Erythrocyte Promotion of Platelet Reactivity Decreases the Effectiveness of Aspirin as an Antithrombotic Therapeutic Modality

The Effect of Low-Dose Aspirin Is Less Than Optimal in Patients With Vascular Disease Due to Prothrombotic Effects of Erythrocytes on Platelet Reactivity

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Background—Aspirin (acetylsalicylic acid, ASA) is widely used for secondary prevention of ischemic vascular events, although its protection only occurs in 25% of patients. We previously demonstrated that platelet reactivity is enhanced by a prothrombotic effect of erythrocytes in a thromboxane-independent manner. This diminishes the antithrombotic therapeutic potential of ASA. Recent data from our laboratory indicate that the prothrombotic effect of erythrocytes also contains an ASA-sensitive component. In accordance with this observation, intermittent treatment with high-dose ASA reduced the prothrombotic effects of erythrocytes ex vivo in healthy volunteers. In the present study, the effects of platelet-erythrocyte interactions were evaluated ex vivo in 82 patients with vascular disease: 62 patients with ischemic heart disease treated with 200 mg ASA/d and 20 patients with ischemic stroke treated with 300 mg ASA/d.

Methods and Results—Platelet activation (release reaction) and platelet recruitment (fluid-phase proaggregatory activity of cell-free releasates from activated platelets) were assessed after collagen stimulation (1 mg/mL) of platelets, platelet-erythrocyte mixtures, or whole blood. Platelet thromboxane A2 synthesis was inhibited by >94% by ASA administration in all patients. Importantly, platelet recruitment followed one of three distinct patterns. In group A (n = 32; 39%), platelet recruitment was blocked by ASA both in the presence and absence of erythrocytes. In group B (n = 37; 45%), recruitment was abolished when platelets were evaluated alone but continued in the presence of erythrocytes, indicating a suboptimal effect of ASA on erythrocytes of this patient group. In group C (n = 13; 16%), detectable recruitment in stimulated platelets alone persisted and was markedly enhanced by the presence of erythrocytes.

Conclusions—In two thirds of a group of patients with vascular disease, 200 to 300 mg ASA was insufficient to block platelet reactivity in the presence of erythrocytes despite abolishing thromboxane A2 synthesis. Platelet activation in the presence of erythrocytes can induce the release reaction and generate biologically active products that recruit additional platelets into a developing thrombus. Insufficient blockade of this proaggregatory property of erythrocytes can lead to development of additional ischemic complications. (Circulation. 1998;97:350-355.)

Key Words: aspirin ▪ blood cells ▪ platelets ▪ cardiovascular diseases ▪ cerebrovascular disorders

Aspirin is the most commonly used therapeutic agent in prevention of vascular ischemic events. However, there is a large group of patients with thrombotic disorders who do not respond to ASA treatment. Several factors may contribute to this clinical situation. Thus ASA-treated platelets can respond to high-dose ADP, collagen, or thrombin via cyclooxygenase-independent mechanisms. Cell-cell interactions are another factor that can modify the response of ASA-treated platelets to agonists. In particular, cell-cell contact between activated platelets and erythrocytes or neutrophils may modulate the effect of ASA on platelets.

We previously demonstrated that erythrocytes markedly increase platelet eicosanoid formation, promote release of intracellular platelet granule components, and induce recruitment of additional platelets from the microenvironment into the forming thrombus. This enhancement of platelet reactivity via cell-cell interactions is readily demonstrated when erythrocytes are brought in close proximity to either
ASA-free or ASA-treated platelets stimulated to overcome the ASA-induced defect.8–10

It is possible that therapeutic ASA regimes directed toward inhibition of platelet function, as evaluated in vitro in platelet-rich plasma, might not reflect therapeutic efficacy in vivo due to the prothrombotic effects of erythrocytes.13,14 For an optimal antithrombotic effect of ASA, we believe the platelet-stimulating activity of erythrocytes must be neutralized. We recently demonstrated that ASA dose–dependently decreases erythrocyte prothrombotic activity toward platelets from normal subjects ex vivo.13 It has not as yet been established whether chronic treatment with moderate doses of ASA (200 to 300 mg/d), can reduce the prothrombotic effects of erythrocytes in patients with chronic vascular diseases. We have now determined the effect of erythrocytes on platelet reactivity in two groups of patients with vascular disease: (1) patients with ischemic heart disease treated with 200 mg ASA/d and (2) patients with ischemic stroke receiving 300 mg ASA/d.

