Antiplatelet Effects of Clopidogrel Compared With Aspirin After Myocardial Infarction: Enhanced Inhibitory Effects of Combination Therapy

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OBJECTIVES
We sought to compare the inhibitory effects of the combination of two doses of aspirin plus clopidogrel with either drug alone on platelet aggregation and activation.

BACKGROUND
Enhanced platelet inhibitory effects of clopidogrel by aspirin on platelet aggregation and activation are suggested by experimental studies but have not been shown in humans.

METHODS
The effects of clopidogrel 75 mg or aspirin 100 (300) mg on platelet aggregation and activation by flow cytometry after stimulation with various agonists were determined in 30 patients with a past history of myocardial infarction.

RESULTS
Clopidogrel alone or in combination with aspirin markedly inhibited adenosine diphosphate (ADP)-mediated platelet aggregation compared with monotherapy with aspirin (24.6 ± 3.3% or 26.6 ± 2.7% vs. 44.7 ± 2.9%; p < 0.001). Combined treatment significantly inhibited collagen-induced aggregation compared with aspirin and clopidogrel (16.4 ± 2.4%, 36.5 ± 4.2% and 59.3 ± 5.1%, respectively; p < 0.001) and resulted in considerable inhibition of aggregation induced by thrombin receptor agonist peptide (TRAP, p < 0.03). Clopidogrel with or without aspirin significantly suppressed expression of platelet activation markers CD 62p, CD 63 and PAC-1 after stimulation with ADP or thrombin (p < 0.001). In addition, the combined treatment was more effective than either agent alone after activation with low dose thrombin (p < 0.05). Both doses of aspirin equally potentiated the platelet inhibitory effects of clopidogrel.

CONCLUSIONS
In this prospective clinical ex vivo platelet study, clopidogrel was more effective than aspirin in inhibiting ADP-mediated platelet aggregation and activation. Clopidogrel in combination with aspirin showed synergistic inhibitory effects after stimulation with collagen and thrombin compared with monotherapies. Thus, this dual antiplatelet treatment strategy deserves further evaluation in clinical trials for secondary prevention of acute myocardial infarction or unstable angina. (J Am Coll Cardiol 2000;36:699–705) © 2000 by the American College of Cardiology

Antiplatelet therapy reduces recurrent ischemic events in patients with cardiovascular disease (1). Aspirin, although most widely used, cost-effective and safe, is a weak platelet inhibitor affecting only the cyclooxygenase pathway. Despite long-term aspirin use, a large number of patients continue to have recurrent events associated with bad outcomes (2). Recurrent events in such patients may reflect natural progression of atherosclerosis or they take place through mechanisms that overcome the aspirin-induced inhibition of platelet function. This may occur by exposure of platelets to high local concentrations of strong agonists, such as collagen or thrombin, or by high levels of platelet released adenosine diphosphate (ADP) or thromboxane A2 from nonplatelet sources (3).

Recent clinical trials with intravenous platelet glycoprotein IIb/IIIa (GP IIb/IIIa) receptor blockers have demonstrated that aspirin monotherapy has been improved upon (4). Further evidence for a combined antiplatelet treatment strategy comes from studies with aspirin and ticlopidine. They have been shown to lower the risk of subacute stent thrombosis and to be safer when compared with conventional anticoagulant therapy (5–8) and aspirin alone (6). In addition, data with clopidogrel, a new thienopyridine derivative lacking the serious hematological side effects of ticlopidine, suggest that its combination with aspirin may be an alternative treatment after coronary stent placement (9). In patients with cardiovascular disease, clopidogrel alone was slightly more effective than aspirin in reducing the combined risk of ischemic stroke, myocardial infarction (MI) or vascular death (10). Although the effects of simultaneous administration of clopidogrel and aspirin on platelet aggregation and function in patients after MI has not been studied, experimental data suggest synergy between both drugs (11–13).

In our study we compared the inhibitory effects of the combination of aspirin and clopidogrel with either drug...
alone on platelet aggregation and platelet activation induced by various agonists in patients with MI. In addition, we analyzed whether the dose of aspirin was affecting the inhibitory potential of the combination therapy.

