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 $111{\text{In}}$-labeled platelets and $125{\text{I}}$-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 $111{\text{In}}$-platelet and $125{\text{I}}$-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 ($\geq2$ 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 $111{\text{In}}$-platelet and $125{\text{I}}$-fibrin deposition on segments of vascular graft but detectably decreased $111{\text{In}}$-platelet and $125{\text{I}}$-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 antithombotic 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 The benefits of aspirin are attributable to its irreversible interruption of thromboxone A2 (TxA2) generation by platelets.1 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%.1,4–9 Ticlopidine, a thiopyridine 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 is a ticlopidine-like thienopyridine that is severalfold more potent and is free of the adverse effects plaguing ticlopidine therapy.\textsuperscript{17,18} Like ticlopidine, clopidogrel is devoid of direct antiplatelet effects and must undergo hepatic metabolic modification to exhibit selective inhibition of ADP-induced platelet aggregation.\textsuperscript{22} Clopidogrel acts by irreversibly inactivating platelet ADP receptor–initiated signaling in a dose-dependent manner.\textsuperscript{10,20,21} 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.\textsuperscript{22} 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.\textsuperscript{13-14}

Clopidogrel and aspirin each inhibit platelet recruitment by interrupting ADP- and TxA\textsubscript{2}-mediated platelet activation, respectively. Presumably, the relative contributions of ADP- and TxA\textsubscript{2}-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.\textsuperscript{20}

**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, platelet-rich thrombi were formed on deployed 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 \textsuperscript{111}In-labeled platelets and homologous \textsuperscript{125}I-labeled fibrinogen. These chronic AV shunts, per se, do not detectably activate platelets or coagulation.\textsuperscript{24,25}

**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 mounted on sterile water–filled noncompliant Duralyn coronary angioplasty balloons (Cordis Corp). The stents were manually crimped onto the deflated balloon and 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) attached to segments of 4-mm-ID silicone rubber medical tubing (Dow Corning, 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\textsuperscript{-1} · d\textsuperscript{-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\textsuperscript{-1} · d\textsuperscript{-1} alone, (2) clopidogrel 0.2 mg · kg\textsuperscript{-1} · d\textsuperscript{-1} with heparin 100 IU/kg bolus and 100 IU/kg infused over 1 hour, (3) clopidogrel 0.2 mg · kg\textsuperscript{-1} · d\textsuperscript{-1} plus aspirin 10 mg/kg administered 2 hours previously, and (4) clopidogrel 0.2 mg · kg\textsuperscript{-1} · d\textsuperscript{-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 antplatelet 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 \textsuperscript{111}Inlabeled fibrinogen (5 \textmuCi), purified and labeled as described previously,\textsuperscript{27} and injected at least 1 hour before the thrombogenic devices were interposed. \textsuperscript{125}I-labeled baboon fibrinogen (5 \textmuCi), purified and labeled as described previously,\textsuperscript{27} 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 A\textsuperscript{I} image processing system (Medtronic) interfaced with the camera, by routines already described.\textsuperscript{27} 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\textsuperscript{-1} · mL\textsuperscript{-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 \textsuperscript{111}In activity that had decayed. Total fibrin accumulated was calculated by dividing the deposited \textsuperscript{111}In activity (counts/min) by the clottable fibrinogen activity (counts · min\textsuperscript{-1} · mL\textsuperscript{-1}) and multiplying by the plasma fibrinogen level (mg/mL).\textsuperscript{26,27}
Platelet deposition in formation of graft and stent thrombus. Autologous $^{111}$In-platelets accumulate rapidly on segments 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 $\approx 60$ minutes. For graft thrombosis, platelet deposition is not significantly 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 thrombosis, 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 $3 \times 10^9$ platelets/mL. 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 (TRAP; a gift from Dr E. Ploey, Cleveland, Ohio), and the secretory granule membrane, P-selectin, a gift from Biogen Inc, Cambridge, Mass. were employed.

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.28,31

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. Factorial ANOVA and ANCOVA were used. A value of $P \leq 0.05$ 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, top; $P > 0.2$).

