Relation Between Bleeding Time and Platelet Connective Tissue Reaction After Aspirin

By J. Hirsh, D. Street, J. F. Cade, and H. Amy

Aspirin prolongs the bleeding time in normal subjects and inhibits platelet release and aggregation with connective tissue and other biological agents. We have investigated one of the possible mechanisms by which aspirin prolongs the bleeding time by comparing the effects of aspirin and placebo on the bleeding time and platelet aggregation with connective tissue in normal volunteers. Two separate studies were performed. Both showed prolongation of the bleeding time and inhibition of the platelet connective tissue reaction after aspirin, but only the second study showed a significant correlation between these changes.

Both studies are reported in detail because the discrepancy between them illustrates some important principles that require consideration when relating the effects of drugs on platelet function in vitro to their effects in vivo. The findings suggest that when particular care is taken to standardize the measurement of the platelet connective tissue reaction in terms of the stimulus used, subject variability, and analysis of results, the prolonged bleeding time after aspirin can be shown to be related to the defect produced in the platelet connective tissue reaction.

The interaction of platelets with connective tissue, adenosine diphosphate (ADP), and thrombin is thought to be of fundamental importance in hemostatic plug formation. A number of antiinflammatory drugs, including aspirin, have been shown to decrease platelet aggregation with connective tissue and with low concentrations of thrombin and to inhibit the secondary wave of aggregation with ADP and with epinephrine. These effects are thought to be due to inhibition by aspirin of release of ADP from platelets. Aspirin has been shown to acetylate numerous plasma and platelet proteins, and it has been suggested that its effect on hemostasis may be related to this acetylation reaction. Aspirin has also been reported to increase the bleeding time in some normal subjects, as well as in patients with an underlying hemostatic defect. It has been suggested that the defect in ADP release produced by aspirin may be responsible for the prolongation of bleeding time. However, others have...
suggested that aspirin may have an additional effect on hemostasis through an effect on the vessel wall itself.\textsuperscript{22,23,25,26}

We have investigated the mechanism of prolongation of the bleeding time produced by aspirin, by relating the changes in bleeding time to the changes in platelet aggregation with connective tissue in normal subjects. Two separate studies were performed, the first having a number of shortcomings that were corrected in the second. Both studies are described in detail because the discrepancy in results illustrates some of the difficulties in using in vitro tests to assess platelet function in vivo.

**MATERIALS AND METHODS**

**Design of Studies**

*Study 1:* Forty normal subjects (all young adult females) were studied using a randomized, double-blind design. Twenty were given placebo and 20 were given aspirin. Tests were performed before treatment in all subjects, after 2 hr (in five of the placebo group and in 12 of the treatment group), after 24 hr (in 18 of the placebo group and in 18 of the treatment group), and after 48 hr (in four of the placebo group and in five of the treatment group). The study was completed within 3 mo.

*Study 2:* Thirteen normal subjects (11 males and 2 females) were studied using a randomized, double-blind cross-over design. All subjects were tested in the morning after a light fat-free breakfast, and smoking was prohibited for at least 24 hr before testing. The subjects were randomly allocated into initial aspirin or placebo groups, and 1 wk later the treatments were crossed over. A period of 1 wk was selected to allow the effects of aspirin to wear off in those subjects who were given aspirin first and placebo second. Bleeding times and platelet aggregation tests with connective tissue were performed before and 24 hr after treatment. The study was completed within 2 wk using a single batch of connective tissue suspension that was frozen in aliquots and thawed immediately before use.

In both studies all subjects agreed not to take any other medication for at least a week before and during the period of the study. No subject had a history of bleeding. Four 300-mg tablets of acetylsalicylic acid were given in a single dose as the active treatment and four 300-mg tablets of sodium bicarbonate were given as the placebo.