METHODS

**Patient selection.** Thirty consecutive patients with stable coronary artery disease and a history of MI older than three months on chronic aspirin treatment were recruited at the cardiology outpatient clinics at the University Hospital Bern. Patients between 37 and 77 years of age without hemorrhagic disorders were enrolled in the study after written informed consent was obtained. Patient characteristics were as follows: former smokers 57%; hypertension 47%; diabetes 17%; mean total cholesterol 5.2 ± 0.9 mmol/L; baseline medication was not changed throughout the study and included lipid lowering drugs in 40%, beta-adrenergic blocking agents in 50%, angiotensin-converting enzyme inhibitors in 47% and calcium antagonists in 27%. The study was performed according to the Declaration of Helsinki and followed protocol approval by the Institutional Review Board of the University Hospital Bern.

**Study design and timing of blood collection.** Patients (n = 30) were assigned to five consecutive treatment periods. At the end of each treatment period, venous blood samples for platelet activation and aggregation experiments were collected. The study consisted of an initial one-week run-in period (phase 1) where patients were treated with aspirin 100 mg daily. Then, clopidogrel 75 mg daily for seven days (phase 2). After a washout period of seven days for clopidogrel and aspirin, clopidogrel 75 mg daily was given alone for seven days (phase 3). After a washout period for clopidogrel and treatment with aspirin 300 mg for 14 days, the fourth measurements were performed (n = 12, phase 4). The final phase (n = 12) consisted of combined treatment with aspirin 300 mg and clopidogrel 75 mg daily for seven days. The Institutional Review Board felt that temporary discontinuation of aspirin and clopidogrel for an untreated control group was ethically not acceptable since it would add little additional information to this comparative study. Treatment periods were chosen to assess effects at the steady state of chronic drug administration (14). Since platelet functions return to normal approximately 10 days after stopping therapy with both drugs, corresponding to the lifespan of platelets, washout periods for either drug consisted of 14 days between phases 2 and 3 and 3 and 4, respectively.

**Studies on Platelets**

**Platelet aggregation in platelet rich plasma.** Thirty mL of whole blood were anticoagulated with 3.8% (wt/vol) sodium citrate (1 vol:9 vol). Platelet-rich plasma (PRP) was prepared by centrifugation at 800 × g for 2 min at room temperature. Platelet count of PRP was adjusted to 300,000/μL using platelet-poor plasma (PPP) obtained by further centrifugation (2,000 × g 15 min). The aggregometer (PAYTON, Dual Channel, Baxter, Switzerland) was calibrated for the difference in light transmission between PPP and PRP. Platelets were stimulated with ADP (Sigma, Switzerland) at concentrations of 3, 6 and 20 μmol/L, with collagen type 1 (Horn, Nycomed, Munich, Germany) at concentrations of 1.5, 4 and 40 μg/mL and with a thrombin receptor agonist peptide (TRAP 14-mer, SFLLRNPNDKYEFP, Fluka, Switzerland) at concentrations of 8, 10 and 15 μmol/L. Results were expressed as percent aggregation calculated linearly as percent light transmission between the optical densities of PPP and PRP. If necessary, collagen was added in additional doses to facilitate construction of a dose-response curve and calculation of ED50 values (defined as the concentration at which there is 50% aggregation). Arachidonic acid-induced aggregation (1 mmol/L, Servotec) was used to assess aspirin intake.

**Murine mAbs used in this study.** Platelet alpha-granule secretion was assessed using an anti-P-selectin mAb, CD 62p (P-selectin, Becton Dickinson); platelet lysosome degranulation was assessed using mAb CD 63 (Immunotech). Platelets were identified in PRP and whole blood using mAb 7H2, directed against GP IIIa (kindly provided by Dr. B. S. Coller, Mount Sinai Hospital, New York). PAC-1, a murine IgM-kappa mAb, binding to a conformation-dependent determinant on activated GP IIb/IIIa complexes unoccupied with adhesive proteins, was purchased from Becton Dickinson.

