Platelet-Activating Factor Acetylhydrolase in Red Cell Membranes

Does Decreased Activity Impair Erythrocyte Deformability in Ischemic Stroke Patients?

Hidemi Yoshida, PhD; Kei Satoh, MD; and Shigeru Takamatsu, MD

Background and Purpose: Platelet-activating factor acetylhydrolase hydrolyzes platelet-activating factor (1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine). It also hydrolyzes oxidized derivatives of phosphatidylcholine that have a short-chain acyl residue at the sn-2 position. This enzyme may act mainly in the degradation of oxidized phospholipids and may play a role in maintaining erythrocyte deformability. Therefore, we assessed the activity of red cell membrane platelet-activating factor acetylhydrolase in patients with ischemic stroke and studied the relation of the enzyme activity to red cell deformability.

Methods: Enzyme activity was measured in the detergent extract of red cell membranes from 38 patients with cerebral thrombosis and 38 age-matched healthy volunteers. Red cell filterability, an index of red cell deformability, was also measured.

Results: The enzyme activity in patients and control subjects was 100 ± 74 and 148 ± 128 nmol/g protein per minute (2.68 ± 2.11 and 3.79 ± 2.46 pmol/10⁶ cells per minute) (mean ± SD), respectively, and the difference was significant (p < 0.05 by the Mann-Whitney U test, two-sided test). Enzyme activity was correlated positively with red cell filterability in the patients (n = 20, r = 0.565, p < 0.01).

Conclusions: Red blood cells from stroke patients have lower levels of platelet-activating factor acetylhydrolase activity when compared with those from healthy subjects. This may result in the accumulation of oxidized lipids in the cell membrane and lead to impaired red cell deformability in patients with cerebral thrombosis. (Stroke 1993;24:14–18)

KEY WORDS • erythrocytes • platelet-activating factor • thrombosis

Impairment of the rheological properties of red blood cells (RBCs) leads to deterioration of the flow dynamics of the cerebral microcirculation. Abnormal hemorheological behavior results from a failure of the normal interactions between vessel wall and bloodstream. Erythrocytes are obliged to pass through small capillaries by undergoing deformation, because these vessels have a diameter smaller than that of the corpuscle. Erythrocyte deformability is regarded as a factor related to cerebral ischemia. A clinical investigation of RBC deformability in patients with cerebral thrombosis recently revealed a close association of the extent of deformability with the severity of this disease. These facts suggest the necessity for studies focusing on the constituents of RBCs that influence their deformability. The erythrocyte membrane contains a variety of phospholipids, and accumulation of their oxidative products may lead to deterioration of the rheological properties of the cells. We have studied the oxidative phospholipid-related metabolism of RBCs from cerebrovascular patients in an attempt to find more effective measures for combating stroke.

Platelet-activating factor (PAF; 1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine) is a potent bioactive phospholipid that plays a critical role in a variety of pathological conditions including stroke and brain injury. PAF is degraded by a specific enzyme, PAF acetylhydrolase (EC 3.1.1.47), which has been purified from human plasma and characterized. This enzyme can also hydrolyze the oxidized derivatives of phosphatidylcholine, which have a short-chain acyl residue at the sn-2 position of the molecule. Stafforini et al have reported the presence of PAF acetylhydrolase in human RBCs and suggested that it could hydrolyze oxidized phospholipids in RBC membranes. Various activities in a PAF acetylhydrolase family are grouped into at least four species, according to their kinetics and physical behavior: the extracellular plasma activity, intracellular tissue, leukocyte, and erythrocyte activities. Erythrocyte PAF acetylhydrolase is distinct from the other three enzymes. We previously showed the existence of PAF acetylhydrolase in the RBC membrane; however, its nature relative to the cytosolic enzyme is still unknown.

Our previous investigation showed a low enzyme activity in the cytosol of RBCs from patients with...
cerebral thrombosis. Because the degradation of oxidized phospholipids may proceed more effectively in membranes than in the cytosol, investigation of membrane PAF acetylhydrolase activity could be useful for clarifying the rheological properties of erythrocytes. In this study, we evaluated RBC membrane PAF acetylhydrolase activity in patients with ischemic stroke.

**Subjects and Methods**

The subjects studied were 38 consecutive patients (20 women and 18 men) with a history of cerebral thrombosis, who ranged in age from 42 to 82 years (mean±SD, 65±9.4 years), and 38 healthy volunteers (22 women and 16 men) aged 50 to 79 years (mean±SD, 64±8.7 years) as a control group.