Deployed metallic endovascular stents in AV shunts flowing at 100 mL/min also produced thrombus that reached plateau values by $\approx 60$ 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, $^{111}$In-platelet deposition decreased from $2.56 \pm 0.96 \times 10^9$ to $1.76 \pm 0.57 \times 10^9$ platelets ($P < 0.01$; Figure 1, bottom).

Control endarterectomized aortic segments produced substantial thrombus by 60 minutes, ie, platelet deposition averaged 1.6±0.39×10^{9} platelets/cm, and mean fibrin accumulation was 1.1±0.3 mg/cm.

Baseline platelet concentrations averaged 285±60×10^{9} platelets/cm, 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 thrombogenic devices, platelets remaining in the circulation did not change aggregatory responsiveness or express activation

![Figure 1. Platelet deposition in formation of graft and stent thrombus. Autologous $^{111}$In-platelets accumulate rapidly on segments 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 $\approx 60$ minutes. For graft thrombosis, platelet deposition is not significantly 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 thrombosis, aspirin therapy produces intermediate reduction in platelet deposition.](http://circ.ahajournals.org/)

![Table 1. Effects of Clopidogrel on Ex Vivo Platelet Aggregation and Bleeding Time](http://circ.ahajournals.org/)

<table>
<thead>
<tr>
<th>Oral Dose, mg/kg</th>
<th>ADP, $\mu$mol/L</th>
<th>Collagen, $\mu$g/mL</th>
<th>TRAP, $\mu$mol/L</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>3.8±25</td>
<td>3.1±0.7</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>
</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>
</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>
</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>
</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>
</tr>
<tr>
<td></td>
<td>$P &lt; 0.001$</td>
<td>$P = 0.004$</td>
<td>$P = 0.002$</td>
<td>$P &lt; 0.001$</td>
</tr>
<tr>
<td></td>
<td>($P = 0.37$)</td>
<td>($P = 0.7$)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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Three hours after high-dose clopidogrel

Baseline 4.36 ± 1.21 (27) 3.06 ± 1.03 (13) 2.56 ± 0.96 (7) 0.56 ± 0.27 (6) 3.1 ± 0.7 (14)

Six days after low-dose clopidogrel

<table>
<thead>
<tr>
<th></th>
<th>Platelets x 10⁶</th>
<th>Fibrin, mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clopidogrel 0.2 mg/kg</td>
<td>3.16 ± 0.84 (6)</td>
<td>1.7 ± 0.42 (6)</td>
</tr>
<tr>
<td>Clopidogrel 0.2 mg·kg⁻¹·d⁻¹ + aspirin 10 mg/kg</td>
<td>2.07 ± 0.41 (6)</td>
<td>1.85 ± 0.28 (6)</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>
</tr>
<tr>
<td>Clopidogrel + aspirin + heparin</td>
<td>2.01 ± 1.03 (5)</td>
<td>1.39 ± 0.33 (5)</td>
</tr>
</tbody>
</table>

Numbers in parentheses are numbers of observations. P₁ indicates control vs treatment; P₂, clopidogrel vs combinations.

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).
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