**Flow cytometry for measurement of platelet surface antigen expression.** The procedures for ex vivo detection of activated platelets have been described previously by Michelson (15), Warkentin (16) and us (17). Platelet-rich plasma was prepared immediately after venipuncture of 8.5 mL blood drawn into a 10 mL Monovette containing 1.5 mL of acid citrate dextrose (citric acid 38 mmol/L, sodium citrate 74.8 mmol/L, dextrose 124 mmol/L; pH 5.0) by centrifugation with 800×g for 2 min at room temperature. Platelets were counted (Sysmex K-1000) and adjusted to 300,000/μL with sterile filtered modified Tyrode’s buffer (137 mmol/L NaCl, 2.8 mmol/L KCl, 1 mmol/L MgCl2, 12 mmol/L NaHCO3, 0.4 mmol/L Na2HPO4, 0.35% bovine serum albumin, 10 mmol/L HEPES, 5.5 mmol/L glucose, pH 7.4). Platelet-rich plasma was then diluted 1:4 with modified Tyrode’s buffer. This dilution of blood into buffer decreases the platelet concentration, thereby reducing platelet-to-platelet interaction af-
ter addition of agonists (15). Diluted PRP was aliquoted into polypropylene tubes containing the agonists at the following final concentrations: ADP 0.1 μmol/L to 100 μmol/L (Sigma), human and equine fibrillar collagen type I 25 μg/mL to 75 μg/mL (Horm, Nycomed) and thrombin 0.05 U/mL to 0.5 U/mL (Diagnotec). Controls were effected in the absence of agonists. After incubation for 15 min, the platelets were fixed by adding paraformaldehyde 1% in phosphate buffered saline (PBS; ratio 1:1) and incubated with mAbs CD62p, CD63 and 7H2 at a final concentration of 10 μg/mL for 30 min. Controls were effected in the absence of primary antibody using PBS. Bound antibody was measured using fluorescein isothiocyanate (FITC)-conjugated F(ab')2 fragments of rabbit anti-mouse immunoglobulins (Dako) at a final concentration of 1.5 μg/mL after incubation for another 30 min.

Whole blood was used for PAC-1 platelet labeling, diluted 1:20 with modified Tyrode's buffer and aliquoted into polypropylene tubes containing PAC-1-FITC antibody (20 μg/mL; Becton Dickinson) and agonists at the predetermined final concentrations (ADP 0.01 to 10 μmol/L, collagen type I 25 to 75 μg/mL and thrombin 0.01 to 0.1 U/mL). The samples were incubated without stirring for 15 min, platelets were fixed by adding cold paraformaldehyde 1% (ratio 1:5) and analyzed within 2 h on FACScan (Becton-Dickinson). Fluorescence histograms were obtained for 10,000 cells gated per sample, data being analyzed using Cell Quest software (Becton-Dickinson). Antibody binding was expressed as the percentage of platelets positive for antibody. The gate for activated platelets was identified by mAb 7H2 and was set so as to include <1% of the events seen when identical platelet samples were incubated with the control murine immunoglobulin (IgM or IgG) used at the same concentration as the murine mAb. Percentages of activation of unstimulated platelets in PRP and whole blood were as follows (given as mean ± SEM): CD 62p, 1.0 ± 0.1%; CD 63, 1.7 ± 0.6% in PRP and PAC-1, 2.3 ± 0.4% in whole blood, respectively. These values are within our normal ranges from blood samples obtained from normal subjects.

Statistical analysis. Continuous variables were expressed as mean ± SEM. For comparisons between two treatment regimens in the same patient group, we used the Wilcoxon signed rank test. For comparison of the effects of different agonist concentrations within treatment groups, the Friedmann test was used. A value of p < 0.05 was considered to indicate a significant difference.

