Trial of Repeated Low-Dose Aspirin in Diabetic Angiopathy

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We compared the ability of aspirin to suppress platelet aggregation and thromboxane synthesis in ten normal subjects and ten patients with diabetic angiopathy and high rate of entry of new platelets into the circulation. When single doses of 100 to 1,000 mg aspirin were ingested daily for 1 month, there were time gaps between doses in which platelets from diabetics and normals aggregated and formed thromboxane in vivo in response to the combination of arachidonic acid plus collagen. Similar gaps were also found for diabetics, but not for normals, following four daily doses (every six hours) of 25 or 100 mg. Our data show that dose schedules of aspirin which may suffice in normals are not effective in patients with diabetic angiopathy, presumably because these patients have a high rate of entry of new platelets into the circulation. We suggest that continual suppression of platelet thromboxane synthesis and aggregation by low-dose, "slow-release" preparations of aspirin would be an ideal long-term approach for the prevention of thrombosis in patients with a high rate of entry of new platelets into the circulation.

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There is a large body of evidence which suggests that blood platelets are involved in arterial thrombosis and atherosclerosis and that drugs which inhibit platelet function may prevent these disorders. The antithrombotic potential of aspirin is believed to be related to its ability to suppress the synthesis of the cyclic endoperoxides, prostaglandins G2 and H2, and thromboxane A2 (TXA2), which are potent inducers of platelet aggregation and secretion as well as vasoconstriction. However, in spite of extensive experimental and clinical studies, proper dosage and scheduling of this drug remain to be determined.

In many clinical trials, aspirin has been administered with widely different doses and schedules. Studies in normal subjects have suggested that after the administration of aspirin, the recovery by circulating platelets of the ability to synthesize prostaglandins and thromboxane, as well as the ability to aggregate and secrete, is related to the entry of new platelets into the circulation. Patients with diabetic angiopathy often have an abnormally short platelet survival time and high rate of entry of new platelets into the circulation. To inhibit their potential thrombotic role, these new platelets should be continually contacting aspirin. Therefore, we decided to determine whether repeated low doses of aspirin could accomplish this in normal subjects and in patients with diabetic angiopathy.

MATERIALS AND METHODS

Subjects

Because of the complexity of the study, only highly cooperative patients were chosen. Ten diabetic patients (six men, four women, 39 to 51 years old) and ten control subjects matched for sex, age, and weight were studied. These diabetic patients were selected because preliminary studies showed that they had an abnormally high rate of entry of new platelets into the circulation. We determined this by measuring malondialdehyde (MDA) formed by their platelets in response to N-ethylmaleimide (NEM) following the method of Catalano et al. An abnormally high entry of new platelets into the circulation determined in this manner correlates with a short platelet survival time determined by radioisotopic methods. Six patients had microangiopathy. Two of them had background retinopathy (i.e., hemorrhages, microaneurysms, and hard and soft exudates without any evidence of neovascularization as determined by fluorangiography): two had extensive neovascularization and two had undergone photocoagulation during the previous year. Four patients had macrovascular complications as judged by the presence of S-T wave depression on EKG (four); pathological Q waves (three); absence of peripheral pulses (one), presence of bruits over the carotid vessels (two), or history of angina pectoris or myocardial infarction (three). Patients with macrovascular complications were taking calcium channel blocking drugs. No patient had macro- or microalbuminuria. None of the patients or volunteers smoked. Three patients (1 with macro- and two with microangiopathy) were diagnosed as Type I diabetics because of the occurrence of at least one episode of ketoacidosis and an abnormal C-peptide response to arginine (less than 0.06 pmol/mL C-peptide following intravenous administration of 0.5 g/kg body weight of arginine over a 30-minute period; sensitivity of the method, 0.06 pmol/mL). No difference in the parameters reported in the results section was found between patients with micro- and macrovascular complications or between ketotic and nonketotic subjects. All the patients had been on a 1,500 Kcal diet with a polyunsaturated/saturated fatty acid ratio > 1 for at least a year and all were on twice-daily injections of regular plus intermediate monocomponent insulin (46 to 75 U/d, mean 59.2 ± 18.2 SD). None of the controls had a history of disease known to alter platelet aggregation or turnover. Because aspirin is present in so many "over-the-counter" medications, all subjects were asked to avoid cold remedies. In subjects in whom psychoactive drugs were required, seven control and three diabetics, the choice was restricted to Valium. Magnesium and aluminum hydroxides (Maalox) were taken when required for gastric discomfort. Neither patients nor controls had taken any other medication for at least ten days before donating blood. There was no difference between diabetic (188.2 ± 15.7 mg/dL, SEM) and control (196.7 ± 14.9) subjects in serum cholesterol or triglyceride levels (88.7 ± 12.1 mg/dL for patients and 96.9 ± 13.5 for normals). The body mass index for controls was 26.3 and for patients 25.7. Fasting plasma glucose concentrations of patients ranged between 125 mg/dL and 240 mg/dL (mean 187 ± 29). Glycosylated hemoglobin determined by the method of Rahbar was between 7.1% and 11.4% (mean 9.0 ± 1.1%) with normal values in our laboratory being 7.1 ± 0.5%. The average duration of diabetes was 8.4 years (range 7 to 13 yrs). Informed consent was obtained from all patients and volunteers after...
approval of the local Human Investigation Committee, and the studies were carried out according to the Principles of the Declaration of Helsinki.

