AJvW-2, an Anti-vWF Monoclonal Antibody, Inhibits Enhanced Platelet Aggregation Induced by High Shear Stress in Platelet-Rich Plasma From Patients With Acute Coronary Syndromes

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Abstract—The platelet aggregation that is dependent on von Willebrand factor (vWF) is important in the thrombogenesis that occurs under conditions of high shear stress, eg, during acute coronary syndromes (ACSs). A monoclonal antibody, AJvW-2, directed against the A1 domain of human vWF specifically blocks the interaction between plasma vWF and platelet glycoprotein (GP) Ib. To evaluate the association between the vWF-GPIb interaction and the enhanced shear-induced platelet aggregation (SIPA) observed in ACSs, we tested the effect of this antibody on platelet aggregation. Platelet-rich plasma was prepared from the citrated blood of 12 patients with unstable angina (UAP) and 20 patients with acute myocardial infarction (AMI) who were admitted within 3 hours of the onset of cardiac symptoms and from 18 controls. We observed the following: (1) 1.7-fold higher plasma levels of vWF and ristocetin cofactor activity in UAP patients and (2) 2.8-fold higher levels in the AMI group than in controls. Using a cone-and-plate viscometer, we measured the mean value of SIPA under high-shear conditions (108 dyne/cm²) and found them to be 1.3-fold higher in the UAP group and 2.0-fold higher in the AMI group than in controls. The high SIPA in all groups was completely inhibited by 10 µg/mL AJvW-2. Under low-shear conditions (12 dyne/cm²), platelet aggregation was increased only in the AMI group, but this was unaffected by AJvW-2. We observed a significant correlation in both ACS groups between high SIPA and the plasma vWF level or vWF larger multimers. These findings suggest that the vWF-GPIb interaction is important in coronary occlusion and that inhibition of this interaction (with the use of AJvW-2) may prevent further events in the coronary arteries. (Arterioscler Thromb Vasc Biol. 1999;19:877-882.)

Key Words: platelet aggregation ■ shear stress ■ glycoprotein Ib ■ von Willebrand factor ■ acute coronary syndromes

Activation of platelets induced by physical shear stress or by physiological agonists is important in arterial thrombogenesis.1,2 Increased levels of plasma von Willebrand factor (vWF) in patients with thrombotic disorders have suggested that this protein may be involved in thrombosis. For example, in survivors of acute myocardial infarction (AMI), the level of plasma vWF may be an index of the increased risk for reinfarction and mortality.3 In addition, an enhanced plasma vWF concentration is thought to be an independent predictor of such acute coronary syndromes (ACSs) as unstable angina (UAP).2 Most importantly, high physiological and/or pathological shear stress can induce vWF-mediated platelet aggregation in vitro1,2,5,6 and may stimulate the interaction between plasma vWF and glycoprotein (GP) Ib on platelets in vivo, eg, in animal models with coronary artery occlusion.7 To further clarify the molecular mechanism for arterial thrombogenesis and to seek an effective strategy for its prevention or treatment, efforts have focused on developing simple methods for monitoring vWF-platelet interactions in the blood of patients with ACSs.2 The recent development of a cone-and-plate viscometer to monitor platelet aggregation under various shear conditions in the absence of an agonist2,5,8 has been used to study the mechanism of vWF-dependent shear-induced platelet aggregation (SIPA). It was shown that a rotation of 70 to 108 dyne/cm² in this instrument can induce vWF-dependent platelet aggregation, whereas a rotation of 10 to 40 dyne/cm² can induce platelet aggregation mediated by the binding of fibrinogen to GPIIb/IIIa.2,5 On the other hand, it was recently reported that the blockade of platelet GPIIb/IIIa receptors by monoclonal antibodies (MoAbs) like c7E3 Fab or by antagonists such as MK-383, Ro 44-9883, Ro 43-8857, and DMP728 specifically

