Inhibition of Platelet Aggregation by Aspirin Progressively Decreases in Long-Term Treated Patients

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OBJECTIVES We sought to investigate, during a two-year follow-up period, the effects of aspirin on platelet aggregation.

BACKGROUND The platelets of patients given aspirin may be less sensitive to antiplatelet treatment, although the extent of such phenomenon over long-term follow-up is unclear.

METHODS Adenosine diphosphate (ADP) and collagen-induced platelet aggregation was periodically monitored before and after 2, 6, 12, and 24 months of treatment with aspirin (n = 150) or ticlopidine (n = 80) in patients matched for gender, age, and risk factors for atherothrombosis.

RESULTS Compared with baseline values, two months of aspirin treatment significantly inhibited platelet aggregation; thereafter, this inhibitory effect progressively decreased. At 24-month follow-up, collagen-induced platelet aggregation was significantly higher than that observed at two months (p < 0.05); a more pronounced difference was observed when collagen-induced lag phase was considered (p < 0.01). Restoration of platelet aggregation was less evident when ADP was used as an agonist. Conversely, the inhibition induced by ticlopidine was constant throughout follow-up with both agonists.

CONCLUSIONS The study demonstrates that a long-term treatment with aspirin is associated with a progressive reduction in platelet sensitivity to this drug. (J Am Coll Cardiol 2004;43: 979–84) © 2004 by the American College of Cardiology Foundation

Acetylsalicylic acid (aspirin) is an antiplatelet agent that inhibits platelet cyclooxygenase-1 (COX-1) and, as a consequence, prevents the formation of the pro-aggregatory substance, thromboxane A2 (TXA2). Although the clinical efficacy of aspirin in acute coronary syndromes has been well established, the evidence of its beneficial effects after myocardial infarction is based on a meta-analysis (1). It is also noteworthy that meta-analysis of antiplatelet therapy demonstrated a progressive decrease of aspirin’s clinical efficacy, particularly after two years of treatment (1). However, it has never been investigated whether this phenomenon is simply due to chance or whether prolonged treatment might provoke a reduced sensitivity to aspirin. Furthermore, previous studies have demonstrated an increased risk of cardiovascular events in patients with either acute coronary syndromes or undergoing invasive strategies and who were taking aspirin (2,3). Two previous studies analyzed the behavior of platelet aggregation in patients undergoing prolonged aspirin treatment, but the results were divergent (4,5). To further explore this issue, we have analyzed platelet aggregation in response to several agonists in patients treated with aspirin or ticlopidine for at least 24 months. We report, for the first time, that while the platelets of patients taking ticlopidine show a similar rate of inhibition during follow-up, the platelets of patients taking aspirin become less sensitive to antiplatelet treatment.

METHODS

Between 1997 and 2001, patients with clinical evidence of atherothrombosis were studied to check for platelet aggregation before and after antiplatelet treatment. Platelet aggregation was performed to analyze whether platelets were sensitive to the antiplatelet treatment chosen by the family doctor. Evaluation of platelet aggregation was retrospectively performed according to the following criteria: 1) accomplishment of an in vitro platelet aggregation test before and during aspirin (100 or 330 mg/day) or ticlopidine (250 mg/day) treatment, following a precise monthly schedule (2 months ± 10 days, 6 months ± 15 days, 12 ± 1 month, and 24 ± 1 month); 2) non-concurrent therapy with any other drug known to interfere with platelet function (e.g., non-steroidal anti-inflammatory drugs, corticosteroids, and the like); 3) compliance to aspirin treatment, as documented by the absence or more than 90% reduction of an in vitro platelet response to arachidonic acid at every test performed; and 4) the absence of acute coronary syndromes or coronary intervention in the previous three months or any other disease linked to platelet function.

Among the 176 patients taking aspirin, 150 (64 men, mean age 58 years [range 52 to 74 years]; 86 women, mean age 62 years [range 53 to 78 years]) met the inclusion criteria. Twenty-six patients who responded to arachidonic acid despite aspirin treatment (4 patients at 2 months; 5

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patients at 6 months, 8 patients at 12 months, and 9 patients at 24 months) were not included in the study and analyzed separately.

Eighty out-patients matched for gender, age, and risk factors for atherosclerosis and treated with ticlopidine (250 mg/day) instead of aspirin were enrolled with the same inclusion criteria and used as the control group.