Materials

Adenosine triphosphate (ATP), adenosine diphosphate (ADP) and aspirin (crystalline) were from Sigma Chemical, St Louis. ATP and ADP were dissolved in distilled water and stored in small aliquots at ~20°C. Collagen (col) was from Kollagen reagent Horm, from Hormon-Chemie, Munich; arachidonic acid (AA, >99% pure) was from Nuchek Prep., Elysian, Minn, and the sodium salt was prepared with 100 mmol/L NaHCO3. The luciferin-luciferase reagent (ChronoTime 395) was from Chrono-Log, Haverton, Penn. NEM and MDA were from Eastman-Organic Chemicals, Rochester, NY. Highly specific antibodies to TxB2 (Seragen, Boston) showed less than 0.05% cross-reactivity with prostaglandins E1, D2, and 6-Keto-PGF1α. Aspirin used in in vitro aggregation studies was dissolved fresh daily in 0.3 mol/L sodium acetate and kept at 20°C. Collagen (col) was from Kollagen reagent Bayer Italia (Bayer Italia, Milano, Italy); Aspirina Pediatrica (100-mg tablets, Bayer Italia) and Vivin C (330-mg tablets of aspirin, Menarini, Firenze, Italy) were used.

Studies on Platelet Aggregation, Secretion, TxB2 Synthesis and MDA Production

Nine volumes of blood were drawn into one volume of trisodium citrate (3.8%) and platelet-rich plasma (PRP) was prepared by centrifuging blood at 200 g for 15 minutes at room temperature. PRP was then held at room temperature and used within two hours after preparation. Platelet-free plasma (PFP) was obtained by centrifuging fresh PRP in an Eppendorf centrifuge (Brinkman Instruments, Westbury, NY) at 12,000 g for five minutes at room temperature. Platelet counts, determined by phase contrast microscopy, were adjusted to 3 × 10^6/mL by diluting the PRP of both volunteers and patients with PFP. Platelet aggregation and secretion was monitored in a lumi-aggregometer (ChronoLog, Haverton, Penn).4 For each sample and its control, arachidonic acid (1 mmol/L) or an equal volume of vehicle was added in microliter amounts to 0.5 mL of PRP which had been stirring at 1,000 rpm at 37°C for one minute. Fifteen seconds later, collagen 1 μg/mL or its vehicle was added to the mixture and the extent of aggregation and secretion was determined after three minutes. Secretion of ATP was monitored using 50 μL of firefly luciferase and luciferin reagent (10 mg/mL). The sensitivity was such that concentrations of released ATP as low as 0.1 μmol/L could be detected. TxB2 was measured by radioimmunoassay11 in aliquots of the supernatant solutions after completion of tests for platelet aggregation and secretion. Sensitivity of the assay was such that as little as 0.5 pmol TxB2 could be detected. Platelet MDA production after stimulation with NEM (50 mmol/L) was determined in platelets washed and resuspended in protein-free buffer as described by Catalano et al.25

Clinical Protocols

Phase 1: Trials of single daily doses of 100, 330 or 1,000 mg aspirin. Blood from all the patients and normal volunteers was collected two days and one day before the initiation of the study. The subjects were then instructed to ingest a 100-mg tablet of aspirin with the evening meal once a day for 1 month, and blood samples were collected twelve to fifteen hours after the first dose and every seven days thereafter. Ingestion of aspirin by all subjects was then stopped and 1 month later a similar trial was initiated with ingestion of 330 mg aspirin daily for 1 month. One month following the end of the 330-mg trial, a similar trial with 1,000 mg daily was carried out.

