Modification of Platelet Aggregation by Leukocytes in Acute Ischemic Stroke

Armin J. Grau, MD; Rainer Sigmund; Werner Hacke, MD

Background and Purpose Platelet aggregation plays an important role in the pathogenesis of thromboembolic cerebrovascular disease. Leukocytes can efficiently stimulate as well as inhibit platelet aggregability. We studied the influence of leukocytes on collagen-induced platelet aggregation in patients with acute ischemic stroke.

Methods We investigated 23 patients within 2 days after stroke and 23 healthy age- and sex-matched control subjects and determined collagen-induced platelet aggregation in platelet-rich plasma with or without addition of polymorphonuclear or mononuclear leukocytes.

Results Platelet aggregation without leukocytes tended to be lower in patients than in control subjects (P=.06). Mononuclear leukocytes reduced (P=.018) and polymorphonuclear leukocytes tended to reduce (P=.06) platelet aggregation in patients. Leukocytes did not significantly alter platelet aggregation in control subjects. In the presence of either mononuclear or polymorphonuclear leukocytes, platelet aggregation was significantly lower in patients than in control subjects (P=.004 and P=.008). The ratio of polymorphonuclear leukocytes to platelets in venous blood was higher in patients than in control subjects (P<.001).

Conclusions Mononuclear—and less clearly polymorphonuclear—leukocytes possess a platelet aggregation-inhibiting potential in the early stages after ischemic stroke, a feature with possible antithrombotic effects. (Stroke. 1994;25:2149-2152.)

Key Words • cerebral ischemia • leukocytes • platelet aggregation

A large body of evidence indicates that platelet function is altered in patients with acute cerebral ischemia. Measurements of release products from alpha-granules and from dense bodies have shown platelet activation, whereas results from platelet aggregation studies are at variance in acute ischemic stroke. Platelet aggregation was mainly measured in platelet-rich plasma (PRP), a system investigating platelets isolated from other blood cells. Leukocytes may be important in the control of platelet aggregability because these cells can efficiently stimulate platelet activation. Arterial thrombi are frequently composed of significant numbers of leukocytes, and platelet aggregates formed in whole blood contained leukocytes in addition to erythrocytes. Leukocytes could potentially be involved in both the formation and dissolution of thrombi. Currently, leukocytes are discussed as important mediators in cerebral ischemia. However, the modifying effect of unstimulated leukocytes on platelet aggregation has not yet been studied in ischemic stroke.

We used PRP with or without addition of polymorphonuclear leukocytes (PMNs) or mononuclear leukocytes (MNs) to assess the influence of leukocytes on collagen-induced platelet aggregation in patients with acute ischemic stroke. Collagen was chosen as stimulus because it is an important platelet activator present in atherosclerotic plaques. In addition, we measured the aggregability of PMNs and MNs induced by the tumor promoter phorbol myristate acetate (PMA) because the formation of leukocyte aggregates may compromise microvascular flow and may thereby contribute to the pathogenesis of cerebral ischemia.

Subjects and Methods We investigated 23 patients (10 women, 13 men; mean±SD age, 66±12 years) within 2 days after ischemic stroke. Exclusion criteria included trauma, surgery, and acute vascular diseases within 4 weeks before stroke and medication that would influence platelet (eg, aspirin or heparin) or leukocyte (eg, steroids and nonsteroidal anti-inflammatory agents) function. All patients received a computed tomographic scan to exclude cerebral hemorrhage. None of the patients had a febrile infection within 1 week before or concomitant with stroke. The control group consisted of 23 healthy subjects (10 women, 13 men; mean±SD age, 61±11 years). The protocol of this study was approved by the Institutional Review Committee, and subjects gave informed consent.

After minor stasis we collected 25 mL of venous blood from an antecubital vein into plastic syringes with sodium citrate as anticoagulant (Sarstedt) using 19-gauge butterfly needles (Braun). The cell concentration in PRP (170g, 10 minutes) was adjusted to 2×10^10 platelets per microliter (final concentration) by addition of platelet-poor plasma (PPP) (150g, 10 minutes). PRP was kept at room temperature until the experiments were performed. Half of the remaining blood was used for the isolation of PMNs and MNs. We isolated PMNs by centrifugation (800g, 15 minutes) of diluted blood on a Percoll gradient (55% and 74%; Pharmacia) with a consecutive hypotonic lysis in sterile distilled water for 20 seconds. For the isolation of MNs, we used a Ficoll (Biochrom) gradient centrifugation (800g, 15 minutes), and finally both cell layers were washed twice in Dulbecco's phosphate-buffered saline (DPBS) without Ca^2+/Mg^2+ (Sigma) (170g, 7 minutes). Cell concentrations were adjusted to 2×10^4 PMNs per microliter and 5×10^4 MNs per microliter; thus, cell ratios were 1:100 (ratio of PMNs to platelets) and 1:400 (ratio of MNs to platelets) in aggregation experiments. Reagents used for experiments with MNs contained less than 0.03 U/mL of endo-
The maximal aggregation of platelets without leukocytes tended to be lower in patients than in control subjects after a short lag time.