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inhibits the development of thrombotic and ischemic disorders.\textsuperscript{9,10} In particular, the administration of c7E3 Fab in patients at high risk for ischemic complications significantly reduced the incidence of such complications after coronary angioplasty or atherectomy.\textsuperscript{11} However, these patients exhibited an increased risk of bleeding during and after the administration of c7E3.\textsuperscript{11}

The anti–human vWF MoAb AJvW-2 reacts with an epitope that is present in the A1 domain of vWF of humans as well as that of many other species, including the pig, rabbit, dog, guinea pig, and rat.\textsuperscript{12} This antibody inhibits the vWF-mediated aggregation and adhesion of human blood platelets induced by high shear stress without increasing the risk of bleeding.\textsuperscript{12} Assay of the ability of AJvW-2 to inhibit SIPA in the blood of patients with ACSs may therefore reveal whether antithrombotic therapy specifically targeting the vWF-GPIb interaction prevents new events in the coronary arteries. Thus, to clarify the involvement of the vWF-GPIb interaction in ACSs, we assayed the effect of AJvW-2 on both the vWF- and fibrinogen-dependent platelet aggregation in the blood of patients with UAP and AMI.

**Methods**

**Patient Population**

This investigation conformed with the principles outlined in the Declaration of Helsinki.\textsuperscript{13} Written, informed consent was obtained from each participant, and the study protocol was approved by the institutional ethics committee.

We studied 32 Japanese patients (19 men and 13 women; mean age, 65±6 years) with ACSs, including 12 patients with UAP (7 men and 5 women; mean age, 62±7 years; range, 48 to 78 years) and 20 patients with AMI (12 men and 8 women; mean age, 68±5 years; range, 44 to 81 years). UAP in this study included Braunwald type B\textsuperscript{14}; transient ST-segment depression or T-wave inversion was documented in all patients. Significant arterial narrowing (>50% narrowing of the lumen of 1 or more major coronary arteries) was confirmed by coronary angiography. The diagnosis of AMI was based on the occurrence of ischemic chest pain exceeding 30 minutes accompanied by ST-segment elevation on the ECG and a subsequent increase in serum creatine kinase to a level 2.5 times the normal value. Coronary artery occlusion (Thrombolysis in Acute Myocardial Infarction [TIMI] grade 0 or 1 flow)\textsuperscript{15} was confirmed by angiography performed within 90 minutes of admission. As controls, blood was obtained from 18 age-matched volunteers (11 men and 7 women; mean age, 64±11 years; range, 38 to 79 years) with normal coronary arteries, as confirmed by angiography, and included individuals with mild hypertension or hyperlipidemia. None of the subjects had taken aspirin or other agents known to alter platelet function for at least 2 weeks before the study, and none had taken any other agent known to affect native blood conditions, including antihypertensive agents (eg, angiotensin-converting enzyme inhibitors, Ca\textsuperscript{2+}-channel antagonists, \(\beta\)-adrenoceptor antagonists, or diuretics), isosorbide dinitrate or other NO donors, or oral hypoglycemic agents (eg, sulfonylureas). To avoid any influence other than those due to the coronary events, patients with a glycosylated
hemoglobin value of >7.0%, inflammatory disorders, renal dysfunction, or heart failure, as well as pregnant or menstruating women, were excluded from study.

**Blood Sampling**

Blood samples were obtained from UAP patients within 3 hours of an episode of ischemic chest pain accompanied by the ECG changes described above and from AMI patients within 3 hours of symptom onset. Blood was obtained by venipuncture at the time of admission before the patient had received any anticoagulants and/or antiplatelet agents. After the application of a light tourniquet, a Luer-lock 19-gauge intravenous cannula was inserted into a forearm vein. Blood samples to which sodium citrate had been added (citrate/whole blood, 1:9 vol/vol; final concentration, 0.38%) for the measurement of SIPA were centrifuged at room temperature for 10 minutes at 120 g to obtain platelet-rich plasma (PRP) and for 15 minutes at 300 g to platelet-poor plasma (PPP).

