Early Potent Antithrombotic Effect With Combined Aspirin and a Loading Dose of Clopidogrel on Experimental Arterial Thrombogenesis in Humans

Yves Cadroy, MD, PhD; Jean-Pierre Bossavy, MD, PhD; Claire Thalamas, MD; Luc Sagnard, MD; Kjell Sakariassen, PhD; Bernard Boneu, MD

Background—We conducted a double-blind, randomized, crossover study to assess the antithrombotic effects of the combination of aspirin (acetylsalicylic acid, ASA) and clopidogrel, with or without a loading dose, versus ASA alone in a model of arterial thrombosis in humans.

Methods and Results—Eighteen male volunteers received the following 3 regimens for 10 days separated by a 1-month period: (1) 325 mg ASA daily, (2) 325 mg ASA + 75 mg clopidogrel daily, (3) 325 mg ASA daily + 300-mg clopidogrel loading dose on day 1 and + 75 mg clopidogrel per day on days 2 to 10. The antithrombotic effect was measured 1.5, 6, and 24 hours after drug intake on day 1 and 6 hours after drug intake on day 10. Arterial thrombus formation was induced ex vivo by exposing a collagen-coated coverslip in a parallel-plate perfusion chamber to native blood for 3 minutes at an arterial wall shear rate. Without a loading dose, clopidogrel + ASA developed an antithrombotic effect within 6 hours after the first intake. It was superior to that produced by ASA, but it was moderate (P ≤ 0.03). However, with the loading dose, the antithrombotic effect of clopidogrel + ASA appeared within 90 minutes, and after 6 hours it was comparable to that on day 10. On day 10, clopidogrel + ASA decreased platelet thrombus formation by ≈ 70%, and the effect was significantly more potent than that produced by ASA alone (P < 0.001).

Conclusions—This study confirms the synergistic antithrombotic effects of a combined ASA and clopidogrel therapy and shows the early benefit obtained with a loading dose of clopidogrel. (Circulation. 2000;101:2823-2828.)

Key Words: aspirin ▪ blood flow ▪ collagen ▪ platelets ▪ thrombosis

A

spirin (acetylsalicylic acid, ASA) and ticlopidine interfere with different and complementary important pathways of platelet activation, that is, arachidonic acid and ADP pathways, respectively.1 Recent clinical studies have demonstrated that a combination of ASA and ticlopidine markedly reduced thrombotic events occurring during coronary artery stenting.2–9 However, ticlopidine therapy has adverse effects. Clopidogrel is a thienopyridine that is more potent than ticlopidine and produces fewer adverse effects.10,11 In addition, clopidogrel also has been shown to increase the antithrombotic effect of ASA in recent experimental studies performed in animals.12,13 However, no published clinical or experimental study has investigated the antithrombotic effect of the combination of ASA and clopidogrel in humans.

The antithrombotic effect of drugs can be experimentally investigated in humans with the use of an ex vivo model of thrombogenesis that closely mimics relevant clinical situations.14,15 In this model, native blood is drawn from healthy volunteers through a parallel-plate chamber device, where it interacts, in well-established flow conditions, with collagen, a relevant thrombogenic surface. Blood flow conditions mimic wall shear rates encountered in moderately stenosed arteries (2600 s⁻¹). The efficacy of antithrombotic drugs is determined by quantifying the respective thrombus content in platelets and fibrin by immunoenzymatic methods.16 This model has been used to investigate a number of different antithrombotic strategies.17–21 Thus, we previously showed that combined ASA and ticlopidine therapy potentiates in a synergistic manner the antithrombotic effect of each drug alone.21 By using this ex vivo model of acute initial thrombus formation, we designed a randomized, non–placebo-controlled, double-blind study to determine the antithrombotic effects of combined clopidogrel + ASA therapy versus ASA.

A major clinical goal of antithrombotic therapy, notably in patients undergoing coronary stent implantation, is to be effective as soon as possible to prevent early stent thrombosis. Clopidogrel must undergo hepatic modification to cause...
selective inhibition of ADP-induced platelet aggregation.10
Thus, inhibition of platelet aggregation is noted 2 hours after the
administration of 75 mg, but it reaches a steady state
within 3 to 7 days. The onset of action of the combination
of clopidogrel+ASA is not known. Therefore, we also studied
the pharmacodynamics of the antithrombotic effect of the
combined clopidogrel+ASA therapy. In this regard, whereas
clopidogrel is indicated for the reduction of atherosclerosis
events at a dose of 75 mg daily, we tested whether an initial
loading dose of 300 mg given on the first day of treatment
accelerates its onset of action.

