Do Patients With Thromboembolic Disease Have Circulating Platelet Aggregates?

By Fred H. Kohanna, Marianne H. Smith, and Edwin W. Salzman

Reports of circulating platelet aggregates (ie, microemboli) in thromboembolism and other vascular disorders are based on a method (Wu and Hoak, 1974) in which venous blood is collected via scalp vein needle and tubing into either formaldehyde, which fixes aggregates, or EDTA, which disperses them. The ratio of platelet counts in platelet-rich plasma (PRP) from the two blood samples after centrifugation is interpreted as a measure of platelet aggregates in the circulation in vivo. We compared this standard Wu and Hoak technique with a modified one, in which blood was drawn directly into a syringe, and with a third method that avoided centrifugation by counting single platelets in whole blood. Both modified techniques could detect aggregates generated in vitro with adenosine diphosphate (ADP). In 12 normal subjects, the three methods were equivalent, but in 37 patients with thromboembolic disorders, the standard Wu and Hoak method gave a lower ratio than the other methods. Similar results were found in a subset of eight patients with myocardial infarction. Heparin treatment of patients did not influence the results. The data suggest that formation of platelet aggregates occurred during venipuncture. Platelets may be hyperactive in patients with thromboembolic disease and may form aggregates in vitro during collection, but the concept of chronic microembolism in such patients should be reassessed.

CIRCULATING PLATELET AGGREGATES have been reported in a variety of clinical settings, including myocardial infarction, stroke, peripheral arteriosclerosis, deep vein thrombosis, sickle cell anemia, and diabetes mellitus, which suggests that chronic states of platelet microembolization are common in thrombotic disorders and related conditions. Persistent circulation of platelet aggregates would be surprising, however, as circulating platelet aggregates larger than capillary diameter would be expected to be filtered out in the microcirculation and to result in impaired organ perfusion. As end-organ dysfunction is not usually a feature of illnesses in which circulating platelet aggregates have been reported, one must question whether the demonstrated aggregates are in fact present in vivo. Methods for demonstrating circulating platelet aggregates have included the use of blood filters, ultrasonic detection, and fixation of platelets during venipuncture. The last method, developed by Wu and Hoak, has been used most extensively, despite possible artifacts of sample collection and processing and lack of direct evidence that platelet aggregates were present in the patient’s circulation. To examine the latter question, we studied patients with thromboembolic disorders and compared the Wu and Hoak technique with two modified methods designed to minimize platelet activation during blood collection and platelet counting.

MATERIALS AND METHODS

Twelve normal volunteers (3 men and 9 women) and 37 patients (20 men and 17 women) with a variety of thromboembolic disorders were studied after informed consent was obtained (Table I). Eighteen patients were receiving intravenous heparin in full therapeutic dosage at the time of study. Ten patients received low-dose subcutaneous heparin for prevention of venous thromboembolism, and nine patients did not receive any heparin. None of the 37 patients studied was receiving antiplatelet drug therapy. All patients were studied within five days of diagnosis.

Standard Wu and Hoak Technique (SWH)

With the aid of a venous tourniquet applied immediately before venipuncture, blood (1 mL) was collected from an antecubital vein, using a 19-gauge scalp-vein needle and plastic tubing, into syringes containing either buffered Na₂ EDTA-formaldehyde solution (4 mL, formaldehyde 1.0% wt/vol, Na₂ EDTA 10⁻² mol/L, pH 7.36), which fixes aggregates, or buffered EDTA alone (4 mL, 10⁻³ mol/L, pH 7.26), which disperses them (Fig 1). Samples were collected in triplicate into syringes, which were weighed before and after addition of the blood to determine the precise volume of blood collected (ie, correcting for pipetting error) and to allow for subsequent correction of platelet counts using the following formula: \( \Delta W + 4/\Delta W + (\text{Mean platelet count}/5) = \text{Corrected platelet count} \), where \( W \) = postweight - preweight of syringe; \( \Delta W = \text{weight in grams of the EDTA-formaldehyde or EDTA solution} \); and \( S \) = the dilution of the whole blood (ie, 1:5). The samples were then transferred from the syringes to polystyrene test tubes for centrifugation at 22°C for 8 minutes at 220 g to remove RBCs and platelet aggregates, if present. Platelet counts in platelet-rich plasma (PRP) were determined with a Clay Adams Ultraflo-100 cell counter coupled with the Nuclear Data-60 pulse-height analyzer. Cells in the size range from 5.2 \( \mu \)m to 25.1 \( \mu \)m were counted for 20 seconds in duplicate, and the mean platelet count was determined. This cell size range contains predominantly single platelets, but might also include some platelet doublets and triplets. Mean platelet counts were then corrected for prior pipetting error as previously described. The platelet aggregate ratio was calculated from the formula: platelet count in EDTA-formaldehyde/Platelet count in EDTA.
Table 1. Number of Patients Studied