Patients were considered hypertensive if blood pressure was >140/90 mm Hg in at least three different measurements when patients were in a supine position for at least 10 min or if they were treated with antihypertensive drugs. Patients were considered hypercholesterolemic if serum cholesterol was >240 mg/dl or if they were treated with lipid-lowering agents, and diabetic if they received treatment with oral antidiabetics or insulin.

No difference in the prevalence of hypertension (45% vs. 41%), hypercholesterolemia (31% vs. 34%), diabetes (7.2% vs. 8.5%), and smoking (15.3% vs. 16.4%) was observed between ticlopidine and aspirin-treated patients, respectively.

Blood samples anticoagulated with sodium citrate (ratio 9:1) were taken from each patient after at least 12 h of fasting. As previously described (6), platelet aggregation (according to Born’s method) was evaluated considering the maximal percentage (Mx%) of platelet aggregation in response to 2 μmol/l adenosine diphosphate (ADP) and 1 mmol/l arachidonic acid, and Mx% as well as the lag phase in response to 2 μg/ml collagen. The lag phase was defined as the delay time occurring between the addition of collagen and the beginning of the aggregation curve; the Mx% was calculated as the light transmission difference between platelet-rich plasma and platelet-poor plasma (100%). All of the platelet agonists used and the aggregometer were from Helena BioSciences (Sunderland, United Kingdom); concurrent control studies were performed to ensure that all agonists retained the same level of activity during the whole study.

**Statistical analysis.** All data are reported as the mean value ± SD and, where appropriate, as median values and ranges. To compare the groups, analysis of variance was performed for data normally distributed (lag phase), and post hoc analysis (Dunnett’s test) was used to test for changes from baseline to post-treatment times. The Kruskal-Wallis test was performed for skewed data (Mx% of platelet aggregation) to compare the different responses obtained before and after 2, 6, 12, or 24 months treatment. The Wilcoxon test was performed to further confirm the significant differences observed between the two groups. Significance was accepted at p < 0.05. The statistical package used was SPSS version 9.0 for Windows (SPSS Inc., Chicago, Illinois).

### RESULTS

The collagen-induced lag phase before and after aspirin treatment is reported in **Figure 1**. Compared with baseline, the lag phase was significantly prolonged at two months (76.6 ± 46.1 s vs. 36.1 ± 18.1 s, p < 0.001); such a phenomenon tended to progressively decrease at 6 (65.5 ± 31.5 s, p = NS vs. 2 months) and 12 months (58.5 ± 39.8 s, p < 0.05 vs. 2 months) and became similar to that observed at baseline after 24 months (42.5 ± 23.8 s, p < 0.001 vs. 2 months). At 24 months, 42% of patients, the lag phase values returned to baseline.

The Mx% of platelet aggregation in response to collagen decreased from 88.2 ± 21.8% to 37.9 ± 24.4% (p < 0.001) at 2 months. A progressive reduction of the aspirin effect was observed at 6 (46.1 ± 27.1%, p = NS vs. 2 months), 12 (48.7 ± 27.6%, p = NS vs. 2 months), and 24 months of therapy (61.9 ± 23.9%, p < 0.05 vs. 2 months) (**Fig. 2**). At 24 months, 43% of patients had the same light transmission values as those observed at baseline.

The reduced sensitivity to aspirin during follow-up was observed also when ADP was used as an agonist, but its behavior was different. Compared with baseline values (85.1
the rate of aggregation inhibition was less than that found with collagen, with values of 67.2 ± 19.3% at 2 months, 65.1 ± 19.1% at 6 months, and 68.3 ± 18.4% at 12 months. After 24 months of therapy, the rate of inhibition decreased (71.2 ± 16.2%, p < 0.05 vs. 2 months) (Fig. 3), with 41% of patients whose values returned to baseline. A separate analysis has been performed after inclusion of the 26 patients who responded to arachidonic acid despite aspirin treatment. The results showed no changes when this group of patients was included in the statistical analysis (data not shown).