Methods

Subjects
The study population consisted of 18 healthy white male volun-
teers 20 to 30 years of age. They had no history or clinical signs
of any disease and were not taking any medication known to
affect blood coagulation or platelets during the study period. The
volunteers each smoked <10 cigarettes per day, and they did not
smoke on the day of the perfusion experiments. Clinical chemis-
try and hematological and hemostatic laboratory values were
within the normal ranges. All subjects gave written informed
consent to the protocol, which was approved by the local Human
Subjects Committee (Comité Consultatif de Protection des Per-
sonnes dans la Recherche Biomédicale, Toulouse).

Study Design
This monocentric, randomized, non–placebo-controlled, double-
blind study was carried out in the Center for Clinical Investigation at
Hôpital Purpan, Toulouse, France. After selection for the trial, an
initial perfusion experiment was performed on each volunteer who
satisfied the inclusion and exclusion criteria: This perfusion exper-
iment was considered as the baseline experiment. Each volunteer then
randomly received 1 of the 3 tested regimens in a 3-period crossover
design: either (1) 325 mg ASA daily for 10 days, (2) 325 mg ASA
plus 75 mg clopidogrel daily for 10 days, or (3) 325 mg ASA plus
300 mg clopidogrel on day 1, followed by 325 mg ASA plus 75 mg
clopidogrel daily for the last 9 days. The 3 regimens were given
separately with a washout period of 3 to 6 weeks between each of
them. All drugs were supplied by Sanofi Recherche in a blister-pack
for 15 to 20 hours before use.21

Preparation of Thrombogenic Surface
Equine collagen (Collagen Reagent Horn, Nycomed) was spray-
coated onto Thermanox plastic coverslips (Miles Laboratories) to a
final density of 0.5 μg/cm². They were stored at room temperature
for 15 to 20 hours before use.21

Perfusion Experiments
Perfusion experiments were performed with a parallel-plate perfu-
sion chamber device at 37°C.14,15 After blood sample collection,
native blood was drawn directly from an antecubital vein of the
volunteers through a 19-gauge infusion set (Ohmeda) over the
collagen-coated coverslip positioned in the parallel-plate perfusion
chamber. The blood flow rate was maintained at 10 mL/min by a
peristaltic roller pump (Multiperpx LKB, Pharmacia) placed distal
to the chamber. The wall shear rate was 2600 s⁻¹, which corresponds
to that encountered in moderately stenosed arteries. The blood
perfusion experiment lasted for 3 minutes and was followed by a
30-second perfusion of PBS at the same flow rate to wash out blood
from the flow channel. The coverslip covered by thrombotic deposits
was placed in a plasmin solution and processed as described below.

Immunological Determination of Fibrin Deposition
Fibrin deposition was quantified by immunological determination
of fibrin degradation products of plasmin-digested thrombi.16 After
perfusion, the thrombus was immediately incubated in 2 mL of a
plasmin solution (Chromogenix, 0.7 U/mL, in Tris-buffered saline,
pH 7.4) for 30 minutes at 37°C. Plasmin digestion was stopped by
aprotinin (2000 kIU/mL, Bayer Pharma). The solution was centri-
fuged (4°C, 4300g, 15 minutes) and the supernatant frozen at −80°C
for measurement of fibrin degradation products and P-selectin levels
(see below). Fibrin degradation products were measured with the use
of an immunoenzymatic assay (Asserachrom D-Di, Stago). The
amount of deposited fibrin is directly determined from the levels of
fibrin degradation products expressed in fibrin equivalent units as
indicated by the manufacturer.

