Platelet Ultrastructure and Secretion in Acute Ischemic Stroke

Rajiv Joseph, MD, J.M. Riddle, PhD, K.M.A. Welch, MD, and G. D'Andrea, MD

We used transmission electron microscopy to count the organelles (dense bodies, alpha granules, and mitochondria) contained within platelets from 11 acute ischemic stroke patients and 12 healthy controls. We randomly selected for evaluation 25 platelet profiles in ultrathin sections cut from three separate blocks. Compared with those from controls, platelets from stroke patients contained significantly fewer alpha granules (p<0.001) and mitochondria (p<0.02) and showed a trend toward fewer dense bodies. Supportive of our previous studies, the amount of adenosine triphosphate secreted following stimulation by collagen also tended to be greater in platelets from stroke patients. These observations support the presence of increased platelet secretion associated with acute cerebral infarction and raise the possibility that platelet secretion may be of separate importance to the mechanical occlusion of blood vessels by platelet aggregates in the pathogenesis of cerebral infarction. (Stroke 1989;20:1316-1319)

Platelet secretion may be of separate importance to the mechanical occlusion of blood vessels by platelet aggregates in the pathogenesis or propagation of acute cerebral infarction. Experiments in animals support the concept that vasoactive substances released from platelet organelles may contribute to stroke worsening by having access to cerebral tissue through a disrupted blood-brain barrier. The evidence for this in human cerebral infarction is derived from recent studies showing increased platelet alpha granule and dense body secretion in whole blood, unassociated with an increase in platelet aggregation. We studied platelet ultrastructure, in particular the secretory organelles, in patients with acute ischemic stroke.

Subjects and Methods

We studied 12 healthy controls (eight women and four men) ranging in age from 29 to 74 (mean±SD 55±13) years and 11 acute ischemic stroke patients (five women and six men) ranging in age from 25 to 90 (61±19) years. All subjects were free of medication that affects platelets (aspirin and nonsteroidal anti-inflammatory drugs) for at least 10 days prior to study. Eight stroke patients had large cortical infarcts attributed to major-vessel occlusion, and the other three had small subcortical infarcts attributed to small-vessel occlusion. The diagnosis of stroke was based on a history of the sudden onset of a fixed focal neurologic deficit. Computed tomography was done in all 11 stroke patients to exclude space-occupying lesions and hemorrhage. Five stroke patients were studied ≤24 and six were studied 36–96 hours after the onset of neurologic dysfunction. Four stroke patients suffered mild hypertension, one had diet-controlled diabetes mellitus, and six had neither. All but one of the four hypertensive stroke patients were free of specific drug therapy; this one patient received 160 mg propranolol daily for 6 months prior to study. There were six smokers and five nonsmokers among the stroke patients.

Whole blood was collected and processed using solutions that optimally retain the organelles of platelets. The anticoagulant, which contained citric acid, sodium citrate, and dextrose (each at 0.1 mol/l) was placed in a syringe and mixed with whole blood (1:9 anticoagulant:whole blood). Platelet-rich plasma was prepared by centrifuging the anticoagulated blood at 190g for 15 minutes at room temperature (refrigerated centrifuge, International Equipment Co., Needham Heights, Massachusetts), and 4–5 ml of platelet-rich plasma was mixed with equal parts of White's saline (pH 7.4) containing 0.6% glutaraldehyde; the mixture was kept at room temperature for 15 minutes. The partially fixed platelets were then centrifuged at 800g for 20 minutes at room temperature to form a pellet. The pellet was fixed at room temperature for another 60 minutes after adding White's saline containing 3% glutaraldehyde.
TABLE 1. Number of Platelet Organelles and ATP Release in Healthy Controls and Acute Stroke Patients

<table>
<thead>
<tr>
<th></th>
<th>Controls (n=12)</th>
<th>Patients (n=11)</th>
<th>p</th>
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</thead>
<tbody>
<tr>
<td><strong>Organelles (number per 25 platelet profiles)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dense bodies</td>
<td>17±6</td>
<td>15±4</td>
<td>NS</td>
</tr>
<tr>
<td>Alpha granules</td>
<td>228±43</td>
<td>185±34</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>30±10</td>
<td>22±5</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td><strong>ATP release (pM/900 µl diluted whole blood)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 µg collagen/ml</td>
<td>826±294</td>
<td>899±376</td>
<td>NS</td>
</tr>
<tr>
<td>2 µg collagen/ml</td>
<td>526±229</td>
<td>657±313</td>
<td>NS</td>
</tr>
</tbody>
</table>

Data are mean±SD. NS, not significant.

The glutaraldehyde fixative was removed and replaced with a cacodylate buffer containing 0.2 M sucrose. The pellet was immersed in buffer for 15–30 minutes at 4° C, exposed to a solution of 1% buffered osmic acid for 90 minutes at room temperature, and then dehydrated in a series of graded ethanol solutions. The pellet was next broken into smaller pieces that were embedded in Maraglas. Five plastic blocks were ordinarily prepared from each pellet. Ultrathin sections were cut from three blocks using an ultratome (Model 8801A, LKB-Produkter AB, Stockholm, Sweden) and a diamond knife. Each section was mounted on a 200-mesh copper grid and doubly stained with uranyl acetate and lead citrate.

Using a transmission electron microscope, we photographed platelets present at five random locations on a grid at ×7,000; 10–20 photographs were usually produced per pellet. This method of selection was chosen to minimize the chance of photographing serial sections of a single platelet. The photographic negatives were used to make an ×2.9 enlargement (final print magnification ×20,300) that was printed as an 8×10-in. black-and-white photograph.