All subjects were informed about the study, and written consent was obtained. At least 3 months had elapsed since the stroke in the cerebral thrombosis group. Computed tomography was performed in all subjects of this group, and patients with neurological deficits attributable to either lacunar infarction or cerebral embolism were excluded. The patients had been treated with several agents for improving the cerebral circulation, such as pentoxifylline, dihydroergotoxine mesylate, nicardipine hydrochloride, or trapidil. However, they had not received aspirin. The capacity of patients to perform normal daily activities was evaluated using an activity of daily living test (ADL-T). This test involves the evaluation of various aspects of daily life. The highest possible score is 48 points; patients with a score of 0–12 are mostly bedridden, those scoring 13–24 can raise themselves in bed, those scoring 25–36 can handle a wheelchair, and those scoring 37–48 can walk unaided. The number of patients with ADL-T scores of 0–12, 13–24, 25–36, and 37–48 was 12, 9, 7, and 10, respectively. Twenty of the stroke patients consented to the measurement of RBC filterability, which necessitated the collection of an extra 12 ml of blood. They consisted of 12 women and eight men, with an average age of 67±9.6 (range, 47–81) years.

The control subjects had no abnormalities on physical examination (including blood pressure), urinalysis, electrocardiogram, chest x-ray film, and biochemical and hematological screenings. They had not been taking any medications for at least the preceding 4 weeks. Erythrocyte filterability was measured in all control subjects.

Fasting venous blood was collected into tubes containing 5.5% (vol/vol) 77 mM ethylenediaminetetraacetic acid–2Na solution. A membrane-free RBC cytosolic fraction and hemoglobin-free RBC membranes (ghosts) were prepared as described previously.19 From 5 ml of blood, RBCs were sedimented by centrifugation at 1,500g for 10 minutes, and the buffy coat was aspirated along with the plasma. The RBCs were washed three times with 10 ml of 20 mM phosphate-buffered saline (pH 7.4) and finally suspended in an equal volume of the same buffer. A portion of this suspension was used for counting RBCs with a Celltac MEK-4150 (Nihon Kohden, Tokyo). Another portion (1 ml) was pipetted into a 10-ml polycarbonate centrifuge tube and centrifuged as above. The sedimented RBCs were lysed by mixing with 10 ml of cold 7 mM sodium phosphate buffer (pH 7.4) and let stand at 4°C for 20 minutes. The mixture was centrifuged at 20,000g for 20 minutes at 4°C, and the supernatant was pipetted out. A portion of the supernatant was diluted four times with the lysis buffer, and the diluted hemolysate was used for the PAF acetylhydrolase assay. The lysed RBC membranes were washed up to six times with 10 ml of the lysis buffer in the manner described above. Finally, the sedimented ghosts were suspended in 1 ml of the lysis buffer. Detergent extraction of the ghosts was performed as described previously.17 To 480 µl of the ghost suspension we added 20 µl of detergent solution (2.5% [vol/vol] polyoxyethylene sorbitan monolaurate [Tween 20] in the lysis buffer), and the mixture was kept on ice for 20 minutes. Then the mixture was centrifuged at 20,000g for 20 minutes at 0°C, and the supernatant thus obtained was used for the PAF acetylhydrolase assay.

The PAF acetylhydrolase activity in plasma, the diluted hemolysate, or the ghost extract was assayed according to the method of Stafforini et al.11 Plasma was diluted 50-fold with 0.1 M N-2-hydroxyethyl piperazine-N'-2-ethanesulfonic acid (HEPES) buffer (pH 7.2). Each assay mixture contained 5–10 µl of a specimen and 5 µl of substrate; the total volume was adjusted to 50 µl by adding HEPES buffer (pH 7.2 for plasma, pH 7.4 for RBCs). The substrate used was 800 µM [2-acetyl-[3H]PAF (New England Nuclear, Boston, Mass.) dissolved in HEPES buffer (pH 7.4) containing 2.5 mg/ml human serum albumin. The specific activity was adjusted to 833 Bq/nmol (5.0×10^6 disintegrations per minute per nanomole). After incubation at 37°C for 30 minutes, the reaction was stopped by adding 50 µl of 10 M acetic acid. This mixture was then applied to an octadecylsilica gel cartridge (Easy Chromato C18; Gasukuro Kogyo Inc., Tokyo). The cartridge was washed three times with 1.0 ml of 0.1 M sodium acetate, and the radioactivity in the washings was measured by scintillation spectrometry using a liquid scintillation system (LSC-3500; Aloka Co., Ltd., Tokyo). The enzyme activity in RBC membranes was corrected by the factor of 1.33 in all subsequent assays. The details of this method have been described elsewhere.17,18 Intra-assay and interassay coefficients of variation were 3.4% (n=10) and 5.0% (n=15, in three different assays) for plasma, 5.9% (n=5) and 8.0% (n=9, in three different assays) for hemolysate, and 3.9% (n=5) and 7.6% (n=9, in three different assays) for the ghost extract, respectively. The total protein content in the ghost extract or the diluted hemolysate was determined by the method of Lowry20 using bovine serum albumin as a standard.