Immunological Determination of Platelet Deposition
Platelet deposition was quantified by measurement of a specific
platelet α-granule membrane protein, P-selectin.16 After centrifuga-
tion of the plasmin-digested thrombus, the pellet was dissolved in
400 μL of a lytic buffer, frozen, thawed 3 times, and then sonicated
(4°C, 20 kHz) for 270 seconds. The lytic buffer is made of PBS
containing 1% Triton X-100 (Merck), 16 mmol/L octyl-β-D-
glucopyranoside (Boehringer Mannheim), 1 mmol/L EDTA
(Merck), 20% sodium azide (Merck), 10 μmol/L pepstatin A
(Sigma), 10 μmol/L leupeptin (Sigma), 100 kIU/mL aprotinin, and
0.1 mmol/L PMSF (Sigma). All samples of dissolved pellets were
stored at −80°C until assayed for P-selectin by immunoenzyme
assay (Bender MedSystems). The level of P-selectin was measured
both in the dissolved pellet and in the supernatant of the plasmin-
digested thrombus. The total number of platelets deposited was
calculated by dividing the amount of P-selectin present in the
thrombus by that present in nonactivated platelets of healthy blood
donors (321±14 ng/10⁶ platelets, n=26). Results are expressed as
the number of platelets deposited per square centimeter.

Other Laboratory Procedures
Red cell, leukocyte, and platelet counts and hemoglobin and hemat-
ocrit were measured by an electronic counting device (Model S plus,
Coulter Electronics). For platelet aggregation tests, blood was
collected into a citrated Vacutainer (Becton Dickinson, ref 367704)
of 5 mL. Before testing, each sample was centrifuged for 15 minutes.
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of 5 mL. Before testing, each sample was centrifuged for 15 minutes.
Results

Study Population

Eighteen healthy male, white subjects, 20 to 30 years of age (mean age 24 years), were enrolled in the study from April 1998 through February 1999 and randomly assigned to the different treatments. None prematurely stopped the trial. All 18 volunteers completed the study according to the protocol.

Antithrombotic Effect of ASA and Clopidogrel

1

ASA on Day 10

The effects of the 3 antithrombotic treatments on collagen-induced thrombus formation 10 days after their first administration are shown in Figure 1. Compared with baseline, ASA had a modest but significant antithrombotic effect: Platelet and fibrin deposition were inhibited by 24% and 35%, respectively ($P < 0.002$ and $P < 0.03$, respectively). However, in volunteers treated with clopidogrel + ASA, the antithrombotic effect was much greater, since clopidogrel + ASA decreased platelet thrombus formation and fibrin deposition by 71% and 74%, respectively. This effect was significantly more potent than that produced by ASA alone ($P < 0.001$), and it was comparable whether or not a 300-mg clopidogrel loading dose had been given ($P > 0.10$).

Pharmacodynamics of Antithrombotic Effect of ASA and Clopidogrel + ASA

The pharmacodynamics of the antithrombotic effect of the 3 antithrombotic treatments on collagen-induced thrombus formation are shown in Figures 2, 3, and 4. Interestingly, ASA did not fully express its antithrombotic effect on the first day of administration. Its effect on platelet deposition was delayed: On day 1, ASA decreased platelet deposition by <10% ($P = NS$), whereas on day 10 it was decreased by 24% ($P = 0.03$). However, the effect of ASA on fibrin deposition was not time-dependent: The reduction of fibrin deposition was comparable on the first and tenth days of treatment (35%, $P \leq 0.010$).

Without a loading dose of clopidogrel, clopidogrel + ASA developed an antithrombotic effect within 6 hours after the first dose. This effect was moderate, since at this time period platelet and fibrin deposition were reduced by 34% and 60%, respectively ($P = 0.042$ and $P < 0.001$, respectively) as compared with 71% and 74%, respectively, on day 10. However, it was significantly superior to that produced by ASA alone ($P < 0.030$).

With the 300-mg clopidogrel loading dose, the antithrombotic effect of clopidogrel + ASA appeared within 90 minutes after the first dose. As early as 6 hours after the first administration, it was very potent (61% and 75% of platelet and fibrin reduction, respectively, $P < 0.001$) and comparable to that obtained after 10 days of treatment. As compared with ASA alone, this treatment was significantly more potent for preventing platelet deposition on collagen-coated surfaces at each time point ($P \leq 0.040$). It also prevented fibrin deposition significantly more than ASA at 6 and 24 hours and at 10 days after the first dose ($P \leq 0.004$). It was also superior to clopidogrel + ASA given without the 300-mg loading dose at each time point on the first day of treatment ($P \leq 0.030$).