We measured platelet aggregation in a four-channel aggregometer (PAP4, Biodata Corp) at a speed of 1000 rpm. For mixed cellular suspensions, PRP plus MNs or PMNs (0%) and PPP plus MNs or PMNs (100%) were used for calibration. When we measured platelets alone, buffer was substituted for leukocytes. In parallel with other investigators,\(^4\) we chose this approach because leukocyte counts after experiments were not significantly different from initial values, as evidenced by cell counting in a Neubauer chamber. Leukocyte-leukocyte aggregates were not observed microscopically. In siliconized glass tubes, PRP was warmed to 3\(^\circ\)C and incubated for 2 minutes with leukocyte suspensions or DPBS before the addition of collagen (0.3 \(\mu\)g/mL; collagen reagent Horm, Nycomed). This comparatively low concentration of collagen led to submaximal platelet aggregation in the majority of preliminary experiments with young and healthy volunteers. In three patients and two control subjects, this concentration of collagen did not induce a measurable platelet aggregation, although the subjects denied an intake of aggregation-inhibiting medications.

Results from these experiments had to be excluded. The experiments were done at least in duplicate but mostly in triplicate or quadruplicate. The coefficient of variation of intra-individual results of maximal aggregation was 0.08 for PRP and 0.12 for mixed cellular suspensions. Experiments were carried out within 3 hours after venipuncture. The aggregometer indicated maximal values of cell aggregation and the slope expressing the velocity of aggregation. The lag time after addition of PMA, an interval after which only minor changes in aggregation were seen. Cell concentrations in patients and control subjects (Table 1). There were no differences in the lag time between groups or between experimental conditions with and without leukocytes (data not shown).

Leukocyte and PMN counts in whole blood were significantly higher in patients than in control subjects, whereas MN and platelet counts were not different. Therefore, the ratio of platelets to PMNs was decreased in patients compared with control subjects (Table 2). Changes in optical density during PMA-induced aggregation of isolated PMNs did not result in differences between patients (n = 20; 61.5 ± 11.1%) and control subjects (n = 22; 60.0 ± 8.6%). A similar result was obtained for MNs (47.9 ± 10.6% in 18 patients versus 47.9 ± 12.1% in 21 control subjects).

**Discussion**

Measuring platelet aggregation in PRP has been criticized because cells are deprived of parts of their natural environment.\(^3\) Whole blood aggregometry better reflects the state of platelets in vivo. This technique, however, does not allow quantification of the influence of unstimulated blood cells on platelet aggregation using standardized cell concentrations. Therefore, we used PRP with and without leukocytes to elucidate the interference of leukocyte subpopulations with platelet aggregation. Because leukocyte stimulation can have substantial impact on platelet aggregation, particular care was taken to minimize artificial cell activation. The fact that only one platelet aggregating substance was tested may be criticized; in our acutely ill patients, however, we were restricted to a small blood sample, which did not allow further analyses.

### Table 1. Collagen-Induced Platelet Aggregation With and Without Leukocytes in Patients and Control Subjects

<table>
<thead>
<tr>
<th>Cells</th>
<th>Maximal Platelet Aggregation, %</th>
<th>Slope</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Patients</td>
<td>Control Subjects</td>
</tr>
<tr>
<td>n Mean±SD</td>
<td>n Mean±SD</td>
<td>n Mean±SD</td>
</tr>
<tr>
<td>Platelets</td>
<td>23 63±15</td>
<td>23 72±17</td>
</tr>
<tr>
<td>Platelets+MNs</td>
<td>22 52±21</td>
<td>23 70±19</td>
</tr>
<tr>
<td>Platelets+PMNs</td>
<td>23 55±24</td>
<td>23 78±29</td>
</tr>
</tbody>
</table>
Results from platelet aggregation studies in ischemic stroke differ widely and are dependent on methodical approach, stimulus, and time interval after ictus. In our experiments platelet aggregation showed a nonsignificant trend toward lower values in acute stroke. Similarly, former studies reported unchanged or reduced collagen-induced platelet aggregation in PRP or whole blood after stroke. Circulating platelet aggregates occurred more frequently after ischemic stroke and transient ischemic attack, suggesting systemic hyperaggregability and platelet consumption. Together with results from aggregation studies, these findings support the hypothesis that active platelets have aggregated before or in response to the ischemic injury, leaving circulating platelets that tend to be more refractory to collagen-induced aggregation.