**Methods**

**Patients and Control Subjects**

Sixty-two IHD patients (mean age, 65±10 years; range, 39 to 83; men/women, 52/10) treated with 200 mg ASA/d (Adiro, Bayer) for >3 months were studied. Clinical diagnoses included myocardial infarction (n=17), unstable angina (n=39), stable angina (n=3), or congestive heart failure (n=3) (Table 1). Diagnostic procedures included ECG, exercise bicycle tests, echocardiography, cardiac catheterization, and serum enzyme analyses.

Twenty patients with ischemic stroke (CVD) (mean age, 66±11 years; range, 44 to 79; men/women, 12/8) were evaluated after >3 months of treatment with 300 mg ASA/d (sustained-release, Tromal, Madaus Cerafarm, SA). Eleven of the patients had atherosclerotic brain ischemia, 8 lacunar infarct, and 1 transient ischemic attack (TIA) (Table 2). All of the patients underwent Doppler sonography, computed or magnetic resonance imaging, and cerebral angiography when appropriate. Functional evaluation of patients was performed using the Rankin scale (mean of the group, 1.5; range, 1 to 3) and the Barthel index (mean, 94.8; range, 65 to 100). Patients with cerebral ischemia due to embolic occlusion were excluded.

Compliance with aspirin treatment in the patients was ascertained by a personal interview with each before venesection.

Normal ASA-free subjects were evaluated concomitantly as control subjects for platelet recruitment (n=30), platelet 5HT release (n=106), and platelet TXA2 synthesis (n=64). In addition, these parameters were determined in 23 normal subjects, 2 hours after ingestion of a single dose of ASA (500 mg). Blood chemistries and hematological parameters were in the normal range for all subjects (Dax-72 autoanalyzer, Bayer Diagnostics; Sysmx-NE-8000 autoanalyzer, Toa Medical Electronics). Volunteers had not taken any medication for at least 15 days before blood collection or administration of the single dose of ASA.

**Selected Abbreviations and Acronyms**

ASA = acetylsalicylic acid (aspirin)
CVD = cerebrovascular disease
HID = ischemic heart disease
PPP = platelet-poor plasma
PRP = platelet-rich plasma
RBC = red blood cell(s)
TXA2/B2 = thromboxane A2/B2
WB = whole blood
5HT = serotonin

**Blood Cell Collection and Processing**

Citrate-anticoagulated venous blood (129 mmol/L; 9:1 vol:vol) was collected from patients and control subjects after an overnight fast into siliconized glass tubes (Vacutainer; Becton Dickinson), according to a protocol approved by the Institutional Review Board. Blood samples from patients were obtained before daily ASA intake.

PRP and PPP were prepared by differential centrifugation (200g and 2500g, respectively, 15 minutes, 22°C). After removal of PRP, PPP, anduffy coat, 1 mL erythrocytes were removed from the central area of the erythrocyte zone in the tubes.

SHT release was used as a marker of the platelet release reaction to monitor platelet activation. Platelets were radiolabeled with serotonin (13C-5HT, 55 mCi/mmol, Amersham International Plc) as previously described.8,15 Supernatants of PRP, PRP plus erythrocytes, or whole blood to which only agonist buffer was added served as control, and SHT content was subtracted from sample values.15

Production of platelet TXA2 was measured in patients and control subjects. Supernatants of collagen–stimulated whole blood were studied by radiommunoassay of the stable TXA2 metabolite TXB2.9 Agonist buffer alone served as control.

**Measurement of Platelet Activation and Recruitment**

Platelet activation and recruitment were independently evaluated using the activation-recruitment system, a two-stage in vitro procedure previously described.8–10 Platelets (1.8×105/mL) (PRP), platelets plus erythrocytes (PRP+RBC, hematocrit 40%), or WB was incubated (10 minutes, 37°C). Collagen (1 µg/mL) was added as platelet agonist and the tube contents mixed by inversion (10 seconds). A cell-free releasate, obtained within 1 minute by centrifugation (13 000g), was either used for biochemical studies to assess platelet activation (SHT, TXB2), or as an inducer of platelet aggregation in a PRP assay system (recruitment), evaluated by optical aggregometry.8–10 Recruitment was expressed as maximal height (mm) of the

**TABLE 1. Patients With Ischemic Heart Disease**

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>n</th>
<th>%</th>
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<tr>
<td>Myocardial infarction</td>
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<td>Unstable angina</td>
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<td>63</td>
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<td>Stable angina</td>
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<td>4.8</td>
</tr>
<tr>
<td>Congestive heart failure</td>
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<td>4.8</td>
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<td>Atrial fibrillation</td>
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<tr>
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<td>54.8</td>
</tr>
<tr>
<td>Intermittent claudication</td>
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<td>11.3</td>
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<tr>
<td>Coronary artery bypass grafting</td>
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<tr>
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<td>1.6</td>
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<tr>
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<tr>
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<tr>
<td>Calcium channel blockers</td>
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<td>45.6</td>
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<tr>
<td>β-Adrenergic blockers</td>
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<tr>
<td>Antihypertensives</td>
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<td>Hypoglycemics</td>
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</tr>
<tr>
<td>Lipid-lowering agents</td>
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<td>14.5</td>
</tr>
<tr>
<td>Sedatives or hypnotics</td>
<td>18</td>
<td>29</td>
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**TABLE 2.** Medications

