Myocardial Ischemia and Infarction

Clopidogrel Inhibits Platelet-Leukocyte Interactions and Thrombin Receptor Agonist Peptide-Induced Platelet Activation in Patients With an Acute Coronary Syndrome

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OBJECTIVES We sought to characterize the effects of clopidogrel on the activation of circulating platelets, the activation and aggregation of ex vivo platelets, and the interactions with leukocytes in patients with a non–ST-segment elevation in acute coronary syndromes (ACS).

BACKGROUND The significant benefits of clopidogrel in cardiovascular trials suggest that blockage of the P2Y12 receptor may be associated with important biologic consequences.

METHODS Blood samples obtained from 23 ACS patients before and 24 h after a loading dose of clopidogrel (300 mg) were analyzed by whole-blood flow cytometry, light transmission aggregometry in platelet-rich plasma, and plasma enzyme-linked immunoassays. A thrombin receptor agonist peptide (TRAP) and adenosine diphosphate (ADP) were used as agonists. Normal individuals pretreated with aspirin served as controls.

RESULTS Clopidogrel attenuated platelet aggregation to both ADP (10 μmol/l) and TRAP (10 μmol/l) by 22% and P-selectin expression by 16% and 25%, respectively. The drug decreased the excess platelet-monocyte and platelet-neutrophil conjugates found in the blood of ACS patients (p < 0.01) and prevented their formation ex vivo with agonist stimulation. Plasma levels of soluble CD40L were reduced by 27% (p < 0.001) and of soluble P-selectin by 15% (p < 0.001).

CONCLUSIONS Clopidogrel attenuates the agonist effects of ADP and TRAP on platelet secretion, aggregation, and formation of platelet-monocyte and platelet-neutrophil conjugates in patients with ACS. These effects may all contribute to the clinical benefits of the drug in these syndromes. (J Am Coll Cardiol 2004;43:1982–8) © 2004 by the American College of Cardiology Foundation

Clopidogrel is a thienopyridine that specifically acts on the platelet P2Y12 purinergic receptor (1). Clinical trials have consistently shown that the drug prevents acute ischemic events (2–5), although it only partially blocks the adenosine diphosphate (ADP) receptor, with a high degree of inter-individual variability (6,7). As indirect effects of the drug could contribute to the benefits, this study looks at mechanisms other than the inhibition of ADP-induced platelet aggregation that could be useful in patients with a non–ST-segment elevation acute coronary syndrome (ACS).

Circulating platelet-leukocyte conjugates are considered a sensitive marker of the interactions that exist between inflammation and thrombosis. The interactions elicit white cell activation with upregulation of CD11b/CD18, cytokine production, and expression of tissue factor and procoagulant activity in monocytes (8–11). The conjugates are elevated in unstable angina (12,13); acute myocardial infarction, where they precede the elevation of creatine kinase-MB isoenzyme (14); during cardiopulmonary bypass surgery (15); and during percutaneous coronary interventions (16). CD40L is secreted by platelets and lymphocytes and binds the receptors present on numerous cells that are involved in the processes of atherosclerosis, plaque inflammation, and thrombosis (10,11,17).

Previous studies have suggested some anti-inflammatory properties of clopidogrel. A reduction in the number of platelet-leukocyte interactions has been described (18,19), and one study reported a special benefit of the drug in reducing the augmented risk of percutaneous interventions in patients with elevated levels of C-reactive protein (20).
METHODS

Study population. Twenty-three patients (7 women and 16 men; 58 ± 10 years of age) were studied within 24 h of hospital admission for a non–ST-segment elevation ACS, and 20 normal individuals served as controls for the platelet-leukocyte conjugate studies. An ACS was defined as chest pain lasting 5 min or more at rest, with the presence of an ischemic ST-T segment or an increase in blood levels of troponin T. At the time of the study, all patients had received a 160-mg dose of aspirin and an intravenous bolus of 60 U/kg of unfractionated heparin; they all received an infusion of heparin for at least 6 h at a rate of 12 U/kg/h, titrated to an activated partial thromboplastin time of 1.5 to 2.5 times the upper limit of normal. No other thromboactive drugs, including glycoprotein (GP) IIb/IIIa antagonists, were used. Other cardiovascular drugs included beta-blockers in 19 patients, nitrates in 13 patients, and calcium antagonists in 6 patients. Sixteen patients had previous statin therapy, and a statin was initiated at hospital admission for the purpose of this study. The local ethics committee approved the study, and all patients signed an informed consent form.