For the measurement of RBC filterability, cells were suspended in autologous plasma at 10^10 cells per microliter to approximate the low hematocrit in the capillaries.21 This suspension was filtered through a polycarbonate membrane (Nucleapore Corp., Pleasanton, Calif.) with 3-µm pores at 25°C under a negative filtration pressure of 20 cm H2O.22 The filterability index was expressed in terms of the volume (ml) of the suspension that passed through the membrane in 1 minute. This measurement was performed in triplicate, and the intrasuspension precision was 5.0% (n=10).

Data are expressed as mean±SD. Statistical significance was tested by the Mann-Whitney U test or by Student’s t test when applicable, unless otherwise specified. All probability values were based on two-sided tests.
TABLE 1. Laboratory Findings of Patients With Cerebral Thrombosis and Control Subjects

<table>
<thead>
<tr>
<th>Item</th>
<th>Stroke patients (n=38)</th>
<th>Control subjects (n=38)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Red cell count (10^6/μl)</td>
<td>408±42</td>
<td>427±51</td>
</tr>
<tr>
<td>Hemoglobin (g/dl)</td>
<td>13.1±1.3</td>
<td>13.4±1.6</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>37.5±3.5*</td>
<td>40.3±4.3</td>
</tr>
<tr>
<td>MCV (fl)</td>
<td>92.1±3.2*</td>
<td>94.3±3.5</td>
</tr>
<tr>
<td>MCH (pg)</td>
<td>32.2±1.6†</td>
<td>31.4±1.6</td>
</tr>
<tr>
<td>MCHC (g/dl)</td>
<td>35.0±0.96†</td>
<td>33.2±1.0</td>
</tr>
<tr>
<td>White cell count (10^9/μl)</td>
<td>5.5±1.5</td>
<td>5.6±1.8</td>
</tr>
<tr>
<td>Total protein (g/dl)</td>
<td>6.8±0.44‡</td>
<td>7.3±0.44</td>
</tr>
<tr>
<td>Albumin (g/dl)</td>
<td>4.0±0.37†</td>
<td>4.2±0.35</td>
</tr>
<tr>
<td>Total cholesterol (mg/dl)</td>
<td>191±41</td>
<td>195±40</td>
</tr>
<tr>
<td>HDL cholesterol (mg/dl)</td>
<td>42±12†</td>
<td>49±12</td>
</tr>
<tr>
<td>LDL cholesterol (mg/dl)</td>
<td>124±37</td>
<td>115±38</td>
</tr>
<tr>
<td>Triglycerides (mg/dl)</td>
<td>123±59</td>
<td>119±55</td>
</tr>
<tr>
<td>Fasting blood glucose (mg/dl)</td>
<td>85±9.1‡</td>
<td>96±12</td>
</tr>
</tbody>
</table>

Data are mean±SD. MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; HDL, high density lipoprotein; LDL, low density lipoprotein. LDL cholesterol concentration was calculated by the formula of Friedewald et al.23

* p<0.01, † p<0.05, and ‡ p<0.001 vs. control group by Student’s t test (two-sided tests).

Results

The mean ADL-T score of the stroke patients was 22.9±14.4 points. Table 1 summarizes the laboratory findings of the patients and control subjects. Differences between the patients and control subjects were observed with regard to hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, total protein, albumin, high density lipoprotein cholesterol, and fasting blood glucose. However, although these differences were statistically significant, they were not clinically important. The mean RBC filterability index values in the patients and control subjects were 0.46±0.14 and 0.57±0.15 ml/min, respectively, and the difference was significant (p<0.01). There were no significant differences of RBC filterability indexes among the subgroups of patients with different ADL-T scores.

Table 2 summarizes the PAF acetylhydrolase activity levels in RBC membranes, RBC cytosol, and plasma from the stroke patients and control subjects. All the differences between patients and control subjects were significant (p<0.05, Mann-Whitney U test). There were no significant differences of PAF acetylhydrolase activity among the subgroups of patients with different ADL-T scores.