RESULTS

Platelet aggregation studies. As shown in Figure 1A, ex vivo platelet aggregation was induced by addition of ADP 3, 6 and 20 μmol/L in a dose dependent manner (p < 0.001). Adenosine diphosphate-induced aggregation was significantly inhibited in patients treated with aspirin 100 mg/day, clopidogrel 75 mg/day and aspirin 100 mg/day plus clopidogrel 75 mg/day. Results represent mean ± SEM. *p < 0.05 versus aspirin (Wilcoxon signed rank test). (B) Effects of different aspirin doses on ADP-induced platelet aggregation (percentage of light transmission in platelet-rich plasma) in 12 patients with aspirin 100 mg/day, aspirin 300 mg/day, aspirin 100 mg/day plus clopidogrel 75 mg/day and aspirin 300 mg/day plus clopidogrel 75 mg/day. Results represent mean ± SEM. *p < 0.05 versus aspirin (Wilcoxon signed rank test). ADP = adenosine diphosphate.
induced platelet aggregation slightly decreased from 5.5 ± 1.5 \mu g/mL in the aspirin 100 mg treated group to 2.5 ± 0.4 \mu g/mL in the clopidogrel 75 mg group (p < 0.05) and significantly increased to 22.4 ± 4.0 \mu g/mL in patients receiving the combined treatment (p < 0.001). Again, no differences in collagen-induced platelet aggregation (4 \mu g/mL) was seen between aspirin 100 mg and 300 mg alone (31.7 ± 7.7\% vs. 33.0 ± 5.1\%) or their combination with clopidogrel 75 mg, respectively (14.5 ± 4.5\% vs. 18.2 ± 4.3\%).

The thrombin receptor agonist peptide (TRAP) induced aggregation was not significantly different at the lowest agonist concentration (8 \mu mol/L) between all groups but tended to be lower in the group with combined treatment at the 10 \mu mol/L TRAP dose. At the 15 \mu mol/L TRAP dose where half-maximal aggregations were surpassed, the combination of aspirin 100 mg and clopidogrel 75 mg significantly reduced platelet aggregation compared with aspirin 100 mg or clopidogrel 75 mg alone (Fig. 3). No difference in TRAP-induced platelet aggregation (15 \mu mol/L) was seen between aspirin 100 mg and 300 mg alone (61.2 ± 3.8\% vs. 58.0 ± 3.9\%, respectively) or their combination with clopidogrel 75 mg (54.2 ± 3.4\% vs. 53.0 ± 3.7\%, respectively).

Aspirin alone or in combination with clopidogrel fully inhibited arachidonic acid–induced aggregation in all patients (3.6 ± 0.6\%), whereas clopidogrel alone did not inhibit arachidonic induced aggregation (74 ± 3.2\%, p < 0.001).

**Activation markers after platelet stimulation.** The expression and induction of CD62p indicating release reaction of alpha-granules, CD63 indicating lysosome secretion and PAC-1 directed against activation-dependent epitopes on GPIIb/IIIa were measured as markers of platelet activation. Interestingly, to achieve similar levels of activated platelets after agonist stimulation, lower ADP and thrombin concentrations were required for PAC-1 compared with platelet P-selectin or CD63 mAbs.

As shown in Figure 4, A to C, clopidogrel alone or combined with aspirin had a significant effect on the percentage of platelets positive for the three mAbs when compared with aspirin alone after stimulation with ADP. The combined therapy compared with clopidogrel alone equally suppressed the activation markers. These effects were seen independently of the ADP concentration used. When the groups treated with aspirin 100 mg and aspirin 300 mg were compared, the results were concordant for all three antibodies showing no difference in platelet activation except for a slightly lower P-selectin expression with aspirin 300 mg at the highest ADP concentration (100 \mu mol/L, Fig. 5). There were no differences in levels of activation epitopes for all three mAbs between the two doses of aspirin when they were combined with clopidogrel.