Blood sampling. Blood samples were obtained from an antecubital vein using a 21-gauge butterfly needle. The blood was collected in a plastic tube containing D-phenylalanyl-prolyl-arginine chloromethyl ketone (final concentration of 40 μmol/l), after having discarded the first 2 ml of free running blood, and was processed within the following 10 to 15 min. Patients were studied before and 24 h after an oral loading dose of 300 mg clopidogrel. Platelet aggregation studies. Platelet aggregation was measured in platelet-rich plasma (PRP) using light transmission aggregometry. Platelet-leukocyte aggregates. Whole blood was mixed gently with PPP at a ratio of 1:4 was added in an Eppendorff tube containing a saturating concentration of anti-CD62P-PE (mouse immunoglobulin [Ig]G1), 10 μmol/l ADP, or 10 μmol/l TRAP (final concentrations), or an equal volume of Tyrode’s buffer solution (pH 7.4). After a 30-min period of incubation in static conditions at 37°C, formaldehyde diluted in a Tyrode’s buffer solution (1.0% final concentration) was added to stop the reaction. The fixed samples were analyzed within the following hour using a Coulter EPICS XL flow cytometer (Beckman–Coulter, Miami, Florida). The instrument computer system was used to quantify fluorescein isothiocyanate (FITC) fluorescence-positive platelets as the percentage of the total number of platelets.

P-selectin expression. Whole blood gently mixed with PPP at a ratio of 1:4 was added in an Eppendorff tube containing a saturating concentration of anti-CD62P-PE (mouse immunoglobulin [Ig]G1), 10 μmol/l ADP, or 10 μmol/l TRAP (final concentrations), or an equal volume of Tyrode’s buffer solution (pH 7.4). After a 30-min period of incubation in static conditions at 26°C, formaldehyde diluted in a Tyrode’s buffer solution (1.0% final concentration) was added to stop the reaction. The fixed samples were analyzed within the following hour using a Coulter EPICS XL flow cytometer (Beckman–Coulter, Miami, Florida). The instrument computer system was used to quantify fluorescein isothiocyanate (FITC) fluorescence-positive platelets as the percentage of the total number of platelets.

Platelet-leukocyte aggregates. Whole blood was mixed gently in an Eppendorff tube containing a saturating concentration of anti–CD14-PC5 (mouse IgG2a) and anti–CD42a-FITC (mouse IgG1), or their negative monoclonal antibodies, and either ADP or TRAP, or an equal volume of Tyrode’s buffer solution. The reaction was stopped after a 15-min period of incubation in static conditions at 37°C. The fixed samples were kept for a period of 60 min at room temperature before red cells were lysed by adding a Beckman–Coulter solution at a ratio of 1:10. Dual-color flow cytometric analyses were then performed within 40 to 60 min, detecting anti–CD14-PC5 at a bandpass filter of 675 nm and anti–CD42a-FITC at 525 nm. Platelets were stained with anti–CD42a-FITC, and monocytes or neutrophils with anti–CD14-PC5. Because monocytes have a higher expression of CD14 (lipopolysaccharide receptor) but a lower granularity compared with neutrophils, they can readily be distinguished from neutrophils by a higher mean fluorescence intensity and a lower side scatter for cell granularity (Fig. 1). Particles positive to both anti–CD42a-FITC and anti–CD14-PC5 were identified as platelet-monocyte or platelet-neutrophil conjugates, depending on the intensity of anti–CD14-PC and granularities. The conjugates were assessed as the percentage of the CD42a-FITC and CD14-PC5–positive particles, reflecting the percentage of monocytes/neutrophils with bound platelets, and as the mean intensity of CD42a-FITC per particle, reflecting the number of platelets bound per monocyte-neutrophil conjugate.

Enzyme-linked immunosorbent assay. A tube of fresh whole blood was centrifuged at 2,200 g (3,500 rpm) for 15 min; the plasma was saved at –70°C for subsequent batch analysis by ELISA of soluble P-selectin (Bender Med
Systems, Vienna, Austria) and soluble CD40L (R&D Systems, Minneapolis, Minnesota), according to the manufacturer’s instructions.

Statistical analyses. All results are given as the mean value ± SD. The data obtained before and after clopidogrel administration were compared using paired Student’s t-test for within the same patients and by the unpaired t-test for between groups of patients. Potential interactions between clopidogrel and other cardiovascular drugs were analyzed using analysis of variance. The SPSS software (Version 10, SPSS Inc., Chicago, Illinois) was used for all analyses. A value p ≤ 0.05 was considered statistically significant in all cases.