Figure 1. Effect of antiplatelet treatment on deposition of platelets and fibrin on collagen-coated coverslips. Surface was exposed for 3 minutes to nonanticoagulated blood at shear rate of 2600 s$^{-1}$. Volunteers were given for 10 days either 325 mg ASA daily, 325 mg ASA + 75 mg clopidogrel daily, or 325 mg ASA + 300 mg clopidogrel per day on days 2 to 10. Baseline perfusion was performed with blood from volunteers before administration of ASA or ASA + clopidogrel. Values are mean $\pm$ SEM (n=18). **$P < 0.01$ vs ASA.

Figure 2. Effect of ASA on deposition of platelets and fibrin on collagen-coated coverslips. Surface was exposed for 3 minutes to nonanticoagulated blood at shear rate of 2600 s$^{-1}$. Volunteers were given 325 mg ASA daily for 10 days, and perfusion experiments were performed 1.5, 6, and 24 hours after drug intake on day 1 and 6 hours after drug intake on day 10. Baseline perfusion was performed in volunteers before ASA administration. Values are mean $\pm$ SEM (n=18). *$P < 0.05$, **$P < 0.01$ vs baseline.
Effect of Treatment on Platelet Aggregation
Platelet aggregation was evaluated on blood samples drawn from volunteers before each perfusion experiment. Results are shown in the Table. ASA inhibited collagen-induced (P<0.010) but not ADP-induced platelet aggregation. This inhibition was comparable on days 1 and 10. On day 1, the inhibition was time dependent: ASA inhibited collagen-induced platelet aggregation at 6 hours but not at 1.5 and 24 hours after drug administration.

ADP- and collagen-induced platelet aggregation were significantly more inhibited by the combination of clopidogrel+ASA than by ASA alone; this inhibition was maximum 10 days after the drug was given, and, at this time period, it was comparable with or without the 300-mg loading dose of clopidogrel administered. However, on day 1, the inhibition of platelet aggregation was time dependent. ADP-induced platelet aggregation was inhibited more quickly when a 300-mg loading dose of clopidogrel was given. Interestingly, with or without a loading dose of clopidogrel, collagen-induced platelet aggregation was less inhibited at 24 hours than at 6 hours after the drug was administered.

Finally, arachidonic acid–induced platelet aggregation was fully inhibited in all 18 volunteers, with all 3 tested regimens, and at all time points.

Discussion
By using a model of human thrombogenesis, we show in the present study that the antithrombotic effect of the combination of clopidogrel and ASA is significantly more potent than the antithrombotic effect of ASA alone. Second, we show that when clopidogrel is given with a 300-mg loading dose on the first day of treatment, the optimal antithrombotic effect of clopidogrel+ASA is obtained within 6 hours after the first dose.

With or without a 300-mg loading dose, clopidogrel increased the antithrombotic effect of ASA (Figure 1). Comparable findings have been shown in recent experimental studies performed in animals.2,3 Previously, we also showed that combined ASA and ticlopidine therapy dramatically potentiated the antithrombotic effect of each drug alone. Taken together, these results indicate that both ADP and thromboxane A2 play a major and synergistic role in mediating platelet thrombus formation and that both pathways must be simultaneously blocked to achieve a maximum antithrombotic effect.

Thienopyridine derivatives have a delayed onset of action.10 Thus, pharmacological studies have indicated that maximum inhibition of ADP-induced platelet aggregation occurs only after 3 to 5 days of oral administration of 250 mg BID of ticlopidine.22 In the present study, the antithrombotic effect of clopidogrel+ASA was time dependent. However, it is interesting to note that when a 300-mg loading dose of clopidogrel was given on day 1, the antithrombotic effect of the combined clopidogrel+ASA therapy appeared within 90 minutes after oral intake and that at 6 hours it was comparable to that seen on day 10. This finding is particularly important in patients undergoing coronary stent implantation or having acute coronary syndrome, in which effective antithrombotic effects are needed as early as possible to prevent thrombosis.
Effects of Aspirin and Clopidogrel on Platelet Aggregation