At the completion of each of these studies blood samples were collected every other day for ten days to measure the formation of MDA by platelets.

Phase 2: Trials of multiple doses of 25 or 100 mg aspirin. Preliminary evidence from Phase I indicated that single daily doses between 100 mg and 1,000 mg aspirin did not suppress platelet thromboxane synthesis, aggregation, or ATP secretion for 24 hours. Thus, there were long periods between doses during which significant platelet activity was present. Therefore we looked for a dosage schedule which would suppress platelet activity continually and two new studies were carried out. In both these studies, after a single loading dose of 100 mg aspirin, 25 mg or 100 mg aspirin were ingested four times daily for 4 weeks. In all subjects blood was collected before the initiation of the study, the day following the first four-times-daily dose schedule, and every week thereafter. Doses were taken at 6 am, 12 noon, 6 pm and 12 midnight. At each visit, blood was collected from each subject at 12 noon and 2 pm (ie, six hours after the morning dose and two hours after the 12 noon dose). As in the Phase I studies, at the end of 4 weeks the drug was discontinued and blood was collected every second day for ten days to measure platelet MDA formation.

Statistical Analysis

Student’s t test for paired and grouped data was used as appropriate. Nonparametric comparisons showed significant differences similar to those found by Student’s t test. Regression analysis was done using a 58C Texas Instrument calculator.

RESULTS

Platelet Aggregation and Secretion After Single Daily Doses of Aspirin (Phase I)

Twelve to fifteen hours after ingestion of the first dose of aspirin (100, 330, or 1,000 mg), platelets from normal or diabetic subjects did not aggregate or secrete ATP in response to AA (1 mmol/L). However, AA enhanced aggregation and secretion in response to collagen (1 μg/mL) (Fig 1) or to ADP (10 μmol/L) (data not shown). No difference in the amount of the enhancement was found regardless of the dosage of aspirin ingested (Table 1). In platelets from both normals and diabetics preincubation of PRP samples with aspirin (0.5 mmol/L) for 30 minutes at 37°C abolished the ability of AA to enhance platelet aggregation and secretion in response to collagen. Controls employing similar volumes of the vehicle for aspirin had no effect. Results similar to those obtained after the first dose of aspirin were found at each weekly measurement for each subject.

TxB2 Formation After Single Doses of Aspirin (Phase I)

In samples obtained before the administration of aspirin, platelets from diabetics formed 1.5 to 2 times as much thromboxane as those from normals when challenged with AA alone (1 mmol/L) (998 pmol ± 116/3 × 10^8 platelets in normals, n=1738 ± 204 in diabetics, p<0.01). Likewise, there was greater TxB2 formation (p<0.05) in platelets from diabetics than in those from normals in response to the combination of collagen (1 μg/mL) plus AA (1 mmol/L) (Table 2). Mean concentration of TxB2 in PFP collected after ingestion of aspirin was below the limit of detection and
Aggregation and secretion studies following the administration of 100 mg of aspirin in single daily doses. In all cases AA was used at 1 mmol/L and collagen at 1 μg/mL. The secretion in PRP samples from diabetic patients collected 12 to 15 hours after ingestion of AA (1 mmol/L), either employed alone or in combination with collagen (1 μg/mL) (Table 3). These platelets did not aggregate or secrete ATP in response to AA (1 mmol/L). In addition, AA did not enhance aggregation and secretion in response to collagen (1 μg/mL) or ADP (10 μM). PRP obtained from normal subjects six hours after the administration of 25 mg or 100 mg aspirin behaved similarly with respect to aggregation and secretion and only formed negligible amounts of TxB2 (Table 3). In contrast, in PRP samples collected from diabetic patients, TxB2 was formed in significant amounts (Table 3) and the synergistic effect of AA plus collagen or ADP on platelet aggregation and secretion was observed (data not shown). Incubation in vitro of these PRP samples from diabetics with aspirin (0.5 mmol/L) for 30 minutes at 37°C suppressed platelet TxB2 synthesis and the enhancement by AA of aggregation and secretion in response to collagen. Controls (vehicle for aspirin) were without effect. Similar results were obtained at each weekly measurement for each subject.

