Platelets Treated with Ticlopidine Are Less Reactive to Unusually Large von Willebrand Factor Multimers than Are Those Treated with Aspirin under High Shear Stress

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Ticlopidine · Aspirin · von Willebrand factor · Cerebral ischemia

Abstract
Much attention has recently been focused on the interaction between unusually large von Willebrand factor multimers (UL-VWFM) and platelets under high shear stress in pathological thrombus formation. The antiplatelet drugs acetylsalicylic acid (aspirin) and a thienopyridine derivative (ticlopidine) are commonly used to treat cerebral ischemia but exert different effects on high-shear-stress-induced platelet aggregation (H-SIPA) in the plasma. To examine the effects of these drugs in the absence of plasma factors, we studied H-SIPA using washed platelets (WPs) and purified UL-VWFM. WPs were prepared from the blood of 9 aspirin-treated and 11 ticlopidine-treated patients with cerebral ischemia, and H-SIPA in the presence of UL-VWFM was measured using a cone plate aggregometer. Plasma levels of VWF antigen with its multimer analysis, ristocetin cofactor and VWF-cleaving protease (ADAMTS13) activity were also measured. Forty-six healthy volunteers from 2 age groups, 20–40 years (n = 20) and 41–60 years old (n = 26), were also tested as controls. H-SIPA was significantly inhibited for ticlopidine-treated platelets, but it was observed to a lesser extent for aspirin-treated platelets. For both groups, no difference in the plasma levels of VWF antigen, ristocetin cofactor and ADAMTS13 activity was noted. All patients possessed UL-VWFM, and it was detected in healthy volunteers with increasing frequency with increasing age. Under plasma-free conditions, platelets from aspirin-treated patients exhibit marginal but significant inhibition of H-SIPA. Furthermore, the presence of UL-VWFM in the plasma of patients and normal volunteers is directly related to their age rather than being a consequence of underlying disease.

Introduction

Antiplatelet drugs such as aspirin and thienopyridine derivatives are commonly used to treat patients with cerebral ischemia to prevent the occlusion of brain arteries. Aspirin inhibits thromboxane A\textsubscript{2} synthesis [1], and the thienopyridine derivatives ticlopidine and clopidogrel block the ADP receptor P2Y\textsubscript{12} [2–4].

In 1994, Uchiyama et al. [5] reported that high-shear-stress-induced platelet aggregation (H-SIPA) using platelet-rich plasma (PRP) is enhanced in patients with cere-
bral ischemia due to the increase in large von Willebrand factor (VWF) multimers and that the enhancement of H-SIPA can be corrected by taking ticlopidine but not low-dose aspirin. H-SIPA is mediated by the interaction of the platelet receptors glycoprotein (GP) Ibα and GP Iib/IIIa with VWF, which is a plasma GP exclusively synthesized in vascular endothelial cells and secreted into the circulation as unusually large VWF multimer (UL-VWFM). UL-VWFM most actively interacts with platelets and induces the formation of platelet thrombi under high shear stress conditions [6]. In the normal circulation, however, UL-VWFM is rapidly cleaved and degraded into smaller VWFM by the plasma protease VWF-cleaving protease (ADAMTS13), which attacks the Tyr842–Met843 bond [7].

In this study, we analyzed the effect of two antiplatelet drugs on H-SIPA using a washed platelet (WP) system and examined the UL-VWFM of patients with cerebral ischemia using SDS-0.9% agarose gel electrophoresis.

### Materials and Methods

#### Subjects

Twenty patients with cerebral ischemia who were being treated with antiplatelet drugs were enrolled in this study as listed in Table 1 after informed consent had been obtained. Diagnoses were made based on neurological examinations, routine laboratory data, brain computed tomography, brain magnetic resonance imaging and cerebral angiography. None of the patients had findings of major vessel occlusions and neurological symptoms at the time of registration. They were not given any antiplatelet drugs for 2 weeks and any anticoagulants or fibrinolytic drugs for 24 h prior to the study. Then the patients were randomly allocated to receive 200 mg ticlopidine (Panaldin®, Daiichi Pharmaceutial Corp.) or 81 mg aspirin (Bufferin®, Lion Corp.) by choosing the envelope including a card with a drug name, ticlopidine or aspirin. There was no statistically significant difference between these two groups concerning the demographics such as age, gender and clinical symptoms.

For each patient, 18 ml of blood was withdrawn by venipuncture from an antecubical vein using a 21-gauge needle before and days 7 after oral administration of ticlopidine or aspirin; the patient was then anticoagulated with a 1/10th volume of 3.8% Na2-citrate. We chose day 7 after oral administration as the 2nd examination point because ticlopidine inhibits platelet aggregation after 3–5 days of use [8]. Five milliliters of the citrated blood were subsequently centrifuged at 3,000 × g for 15 min at 4 °C, and the platelet-poor plasma was separated and stored in aliquots at −80 °C until use. The remaining 15 ml was used to prepare WPs as described below.