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### 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>
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<tr>
<td><strong>Stent thrombosis</strong></td>
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<tr>
<td>Platelet deposition</td>
<td>×10⁹</td>
<td>2.56±0.96 (7)</td>
<td>1.82±1.30 (3)</td>
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<td></td>
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<td>0.3</td>
<td>0.009</td>
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<tr>
<td>Fibrin accumulation, mg</td>
<td></td>
<td>0.56±0.27 (6)</td>
<td>0.42±0.08 (3)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.42</td>
<td>0.04</td>
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<tr>
<td><strong>Graft thrombosis</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Platelet deposition</td>
<td>×10⁹</td>
<td>4.36±1.21 (27)</td>
<td>3.52±1.42 (3)</td>
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<td></td>
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<td>0.3</td>
<td>0.03</td>
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<tr>
<td>Fibrin accumulation</td>
<td>(mg)</td>
<td>3.06±1.03 (13)</td>
<td>2.34±0.23 (3)</td>
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<td></td>
<td></td>
<td>0.3</td>
<td>0.0007</td>
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<td>BT, min</td>
<td></td>
<td>3.1±0.7 (14)</td>
<td>4±0.5 (3)</td>
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<tr>
<td></td>
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<td>0.05</td>
<td>0.03</td>
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<td><strong>Platelet aggregation (AC₅₀)</strong></td>
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<tr>
<td>ADP, μmol/L</td>
<td></td>
<td>5.1±2.8 (18)</td>
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<td>Collagen AC₅₀, μg/mL</td>
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<td>2.2±1.0 (16)</td>
<td>7.2±3.4 (10)</td>
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<tr>
<td>TRAP₁–₆ AC₅₀, μmol/L</td>
<td></td>
<td>38±25 (18)</td>
<td>35.6±20.8 (10)</td>
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<tr>
<td></td>
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<td>0.8</td>
<td>0.5</td>
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<td><strong>Expression of activation epitopes</strong></td>
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<tr>
<td>LIBS</td>
<td></td>
<td>1440±476 (16)</td>
<td>1890±540 (7)</td>
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<td>1200±192 (7)</td>
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<td>LIBS after ADP</td>
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<td>20 500±8900 (17)</td>
<td>6250±3400 (7)</td>
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<td>7100±4800 (7)</td>
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<td>LIBS after TRAP₁–₆</td>
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<td>7570±7210 (9)</td>
<td>2100±820 (7)</td>
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<td>2710±1270 (4)</td>
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<td>P-Selectin</td>
<td></td>
<td>352±339 (17)</td>
<td>162±28 (7)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>164±54</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></td>
<td></td>
<td>0.0022</td>
<td>0.0001</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 \( \cdot \) kg\(^{-1}\) \( \cdot \) 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 clopidogrel and aspirin 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 $\cdot$ kg$^{-1} \cdot$ d$^{-1}$ clopidogrel plus 10 mg $\cdot$ kg$^{-1} \cdot$ d$^{-1}$ aspirin, prolonged the BT to 16.6 minutes (Figure 9 and Table 2; $P$ $<$ 0.0002 compared with baseline control of 3.1 minutes and $P$ $<$ 0.03 compared with 7.7 minutes for clopidogrel alone at 0.2 mg $\cdot$ kg$^{-1} \cdot$ d$^{-1}$). Interestingly, high-dose clopidogrel alone prolonged BT without significantly decreasing 111 In-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.32 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
Clopidogrel Inhibits Thrombogenesis

Figure 9. Effects of clopidogrel and aspirin on platelet deposition and template BTs. Chronic oral clopidogrel inhibits platelet deposition plateauping at intermediate levels for doses of ≥0.2 mg · kg⁻¹ · d⁻¹ while minimally prolonging BT. By contrast, high-dose clopidogrel maximally prolongs template BT results. Addition of aspirin substantially decreases platelet deposition while adding modest prolongation to BT measurements. Clopidogrel at doses of 2.0 and 20 mg/kg does not significantly decrease platelet deposition compared with chronic low doses but maximally prolongs BT results.

The present study demonstrates that high-dose clopidogrel (≥2 mg/kg) produces immediate, irreversible inactivation of platelet ADP-receptor signaling that dissipates over 6 days, the life span of baboon platelets. One-hundredth that dose (0.2 mg · kg⁻¹ · d⁻¹) produces cumulative, irreversible inhibition of platelet ADP-receptor function that is complete after 5 to 6 days. Aspirin, which irreversibly inactivates the generation of TxA₂, by platelets within 1 to 2 hours after oral dosing, significantly enhances the antithrombotic effects of clopidogrel. It follows that enhancement by aspirin of the antithrombotic effects of clopidogrel represents additive, concurrent, irreversible interruption of the both TxA₂ and ADP pathways of platelet recruitment.

The thrombotic models used in the present study constitute 3 different thrombotic processes composing variable proportions of the 3 platelet recruitment pathways. It seems likely that the most favorable antithrombotic versus antihemostatic strategy for complex resistant thrombotic processes involves tailoring combinations of pathway-specific inhibitors appropriate for the specific thrombotic process. Various thrombotic processes in humans also appear to exhibit differences in their relative dependence on ADP, TxA₂, and thrombin pathways 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 ŵmβ₃ 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.

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

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