**Plasma vWF Antigen Levels and Ristocetin Cofactor Activities**

Plasma was separated by centrifugation from venous blood that had been collected in a 1/9 volume of 50 mmol/L EDTA, 3.2% trisodium citrate, 10 mmol/L leupeptin (Sigma Chemical Co), and 60 mmol/L N-ethylmaleimide (Sigma) and then frozen. The concentration of vWF antigen in each sample was determined by a sandwich ELISA using a rabbit anti-human vWF polyclonal antibody (DAKO). The concentration of vWF antigen in a plasma sample pooled from 100 healthy donors was defined as 100%.

Ristocetin cofactor activity was determined in PPP from patients and controls by measuring the platelet aggregation induced by a fixed concentration (1.2 mg/mL) of ristocetin (Sigma) with the use of an assay kit (Hoechst-Behring). Washed platelets (3.0 × 10⁵/mL in buffered saline, pH 7.2) prepared from healthy donors and plasma were added to the aggregometer cuvette. The aggregometer (NBS HEMA TRACER 801, Niko Bioscience, Inc) was calibrated with PPP pooled from healthy donors, and the maximal aggregation achieved with 1.2 mg/mL ristocetin was measured in the individual plasma samples. Maximal ristocetin cofactor activity in a plasma sample pooled from 100 healthy donors was defined as 100%.

**vWF Multimer Analysis**

vWF multimers in citrated plasma and platelet lysates were analyzed by thin-layer agarose electrophoresis in the presence of SDS, with
larger multimers defined as bands larger than the tenth to eleventh band from the bottom of the gels. The amount of these larger multimers was calculated densitometrically (Fluor Imaget SI, Molecular Dynamics) and expressed as a percentage of that in the controls.

**Preparation of MoAb**
AJvW-2, a murine anti– human vWF MoAb, was prepared as described previously. The high shear–induced platelet aggregation and adhesion between platelets and collagen-coated glass in samples from healthy donors can be completely inhibited by AJvW-2 at a concentration of 10 μg/mL, which we used as Fab fragment in the following studies.

**Determination of SIPA**
SIPA was measured by a modified cone-and-plate–type viscometer (Toray, Inc) within 30 to 40 minutes after blood drawing. In brief, citrated PRP (adjusted to 3.0×10^5 platelets/μL) was incubated for 10 minutes at room temperature in the presence or absence of AJvW-2 (10 μg/mL), and 400 μL was applied to the surface of a polymethylmethacrylate plate. The rotation rate of the cone was increased from 0 to 200 rpm or 1800 rpm for 15 seconds, corresponding to shear stresses of 12 or 108 dyne/cm², respectively, and then kept constant for the following 345 seconds. Aggregation was continuously monitored by recording the intensity of transmitted light (ITL) through the platelet suspension. Platelet aggregation was calculated as log (A/C)/log (A/B)<100 (in percent), in which A is the ITL of PRP; B, the ITL of PPP; and C, the ITL of PRP under shear stress.

**Statistical Analysis**
Results are expressed as mean±SEM. Spearman’s correlation coefficient was used to evaluate the relation between the extent of SIPA and the amount of vWF larger multimer or plasma vWF antigen. Statistical significance was evaluated using an ANOVA followed by Scheffe’s procedure with SuperANOVA software (Abacus Concepts) on a Macintosh computer. A level of P<0.05 was considered statistically significant.

**Results**

**Shear-Induced Platelet Aggregation**
SIPA was determined in 12 patients with UAP, in 20 patients with AMI, and in 18 control subjects. Under high-shear conditions (108 dyne/cm², SIPA was 1.3-fold higher in the UAP group (39±6%, P<0.01) and 2.0-fold higher in the AMI group (59±4%, P<0.01) than in the control group (29±6%, Figures 1 and 2). SIPA induced by high shear in all groups, however, was completely inhibited by 10 μg/mL AJvW-2 (P<0.001, Figure 2). Under low-shear conditions (12 dyne/cm²), SIPA was increased in the AMI group (41±7%) but not in the UAP group (24±5%, P<0.01 versus AMI group), compared with the controls (24±7%, P<0.01 versus AMI group, Figures 3 and 4). The SIPA induced by low shear in all groups was not affected by AJvW-2 (Figure 4).