We analyzed whether the sensitivity to aspirin was dependent on its dosage, but throughout the follow-up, no difference between patients given 100 mg/day or 300 to 330 mg/day aspirin with either agonist was observed (Fig. 4). Platelet sensitivity to aspirin was similar both in hypertensive and hypercholesterolemic patients (Fig. 5). The influence of smoking on platelet responses during aspirin treatment was not taken into consideration, as 95% of patients stopped smoking concomitantly with the beginning of antiplatelet treatment; none of them resumed smoking during follow-up.

During the two-year follow-up, the aggregometric responses of patients treated with ticlopidine (250 mg/day) showed a significant reduction at every time point analyzed (Table 1), the highest inhibition being found when ADP was used as an agonist. There were, however, a certain number of patients who did not show any change of ADP-induced platelet aggregation during follow-up; therefore, they were considered non-responders (8.7%).

**DISCUSSION**

Previous studies have demonstrated that patients taking aspirin have platelets that may respond to common agonists (7). By using an arbitrary unit of platelet resistance, Gum et al. (7) found that about 10% to 20% of patients given aspirin completely responded to one or more agonists immediately after the beginning of treatment. The uniqueness of our study is that we performed long-term follow-up and periodic monitoring of the platelet response to agonists. In this study, we demonstrated that the inhibition of platelet aggregation by aspirin progressively decreased no matter which agonist was used. The loss of antiplatelet effect...
started after 6 to 12 months of treatment and was more evident thereafter. It must be emphasized, however, that the reduced sensitivity to aspirin treatment was more evident using collagen as an agonist; this could be dependent on the sensitivity of the assay, as aspirin inhibits collagen-induced platelet aggregation potently but has only a modest effect on ADP-induced platelet aggregation (8).

Two previous studies (4,5) have already analyzed the behavior of platelet aggregation during a long period of follow-up. Berglund and Wallentin (4) randomized 193 patients with unstable angina to placebo or 75 mg/day aspirin and performed repeated measurement up to 24 months of follow-up. In the aspirin group, no change of ADP-induced platelet aggregation was detected, whereas collagen-induced platelet aggregation was significantly inhibited throughout follow-up. A potential limitation of this study was that in about half of the patients, baseline platelet aggregation was not performed; furthermore, blood sampling was obtained between 1 and 2 PM, thus a comparison of these results with those of the present study is quite difficult. Helgason et al. (5) studied 306 aspirin-treated patients with previous ischemic stroke and monitored platelet aggregation up to six months. Of the 306 patients recruited, 228 showed immediate inhibition of platelet aggregation and 78 had partial inhibition. During follow-up, only 119 patients with immediate and complete inhibition underwent repeat testing. Of these, 39 patients (32.7%) had lost part of this inhibitory effect, suggesting a reduced sensitivity to aspirin. This finding is in accordance with the results of the present study, indicating that platelet sensitivity to aspirin is progressively lost during long-term treatment.

An important point that has not yet been considered is whether platelet "resistance" to an antiplatelet drug is specific. To this purpose, we investigated whether a sort of resistance to ticlopidine may be also observed by time. We found that in patients taking ticlopidine, which inhibits platelet aggregation in response to ADP (9), platelet inhibition was constant over time, suggesting that the progressive reduction of the antiplatelet effect may be drug specific. This comparison, however, is limited by the fact that the ticlopidine-treated group was not included in a randomized study, but served only as a control group. Direct comparison between aspirin and ticlopidine should be tested in a future study. However, it should be noted that also in the group taking ticlopidine, a certain number of patients (8.7%) did not respond to treatment. This finding is consistent with the results of a recent study showing that after one month of treatment with clopidogrel, another ADP receptor antago-

Figure 5. Platelet aggregation (mean ± SEM) analyzed as lag phase and maximum percentage of aggregation (Mx%) in response to collagen (2 µg/ml) and as Mx% in response to adenosine diphosphate (ADP) (2 µmol/l) at baseline and throughout follow-up in hypertensive and hypercholesterolemic patients.