RESULTS

Platelet aggregation and secretion. Platelet counts in patients were 156 ± 36 × 10^9/l before the administration of clopidogrel and 158 ± 39 × 10^9/l afterward; leukocyte counts were also unchanged: 7.1 ± 2.0 × 10^9/l and 7.0 ± 1.9 × 10^9/l, respectively. When ADP was added ex vivo in the basal state, it produced 52% platelet aggregation; TRAP, a more potent agonist, induced 77% platelet aggregation. Membrane P-selectin expression was detected in 45% of platelets with ADP and in 72% with TRAP. The administration of clopidogrel in patients with ACS resulted in a 22.6% absolute reduction (44% of the pre-administration level) in the number of aggregating platelets in response to ADP and in a 21.3% reduction (28% of the pre-administration level) in response to TRAP. The number of platelets expressing P-selectin was reduced by 15.6% and 25.1%, respectively (35% of the pre-administration number for both ADP and TRAP, p ≤ 0.01) (Table 1).

The drug also significantly reduced both soluble CD40L and soluble P-selectin in plasma, by 27% and 15%, respectively (p ≤ 0.001) (Fig. 2).

Platelet-leukocyte conjugates. Figure 1 illustrates a typical flow cytometric display of platelet-monocyte and platelet-neutrophil conjugates obtained in one patient with ACS. The mean results in controls and patients are shown in Figures 3 and 4. At baseline, before the administration of clopidogrel and the addition of an agonist in blood, there were significantly more platelet-monocyte and platelet-neutrophil conjugates and more platelets per conjugate in patients than in controls. The number of platelet-monocyte conjugates was particularly high, exceeding by threefold that of platelet-neutrophil conjugates. Adenosine diphosphate intensified the formation of

Table 1. Platelet Aggregation and P-Selectin Expression Induced by ADP (10 μmol/l) or TRAP (10 μmol/l) in Patients With an Acute Coronary Syndrome Before and 24 Hours After Administration of Clopidogrel (n = 21)

<table>
<thead>
<tr>
<th></th>
<th>ADP-Induced Platelets</th>
<th>TRAP-Induced Platelets</th>
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<tbody>
<tr>
<td></td>
<td>Aggregation</td>
<td>P-Selectin Expression</td>
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<td></td>
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<tr>
<td>Pre-drug (%)</td>
<td>51.5 ± 16.5</td>
<td>44.8 ± 15.8</td>
</tr>
<tr>
<td>Clopidogrel (%)</td>
<td>28.9 ± 23.3*</td>
<td>29.2 ± 17.1*</td>
</tr>
<tr>
<td>Inhibition (%)</td>
<td>44%</td>
<td>35%</td>
</tr>
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|                | Aggregation           | P-Selectin Expression  |
|                |                        |                        |
| Pre-drug (%)   | 76.9 ± 7.6†           | 72.2 ± 4.6‡            |
| Clopidogrel (%)| 55.6 ± 17.5*          | 47.1 ± 22.3*           |
| Inhibition (%) | 28%                   | 35%                    |

*p < 0.01 vs. pre-drug. †p < 0.01 vs. ADP-induced platelet aggregation. ‡p < 0.01 vs. ADP-induced P-selectin expression in the same row. Data are expressed as the mean value ± SD.

ADP = adenosine diphosphate; TRAP = thrombin receptor agonist peptide.
these aggregates and the density of platelets per aggregate. Once again, monocytes were more active than neutrophils, with more than 80% of monocytes and half that amount of neutrophils, in forming conjugates. Responses to TRAP exceeded those of ADP, although there were no differences between ACS patients and controls; more than 90% of monocytes and more than 70% of neutrophils formed conjugates in the two groups. Platelet activity per monocyte was also twice as intense as the activity per neutrophil.

Clopidogrel completely reverted the number of spontaneous conjugates present in the circulation in ACS patients to control values and further prevented nearly all agonist effects of ADP and 50% of the effects of TRAP. The changes in the density of platelets per conjugate followed the same pattern as the changes in the number of conjugates (Figs. 3 and 4).