Return of MDA Formation in Circulating Platelets From Normal and Diabetic Subjects Following Cessation of Aspirin Ingestion

In both patients and normal volunteers, ingestion of aspirin was stopped after 4 weeks and the return of MDA formation by circulating platelets was determined. Fifty percent return of MDA formation in normals was achieved in 4.7 ± 0.2 days (range 3.5 to 5) while in diabetic patients it was achieved in significantly less time; 2.4 ± 0.2 days, p < 0.05 (Fig 2). Both in normals and diabetics no differences were found in the recovery of MDA formation with respect to the dosage or scheduling of aspirin and no correlation could

### Table 1. Aggregation and ATP Secretion in Response to the Combination of Collagen (1 μg/mL) Plus AA (1 mmol/L) by Platelets in PRP From Normal and Diabetic Subjects 12 to 15 hours After the Ingestion of Single Daily Doses of Aspirin (mean ± SEM)

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Before Aggregation (LTU)</th>
<th>After Ingestion of Aspirin (mg)</th>
<th>ATP secretion (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(100)</td>
<td>(330)</td>
</tr>
<tr>
<td>Normal</td>
<td>6.4 ± 1.3</td>
<td>3.7 ± 0.3*</td>
<td>3.2 ± 0.2*</td>
</tr>
<tr>
<td>(n = 10)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATP secretion</td>
<td>3.3 ± 0.3</td>
<td>2.1 ± 0.3*</td>
<td>2.0 ± 0.2*</td>
</tr>
<tr>
<td>Diabetics</td>
<td>6.8 ± 1.2</td>
<td>4.1 ± 0.4*</td>
<td>4.5 ± 0.4*</td>
</tr>
<tr>
<td>(n = 10)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATP secretion</td>
<td>3.6 ± 0.2</td>
<td>2.8 ± 0.2*</td>
<td>2.9 ± 0.2*</td>
</tr>
</tbody>
</table>

n = number of subjects tested.

LTU, light transmission units; 1 unit = 10% light transmission.

*p < 0.05 before vs after aspirin (both normals and diabetics). All other comparisons are not statistically significant (p > 0.05).
be demonstrated between the time at which platelets had recovered 50% of their ability to form MDA and microangiopathy \( r = 0.31 \), macroangiopathy \( r = 0.43 \) or glycosylated hemoglobin \( r = 0.50 \).

**DISCUSSION**

When used singly at appropriate concentrations in vitro, all naturally occurring aggregating agents induce full platelet aggregation and secretion. Such aggregation and secretion may also be induced by combinations of small amounts of agents at concentrations that are ineffective when used alone. This synergistic effect has been demonstrated in vivo and is likely to play a major role in the early stages of hemostasis and thrombosis. Animals, such as mice, which are very resistant to the thrombotic challenge of intravenous injection of large amounts of single aggregating agents, died with massive occlusion of the lungs by platelet thromboemboli after the injection of very low doses of combinations of aggregating agents.

Prostaglandin endoperoxides and thromboxane must play a critical role in the synergistic effect of combinations of agents in vivo as well as in vitro. For example, extremely low levels of thromboxane in vitro, in combination with other aggregating agents, are sufficient to induce full aggregation and secretion. In addition, aspirin and other cyclooxygenase inhibitors protect mice against the thrombotic effects of intravenous injections of combinations of aggregating agents. Thus aspirin must abolish the synthesis of prostaglandins and thromboxane to achieve a maximal antithrombotic effect. We previously showed that the combination of collagen plus AA is the most potent for revealing the presence of the very small numbers of platelets able to form prostaglandins and thromboxane. Therefore we used this combination to evaluate the effectiveness of single daily doses of aspirin and found that regardless of the dosage of aspirin employed, 12 to 15 hours after the administration of a single dose, when about 98% of thromboxane formation was inhibited, the residual 2% sufficed to enhance aggregation and secretion of platelets from normals (Table 1). Similar results were found in platelets from diabetics (Tables 1 and 2). Thus, we did not find a single daily dose of aspirin in the range of 100 mg to 1,000 mg able to suppress the synthesis of PG endoperoxides/thromboxane for a full 24 hours. This suggested that the antithrombotic effect of a single large dose of aspirin is limited in time (will not last for

![Fig 2. Return of MDA formation in platelets from normal subjects or diabetic patients after cessation of a regimen of 100 mg aspirin for 1 month. Each point is the mean ± SEM of the data from all the subjects studied. The arrows indicate the time at which circulating platelets had recovered 50% of their original (before aspirin) ability to form MDA.](image-url)
more effective.

