Possible existence of platelet activation before the onset of cerebral infarction

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Received 11 January 1999; received in revised form 29 November 1999; accepted 7 January 2000

Abstract

To study the existence of platelet activation before the onset of cerebral infarction, the ultrastructural features of platelets (7-day survival) and coagulation-fibrinolytic markers (70–100-min life span) were measured 2–12 h (acute phase), 7 days (subacute phase) and 6 months (chronic phase) after onset in 18 patients with cerebral infarction. Seven patients with atherosclerosis but without cerebral infarction and eight healthy subjects were studied as controls. Ultrastructural study included folds, pseudopods, vacuoles and centralization in addition to immunochemical staining such as platelet peroxidase and fibrinogen. Furthermore, β-thromboglobulin, platelet factor-4, thrombin antithrombin complex and α2-plasmin inhibitor plasmin complex were examined as coagulation-fibrinolytic markers. Ultrastructural study of circulating platelets demonstrated no difference between acute and chronic phases and little difference between cerebral infarction and atherosclerosis, although plasma coagulation-fibrinolytic markers showed an increase in cerebral infarction at the acute phase but no difference among the chronic phase of cerebral infarction, atherosclerosis and normal healthy subjects. It is considered that shape change in circulating platelets was caused by pre-existed atherosclerosis rather than the thrombotic event itself though coagulation-fibrinolytic markers were derived from the thrombotic event. © 2000 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Platelet activation; Cerebral infarction; Atherosclerosis; Ultrastructure; Platelet peroxidase

1. Introduction

Although morphological analysis of platelets in patients with cerebral infarction demonstrated platelet shape change as a result of a thrombotic event [1], little is known about the ultrastructure of platelet in atherosclerosis. It remains controversial whether the shape change observed in platelets obtained from peripheral blood is a result of a thrombotic event or it represents the remaining platelets that escaped the thrombotic event. As injured platelets are removed rapidly by the spleen, ultrastructural shape change observed during the subsequent several days seems to be due to pre-existing atherosclerosis rather than the thrombotic event itself. Although it was reported that shape changes such as folds, pseudopods, ballooning and decreased α-granules were observed in platelets obtained from injured vessels [2,3], the result alone cannot fully explain the question described above. Recently, proteins released from platelets, β-thromboglobulin (β-TG) and platelet factor-4 (PF-4), were reported to be useful markers for platelet activation [4]. They are removed from circulation with a half life of 70–100 min. Some reports demonstrated significant increases in plasma β-TG and PF-4 after the onset of cerebral infarction but others did not [5,6]. As platelet peroxidase (PPO) detected in the dense tubular system is associated with prostaglandin synthesis [7–9], PPO is supposed to play a role in platelet activation. Fibrinogen is localized in the α-granules of resting platelets and released by physical and chemical stimulations [10]. Thus, we examined the ultrastructure of platelets with reference to the localization of PPO and fibrinogen, and the plasma levels of β-TG and PF-4 in patients with cerebral infarction at 3 points: at the acute phase or

Abbreviations: β-TG, β-thromboglobulin; Fbg, fibrinogen; PF-4, platelet factor-4; PIC, α2-plasmin inhibitor plasmin complex; PPO, platelet peroxidase; TAT, thrombin antithrombin complex.

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immediately after onset, at the subacute phase or 7 days after onset, and at the chronic phase or 6 months after onset, in comparison with that in patients with atherosclerosis and healthy subjects, in order to deduce whether platelet shape change observed in peripheral blood after onset is caused by a thrombotic event itself or by pre-existing atherosclerosis.

