</td>
<td></td>
</tr>
<tr>
<td>Collagen AC₅₀, µg/mL</td>
<td>2.2±1.0 (16)</td>
<td>7.2±3.4 (10)</td>
<td>7.2±4.0 (10)</td>
</tr>
<tr>
<td>p</td>
<td>0.0001</td>
<td>0.0001</td>
<td></td>
</tr>
<tr>
<td>TRAP₁–₆ AC₅₀, µmol/L</td>
<td>38±25 (18)</td>
<td>35.6±20.8 (10)</td>
<td>45±23 (10)</td>
</tr>
<tr>
<td>p</td>
<td>0.8</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td><strong>Expression of activation epitopes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LIBS</td>
<td>1440±476 (16)</td>
<td>1890±540 (7)</td>
<td>1200±192 (7)</td>
</tr>
<tr>
<td>p</td>
<td>0.0001</td>
<td>0.0001</td>
<td></td>
</tr>
<tr>
<td>LIBS after ADP</td>
<td>20500±8900 (17)</td>
<td>6250±3400 (7)</td>
<td>7100±4800 (7)</td>
</tr>
<tr>
<td>p</td>
<td>0.0035</td>
<td>0.0012</td>
<td></td>
</tr>
<tr>
<td>LIBS after TRAP₁–₆</td>
<td>7570±7210 (9)</td>
<td>2100±820 (7)</td>
<td>2710±1270 (4)</td>
</tr>
<tr>
<td>p</td>
<td>0.099</td>
<td>0.19</td>
<td></td>
</tr>
<tr>
<td>P-Selectin</td>
<td>352±339 (17)</td>
<td>162±28 (7)</td>
<td>164±54</td>
</tr>
<tr>
<td>p</td>
<td>0.092</td>
<td>0.033</td>
<td></td>
</tr>
<tr>
<td>P-Selectin after ADP/epitopes</td>
<td>2650±720 (17)</td>
<td>1640±384 (7)</td>
<td>1110±270 (7)</td>
</tr>
<tr>
<td>p</td>
<td>0.0022</td>
<td>0.0001</td>
<td></td>
</tr>
</tbody>
</table>

Numbers in parentheses are numbers of observations.
thrombosis (Figures 7 and 8; Table 2; \(P < 0.01\) for all cases), with a BT of 16±11 minutes (Table 2). Thus, by reduction of the chronic clopidogrel dosing to 0.2 mg kg\(^{-1}\) d\(^{-1}\), antithrombotic efficacy was retained while the prolongation of template BTs was minimized (Figure 9). When oral aspirin 10 mg \(\cdot\) kg\(^{-1}\) \(\cdot\) d\(^{-1}\) was administered concurrently throughout the 6-day period of low-dose clopidogrel therapy (0.2 mg \(\cdot\) kg\(^{-1}\) \(\cdot\) d\(^{-1}\)), antithrombotic effects were not observed earlier or more intensely than the effects produced by adding a single dose of aspirin after 6 days of low-dose clopidogrel therapy (Figure 8). Reductions in platelet and fibrin accumulation remained incomplete after 3 days of concurrent aspirin and clopidogrel therapy, compared with the effects on day 6 for stent thrombosis (Table 3 and Figure 7; \(P < 0.05\) in both cases) and graft thrombosis (Figure 8; Tables 2 and 3; \(P < 0.05\) in all cases). ADP- and collagen-induced platelet aggregation and ADP-induced P-selectin and LIBS expression were fully abnormal after 3 days of concurrent 10 mg \(\cdot\) kg\(^{-1}\) \(\cdot\) d\(^{-1}\) aspirin and 0.2 mg \(\cdot\) kg\(^{-1}\) \(\cdot\) d\(^{-1}\) clopidogrel administration (Table 3).

Effects of Combining Heparin With Clopidogrel

Intravenous heparin therapy (100 IU/kg bolus and 100 IU/kg over 60 minutes) after 6 days of oral clopidogrel did not reduce \(^{111}\)In-platelet deposition on stents or vascular graft thromboses (Table 2; \(P > 0.1\) in both cases). Although \(^{125}\)I-fibrin accumulation was not reduced on vascular grafts by the addition of heparin to clopidogrel (\(P = 0.15\)), \(^{125}\)I-fibrin accumulation was decreased on stents by the combination of clopidogrel plus heparin (\(P = 0.0002\)). Importantly, the enhanced antithrombotic effects produced by addition of either aspirin or heparin to clopidogrel were not further augmented for vascular graft thrombosis and stent thrombosis by addition of both aspirin and heparin to clopidogrel (Table 2; \(P > 0.2\) in all cases).