**Platelet Studies**

Blood was collected in plastic tubes containing 3.8\% trisodium citrate using a plastic syringe and a 19-gauge needle. The blood was mixed with citrate in a ratio of nine parts of blood to one part of anticoagulant. The blood was centrifuged at 300 g for 10 min at room temperature to obtain platelet-rich plasma. The platelet-rich plasma was dispensed into plastic tubes, and an aliquot was retained for platelet counting. The remaining blood was centrifuged at 2000 g for 15 min at room temperature to obtain platelet-poor plasma, which was decanted into separate plastic tubes. The platelet count of the platelet-rich plasma was adjusted by dilution with autologous platelet-poor plasma to obtain a count of 200,000/cu mm. Platelet aggregation was measured by a turbidimetric method,\textsuperscript{27,28} using a Payton aggregometer.

**Bleeding Time**

In the first study, the bleeding time was performed by a modified Ivy method. The tourniquet was inflated to 40 mm Hg, and three incisions (3 mm long and 3 mm deep) were made on the volar aspect of the forearm. The bleeding time was taken as the mean of the three individual measurements.

In the second study, the bleeding time was performed by the template method as described by Mielke et al.\textsuperscript{21} The tourniquet was inflated to 40 mm Hg, and two incisions (9 mm long and 1 mm deep) were made on the volar aspect of the forearm. The bleeding time was taken as the mean of the two individual measurements.
Preparation of Connective Tissue Suspension

Connective tissue (Sigma Chemical) was homogenized in a Waring Blendor using a semimicro container that contained the blending assembly in an ice-filled cooling jacket. Five grams of connective tissue were homogenized in 85 ml of 0.9% saline for 30 min. The suspension was centrifuged at 1740 g for 15 min at 4°C, and the supernatant material was aspirated, divided into aliquots, stored at −20°C, and thawed immediately before use. The platelet-aggregating activity of the connective tissue suspension remained stable for a period of at least a month when tested in serial dilutions in four normal controls not in the study, and new batches of suspension were prepared monthly.

Assessment of Platelet Aggregation and Analysis of Results

In the first study, the collagen concentration used in each experiment was that which produced between 30% and 50% maximum aggregation in the same two control subjects who were not in the study. Platelet aggregation was assessed by measuring: (1) the maximum change in optical density units, expressed as a percentage of the difference in optical density between platelet-rich and platelet-poor plasma for each determination (percentage aggregation), and (2) the time in seconds between the addition of connective tissue and the beginning of the upward deflection of the curve (reaction time). The effects of aspirin and placebo on the platelet connective tissue reaction were analyzed by comparing the absolute values of these measurements in the treatment group with those in the placebo group.

In the second study, platelet aggregation with connective tissue was assessed by measuring: (1) the maximum change in optical density units, expressed as a percentage of the difference in optical density between platelet-rich and platelet-poor plasma for each determination (percentage aggregation), (2) the slope of the curve at its steepest point (slope), and (3) the time in seconds between the addition of the connective tissue and the beginning of the upward deflection of the curve (reaction time). Serial dilutions of connective tissue were prepared, and each sample of platelet-rich plasma was tested at at least five dilutions of connective tissue to obtain a percentage aggregation over a range from greater than 75% to less than 25% on each occasion (Fig. 1). The effects of aspirin and placebo on the platelet connective tissue reaction were analyzed by calculating the change in concentration of connective tissue that was required to produce the same percentage aggregation, slope, and reaction time as in the pretreatment sample. Thus, the concentrated connective tissue suspension was considered to have 100% activity, a 1:2 dilution 50% activity, a 1:4 dilution 25% activity, and so on. For example, as shown in Fig. 1, if a 1:14 dilution (7% activity) produced 75% aggregation after placebo and a 1:2 dilution (50% activity) was required to produce 75% aggregation after aspirin, the change in concentration of connective tissue would be +43%. The results of the change in bleeding time produced by aspirin or placebo were then plotted against the change in connective tissue concentration required to produce 75%, 50%, and 25% aggregation (Fig. 2), a slope of 1, 0.75, 0.5, and 0.25 (Fig. 3), and a reaction time of 60 and 90 sec (Fig. 4).