Platelet activation after thrombin stimulation is illustrated in Figure 4, D to F. Clopidogrel alone or in combination with aspirin compared with aspirin alone considerably suppressed the levels of platelet activation as measured by the three mAbs. In addition, the combination therapy compared with clopidogrel or aspirin alone significantly decreased platelet P-selectin expression and degranulation at low concentrations of thrombin, and a trend towards a reduction persisted at high thrombin concentrations. These effects of combined therapy on platelet activation were not seen with PAC-1 where full extent of activation was achieved at intermediate to high concentrations of thrombin. No differences in platelet activation levels between the two aspirin groups were detected with CD 63, CD 62p and PAC-1, except for CD 62p and CD 63 expression at the highest dose of thrombin representing extensive platelet activation (80.8 ± 1.3\% vs. 72.1 ± 2.4\% and 51.4 ± 3.7\% vs. 41.6 ± 3.3\% for aspirin 100 mg vs. aspirin 300 mg [p < 0.05], respectively). However, activa-
tion levels for the three mAbs were identical for both aspirin groups when combined with clopidogrel.

The expression of activation-dependent epitopes measured with the three mAbs were always lower than 20% after activation with human or equine collagen type I with concentrations of 25, 50, 75 μg/mL. Although a trend towards lower levels of platelet activation was detected in patients treated with clopidogrel alone or with the combination of clopidogrel with aspirin, the dose response curves were too flat to elicit significant differences between groups (data not shown). With our experimental conditions used, analysis of flow cytometric histograms showed no interference with microaggregate formation, suggesting that the collagen types used in our study were not effective enough to induce platelet activation. Alberio and Dale (19) recently demonstrated that collagen-induced P-selectin expression and upregulation of surface GP IIb/IIIa varies significantly depending on the type of collagen used.

Regarding the safety of clopidogrel, two patients developed a skin rash, which was rapidly reversible after discontinuation of the drug. Platelet count, hemoglobin concentration and leucocyte count remained unchanged throughout the study. There were no bleeding complications noted with each of the treatments.

**DISCUSSION**

This study in postmyocardial infarction patients demonstrates that clopidogrel alone or in combination with aspirin markedly inhibited ADP-mediated platelet aggregation in platelet-rich plasma compared with monotherapy with aspirin. Moreover, clopidogrel in combination with aspirin significantly reduced collagen-induced platelet aggregation compared with both monotherapies, suggesting a synergistic
platelet inhibitory effect. In addition, the combined treatment resulted in a mild inhibition of aggregation induced by stimulation of platelet thrombin receptor and was more effective in inhibiting platelet activation by thrombin. Both doses of aspirin equally potentiated the platelet inhibitory effects of clopidogrel. Preliminary data indicate that full inhibition of platelet activation is present after 2 to 6 h following oral administration of a loading dose of 300 mg or 525 mg (20, 21). The maximal inhibition at 2 h is equivalent to that achieved by a daily dose of 75 mg clopidogrel for seven days (21). Results from a dose-finding study demonstrated that the inhibition of platelet aggregation by clopidogrel was concentration-dependent at low doses, but no differences were seen in patients on daily clopidogrel doses of 50 mg, 75 mg and 100 mg for 7 or 28 days, indicating sustained and plateauphase effects during chronic administration at doses higher than 50 mg (22). A clopidogrel dose of 75 mg was chosen in our study and previously evaluated in the CAPRIE study (10). Although animal models suggest a dose-dependent inhibition of platelet-mediated thrombosis by clopidogrel, this effect is mostly seen within the first hours of intravenous administration (13), whereas it plateaus at intermediate doses after oral administration of clopidogrel for six days as demonstrated by a model of stent and graft thrombosis in baboons (11).

Mechanisms of combination antiplatelet therapy. Although the exact mechanism is not known, the synergy between clopidogrel and aspirin may be secondary to the different platelet activation pathways inhibited by these antiplatelet drugs. Our results suggest that simultaneous antagonism of thromboxane A2 by aspirin and adenosine diphosphate by clopidogrel resulted not only in inhibition of arachidonic acid- and ADP-mediated platelet activation but also in a reduction of collagen- and thrombin-induced platelet activation. Collagen is an important compound of the extracellular matrix of the vessel wall that is exposed after injury. Collagen-induced platelet aggregation at low doses is dependent on ADP release by activated platelets (14), whereas at high concentrations it can become ADP-independent, overcoming inhibition of aggregation by clopidogrel monotherapy as shown by our findings. Potent synergistic effects of clopidogrel and aspirin regarding the inhibition of collagen-induced aggregation, direct release reaction of platelets and platelet thrombus formation have also been demonstrated in preclinical trials in rabbits (12) and nonhuman primates (11). Similar effects were seen in a porcine model of high shear induced stent thrombosis (13). Moreover, in patients after stent implantation, ticlopidine—a prodrug that inhibits platelet function through inhibition of the ADP receptor—was found to have synergistic platelet inhibitory effects when combined with aspirin (23). Consistent with our results in patients after MI, these effects were seen particularly in collagen-induced aggregation but not in ADP-mediated aggregation.