**Drug interactions.** Potential interactions between the use of other cardiovascular drugs and the antiplatelet effects of clopidogrel were explored. Beta-blockers, nitrates, and calcium antagonists had no influence on the amount of inhibition. The reduction in the density of platelets in neutrophil conjugates following clopidogrel was of a lesser magnitude in patients using a statin than in nonusers (14.7 ± 10.3% vs. 26.0 ± 7.2%, \( p < 0.05 \)). The baseline values were similar among patients using a statin versus those not (4.3 ± 1.0% vs. 4.6 ± 0.6%); they were also the same in patients already receiving a statin and in patients in whom the statin was initiated in the hospital. All other parameters, however, including aggregation to ADP and TRAP, P-selectin expression, and the number of platelet-leukocyte conjugates, were unaffected by the use of a statin.

**DISCUSSION**

This study provides new insights on the mechanisms, other than the inhibition of ADP-induced platelet aggregation, which could account for some of the benefits of clopidogrel in patients with an ACS. Importantly, TRAP-induced platelet activation and aggregation and platelet-leukocyte interactions were sensitive to the effects of clopidogrel and
thus were significantly reduced. Clopidogrel also reduced
the presence of spontaneous platelet-monoocyte and platelet-
neutrophil conjugates in the circulation, as well as the
plasma levels of P-selectin and CD40L. All these effects are
believed to be beneficial in patients with ACS and to
contribute to or be associated with controlling the disease.

Platelet aggregation and secretion. Adenosine diphos-
phate is a weak platelet agonist secreted from dense granules
of activated platelets and from damaged vascular tissue and
red blood cells, whereas TRAP is a strong agonist that acts
on platelets through the protease-activated receptors PAR-1
and -4. In the study, the two agonists were used at similar
concentrations. Despite the high specificity of clopidogrel in
blocking the ADP P2Y12 platelet receptor and preventing
ADP effects (1), the reduction of TRAP-induced platelet
aggregation attained 22%, equaling the absolute amount of
reduction in response to ADP. As a consequence, the level
of aggregation achieved by TRAP with clopidogrel was in
the range of that observed with ADP in the control state.
P-selectin expression, a marker of platelet activation, was
similarly reduced with the two agonists, supporting the
significance of the findings on aggregation.

These results are in contrast to those of previous studies
that have generally reported no or minimal inhibition of
TRAP or collagen-induced platelet aggregation with clopi-
dogrel (6,21,22). On the other hand, in line with our
findings, Klinkhardt et al. (19) reported an inhibition of
TRAP-induced P-selectin expression, along with a reduc-
tion in PAC-1 (monoclonal antibody specific for a platelet
activation complex near the fibrinogen receptor) expression.
The putative effects of clopidogrel observed in our study on
different pathways to platelet activation are supported by the
previous documentation of an important role of ADP and
the P2Y12 receptors in amplifying and modulating the
effects of various platelet agonists, including the effects of
thrombin on PAR-1 (23–25). The differences in results
between studies can likely be explained by the experimental
conditions, study populations, and concomitant drug ther-
apy. Patients with ACS enrolled in our study had a high
level of platelet activation, as witnessed by a hyper-
responsiveness to the agonist effects of ADP, compared
with controls, and elevated levels of circulating platelet-
monoocyte and platelet-neutrophil conjugates (Figs. 3 and
4). The platelet studies were performed 24 h after a loading
dose of 300 mg clopidogrel; this dose produces steady-state
inhibition of platelet aggregation in the range of 40% to
60%. Lower doses require many days before reaching the
full effect, and higher bolus doses result in the same extent
of inhibition occurring more rapidly (21).

Platelet-monoocyte and platelet-neutrophil conjugates.
As many as 45% of monocytes and 15% of neutrophils
circulated as heterotypical aggregates in our population of
ACS patients. These figures were significantly greater than
those in control subjects, suggesting a true elevation beyond
that induced by blood manipulation. Furthermore, these
numbers increased to 85% and 45%, respectively, after
stimulation with ADP in ACS patients, again significantly
more than in controls. With TRAP, the stimulation was
already maximal in both groups of patients. The formation
of leukocyte–platelet conjugates is mainly mediated by the
binding of P-selectin expressed on activated platelets to
P-selectin glycoprotein ligand-1 (PSGL-1) on leukocytes.
Other contributing mechanisms are fibrinogen, which
link cross-links activated GP IIb/IIIa receptors on platelets with
integrin MAC-1 (CD11b/CD18) on leukocytes, and the
platelet secretion products CD40L and RANTES (Regu-
lated upon Activation; Normal T cell Expressed and Se-
creted) that bind the respective receptors on monocytes and
neutrophils.