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Full-Dose Heparin</th>
<th>Low-Dose Heparin</th>
<th>No Heparin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myocardial infarction</td>
<td>0</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>Deep vein thrombosis</td>
<td>11</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Pulmonary embolism</td>
<td>4</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Peripheral arteriosclerosis</td>
<td>0</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Arterial embolus or thrombo-</td>
<td>2</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>sis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TIA/stroke</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Prosthetic graft</td>
<td>0</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>18</td>
<td>10</td>
<td>9</td>
</tr>
</tbody>
</table>

Without ADP (control) or with ADP (0.5 μmol/L, 1.0 μmol/L, 5.0 μmol/L, or 10.0 μmol/L) for 2 minutes.

Aliquots (0.4 mL) from the remaining whole blood were then treated in a similar fashion. At 30, 60, 90, 120, or 180 seconds, 0.2-mL aliquots were removed and 1.6 mL placed in either 1.6 mL EDTA or EDTA-formaldehyde solution. Platelets were counted on the Ultraflo 100, as previously described, both before and after centrifugation (120 g for 4 minutes). The ratio of platelet count in EDTA-formaldehyde to that in EDTA alone was then determined in whole blood and PRP.

Statistical Analysis

Comparison of means was performed with the Newman-Keuls multiple range test for comparison of multiple samples. A P value <.05 was considered significant.

RESULTS

Results of In Vitro Validation

ADP-induced platelet aggregates that were detectable by conventional platelet aggregometry could also be detected by both modifications of the SWH (Fig 2).

In 12 normal subjects, the three methods were equivalent [ratio: SWH, 0.93 ± 0.09 (mean ± SD); MWH, 0.94 ± 0.05; WB 0.94 ± 0.07, P > .05].

![Fig 2. Platelet aggregate ratios measured over time at different ADP concentrations in either PRP or whole blood (middle and lower panels) with accompanying aggregometry tracings (upper panel).](http://www.bloodjournal.org/content/bloodjournal/doi/10.1182/blood-2006-06-032266.full/118/23/5860/F11823f2a.png)
CIRCULATING PLATELET AGGREGATES

Fig 3. Platelet aggregate ratios with the standard Wu and Hoak technique and two modified methods in patients with thromboembolic disorders. Values are means ± SEM.

Standard Wu and Hoak ratios for all patients with thromboembolic disorders, however, were significantly lower than with the two modified methods (ratio: SWH 0.88 ± 0.12 v MWH 0.96 ± 0.06 and WB 0.96 ± 0.07, P < .001) (Fig 3). Among 37 patients with thromboembolic disorders, 8 had standard Wu and Hoak ratios less than 0.80 (ie, “abnormal” according to Wu and Hoak).

Because administration of heparin may induce transient platelet aggregation, we examined samples from patients receiving porcine mucosal heparin for evidence of circulating platelet aggregates. Standard Wu and Hoak ratios were lower in patients with thromboembolism, regardless of whether the patients were receiving full-dose intravenous heparin (ratio: SWH 0.90 ± 0.11 v MWH 0.96 ± 0.08 and WB 0.97 ± 0.05, P < .025), low-dose subcutaneous heparin (ratio: 0.84 ± 0.15 v 0.94 ± 0.07 and 0.95 ± 0.07, P < .01), or no heparin (ratio: 0.89 ± 0.08 v 0.97 ± 0.05 and 0.96 ± 0.05, P < .05).

The standard Wu and Hoak ratio was also lower than with the two modified methods in a subset of patients with myocardial infarction (ratio: SWH 0.88 ± 0.12 v MWH 0.96 ± 0.03 and WB 0.96 ± 0.07, P < .05).

DISCUSSION

In 1974, Wu and Hoak11 described a technique that purported to detect circulating platelet aggregates in patients with thromboembolic disorders. Subsequently, the test has become popular and a multitude of investigators have both “confirmed” and added to these results by describing circulating platelet aggregates in thromboembolic states and other types of illnesses, including diabetes, sickle cell anemia, acute infection, and cancer of the lung.2 Circulating platelet aggregates have also been described in normal subjects after a fatty meal17 or cigarette smoking.18

A serious pitfall in interpreting the results of these studies lies in the uncertainty as to whether platelet aggregates are, in fact, present in vivo or rather are formed ex vivo as an artifact of blood collection and processing. Indirect evidence cited in favor of the former interpretation includes a fall in platelet aggregate ratio in rabbits infused with thrombin,11 elevated plasma levels of platelet products (eg, β-thromboglobulin, platelet factor 4, and thromboxane B2),19 and decreased platelet survival in patients with thromboembolic disorders.20 However, other explanations for these phenomena are possible.