Table 1. Collagen (2 µg/ml) and Adenosine Diphosphate (2 µmol/l) Induced Platelet Aggregation During Ticlopidine Treatment

<table>
<thead>
<tr>
<th></th>
<th>Before Treatment</th>
<th>2 Months</th>
<th>6 Months</th>
<th>12 Months</th>
<th>24 Months</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen lag phase (s)</td>
<td>35.5 ± 14.1</td>
<td>46.6 ± 12.3°C</td>
<td>43.7 ± 17.7°C</td>
<td>48.5 ± 19.9°C</td>
<td>41.4 ± 21.5°C</td>
</tr>
<tr>
<td>Collagen (Mx%)</td>
<td>86.1 ± 12.8</td>
<td>79.2 ± 13.8°C</td>
<td>76.7 ± 17.5°C</td>
<td>71.2 ± 17.4°C</td>
<td>76.1 ± 19.2°C</td>
</tr>
<tr>
<td>ADP (Mx%)</td>
<td>86.1 ± 12.1</td>
<td>49.5 ± 17.2°C</td>
<td>45.1 ± 18.2°C</td>
<td>49.6 ± 19.4°C</td>
<td>37.2 ± 26.4°C</td>
</tr>
</tbody>
</table>

*p < 0.05; †p < 0.001 vs. baseline values. Data are reported as the mean value ± SD.
ADP = adenosine diphosphate; Mx% = maximum percentage of platelet aggregation.
nist, 15% of patients who underwent coronary stenting did not show any change of platelet aggregation (10).

Patients taking aspirin have evident clinical benefits, as demonstrated by a more recent meta-analysis (1), but it is also becoming evident that many patients are still at risk of cardiovascular events, despite the regular consumption of aspirin (11). A recent study demonstrated that patients taking aspirin who had elevated urinary excretion of 11-dehydro-thromboxane B2 (TXB2) were at high risk of cardiovascular events, suggesting that aspirin resistance might predispose to atherothrombotic complication (12).

However, the authors did not investigate whether the persistent elevation of 11-dehydro-TXB2 was dependent on activation of platelets or other cellular sources, such as monocytes and macrophages, which are potentially responsible for TXA2 formation. Platelet and extraplatelet mechanisms have been considered in explaining the resistance to aspirin (11). Even if the aim of our study was not to explore the mechanism of aspirin resistance, our findings show that despite aspirin treatment, platelet aggregation in response to common agonists is progressively restored. A genetic basis for explaining individual variations in response to aspirin, such as single-nucleotide polymorphisms of COX-1, may represent an intriguing mechanism that deserves further investigation (11).

Another interesting possibility is provided by a recent study demonstrating that under physiologic conditions, platelets express a negligible amount of COX-2, whereas newly formed platelets have a detectable amount of COX-2, which may contribute to TXA2 biosynthesis (13). Presently, we have no element to support the hypothesis that platelets become less sensitive to aspirin as a consequence of COX-2–dependent TXA2 production; such a suggestion needs to be investigated in a future study.

Even if we did not find any relationship between risk factors at baseline and sensitivity to aspirin during follow-up, we cannot be certain that other factors or modifications of the aforementioned reported risk factors could affect aspirin efficiency; this issue deserves further investigation in a larger number of patients.

**Conclusions.** We have demonstrated that in patients taking aspirin for a long period of time, platelets become progressively less sensitive to the drug. However, the clinical impact of this finding is limited by the lack of evidence that patients who are not sensitive to aspirin are at a higher risk of cardiovascular events. Using an aggregometric test, a recent study demonstrated that patients with “resistance” to aspirin were at a high risk of cardiovascular events (14). Thus, among the patients who were not sensitive to aspirin therapy, the rate of cardiovascular events was 24%, compared with 10% in patients who were sensitive to aspirin.

An intriguing finding of this study was the late divergence of the Kaplan–Meier curves, suggesting that the risk of cardiovascular events increased in relation to the duration of treatment. As the increase in cardiovascular risk was observed after at least one year of treatment, our study may provide insight into the mechanism accounting for the late occurrence of cardiovascular events in aspirin-treated patients and could support recent trials suggesting the need of adding other antiplatelet agents, such as clopidogrel, to aspirin for early and late reduction of cardiovascular events (15). Of particular interest were the findings of the Clopidogrel for the Reduction of Events During Observation (CREDO) trial (16), showing that prolonged treatment with such combined therapy reduced cardiovascular events, with a divergence of Kaplan–Meier curves that was more evident after nine months of follow-up, compared with aspirin–treated patients. Future studies should therefore address the question of whether the superiority of such a combination is dependent on a more complete and efficient inhibition of platelet aggregation or on a protective effect provided by clopidogrel in patients with a progressively reduced sensitivity to aspirin.

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