The association of platelet-leukocyte interactions with
ACS has been well demonstrated in various clinical settings
(12–16). The high proportion of platelet-monoocyte conjugates
observed suggests that they may play an important role in
the mechanisms of ACS, although a cause-effect relation-
ship remains to be documented. Similarly, the presence
of neutrophil conjugates and the platelet hyper-
responsiveness to agonist stimulation may suggest a more
general coronary or systemic inflammatory response. Con-
control of these aggregates with clopidogrel might therefore
contribute to the benefits of clopidogrel. Other antiplatelet
drugs have differential effects. Thus, GP IIb/IIIa antagonists can increase or decrease the number of platelets per conjugate and may increase the total number of conjugates as they activate platelets and promote P-selectin expression (26–28).

Variable effects of clopidogrel have previously been reported. In one study, the drug reduced the ADP agonist effects by 50% (18); in another study, it prevented the increase in platelet-monocyte and platelet-neutrophil conjugates induced by abciximab (19). These findings, however, were not reproduced in 51 patients who were administered 300 mg clopidogrel after successful stent implantation, followed by 75 mg/day; in that study, the number of platelet-leukocyte aggregates was significantly greater after 24 h, as clopidogrel reached its maximal effect on ADP-induced aggregation, whereas PAC-1 binding remained elevated for 30 days (29).

**Soluble platelet secretion products.** Platelet secretion products play important roles in the pathophysiology of ACS, as they mediate numerous interactions between platelets, the coagulation cascade, and the cells involved in inflammation and atherosclerosis. Their pathophysiologic role may differ substantially when expressed on membrane surfaces functioning by cell interaction or when released in blood then operating more systematically. Increased blood levels of soluble CD40L and of P-selectin have been reported in patients with unstable angina (30). However, few studies have looked at the effects of clopidogrel on these secretion products. Clopidogrel, but not aspirin, reduced ADP-induced membrane expression of CD40L in one study in normal volunteers (31). In another study, clopidogrel reduced ADP-induced membrane P-selectin expression but did not influence the soluble form (32). In the Clopidogrel in Unstable angina to prevent Recurrent Events (CURE) trial, long-term administration of clopidogrel had no effect on soluble P-selectin (33). In our study, both membrane P-selectin and soluble P-selectin were significantly reduced within 24 h of the administration of clopidogrel; soluble CD40L levels also decreased.

**Study limitations and conclusions.** All patients in our study were treated with unfractionated heparin, which is a direct pro-aggregant (34) that can also amplify the aggregation induced by other agonists, an effect partly related to activation of the P2Y_{12} receptor (23). Due to the study design, the effects related to heparin are not readily distinguished from those related to the disease. Similarly, the respective effects of clopidogrel and aspirin cannot be separated, as all patients received a combination of the two drugs. Post hoc analyses of data showed no interactions between the use of beta-blockers, calcium antagonists, and nitrates and the effects of clopidogrel. A weak interaction was found with a statin in only one of the multiple tests that were done, making a significant influence of statin therapy on our results unlikely. Although an interaction between atorvastatin and clopidogrel on cytochrome P450 (CYP) 3A4 has been reported (35), the significance of this interaction remains inconclusive (36). Recent studies have shown a variability in the response to clopidogrel in various platelet function tests and suggest that as many as 20% to 30% of patients could be poor responders (7). A similar variability was seen in our study in the various tests performed, including the inhibition of platelet-leukocyte complex formation. Whether this variability in response influences the clinical efficacy of the drug remains to be studied.

Another potential limitation of our study is the absence of a control group of ACS patients randomized to placebo or to no clopidogrel. Such a group could not be studied because of ethical issues. It could have influenced the results quantitatively but was unlikely to affect the general trends and conclusions of the study.

Finally, although clopidogrel only partially inhibited the agonist effects of ADP and TRAP, the inhibition to the two agonists was of the same magnitude in the clinical setting of our study, suggesting that they could both contribute to the global effects of the drug. More complete inhibition of the platelet purinergic receptors could be expected to magnify the benefits of pharmacologic inhibition of the ADP receptors. Such approaches are currently available with ATP analogues (18,22,37) and with a combination of P2Y_{1} and P2Y_{12} blockers (38) and may lead to new perspectives in antiplatelet therapy.

**REFERENCES**


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