RESULTS

Study 1

The results of the first study are summarized in Table 1. There was no significant difference in the pretreatment values for the bleeding time or for either of the two indices of the platelet connective tissue reaction between the placebo and aspirin groups. In the placebo group, there was no significant change at 2, 24, or 48 hr in the bleeding time or in the platelet connective tissue reaction. In the aspirin group, there was a significant increase in the bleeding time at 2 hr. At 24 hr, four subjects showed a considerable prolongation of the bleeding time, but there was wide individual variation so that
Fig. 1. Platelet reaction to increasing dilution of connective tissue (collagen) before and after placebo or aspirin in subject A.G. Platelet reaction has been expressed as percentage aggregation (top right), reaction time (bottom right), and slope (left).

Fig. 2. Relation between change in bleeding time and change in collagen concentration (%) required to produce aggregation of 75% (top), 50% (middle), and 25% (bottom) after placebo (closed circles) and aspirin (open circles). NS, not significant, r, correlation coefficient.
Fig. 3. Relation between change in bleeding time and change in collagen concentration (%) required to produce a slope of 1.00 (top), 0.75 (second from top), 0.50 (second from bottom), and 0.25 (bottom) after placebo (closed circles) and aspirin (open circles). NS, not significant; r, correlation coefficient.

Fig. 4. Relation between change in bleeding time and change in collagen concentration (%) required to produce a reaction time of 60 sec (top) and 90 sec (bottom) after placebo (closed circles) and aspirin (open circles). NS, not significant; r, correlation coefficient.
Table 1. Bleeding Time and Platelet Connective Tissue Reaction After Aspirin and Placebo (Study 1)

<table>
<thead>
<tr>
<th>Test</th>
<th>Placebo</th>
<th>Aspirin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre 2</td>
<td>24 48</td>
</tr>
<tr>
<td>Bleeding time (min)</td>
<td>4.08 (0.03)</td>
<td>5.00 (0.00)</td>
</tr>
<tr>
<td>Aggregation (%/o)</td>
<td>46.5 (3.27)</td>
<td>46.0 (4.32)</td>
</tr>
<tr>
<td>Reaction time (sec)</td>
<td>63 (4.1)</td>
<td>69 (3.6)</td>
</tr>
</tbody>
</table>

Pre, 2, 24, and 48 refer to measurements before and 2, 24, and 48 hr after treatment with either aspirin or placebo. Values are mean, with standard error in parentheses.

*Value after treatment significantly different from that before treatment (paired t test), tp <0.001.
†p <0.001.
‡p <0.05.

the over-all difference was not statistically significant. At 48 hr, the bleeding time in the five subjects tested was normal. There was a significant decrease in both indices of the platelet connective tissue reaction at 2 and 24 hr and in the percentage aggregation at 48 hr.

There was no significant correlation between the prolongation of the bleeding time at 24 hr and the inhibition of the platelet connective tissue reaction as assessed by either index. In fact, the three aspirin-treated subjects with the greatest decrease in the platelet connective tissue reaction showed the least prolongation of the bleeding time, whereas the subject with the greatest prolongation of the bleeding time showed the least decrease in the platelet connective tissue reaction.

Study 2

The results of the second study are summarized in Table 2. All subjects showed a significant prolongation of the bleeding time and impairment of the platelet connective tissue reaction for all three indices after aspirin, but there was no significant change in the bleeding time or in the platelet connective tissue reaction after placebo.

There was a significant correlation between the increase in bleeding time after aspirin and the increase in the connective tissue concentration required to produce 75% and 50% maximum aggregation but not 25% maximum aggregation (Fig. 2). There was also a significant correlation between the increase in bleeding time and the increase in connective tissue concentration required to produce a slope of 1 but not of 0.75, 0.5, and 0.25 (Fig. 3). There was no significant correlation between the increase in bleeding time and the increase in connective tissue concentration required to produce either a 60-sec or a 90-sec reaction time (Fig. 4).