2. Materials and methods

2.1. Patients and controls

Eighteen newly diagnosed patients with cerebral infarction (11 males and seven females, 63.8 ± 8.1 [mean ± SD] years) presenting hemiplegia, who were treated in our hospital between 1995 and 1996, were included in this study. Patients with consciousness disturbance or infectious disease at acute phase were excluded from this study. There were no patients demonstrating any episode of myocardial or pulmonary infarction, primary platelet or coagulation disorder, or any evidence that the cerebral infarction was due to cardiac embolism. No patients were given anticoagulant or antiplatelet drugs during the period of this study. Repeated CT scans demonstrated no evidence of post-infarction hemorrhage in any patients. An age-matched control group consisted of eight normal healthy volunteers (five males and three females, 61.3 ± 10.9 years). These eight volunteers had no diseases that could be detected by physical, urinary, fecal and blood examinations, chest radiography, echocardiogram, and brain CT scan. A second control group consisted of seven patients with atherosclerosis (five males and two females, 73.7 ± 6.8 years), which was diagnosed based on either moderate or severe hypertension in stage 2 [11] and arteriosclerotic retinopathy of grade II or III by Keith-Wagener-Barker classification [12]. These patients had been all treated with calcium antagonist alone and had not demonstrated any evidence of coronary heart disease, cerebrovascular disease or primary platelet disorder. Informed consent was obtained from all patients and control subjects. This study was approved by the ethical committee of Kusatsu Branch Hospital, Gunma University Hospital.

2.2. Ultrastructural study

Venous blood was carefully collected without a tourniquet into a plastic syringe containing acid citrate dextrose solution at three points: at the acute phase or 2–12 h after the onset of cerebral infarction, at the subacute phase or 7 days later, and at the chronic phase or 6 months later in patients with cerebral infarction. Control blood samples were obtained from atherosclerotic patients and normal healthy subjects at 08:00 h. The blood sample was gently transferred into a plastic tube and centrifuged at 190 × g for 15 min to obtain platelet-rich plasma. The platelet-rich plasma was transferred into another plastic tube containing acid citrate dextrose solution and further centrifuged at 800 g for 15 min. The pellet was used for ultrastructural study by the conventional method using an electron microscope (JEM 200CX, JEOL, Japan). Two hundred platelets were observed to evaluate the frequency of folds, pseudopods, vacuoles and centralization. PPO reaction was performed by the method of Breton-Gorius et al. [8]. Immunoelectron gold staining was performed by the method of Cramer et al. [10]. Anti-fibrinogen polyclonal antibody and goat antirabbit immunoglobulin fractions coupled to colloidal gold particles were purchased from Dakopatts (Copenhagen) and Janssen Pharmaceutica (Belgium), respectively.

The degrees of PPO staining and immunogold staining were tentatively expressed as follows [13]: full staining of PPO or immunogold was scored as 3, while intermediate, weak and no stainings were scored as 2, 1 and 0, respectively. The total score was calculated as the sum after counting 100 platelets at random. Ultrastructural analysis was performed by an electron microscopist who did not know the patients’ information.

2.3. Hematological variables

Plasma levels of β-TG, PF-4, thrombin antithrombin complex (TAT) and α2-plasmin inhibitor plasmin complex (PIC) were measured by the enzyme immunoassays in patients with cerebral infarction and atherosclerosis and in normal healthy subjects.

2.4. Statistical analysis

All data are presented as mean ± SD. Unpaired Student’s t-test was used to evaluate the significance of differences between the two categorical variables. Differences were considered significant at P < 0.05.

3. Results

Eighteen patients were evaluable 7 days after cerebral infarction and 11 out of 18 patients were eligible 6 months after cerebral infarction because seven patients dropped out from this study: four patients changed their place of residence in order to live with their children as care givers and three patients developed infectious disease during the period. There were no patients with recurrent cerebral infarction or other thrombotic event until 6 months after the onset of cerebral infarction. Fig. 1 shows typical resting platelets presenting no morphologic change obtained from a normal healthy subject and activated platelets with
Fig. 1. Typical ultrastructure of platelets. (A) Peroxidase reaction in a platelet obtained from a normal subject. Scale bar = 0.5 μm. (B) Peroxidase reaction in a platelet obtained from a stroke patient (5 h after the onset of stroke). Scale bar = 1.0 μm. (C) Immunoelectron gold staining for fibrinogen in a platelet obtained from a normal subject. Scale bar = 0.5 μm. (D) Immunoelectron gold staining for fibrinogen in a platelet obtained from a stroke patient (3 h after the onset of stroke). Scale bar = 0.5 μm.