An 8×10-in. sheet of transparent plastic was divided into 1-in. squares, and the left corner of each square was numbered consecutively. A micrograph showing sectioned platelets at ×20,300 was placed behind the plastic sheet. Several lists of random numbers were generated with a computer, and a starting point in a list was blindly chosen. Each successive number in the list indicated a square, and the number of dense bodies, alpha granules, and mitochondria in sections of platelets in that square were counted. This procedure was repeated using micrographs from the different blocks until 25 platelets were randomly selected; five or more micrographs were usually required.

Release of adenosine triphosphate (ATP) from platelet dense bodies was measured in whole blood using the luciferin-luciferase reagent in response to stimulation with final concentrations of 2 and 4 µg/ml of collagen as previously described. Briefly, firefly luminous organ extracts that have become dark react to added ATP by producing light in a dose-dependent fashion. ATP released from platelets is reliably estimated by this reaction. Aliquots of 900 µl of whole blood diluted 1:1 by volume with 0.9% saline were placed in cuvettes, warmed for 2–3 minutes at 37° C in the aggregometer heater block, and stirred at 1,200 rpm using a Teflon-coated magnetic stirrer bar; 100 µl (4 mg) of luciferin-luciferase reagent (Chrono-Log Corp., Havertown, Pennsylvania) was added. The maximum height of the ATP-release curve was derived from ATP standard concentrations in picomoles per 900 microliters of whole blood diluted 50%.

**Results**

We performed analysis of covariance (adjusting for age and sex) for each measurement, but no age- or sex-related effects were detected and the results were similar to those of a univariate analysis of variance. Therefore, we report only the univariate results.

The numbers of alpha granules (Figure 1) and mitochondria were significantly reduced in platelets from acute ischemic stroke patients compared with controls; the number of dense bodies also tended to be fewer in platelets from stroke patients. ATP release from platelets in diluted whole blood did not differ significantly between stroke patients and controls, although there was a trend toward greater ATP release in the stroke patients (Table 1).

Comparisons among the stroke patients based on the size of the infarct and the time since the onset of stroke did not reveal any significant differences (Table 2). There was no difference in platelet ultrastructure or ATP release between smoking and nonsmoking stroke patients (data not shown). The one stroke patient receiving propranolol had results comparable to those of the other stroke patients.

**Table 2. Comparisons of Number of Platelet Organelles and ATP Release Between Subsets of 11 Stroke Patients**

<table>
<thead>
<tr>
<th>Infarct size</th>
<th>Organelles (no./25 platelet profiles)</th>
<th>ATP release</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dense bodies</td>
<td>Mitochondria</td>
</tr>
<tr>
<td>Large (n=8)</td>
<td>14±4</td>
<td>23±5</td>
</tr>
<tr>
<td>Small (n=3)</td>
<td>19±4</td>
<td>20±3</td>
</tr>
<tr>
<td>Time since stroke</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤24 hr (n=5)</td>
<td>15±6</td>
<td>21±6</td>
</tr>
<tr>
<td>36–96 hr (n=6)</td>
<td>15±2</td>
<td>23±4</td>
</tr>
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</table>

Data are mean±SD.
Discussion
Ultrasound analysis of platelets from patients who had recently suffered cerebral infarction showed features that were both similar to and different from those of platelets from controls. Shared characteristics included the presence of an intact circumfer-

FIGURE 1. Photomicrographs of platelets from (top) patients with acute ischemic stroke with reduced number of alpha granules (arrowheads) compared with platelets from (bottom) control subjects, in which many alpha granules are present. ×23,200.
potential band of microtubules, masses of glycogen particles, an occasional Golgi complex, and the dense tubular system. All components of the organelar zone (dense bodies, alpha granules, and mitochondria) were also observed in platelets from the two groups of subjects. Quantification of the organelles, however, showed that the platelets from patients who had experienced an acute ischemic stroke were altered.

The number of alpha granules in the platelets of acute ischemic stroke patients was significantly reduced. This morphologic finding may correlate with the measured increases in concentrations of plasma $\beta$-thromboglobulin and platelet factor 4 found in such patients since alpha granules are the source of these substances.5,6 Our previous observation that platelet dense body secretion in whole blood is increased in acute stroke patients1 is also reflected in our present results. The significance of the fewer mitochondria in platelets of stroke patients is uncertain, but one possibility is that mitochondria may have been released or consumed to a greater extent in the activated platelets of stroke patients. Platelet secretory products contain large amounts of vasoactive substances including thromboxane, adenosine diphosphate (ADP), ATP, calcium, and serotonin.12 The release of these substances (particularly thromboxane and serotonin) can result in vascular and neuronal injury.13,14 For example, ADP and arachidonic acid, both potent platelet activating agents, injected directly into the carotid artery of rats and rabbits cause functionally significant structural damage2-4 in the ipsilateral middle cerebral artery territory, probably mediated by platelet release products and unassociated with occlusive platelet aggregates.

Our present study supports the view that platelet secretion occurs in close temporal association with ischemic stroke.1 Platelet secretion may be of separate importance to mechanical occlusion of blood vessels by platelet aggregates in the pathogenesis of cerebral infarction.

References

Key Words • adenosine triphosphate • cerebrovascular disorders • platelets
Platelet ultrastructure and secretion in acute ischemic stroke.
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