<table>
<thead>
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<th>Category</th>
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<td>28.7±16.4</td>
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<td>Calcium channel blockers</td>
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<td>Lipid-lowering agents</td>
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<td>14.5±16.4</td>
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<td>Smoking</td>
<td>24±10</td>
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aggregation response using autologous PRP. This procedure represents an in vitro model of platelet thrombus growth. Erythrocyte promotion of platelet reactivity takes place in the absence of lysis and only occurs with metabolically intact erythrocytes.6,7 Patients were classified into three groups on the basis of the effect of aspirin on their platelet reactivity: group A, patients who demonstrated total inhibition of platelet recruiting activity with platelets alone as well as with platelets in the presence of erythrocytes; group B, patients whose platelets alone were devoid of recruiting activity but demonstrated recruitment in the presence of erythrocytes; and group C, patients who demonstrate detectable recruitment even in platelets alone.

Statistical Analyses

The paired Student’s t test was used to compare effects of platelets versus platelets plus erythrocytes from the same donor. Data are expressed as mean ± SD.

To evaluate possible effects of other medications taken in parallel with aspirin on the data obtained, one-way ANOVA plus Duncan’s test was used. The results demonstrated that platelet reactivity was not affected by medications taken in parallel with aspirin (data not shown).

Results

Recruitment in Platelets From Aspirin-Treated Patients With Vascular Disease

In 30 of 62 patients with IHD treated with ASA (200 mg/d), platelet recruiting activity was undetectable when evaluated in platelets alone, in combined suspensions of platelets plus erythrocytes, or in whole blood (group A, data not shown). Moreover, TXA2 synthesis in the patients’ platelets was abolished by ASA, as evaluated in collagen-stimulated whole blood (Table 3). However, 25 IHD patients with null recruiting activity in platelets alone showed detectable recruitment when their platelets were evaluated in the presence of erythrocytes or whole blood (Fig 1, group B). This took place in the setting of total inhibition of platelet TXA2 synthesis (Table 3). The 7 remaining IHD patients had detectable platelet recruitment even when evaluated in platelets alone (Fig 1, group C). Furthermore, recruitment was markedly amplified when measured in the presence of erythrocytes or whole blood (Fig 1, group C). These data indicate that patients can be classified into three groups according to their platelet responsiveness as designated above.

Evidence for three clearly defined groups according to the effects of ASA on platelet recruitment was also obtained in 20 ischemic stroke patients. Only 2 of the 20 CVD patients treated with 300 mg/d ASA had undetectable recruiting activity in the presence or absence of erythrocytes (group A, data not shown). This was accompanied by total absence of TXA2 synthesis (Table 3). Twelve of 20 CVD patients had
complete inhibition of platelet recruitment when measured in platelets alone. However, there was notable recruiting activity in stimulated platelet-erythrocyte mixtures or whole blood (Fig 2, group B), in the absence of TXA2 synthesis (Table 3). Six CVD patients (Fig 2, group C) demonstrated recruitment in platelets alone, which increased fivefold in the presence of erythrocytes or whole blood, again in the absence of TXA2 synthesis (Table 3).

Comparison of samples from patients and ASA-free control subjects (control subjects, Figs 1 and 2) indicated that ASA reduced platelet recruitment in patients with vascular disease, although in patients of groups B and C this did not reach optimal levels. Platelet recruitment was inhibited (≥95%) by a single dose of 500 mg ASA in normal subjects, both in the presence or absence of erythrocytes (not shown).

The effect of intermediate-dose aspirin (200 to 300 mg) on platelet recruitment in our 82 patients with vascular disease was summarized in Fig 3: 39% of patients were classified in group A, 45% in group B, and the remaining 16% in group C.