Table 1
Ultrastructural findings and coagulation-fibrinolytic markers in patients with cerebral infarction and normal subjects

<table>
<thead>
<tr>
<th>Patients with cerebral infarction</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
</tr>
<tr>
<td>Acute phasea,b</td>
<td>B</td>
</tr>
<tr>
<td>2–12 h n = 18</td>
<td>27.0 ± 7.8</td>
</tr>
<tr>
<td>Subacute phasea,b,c</td>
<td></td>
</tr>
<tr>
<td>7 days n =</td>
<td></td>
</tr>
<tr>
<td>Chronic phasea,b,c,d</td>
<td></td>
</tr>
<tr>
<td>6 months n = 11</td>
<td></td>
</tr>
<tr>
<td>PPO score</td>
<td>218 ± 34</td>
</tr>
<tr>
<td>Fbg score</td>
<td>142 ± 36</td>
</tr>
<tr>
<td>β-TG (ng/ml)</td>
<td>74.8 ± 24.6</td>
</tr>
<tr>
<td>PF-4 (ng/ml)</td>
<td>40.1 ± 23.2</td>
</tr>
<tr>
<td>TAT (mg/ml)</td>
<td>5.28 ± 2.60</td>
</tr>
<tr>
<td>PIC (mg/ml)</td>
<td>0.80 ± 0.26</td>
</tr>
</tbody>
</table>

* Acute, subacute and chronic phases show results 2–12 h, 7 days and 6 months after the onset of cerebral infarction, respectively. Data are presented by mean ± SD.

b The values at the chronic and subacute phases are compared with those at the acute phase, (A vs B, A vs C); *P < 0.05, **P < 0.01 by unpaired Student’s t-test.

c The values at the subacute phase are compared with those at chronic phase, (B vs C); §§P < 0.01 by unpaired Student’s t-test.

d The values in patients with atherosclerosis and healthy subjects are compared with those at the acute phase, (C vs D, C vs E); ¶¶P < 0.01 by unpaired Student’s t-test.

e The values in patients with atherosclerosis are compared with those in patients with cerebral infarction at the chronic phase, (D vs E); ¶¶P < 0.01 by unpaired Student’s t-test.

Subacute and chronic phases of cerebral infarction (Table 1). The frequency of pseudopods in patients with atherosclerosis was similar to that in patients with cerebral infarction at the chronic phase but significantly higher than that in normal healthy subjects. The frequency of vacuoles in cerebral infarction at the chronic phase was significantly higher than that in patients with atherosclerosis which was significantly higher than that in normal healthy subjects. The frequency of centralization in patients with cerebral infarction at the chronic phase was significantly higher than both in atherosclerosis and in normal healthy subjects. Strong PPO activity was detected only in the dark tubular system in healthy subjects but weak PPO reaction was scarcely observed in patients with cerebral infarction at the acute, subacute and chronic phases (Fig. 1). Similarly, although fibrinogen was detected exclusively in α-granules in normal healthy subjects, less fibrinogen was observed in patients with cerebral infarction at the acute, subacute and chronic phases. Table 1 shows the total scores of PPO and fibrinogen content of platelets in patients with cerebral infarction and atherosclerosis and healthy subjects. PPO and fibrinogen scores in patients with cerebral infarction at the chronic phase were significantly decreased compared with those in normal healthy subjects (Table 1). However, there was no significant difference between patients with atherosclerosis and normal healthy subjects.
Plasma levels of \( \beta \)-TG, PF-4 and TAT at the acute phase were significantly higher than those both at the subacute and at the chronic phases. In addition, the plasma level of \( \beta \)-TG at the subacute phase was significantly higher than that at the chronic phase and the plasma level of PF-4 at the subacute phase showed a tendency to increase compared with that at the chronic phase. However, there were no significant differences among patients with cerebral infarction at the chronic phase, patients with atherosclerosis, and normal healthy subjects. The plasma level of PIC did not change.