For the control experiments, citrated platelet-poor plasma was also prepared from two groups of healthy volunteers composed of 13 males and 7 females aged 20–40 years and 25 male and 1 female volunteers aged 40–61 years.

#### Preparation of UL-VWFM

Purification of VWF from cryoprecipitate was performed as described elsewhere [9]. Briefly, cryoprecipitate was prepared from 3 liters of outdated fresh frozen plasma, provided from the Japan Red Cross Blood Center by freezing at −80 °C and thawing over-night at 4 °C. After centrifugation at 7,500 × g for 30 min at 4 °C, the cryoprecipitate was then collected and dissolved in 300 ml of 25 mmol/l Tris-HCl buffer (pH 7.3) containing 0.5 mmol/l EDTA-4Na, 150 mmol/l NaCl and 1 mmol/l phenylmethylsulfonyl fluoride. Next, it was centrifuged at 7,500 × g for 15 min at room temperature, and the supernatant was applied to a gelatin-sepharose 4B (Amersham Bioscience) column (Vt = 200 ml) at room temperature to remove fibronectin, after which the fall-through fractions were pooled. Following precipitation with 40% saturated (NH4)2SO4 and centrifugation, the precipitate was separated and dissolved in 50 ml of 20 mmol/l imidazole-HCl buffer (pH 6.5) containing 20 mmol/l e-aminocaproic acid, 1 mol/l NaCl and 10 mmol/l sodium citrate. It was then centrifuged again at 7,500 × g for 15 min, and the resulting supernatant was applied to a sepharose 4B gel filtration column (5 × 100 cm, Amersham Bioscience). The eluate was collected in 8 ml volumes in separate tubes and dialyzed against phosphate-buffered saline (pH 7.3) at 4 °C overnight, and the fractions containing UL-VWFM (fig. 1 and Results) were pooled and kept frozen in aliquots at −80 °C. This purified material was used subsequently throughout this study.

### Table 1. Patient characteristics

<table>
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<th>Age years</th>
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TIA = Transient ischemic attack; RIND = reversible ischemic neurological deficit; DM = diabetes mellitus; HT = hypertension; HL = hyperlipidemia; AP = angina pectoris.
The Effects of Ticlopidine and Aspirin on H-SIPA

Measurement of H-SIPA Using WPs

WPs were prepared under room temperature as follows [10]. First, PRP was produced by centrifugation at 200 g for 10 min using 15 ml citrated blood from patients as mentioned in the Subjects section and from a normal volunteer (blood type 0, Rho(D)+), and then acidified to pH 6.5 with acid citrate dextrose. The resulting platelets were separated from the PRP by centrifugation at 800 g for 10 min in the presence of 1 U/ml apyrase (Sigma-Aldrich) and 1 mmol/l PGE₁. The platelet pellets were next resuspended in HEPES-Tyrode’s buffer (138 mmol/l NaCl, 2.8 mmol/l KCl, 2 mmol/l CaCl₂ and 10 mmol/l HEPES, pH 7.4) at a concentration of 3 × 10⁹/ml and used within 2.5 h.

H-SIPA was measured using an argon-laser-assisted cone platelet aggregometer (Torey Medical Inc., Tokyo, Japan) [11] at room temperature. Before applying constant high shear stress at 10⁸ dyn/cm², the purified UL-VWFM was added to the platelet suspensions at a final concentration of 5 µg/ml in a total volume of 400 µl. For some experiments, H-SIPA was measured in the presence of anti-VWF monoclonal antibody (NMC-4), for which the epitope resides on the VWF A1 domain and inhibits its binding to platelet GP Ib [9].

Additional Assays

Assays for VWF antigen [12] and ristocetin cofactor [13] were performed in addition to SDS-0.9% agarose gel electrophoresis followed by Western blotting with luminographic detection of VWFM [14, 15]. Plasma ADAMTS13 activity was assayed by the modified method of Furlan et al. [16] based on VWFM analysis [17]. The activity of pooled normal plasma was defined as 100%.

Statistical Analysis

Paired and unpaired comparisons between the two groups were performed using the Student’s t test for which p < 0.05 was judged to indicate statistical significance. All experimental data are presented as means ± SD.

Results and Discussion

From SDS-1.2% agarose gel electrophoretic analysis, the initial half void volume fractions (F71–78) were found to possess UL-VWFM and also showed a single 250-kD band by SDS-5% polyacrylamide gel electrophoresis under reducing conditions as shown in the left inset. H-SIPA using normal WPs and the purified VWF fraction. H-SIPA using a mixture of normal WPs at a final concentration of 30 × 10⁹/ml and each purified VWF fraction at a final concentration of 5 µg/ml was dependent on its multimeric size.