Direct evidence for the presence of platelet aggregates that circulate in vivo is lacking. The results of our study indicate that, in normal subjects, who presumably have only discrete single platelets in their circulation, platelet aggregate ratios are in Wu and Hoak’s “normal range” and are not significantly different whether the blood is collected using the standard Wu and Hoak technique or by two modified techniques that control for passage of blood through a length of plastic tubing and for centrifugation. In patients with thromboembolic disorders, however, ratios obtained by the standard Wu and Hoak technique were, as a group, significantly lower than with the two modified techniques, and in eight such patients, they were in the range of values deemed “abnormal” by Wu and Hoak. The fact that low values were not obtained in these patients when this blood was collected directly into a syringe suggests that the passage of blood through tubing was responsible for the lower ratios (ie, greater aggregation) in these patients. Centrifugation of the sample did not appear to be a determinant variable.

Because platelet aggregates are apparently formed in response to the interaction between plastic tubing and blood from some patients with thromboembolism but not in blood from normal donors, it is likely that some patients with thromboembolic disorders have hyperreactive platelets. This view is supported by reports that platelets from donors with thromboembolic disorders, as well as diabetes and hyperlipidemia, show increased responsiveness in vitro to platelet agonists, such as ADP and epinephrine,21,22 and undergo spontaneous aggregation when stirred in vitro.23 A voluminous literature has considered the proposition that platelet reactivity is enhanced in patients with thromboembolic disease.24

The contention that formation of platelet aggregates in vitro in the Wu and Hoak technique may be an artifact of interaction of the blood with surfaces of the collecting system is consistent with the work of Rohrer et al,12 who found a significant interaction between blood and collection tubing leading to measurement of platelet aggregates in greater number (Wu and Hoak technique) in normal subjects when blood was collected at a slower (3.0 ± 1.2 mL/min) rather than a faster (11.4 ± 2.6 mL/min) rate of flow. Similarly, at a constant rate of flow, more platelet aggregates were found when the time interval from venipuncture to sampling was prolonged. They, too, found little or no effect from centrifugation of the blood sample.
Heparin may produce thrombocytopenia in vivo\textsuperscript{25,26} and induce platelet aggregation in vitro\textsuperscript{27,28} but we found that the presence of heparin in the blood of our patients, either full-dose or low-dose, did not alter the pattern of finding low platelet aggregate ratios in patients with thromboembolic disease only when tubing was employed during venipuncture.

Platelet aggregate ratios were not as low in our patients with thromboembolic disorders using the Wu and Hoak technique as have been reported elsewhere.\textsuperscript{11} Higher ratios in our patients may reflect technical factors, such as triplicate sampling and correction for “pipetting” error. Furthermore, our patient group only contained one patient with cerebral ischemic attacks and eight patients with myocardial infarction, two groups that have been noted to have especially low platelet aggregate ratios. However, the reduced platelet aggregate ratios in patients with thromboembolic disorders are by no means a universal finding. Using normal subjects and patients with unstable angina, myocardial infarction, and Hoak technique was employed during venipuncture.

Patients with thromboembolic disease only when tubings have been noted to have especially low platelet aggregate ratios. However, the reduced platelet aggregate ratios in patients with thromboembolic disorders are by no means a universal finding. Using normal subjects and patients with unstable angina, myocardial infarction, and Hoak technique was employed during venipuncture.

Platelet aggregate ratios were not as low in our patients with thromboembolic disorders using the Wu and Hoak technique as have been reported elsewhere.\textsuperscript{11} Higher ratios in our patients may reflect technical factors, such as triplicate sampling and correction for “pipetting” error. Furthermore, our patient group only contained one patient with cerebral ischemic attacks and eight patients with myocardial infarction, two groups that have been noted to have especially low platelet aggregate ratios. However, the reduced platelet aggregate ratios in patients with thromboembolic disorders are by no means a universal finding. Using normal subjects and patients with unstable angina, myocardial infarction, and Hoak technique was employed during venipuncture.

Platelet aggregate ratios were not as low in our patients with thromboembolic disorders using the Wu and Hoak technique as have been reported elsewhere.\textsuperscript{11} Higher ratios in our patients may reflect technical factors, such as triplicate sampling and correction for “pipetting” error. Furthermore, our patient group only contained one patient with cerebral ischemic attacks and eight patients with myocardial infarction, two groups that have been noted to have especially low platelet aggregate ratios. However, the reduced platelet aggregate ratios in patients with thromboembolic disorders are by no means a universal finding. Using normal subjects and patients with unstable angina, myocardial infarction, and Hoak technique was employed during venipuncture.