Neither PMNs nor MNs had a significantly modulating effect on platelet aggregation in our control group. This is in accordance with the findings of Zoucas et al, whereas others describe an inhibitory effect of unstimulated PMNs on platelet aggregation. In patients, MNs—and to a smaller and nonsignificant degree PMNs—decreased collagen-induced platelet aggregation. However, interindividual variability was considerable, and mainly PMNs also stimulated platelet aggregation in some patients. We used comparatively low ratios of leukocytes to platelets and may therefore underestimate the impact of leukocytes on platelet aggregation to some extent. Leukocytes can modulate platelet aggregability by various mechanisms. Stimulated PMNs are able to activate platelets by the release of cathepsin G, platelet-activating factor, and oxygen-derived free radicals. Activated MNs can induce platelet aggregation by tissue factor-mediated thrombin formation. On the other hand, PMNs can inhibit platelet activation by inactivating ADP, and both MNs and PMNs can inhibit platelets by releasing a nitric oxide-like factor. Arachidonic acid-derived metabolites from MNs and PMNs can lead to activation or inhibition of platelets, as can the release of elastase by PMNs. Further investigations are required to identify the mechanisms by which leukocytes reduce collagen-induced platelet aggregation in the majority of patients but lead to an increase in others.

Recently, Uchiyama et al found increased leukocyte and platelet aggregation in whole blood in stroke patients. However, the impact of leukocytes on platelet aggregation was not statistically significant in our study.

### Table 2. Blood Cell Counts and Cell Ratios in Patients and Control Subjects

<table>
<thead>
<tr>
<th></th>
<th>Patients</th>
<th>Control Subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Mean±SD</td>
</tr>
<tr>
<td>Leukocytes†</td>
<td>23</td>
<td>9.3±2.5</td>
</tr>
<tr>
<td>PMNs†</td>
<td>20</td>
<td>7.5±2.4</td>
</tr>
<tr>
<td>MNs†</td>
<td>20</td>
<td>2.3±1.1</td>
</tr>
<tr>
<td>Platelets†</td>
<td>23</td>
<td>230±45</td>
</tr>
<tr>
<td>Platelets/PMNs ratio</td>
<td>19</td>
<td>33±14</td>
</tr>
<tr>
<td>Platelets/MNs ratio</td>
<td>19</td>
<td>123±77</td>
</tr>
</tbody>
</table>

PMNs indicates polymorphonuclear leukocytes; MNs, mononuclear leukocytes.

*By unpaired Student's t test.
†Per nanoliter.
patients. The experimental approach included leukocyte stimulation and pretreatment with an inhibitor of microfilament contraction; this may alter the platelet-modifying potential of leukocytes and explain the differences in our findings. Experimental trauma rapidly led to a suppression of platelet aggregation by PMNs in response to ADP and arachidonic acid. Analogously, the cerebral tissue damage may have led to altered leukocyte function in our patients. Follow-up studies are desirable to clarify this issue; however, the necessary treatment of patients with platelet-inhibiting agents makes this issue rather difficult to investigate. Evaluating leukocyte aggregation with a leukergy test, Galante et al found increased values after stroke, whereas our measurements failed to show differences between groups. Our assay investigates the aggregability of isolated and stimulated leukocytes, whereas the leukergy test evaluates unstimulated cells ex vivo. This may explain the different results.

In the recent discussion about the role of leukocytes in the pathogenesis of cerebral ischemia, clinical studies have focused on the rheological and cytotoxic properties of leukocytes. We recently reported a reduced coagulation may be an important aspect of their activation in acute ischemic stroke, and further investigations are needed in this field.

Acknowledgment

We wish to thank Axel Vogt for help with the manuscript.

References


3. Joseph R, D'Andrea G, Oster SB, Welch KMA. Whole blood leukocyte function in our patients. Follow-up studies are desirable to clarify this issue; however, the necessary treatment of patients with platelet-inhibiting agents makes this issue rather difficult to investigate. Evaluating leukocyte aggregation with a leukergy test, Galante et al found increased values after stroke, whereas our measurements failed to show differences between groups. Our assay investigates the aggregability of isolated and stimulated leukocytes, whereas the leukergy test evaluates unstimulated cells ex vivo. This may explain the different results.

In the recent discussion about the role of leukocytes in the pathogenesis of cerebral ischemia, clinical studies have focused on the rheological and cytotoxic properties of leukocytes. We recently reported a reduced coagulation may be an important aspect of their activation in acute ischemic stroke, and further investigations are needed in this field.

Acknowledgment

We wish to thank Axel Vogt for help with the manuscript.

References


3. Joseph R, D'Andrea G, Oster SB, Welch KMA. Whole blood leukocyte function in our patients. Follow-up studies are desirable to clarify this issue; however, the necessary...
Modification of platelet aggregation by leukocytes in acute ischemic stroke.
A J Grau, R Sigmund and W Hacke

Stroke. 1994;25:2149-2152
doi: 10.1161/01.STR.25.11.2149
Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1994 American Heart Association, Inc. All rights reserved.
Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://stroke.ahajournals.org/content/25/11/2149

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Stroke can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Stroke is online at:
http://stroke.ahajournals.org//subscriptions/