It is known that the inhibition of prostaglandin and thromboxane synthesis by aspirin in circulating platelets is an irreversible process.\(^1\) Therefore the formation of prostaglandins and thromboxane following the administration of aspirin depends on the entry into the circulation of new platelets whose ability to synthesize prostaglandin endoperoxides/thromboxane has not been affected by contact with aspirin. Studies using platelets labelled with \(^3\)Cr indicate that approximately 10% of platelets turnover every day.\(^2\)\(^5\)\(^28\)

Using combinations of collagen plus AA we saw evidence of newly formed platelets 12 to 15 hours after the administration of aspirin. Within that time period approximately 5% to 6% of newly formed platelets would be in the circulation. Since at least 2% to 3% of platelets that have not contacted aspirin are required to produce concentrations of thromboxane sufficient to act synergistically with other agents,\(^1\) we thought that dosing with aspirin four times a day would suffice to continually abolish the synthesis of thromboxane. In addition to its inhibitory effects on platelets aspirin also inhibits the synthesis by endothelial cells of the vasodilator and antiaggregating agent prostacyclin, and this could limit its usefulness.\(^1\)\(^4\) This possible limitation has been countered by observations on the “dose-related” selectivity of aspirin, since single, low, daily doses almost completely suppress TxB\(_2\) synthesis with little apparent effect on prostacyclin formation.\(^29\)\(^33\)\(^34\) There has been a recent report on the anti-thrombotic efficacy of low-dose aspirin.\(^34\) However, the possible role of prostacyclin in thromboresistance is still uncertain.\(^4\)\(^5\)\(^35\)\(^36\)\(^37\)\(^38\)\(^39\) Using a low-dose, four-times-a-day schedule, we found that 25 mg aspirin suppressed thromboxane synthesis and aggregation in response to collagen plus AA by platelets from normals. However, four daily doses of 25 mg or 100 mg still left time intervals in which platelets from diabetic patients aggregated and formed thromboxane in response to the combination. It is unlikely that an impaired sensitivity of circulating platelets to the inhibitory effect of aspirin is involved in this difference since two hours after the administration of 25 mg of aspirin thromboxane synthesis was abolished in platelets from normals as it was in normals. In addition, when incubated in vitro with aspirin, platelets from normals and diabetics behaved similarly with respect to inhibition of prostaglandin and thromboxane synthesis. Our MDA data (Fig 2) show that the daily entrance of new platelets into the circulation of diabetic patients is about twice that of normal subjects. It is conceivable that the minimal amounts of cyclooxygenase/thromboxane required to potentiate aggregation and secretion in response to collagen are related to the abnormally high rate of entry of new platelets into the circulation of these patients. Newly formed platelets are considered to be larger in size than older platelets\(^50\) and the conversion of AA to prostaglandins and thromboxane is reported to be proportional to the size of platelets.\(^51\) More recently there has been a report indicating that in rabbits young platelets make more thromboxane than older platelets.\(^52\) We found increased synthesis of thromboxane by platelets from patients with an abnormally high rate of entry of new platelets into the circulation. It is also possible that the pharmacokinetics of aspirin may differ in diabetics and normals and so influence our observed findings.

Our observations show that dosage schedules of aspirin based on studies in normal subjects are not applicable to patients with diabetic angiopathy and that doses of aspirin given at relatively long intervals allow for a temporal hiatus in which new platelets entering the circulation cause full aggregation and secretion of ATP in response to combinations of aggregating agents. Thus, continual suppression of platelet prostaglandin and thromboxane synthesis may greatly enhance the antithrombotic potential of aspirin. This has previously been suggested for dosage of aspirin to suppress platelet thromboxane formation without affecting endothelial cell prostacyclin synthesis.\(^16\)\(^17\)

Since very frequent oral dosing is not practical, low-dose, slow-release preparations of aspirin which will not allow time gaps in the suppression of prostaglandin endoperoxides and thromboxane synthesis might be the ideal long term approach for the prevention of thrombosis in patients with a high rate of entry of new platelets into the circulation.

In summary, our findings indicate that a small amount of thromboxane can go a long way toward promoting platelet aggregation and a possible thrombotic episode, and that during a course of treatment with high or low dose aspirin there are time periods between doses during which the platelets in PRP from diabetic angiopathic patients can make biologically significant amounts of PG endoperoxides/thromboxane. We suggest that during such time gaps, a thrombotic event could occur. These findings provide the rationale for the continued suppression of cyclooxygenase activity in such patients. This might be accomplished by low-dose, slow-release aspirin.

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