Platelet aggregate ratios were not as low in our patients with thromboembolic disorders using the Wu and Hoak technique as have been reported elsewhere.\textsuperscript{11} Higher ratios in our patients may reflect technical factors, such as triplicate sampling and correction for “pipetting” error. Furthermore, our patient group only contained one patient with cerebral ischemic attacks and eight patients with myocardial infarction, two groups that have been noted to have especially low platelet aggregate ratios. However, the reduced platelet aggregate ratios in patients with thromboembolic disorders are by no means a universal finding. Using normal subjects and patients with unstable angina, myocardial infarction, and Hoak technique was employed during venipuncture.

Platelet aggregate ratios were not as low in our patients with thromboembolic disorders using the Wu and Hoak technique as have been reported elsewhere.\textsuperscript{11} Higher ratios in our patients may reflect technical factors, such as triplicate sampling and correction for “pipetting” error. Furthermore, our patient group only contained one patient with cerebral ischemic attacks and eight patients with myocardial infarction, two groups that have been noted to have especially low platelet aggregate ratios. However, the reduced platelet aggregate ratios in patients with thromboembolic disorders are by no means a universal finding. Using normal subjects and patients with unstable angina, myocardial infarction, and Hoak technique was employed during venipuncture.

Platelet aggregate ratios were not as low in our patients with thromboembolic disorders using the Wu and Hoak technique as have been reported elsewhere.\textsuperscript{11} Higher ratios in our patients may reflect technical factors, such as triplicate sampling and correction for “pipetting” error. Furthermore, our patient group only contained one patient with cerebral ischemic attacks and eight patients with myocardial infarction, two groups that have been noted to have especially low platelet aggregate ratios. However, the reduced platelet aggregate ratios in patients with thromboembolic disorders are by no means a universal finding. Using normal subjects and patients with unstable angina, myocardial infarction, and Hoak technique was employed during venipuncture.

Platelet aggregate ratios were not as low in our patients with thromboembolic disorders using the Wu and Hoak technique as have been reported elsewhere.\textsuperscript{11} Higher ratios in our patients may reflect technical factors, such as triplicate sampling and correction for “pipetting” error. Furthermore, our patient group only contained one patient with cerebral ischemic attacks and eight patients with myocardial infarction, two groups that have been noted to have especially low platelet aggregate ratios. However, the reduced platelet aggregate ratios in patients with thromboembolic disorders are by no means a universal finding. Using normal subjects and patients with unstable angina, myocardial infarction, and Hoak technique was employed during venipuncture.

Platelet aggregate ratios were not as low in our patients with thromboembolic disorders using the Wu and Hoak technique as have been reported elsewhere.\textsuperscript{11} Higher ratios in our patients may reflect technical factors, such as triplicate sampling and correction for “pipetting” error. Furthermore, our patient group only contained one patient with cerebral ischemic attacks and eight patients with myocardial infarction, two groups that have been noted to have especially low platelet aggregate ratios. However, the reduced platelet aggregate ratios in patients with thromboembolic disorders are by no means a universal finding. Using normal subjects and patients with unstable angina, myocardial infarction, and Hoak technique was employed during venipuncture.

Platelet aggregate ratios were not as low in our patients with thromboembolic disorders using the Wu and Hoak technique as have been reported elsewhere.\textsuperscript{11} Higher ratios in our patients may reflect technical factors, such as triplicate sampling and correction for “pipetting” error. Furthermore, our patient group only contained one patient with cerebral ischemic attacks and eight patients with myocardial infarction, two groups that have been noted to have especially low platelet aggregate ratios. However, the reduced platelet aggregate ratios in patients with thromboembolic disorders are by no means a universal finding. Using normal subjects and patients with unstable angina, myocardial infarction, and Hoak technique was employed during venipuncture.

Platelet aggregate ratios were not as low in our patients with thromboembolic disorders using the Wu and Hoak technique as have been reported elsewhere.\textsuperscript{11} Higher ratios in our patients may reflect technical factors, such as triplicate sampling and correction for “pipetting” error. Furthermore, our patient group only contained one patient with cerebral ischemic attacks and eight patients with myocardial infarction, two groups that have been noted to have especially low platelet aggregate ratios. However, the reduced platelet aggregate ratios in patients with thromboembolic disorders are by no means a universal finding. Using normal subjects and patients with unstable angina, myocardial infarction, and Hoak technique was employed during venipuncture. Published results of studies with the Wu and Hoak technique require reinterpretation.

REFERENCES

23. Ten Cate J, Vos J, Oosterhuis H, Prenger D, Jenkins CSP.
Do patients with thromboembolic disease have circulating platelet aggregates?

FH Kohanna, MH Smith and EW Salzman