Platelet activation and surface expression of adhesive glycoproteins play a key role in MI (24) and in thrombotic complications after coronary interventions (25, 26). Evaluation of platelet activation by flow cytometry uses monoclonal antibodies that recognize membrane glycoproteins present only on activated platelets. Previous studies showed that significant platelet activation as measured by surface expression of activated GPIIb/IIIa receptors and P-selectin, occurred in patients after acute coronary syndromes (27) and in patients after stenting receiving anticoagulation therapy compared with combined antiplatelet therapy with ticlopidine and aspirin (26). Our findings in patients after MI show that ADP-induced platelet activation measured with three monoclonal antibodies is significantly reduced by clopidogrel compared with aspirin, but platelet reactivity was not reduced further after ingestion of clopidogrel and aspirin compared with clopidogrel alone. However, a synergistic inhibitory effect on platelet alpha-granule and lysosome degranulation was observed using thrombin as a strong agonist at lower concentrations, but the effect was overpowered at higher concentrations. Since thrombin plays a pivotal role in platelet thrombus formation (28), the simultaneous administration of clopidogrel and aspirin may be beneficial in blocking the effects of physiologic levels of thrombin on platelets in patients with acute coronary syndromes.

Safety. Regarding the safety, reduction in the dose of aspirin is desirable because side effects, in particular those in the gastrointestinal tract, are dose-related. As a separate part of our study, we examined the antiplatelet effects of two aspirin doses (100 mg and 300 mg) in combination with clopidogrel 75 mg. Chronic administration of both doses of aspirin prevents thromboxane production in platelets, and available data suggest little if any difference in clinical efficacy (1). Our results confirm that platelet aggregation and reactivity is not dependent on the maintenance dose of aspirin, particularly when it is combined with an ADP-receptor antagonist. Both drugs lead to a sustained inhibition of platelet aggregation for the life span of the platelet (14). Recently, a large clinical trial showed an excellent safety profile of clopidogrel (10). Compared with aspirin it showed fewer side effects, and the incidence of significant neutropenia was 0.1% compared with a reported incidence of 1–3% for ticlopidine in clinical trials (29). Preliminary results from three nonrandomized trials (9, 30, 31) and the Clopidogrel Aspirin Stent International Cooperative Study trial (32) suggest that simultaneous administration of clopidogrel plus aspirin is safer than ticlopidine plus aspirin after coronary stent implantation. Although these studies suggest similar efficacy of both regimens in reducing coronary events after stenting, they were not sufficiently powered to answer the question of equivalence.

Study limitations. There are several limitations of this study, which was designed as a pilot investigation, and, therefore, has a small sample size of patients with chronic stable coronary disease. In addition, the study is a comparative investigation of different treatments but is lacking an
untreated control group. Although platelet reactivity was measured at steady-state drug administration, treatment periods were too short to assess long-term antiplatelet effects. Despite its wide use, there are also inherent limitations of ex vivo platelet aggregation studies, which are crude indicators of what is happening in vivo. However, using flow cytometric analysis as a second method for the evaluation of platelet reactivity and inhibitory effects of antiplatelet drugs, our findings were consistent with two independent methods.

Conclusions. In this prospective clinical ex vivo platelet study, we found that the combination of two antiplatelet agents acting through different pathways had greater inhibitory effects on platelet aggregation and activation than either agent alone in patients with chronic stable coronary disease. Thus, the concept of dual antiplatelet treatment with clopidogrel and aspirin is promising and deserves further evaluation in large randomized trials for secondary prevention in patients after acute MI or unstable angina.

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