For the patients treated with ticlopidine, H-SIPA was dramatically but not totally inhibited, and the value was 43.8 ± 17.7% before and 21.2 ± 10.5% after treatment (p < 0.001) as shown in figure 2. Furthermore, for the patients treated with aspirin, H-SIPA was also inhibited to

![Fig. 1. Preparation of UL-VWFM. a Chromatographic separation of VWF on a Sepharose 4B column, where the black bar indicates the fractions which contain UL-VWFM. The right inset shows the results of the multimeric analysis performed by SDS-1.2% agarose gel electrophoresis. NP = Normal human plasma. To check the purity of VWF, the purified UL-VWFM fraction was subjected to SDS-5% polyacrylamide gel electrophoresis under reducing conditions as shown in the left inset. b H-SIPA using normal WPs and the purified VWF fraction. H-SIPA using a mixture of normal WPs and each purified VWF fraction at a final concentration of 5 µg/ml final concentration was dependent on its multimeric size.](image-url)
some extent but less than that after ticlopidine treatment, and the value was $54.5 \pm 16.0\%$ before and $44.6 \pm 12.5\%$ after treatment ($p = 0.0017$), which is in contrast to the results of Uchiyama et al. [3], who found a remarkable inhibition of H-SIPA in patients treated with ticlopidine but no significant inhibition in those treated with aspirin using a PRP system.

Since H-SIPA in the PRP system is influenced by the nature of the platelets and multiple plasma factors, we analyzed the plasma VWF of both patient groups. As shown in figure 2, both groups exhibited a remarkably increased level of plasma VWF antigen and ristocetin cofactor compared with young healthy volunteers. The plasma VWF antigen and ristocetin cofactor of patients with cerebral ischemia were significantly unchanged before and after treatment, but a remarkable increase in both was observed in subjects of advanced age. Furthermore, a significant decrease in plasma ADAMTS13 activity compared with young healthy volunteers was noted for both patient groups, which was unchanged before and after treatment. A remarkable decrease was also observed for subjects of advanced age. The reason why the activity of plasma ADAMTS13 decreased in both patients with cerebral ischemia and advanced-age subjects is consid-

![Fig. 2. Comparison of H-SIPA, platelets and VWF. For the patients treated with ticlopidine, H-SIPA was dramatically inhibited after treatment as was the case for patients treated with aspirin, although to a lesser extent. The plasma VWF antigen, ristocetin cofactor and ADAMTS13 activity in patients from both groups were significantly unchanged before and after treatment.](image-url)
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tered to be decreased production of ADAMTS13 in the liver or consumption of ADAMTS13 to degrade very high amounts of VWF antigen. Mannucci et al. [18] speculated that the consumption of ADAMTS13 degraded a large amount of VWF antigen in order to explain the inverse correlation between ADAMTS13 activity and VWF antigen in healthy and various pathological conditions. Decreased activity of ADAMTS13 together with a large amount of VWF antigen may induce the appearance of UL-VWFM in plasma, which may result in a risk factor for cerebral ischemia to occur.

Our assay using the patient’s WPs was successful at determining platelet function because it excluded the effect of plasma factors. Likewise, Sun et al. [19] have recently reported that something in the plasma was responsible for the phenomenon of shear aggregation ‘aspirin resistance’.

Next, we examined the UL-VWFM of patients with cerebral ischemia by SDS-0.9% agarose gel electrophoresis as shown in figure 3. All patients with cerebral ischemia in both groups had UL-VWFM, and this confirmed the observed increase in plasma VWF antigen and ristocetin cofactor in patients with cerebral ischemia. UL-VWFM was also detected in 20 of 26 subjects of advanced age, whereas it was only seen in 3 out of 20 young healthy volunteers.

Cerebral ischemia is caused by platelet thrombosis induced by high shear stress. Recent studies revealed that

Fig. 3. The detection of UL-VWFM by SDS-0.9% agarose gel analysis. All patients with cerebral ischemia in both groups had UL-VWFM, and UL-VWFM was also detected in 20 of 26 of subjects of advanced age, whereas it was only seen in 3 out of 20 young healthy volunteers. NP = Normal human plasma.
VWF and its interaction with the platelet receptors GP Ibα and GP IIb/IIIa play a role in platelet thrombosis induced by this type of stress [4]. In this study, we found that the enhancement of H-SIPA in patients with cerebral ischemia is corrected for by taking ticlopidine and aspirin, and that ticlopidine has a stronger effect than does aspirin. These drugs seem to solely affect platelet function. In addition, subjects of advanced age possessed UL-VWFM more frequently than did young subjects. These results indicate that people of advanced age may be more susceptible to developing cerebral ischemia.

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References