**Serotonin Release in Platelets From Aspirin-Treated Patients With Vascular Disease**

5HT release was measured as a marker of the platelet release reaction in vascular disease patients and in control subjects. Although 5HT release was reduced in ASA-treated patients as compared with a control group of ASA-free subjects (Fig 4), appreciable 5HT release was observed in stimulated platelets alone from ASA-treated IHD and CVD patients. In all patient groups this release was significantly enhanced when platelet-erythrocyte suspensions or whole blood samples were studied (Fig 4). Importantly, this occurred in the setting of total TXA2 inhibition (Table 3). It was noted that 5HT release in whole blood was always less than in platelet-erythrocyte mixtures, probably due to neutrophil inhibition of platelet 5HT release.10

**Discussion**

Thrombotic events in high-risk patients for cardiovascular complications and stroke may be a consequence of increased platelet activation.1 Therefore, inhibition of platelet reactivity is important for prophylaxis of thromboembolic events. Platelet interactions with erythrocytes enhance several aspects of platelet function in vitro.8,9,16-19 Thus in addition to inhibiting platelet function, our data indicate that blocking the capacity of erythrocytes to stimulate platelets should be an effective component of treatment for patients with vascular disease.

The present study indicates that three groups of responses involving platelet recruitment can be clearly delineated in patients with cardiovascular and cerebrovascular diseases who are chronically treated with 200 to 300 mg ASA/d, respectively (Fig 3). Importantly, these responses occurred when TXA2 synthesis was completely blocked in each instance (Table 3). Only group A demonstrated complete inhibition of platelet recruitment ex vivo whether studied in PRP, platelet-erythrocyte suspensions, or whole blood. Therefore, in this group of patients with vascular disease (n=32; 39% of the total 82), the dose of ASA administered was adequate and inhibited the phase of platelet reactivity which leads to an evolving thrombus.

A second group of patients (Figs 1 and 2, group B, n=37; 45%) also had undetectable recruitment but only when their PRP was studied. In the presence of erythrocytes or whole blood, recruitment did occur. Importantly, some patients...
ASA-Treatment in Patients with Vascular Disease

Figure 4. Effect of long-term ASA treatment on platelet 5HT release in patients with vascular disease.14 C–5HT-radiolabeled PRP, platelet-erythrocyte suspensions, or WB were stimulated with collagen (1 μg/mL), and 5HT release was evaluated in cell-free supernatants (see “Methods”). Control subjects are normal subjects before (¬ASA) and 2 hours after administration of 500 mg ASA (+ASA). *P<.002, PRP vs PRP+RBC in all instances.

Prothrombotic Effects of Erythrocytes Decrease Effectiveness of Aspirin

We previously demonstrated that erythrocyte prothrombotic activity is sensitive to ASA in a dose-dependent manner, and is blocked after a single dose of 500 mg ASA in normal subjects.13 However, with the passage of time, erythrocytes “escape” from this initial inhibition. To maintain inhibition, normal subjects require an intermittent dose of 500 mg every 2 weeks, supplemented with a low maintenance dose (50 mg ASA/d).13 This low maintenance dose of ASA was chosen to inhibit platelet TXA2 synthesis while avoiding continuous inhibition of vascular prostaglandin I2 production22 as well as possible gastrointestinal side effects23,24 which may occur during administration of high dose ASA.25

In patient group C (n=13, 16%) there was detectable recruitment even when studied in platelets alone. This was markedly amplified in the presence of erythrocytes (Figs 1 and 2). The low degree of inhibition in platelets alone in this group could be attributed to their platelets being more responsive to collagen stimulation via TXA2 synthesis–independent mechanisms. However, an alternative explanation could be the presence of a larger number of newly formed circulating platelets with an active cyclooxygenase, due to increased platelet turnover. This phenomenon has been reported as early as 4 hours after ASA ingestion.26 It should also be pointed out that collagen stimulation of a mixture of 10% ASA-free platelets with 90% ASA-treated platelets resulted in detectable recruitment—an effect not seen with the same quantity of either type of platelets alone.9 When this platelet mixture was stimulated in the presence of ASA-treated erythrocytes, recruitment was greatly amplified.9 Thus the rapid appearance of an increased number of ASA-free platelets in group C could be responsible for their higher degree of responsiveness. This would suggest that administration of ASA twice a day might improve control of platelet reactivity in this patient group.

The platelet release reaction as evaluated by dense granule secretion (5HT) was significantly reduced by ASA treatment (Fig 4). However, in all instances, 5HT release observed in platelets alone was enhanced approximately threefold by erythrocytes. As a consequence of these platelet-erythrocyte interactions, increased platelet release of 5HT as well as other platelet granule constituents known to promote cell proliferation in the vascular wall27 could contribute to further development of atherosclerotic complications.

We conclude that in a significant number of patients the doses of ASA presently used for antithrombotic treatment (200 to 300 mg) are insufficient to block platelet reactivity as promoted by erythrocytes. Therefore, the optimal dose of aspirin may need to be established for patients with a thrombotic diathesis in order to provide adequate aspirin-mediated inhibition of both platelet reactivity and the prothrombotic effect of erythrocytes. This may serve to reduce the incidence of further vascular occlusive events.

Acknowledgments

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