4. Discussion

In the acute phase of cerebral infarction, the plasma levels of \( \beta \)-TG, PF-4 and TAT were increased when compared with those in normal healthy subjects, suggesting the existence of platelets being destroyed. The frequencies of pseudopods, vacuoles and centralization were increased. In the subacute phase, the plasma level of \( \beta \)-TG remained significantly increased when compared with those in normal healthy subjects and those of PF-4 and TAT were decreased to normal levels, suggesting that platelets were still being destroyed or had been destroyed. The increase in platelet shape change persisted. In the chronic phase, the plasma levels of \( \beta \)-TG, PF-4 and TAT were decreased to normal levels. The frequencies of pseudopods, vacuoles and centralization remained significantly increased when compared with those in normal healthy subjects. The frequency of pseudopods in cerebral infarction at the chronic phase was equal to that in atherosclerosis and higher than that in normal healthy subjects. The frequency of vacuoles in cerebral infarction at the chronic phase was higher than that in atherosclerosis which was higher than that in the normal healthy subjects. The frequency of centralization in cerebral infarction at the chronic phase was higher than that in atherosclerosis which was equal to that in the normal healthy subjects. In contrast, the platelet shape change, and PPO and fibrinogen scores were equal among acute, subacute and chronic phases of cerebral infarction. In atherosclerotic patients, the frequencies of pseudopods and vacuoles were higher than those of normal healthy subjects, though the serum levels of \( \beta \)-TG, PF-4 and TAT were decreased to normal levels. Therefore, platelet shape changes or platelet activation existed in atherosclerotic patients and furthermore, platelet activation may have existed before the onset of cerebral infarction, considering that \( \beta \)-TG and PF-4 have a 70–100-min life span and increase transiently after the onset of cerebral infarction [4–6], platelet survival is about 7 days and injured platelets are rapidly trapped by the spleen. In addition, this study deduced that centralization might be characteris-tic for cerebral infarction or unstable atherosclerosis. Though the transient increase in \( \beta \)-TG, PF-4 and TAT may be attributable to the thrombotic event itself, the consistent increase in pseudopods, vacuoles and centralization and the coincidental decrease in PPO and Fbg may be caused by preexisting atherosclerosis.

The ultrastructural platelet shape change at the acute phase in this study was consistent with a study reported previously [1], which did not describe platelet shape change at the chronic phase. Ultrastructural platelet shape change caused by mechanical or electrical damage [2,3] also resembles the results of our study. Our study revealed that the decrease in PPO and fibrinogen concentrations coincided with the increase in platelet shape change. Peroxidase in platelets is reported to play a role in the synthesis of prostaglandins associated with hemostasis, thrombosis and aggregation [14]. Fibrinogen is synthesized only in megakaryocytes and cannot be taken up by platelets from plasma [15]. In addition, it was reported that the fibrinogen content in \( \alpha \)-granules decreased after platelet activation. Thus, increased pseudopods, vacuoles and centralization, and decreased peroxidase activity and fibrinogen content observed in patients with cerebral infarction at the chronic phase may have been partly caused by atherosclerosis or endothelial injury. It is interesting that centralization was increased in patients with cerebral infarction but not in patients with atherosclerosis. The centralization was reported to be a final process of morphological change in platelet activation in contrast to folds and pseudopods which might be reversible and were separated events from centralization [16]. Since atherosclerotic patients included in this study had no episodes of thrombosis or ischemia, they seemed to have had stable atherosclerosis or plaque. Unstable atherosclerosis or plaque might be associated with centralization, while the stable forms might be associated with pseudopods and vacuoles. Platelet activation observed in hypertensive patients was reversible change and platelets might not be completely activated by stable atherosclerosis alone. Thus, centralization might be a possible marker of unstable plaque or infarction.

Besides platelet activation, various factors such as arterial blood pressure, plasma catecholamine, blood fibrinolytic activity, blood viscosity, and physical and mental stress after waking have been proposed to be associated with the onset of cerebral infarction [17,18]. However, it remains true that crack or endothelial injury is an important trigger of cerebral and myocardial infarctions and closely related to platelet activation [12,19–21]. Recently, some markers of platelet activation were reported to be increased several months after the onset of cerebral infarction [22,23] and other markers were also reported to be increased in hypertensive patients [24,25]. Though ultrastructural studies were not included in those reports, our ultrastructural study...
may be a useful addition to their results. Comparing outcomes among cerebral infarction, atherosclerosis and healthy subjects, circulating platelets might begin to be activated in atherosclerosis before a thrombotic event occurs. Although thrombosis is caused by ulcerated plaque, increased aggregability of platelets, and endothelial injury by platelets [26–30], it is suggested that morphological platelet activation is associated with the increasing risk of cerebral infarction in atherosclerotic patients.

References