In the stroke patients, linear regression analysis showed a significant association between the RBC filterability index and RBC membrane PAF acetylhydrolase activity per gram of protein (n=20, r=0.565, p<0.01) (Figure 1). The relation between the RBC filterability index and the enzyme activity per erythrocyte was also significant (n=20, r=0.530, p<0.05). In the control subjects, neither relation was significant (n=38, r=0.022 and −0.086, respectively). The RBC cytosolic PAF acetylhydrolase activity per gram of protein was also correlated positively with the RBC filterability index in the stroke patients (n=20, r=0.469, p<0.05), but the activity per erythrocyte was not (n=20, r=0.241). These relations were not significant in the control subjects (n=38, r=0.126 and −0.085, respectively). There were no significant correlations among the RBC membrane, RBC cytosolic, and plasma PAF acetylhydrolase activities in both the patients and the control subjects. There was also no significant correlation between these PAF acetylhydrolase activities and the laboratory data shown in Table 1.

Discussion

The RBC membrane activity of PAF acetylhydrolase tended to be lower in stroke patients than in healthy

![FIGURE 1](http://stroke.ahajournals.org/)

**FIGURE 1.** Plot showing red blood cell (RBC) membrane (ghost) platelet-activating factor acetylhydrolase (PAF-AH) activity and RBC filterability (deformability) in patients with a history of cerebral thrombosis.
control subjects, although there was a significant degree of overlap between the two groups (Table 2). In addition, the cytosolic activity was lower in the patients, a finding in accord with data from our previous study. Moreover, the plasma PAF acetylhydrolase activity was higher in the patients with ischemic stroke than in the control subjects, as in our previous study. When we previously identified the PAF-hydrolyzing enzyme in the RBC membrane as an intracellular form of PAF acetylhydrolase, we confirmed the identity of the membrane and cytosolic enzymes using biochemical methods. These data suggest that the decreased RBC membrane enzyme activity in stroke patients may be due to either an intrinsic abnormality or to enzyme consumption induced by augmented lipid oxidation. Any change that results in a lower activity of PAF acetylhydrolase in the RBC membrane could lead to the accumulation of oxidized membrane phospholipids.

Steinbrecher and Pritchard and Stremler et al have demonstrated the hydrolysis of oxidative derivatives of phosphatidylcholine other than PAF by plasma PAF acetylhydrolase, which was attributed to the presence of a short-chain acyl residue at the sn-2 position in these derivatives. The RBC enzyme can also hydrolyze such derivatives as well as that in plasma. Phosphatidylcholine is a source of oxidized phospholipids and is a major component of RBC membrane phospholipids. PAF does not exert any agonistic activity on RBCs, and these cells do not synthesize the PAF molecule. Therefore, the target of this enzyme activity in the RBC membrane is more likely to be oxidized phospholipids than PAF itself. Damage to RBC membranes by lipid peroxidation impairs the rheological properties of erythrocytes, which allow them to circulate in microvascular channels. The capillary hematocrit value is reported to be 8.4% in cat mesentery, 10–14% in hamster cremaster muscle, and 15% in rat mesentery, and 17% in rabbit omentum. In our experiment, the hematocrit of the blood cell suspension used for the filterability studies was 9%, a value comparable to those mentioned above. Our study detected a positive correlation between the PAF acetylhydrolase activity in RBC membranes and the RBC filterability index in the stroke patients (Figure 1). This finding may support the putative role of the RBC membrane enzyme as a scavenger of oxidized phospholipids because phospholipid oxidation and degradation of oxidation products occur mainly in cell membranes, and it may suggest an important role for the membrane enzyme in maintaining RBC deformability.

Ischemic tissue injury is known to be associated with the release of PAF, and a PAF antagonist is reported to be effective in reducing infarct volume. In addition, tissue injury can cause impairment of blood cell rheological properties. These facts may be explained in part by the generation of oxidized phospholipids, because some of these compounds are known to exert their activity through binding to the PAF receptor. It is reported that RBC deformability is lower in stroke patients than in control subjects, although there is some controversy about this finding. Abnormal blood rheological properties are reported to be associated with a poor prognosis after stroke. The question of whether rheological abnormalities cause the cerebral ischemia or vice versa merits further study. Our present results suggest that measurement of the PAF acetylhydrolase activity in erythrocytes may provide useful information concerning the hemorheological state of stroke patients.

In conclusion, PAF acetylhydrolase may play an important role in scavenging oxidized phospholipids in RBC membranes and in maintaining the normal rheological properties of erythrocytes. A decrease in the activity of this enzyme may precipitate various pathological events related to ischemic cerebrovascular disease by retarding RBC deformability in the microcirculation.

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