<table>
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<th>Treatment</th>
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<th>Hour</th>
<th>ADP, %</th>
<th>Collagen, %</th>
<th>Arachidonic Acid, %</th>
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<tr>
<td>Baseline</td>
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<td></td>
<td>83±3</td>
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<tr>
<td>ASA</td>
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<td>81±4</td>
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<td>64±4</td>
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<td>24</td>
<td>54±3</td>
<td>84±2</td>
<td>4±1</td>
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<td>58±3</td>
<td>70±3</td>
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<td>75 mg clopidogrel+ASA</td>
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<td>37±3*</td>
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<tr>
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<td>38±5*</td>
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<td>6</td>
<td>21±3*</td>
<td>37±3*</td>
<td>3±1</td>
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</table>

Results are expressed as % of maximal amplitude of the platelet aggregation curve with ADP (5 μmol/L), collagen (10 μg/mL), and arachidonic acid (1 mmol/L).

*P<0.01 vs ASA.

Surprisingly, the antiplatelet and antithrombotic effects of ASA were also time dependent. ASA inhibited collagen-induced platelet aggregation only at 6 hours after its oral ingestion. In addition, this inhibition was rapidly reversible, since collagen-induced platelet aggregation was no longer inhibited 24 hours after its administration. The combined clopidogrel+ASA therapy also showed a lesser antithrombotic effect at 24 hours than at 6 hours. Likewise, platelet thrombus formation was not prevented on the first day of the ASA administration, but only on day 10. These findings could have a clinical relevance to the frequency of dosing. Also, they suggest that ASA exerts part of its antithrombotic effect by mechanisms that are unrelated to its inability to inactive cyclooxygenase and suppress the synthesis of platelet thromboxane A₂. Indeed, >99% of thromboxane A₂ synthesis is inhibited within 2 hours after intake of 325 mg ASA.1 In our study, arachidonic acid–induced platelet aggregation, which reflects this phenomenon, was inhibited by >90% within 1.5 hours after the administration of ASA, and this inhibition was irreversible. These other contributory mechanisms include non–prostaglandin-dependent effects on platelet function, enhancement of fibrinolysis, and inhibition of plasma coagulation.1

The antiplatelet drug regimens affected coagulation as well, since fibrin deposition was significantly reduced. The apparent anticoagulant effect provided by antiplatelet regimens may be the direct consequence of a reduction in platelet deposition, since fibrin deposition on collagen substrate generally occurs subsequent to platelet thrombus formation.23 It is also possible that this finding is a result of reduced platelet activation, since activated platelets amplify the coagulation cascade by binding activated coagulation factors to form the tenase and prothrombinase complexes.24

Criticisms with respect to the significance and clinical relevance of the ex vivo model of human thrombogenesis used may be raised. In our study, thrombus formation was only determined on a collagen-coated surface. Whereas collagen is an important determinant of the thrombogenicity of ruptured human atherosclerotic lesions,25 there are other components at least as important, notably tissue factor.26 We did not study the antithrombotic effect of clopidogrel+ASA on tissue factor, since we previously showed that ticlopidine+ASA did not inhibit the thrombotic process on tissue factor.21 In addition, we examined the effect of antithrombotic drugs on early acute platelet thrombus formation. Perfusion times were only 3 minutes because thrombus formation in this model is maximum at 3 minutes.16 Also, the study of antithrombotic drugs on the very early events of thrombus formation are important, since they have profound impact on later events. However, longer perfusion times would give additional information on thrombus growth and thrombus stabilization. But, as discussed above, our thrombosis model has previously been shown to be useful in evaluating different antithrombotic agents.17–21 One can note that results obtained with largely used antithrombotic agents appear consistent with clinical data.27

In conclusion, this study confirms that combined ASA and clopidogrel therapy exerts a potent synergistic antithrombotic effect significantly superior to that given by ASA alone. Since clopidogrel is well tolerated, this result warrants clinical trials in which clopidogrel is associated with ASA. In this regard, the present demonstration that a 300-mg loading dose of clopidogrel given on day 1 accelerates the rate with which combined clopidogrel+ASA exerts its antithrombotic effect is important, since a major clinical goal of antithrombotic therapy is to be rapidly effective.

References


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