DISCUSSION

The findings in both studies confirm other reports that aspirin prolongs the
BLEEDING TIME AND PLATELET CONNECTIVE TISSUE

Table 2. Changes in Bleeding Time and Platelet Connective Tissue Reaction After Aspirin and Placebo (Study 2)

<table>
<thead>
<tr>
<th>Test</th>
<th>Placebo</th>
<th>Aspirin</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bleeding time (min)</td>
<td>+0.01</td>
<td>+4.09</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>(1.08)</td>
<td>(2.83)</td>
<td></td>
</tr>
<tr>
<td>Aggregation (%/o)</td>
<td>75</td>
<td>+40.58</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>(3.84)</td>
<td>(18.59)</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>-0.54</td>
<td>+22.08</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>(4.16)</td>
<td>(13.33)</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>-1.15</td>
<td>+12.77</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td></td>
<td>(3.44)</td>
<td>(10.80)</td>
<td></td>
</tr>
<tr>
<td>Slope</td>
<td>1.00</td>
<td>+22.38</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>(2.74)</td>
<td>(15.26)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.75</td>
<td>+15.38</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>(1.64)</td>
<td>(9.64)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.50</td>
<td>+12.31</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>(1.70)</td>
<td>(8.35)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.25</td>
<td>+10.27</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>(1.61)</td>
<td>(6.34)</td>
<td></td>
</tr>
<tr>
<td>Reaction time (sec)</td>
<td>60</td>
<td>+21.00</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td></td>
<td>(3.15)</td>
<td>(14.31)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>+11.08</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td></td>
<td>(1.86)</td>
<td>(8.69)</td>
<td></td>
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</table>

Values are mean changes, with standard deviation in parentheses.

For platelet connective tissue reaction, the changes are in the concentration of connective tissue required to produce per cent aggregation, slope, and reaction times shown. p is derived from paired t test and compares change after placebo with change after aspirin.

bleeding time in normal subjects and inhibits the platelet connective tissue reaction. In addition, the second study demonstrates a direct correlation between the increase in bleeding time and the suppression of the platelet connective tissue reaction produced by aspirin, when this latter reaction is expressed in terms of percentage aggregation or of slope. Such a correlation has not hitherto been described, either because it was not sought or because, as in our first study, less precise methods were used.

The mechanism by which drugs such as aspirin impair hemostasis can be examined by correlating drug-induced changes in the result of in vitro tests with changes in a test of hemostatic function, such as the bleeding time. However, a correlation could be obscured if (1) the stimulus being examined is only one of several factors relevant to hemostasis, (2) if an inappropriate strength of stimulus is used in the in vitro tests, (3) if the strength of the stimulus used for the in vitro test varies during the course of the investigation, and (4) if the subject's response to the stimulus is affected by factors other than the drug being tested. The pharmacologic suppression of platelet function may also be important in the treatment of thrombosis, and it is likely that
these considerations are also relevant in the assessment of the antithrombotic effects of drugs that suppress platelet function.

The discrepancy between our two studies is of interest because it illustrates the importance of the general principles outlined above in relating platelet function as assessed in vitro to platelet function in vivo. The first study had the following shortcomings. (1) A number of different connective tissue suspensions were used over the 3 mo of the study, although all were prepared from the same dried extract. This resulted in wide variation in the platelet connective tissue reaction in the placebo group, despite the fact that the strength of the connective tissue stimulus was standardized against platelets from the same control subjects throughout the 3 mo of the study. (2) The connective tissue reaction was examined using only one strength of stimulus and was analyzed using only two indices of the platelet connective tissue reaction. (3) The effects of aspirin and placebo were compared using a randomized, double-blind design but with different subjects in each group. Thus, the drug effect may have been blurred by individual variations. (4) The bleeding time was performed by the Ivy method, which is less reproducible and less sensitive to aspirin than the modified template method used in the second study.

In the second study, an attempt was made to minimize these sources of error as far as possible. Thus, the effect of the stimulus was examined over a range of connective tissue concentrations, and the strength of the stimulus was rigidly standardized by using frozen aliquots of the same connective tissue preparation during the study, which was limited to a 2-wk period. The effects of variation of response between subjects were minimized by using each as his own control. Variation of the response within subjects was minimized by carefully standardizing factors such as diet, exercise, smoking, and diurnal variation. It was only when these variables were controlled that a direct correlation was found between the effect of aspirin on the bleeding time and the platelet connective tissue reaction and then only when a relatively strong stimulus was used. The results of our second study, therefore, support the hypothesis that the prolongation in the bleeding time produced by aspirin is causally related to suppression of the platelet connective tissue reaction.

REFERENCES


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