The addition of heparin after 6 days of clopidogrel 0.2 mg \(\cdot\) kg\(^{-1}\) \(\cdot\) d\(^{-1}\) did not detectably prolong the BT beyond that produced by clopidogrel alone (Table 3; \(P > 0.4\)). Similarly, platelet aggregation and expression of P-selectin and LIBS induced by either collagen or TRAP were not increased by combining heparin with clopidogrel (\(P < 0.05\) in all cases).

Antithrombotic Benefits Versus Prolongation of Template BTs

Antithrombotic efficacy was compared with changes in the BT by relating \(^{111}\)In-platelet deposition on segments of vascular graft (a measure of thrombus formation) and tem-
plate BT (generally a measure of overall platelet hemostatic function). The minimal regimen producing maximal interruption of graft thrombosis achievable by this therapy, ie, 0.2 mg·kg⁻¹·d⁻¹ clopidogrel plus 10 mg·kg⁻¹·d⁻¹ aspirin, prolonged the BT to 16±11 minutes (Figure 9 and Table 2; P < 0.0002 compared with baseline control of 3.1±0.7 minutes and P = 0.03 compared with 7.7±7.4 minutes for clopidogrel alone at 0.2 mg·kg⁻¹·d⁻¹). Interestingly, high-dose clopidogrel alone prolonged BT without significantly decreasing ¹¹¹In-platelet deposition (Figure 9).

Discussion

This study in baboons demonstrates that clopidogrel inhibition of ADP-mediated platelet recruitment produces irreversible, dose-dependent, intermediate reduction in thrombus forming on segments of prosthetic vascular grafts, endovascular metallic stents, and endarterectomized aorta within 3 hours of oral dosing at ≥2 mg/kg, and cumulative for chronic daily dosing at 0.2 mg/kg, with corresponding inhibitory effects on ex vivo platelet activation induced by ADP and prolongation of template BT measurements. Clopidogrel in combination with aspirin markedly inhibits platelet-dependent thrombus formation while modestly impairing platelet hemostatic plug-forming capability.

Under arterial flow conditions, platelet recruitment into formation of thrombus proceeds via 3 independent pathways: TxA₂ generation, ADP secretion, and thrombin production. In patients with symptomatic atherosclerotic vascular disease, selective irreversible inactivation of platelet TxA₂ generation by aspirin reduces the risk of thrombo-occlusive episodes by 20% to 25%,¹ and selective irreversible inactivation of platelet ADP-receptor–mediated platelet recruitment by clopidogrel is more effective than aspirin and at least as safe.²² The antiplatelet effects of clopidogrel are attributable to
of platelet recruitment. Now that mechanism-specific platelet inhibitors are available, it will be possible to define the relative contribution of each pathway to specific thrombogenic processes and select the most favorable antiplatelet therapy.

In extrapolation of these preclinical findings in nonhuman primates to patients, doses of clopidogrel 1 mg/d plus aspirin ≥1 mg · kg⁻¹ · d⁻¹ are predicted to produce additive antithrombotic benefits when evaluated in controlled clinical trials, ie, 50% to 60% risk reduction in vaso-occlusive episodes in patients with symptomatic atherosclerotic vascular disease. These preclinical results also suggest that an initial loading dose of clopidogrel will produce full antithrombotic effects within 3 hours.

Clopidogrel and aspirin have highly favorable therapeutic ratios when antithrombotic benefit is compared with hemorrhagic burden. By contrast, safety may be limiting in the proposed long-term use of platelet integrin α₃β₁ fibrinogen receptor antagonists. The present demonstration that aspirin enhances the antithrombotic efficacy of clopidogrel with little hemostatic impairment strengthens the rationale for performing controlled clinical trials comparing antithrombotic and hemorrhagic effects of clopidogrel 75 mg/d plus aspirin 325 mg/d versus aspirin 325 mg/d alone in patients at risk of vascular thrombo-occlusion.

References

Clopidogrel Inhibition of Stent, Graft, and Vascular Thrombogenesis With Antithrombotic Enhancement by Aspirin in Nonhuman Primates
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