**Plasma Levels of vWF Antigen and Ristocetin Cofactor Activity**
The plasma level of vWF antigen was significantly higher in the UAP (159±31%) and AMI (257±27%) groups than in the control group (93±13%, P<0.001, Figure 5A). Ristocetin cofactor activity was also elevated in the UAP (149±25%) and AMI (249±22%) groups compared with the controls (89±9%, P<0.001, Figure 5B). Significant differences in both parameters were observed between the UAP and AMI groups (P<0.001, Figure 5).

**vWF Multimer Analysis**
When we analyzed plasma vWF multimers in the UAP and AMI patients and controls, we observed higher levels of these multimers in both the UAP (118±15%, P<0.05) and AMI (159±24%, P<0.01) groups than in the controls (100%). The amount of these multimers in the UAP and AMI patients could be densitometrically correlated with the extent of SIPA induced by high shear (UAP, r=0.622, P<0.001; AMI, r=0.915, P<0.0001). Significant correlations were also observed in both groups between the plasma level of vWF antigen and the extent of high shear–induced SIPA (UAP, r=0.536, P<0.05; AMI, r=0.686, P<0.01). In contrast, there was no significant correlation, in any of these groups, between the plasma vWF or vWF multimer level and the extent of SIPA induced by low shear stress (data not shown).

**Discussion**
The present study showed that plasma levels of vWF antigen, ristocetin cofactor activity, and SIPA under high-shear conditions were elevated in the patients with UAP and AMI, particularly during the very acute phase after symptom onset. We also found that the amount of larger vWF multimers in patients with ACSs exceeded that in controls and that there was significant correlation in ACSs between the plasma level of vWF or the amount of larger vWF multimers and the extent of SIPA induced by high shear.

Plasma levels of vWF are elevated in patients with coronary artery disease as well as in patients with other disorders, including diabetes mellitus, IgA nephropathy, and malignant hypertension. After endothelial damage, larger multimers of vWF are released from endothelial cells and can mediate the adhesion of platelets to the vessel wall. In disorders such as coronary artery disease, platelet adhesion mediated by the vWF-GPⅡb/Ⅲa interaction is the initial step in thrombus formation, followed by platelet aggregation, which is initially mediated by the vWF-GPⅡb/Ⅲa interaction. Subsequent linkage of platelets to one another by fibrinogen or by vWF bound to the activated GP IIb/Ⅲa complex may then lead to arterial thrombosis.

Recent data indicate that plasma vWF and platelet GPⅡb are essential in the response to varying hemodynamic conditions and that vWF-dependent platelet aggregation mediated by the vWF-GPⅡb interaction is necessary to functionally complement the activated GPⅡb/Ⅲa in the presence of elevated fluid dynamic forces. In contrast, it has been
shown that high shear–induced aggregation of washed platelets increases as a function of the concentration of exogenous purified vWF. In addition, because wall shear rates of 3000 to 10 000 s⁻¹ have been measured at the top of plaques, causing a 50% occlusion of the coronary arteries, vWF-GPIIb binding may lead to arterial occlusion in a patient with atherosclerosis and precipitate a disease such as an ACS. It should therefore be possible to examine the association between the plasma vWF concentration and the vWF-GPIIb interaction by blocking vWF binding to GPIIb, where flow generates higher shear rates, to prevent ACSs.

SIPA induced by high shear stress has also been shown to be enhanced in patients with stable effort angina who have a 50% to 75% narrowing of the coronary arteries; this is especially observed after treadmill exercise when the plasma levels of catecholamines and vWF are elevated. These results further suggest that SIPA induced by high shear in patients with ACSs may be due, at least in part, to elevated plasma vWF levels, especially that of the larger vWF multimers released by the vasculature. Because the expression of GPIIb on platelets is not upregulated in patients with AMI, the enhancement of the high shear–induced SIPA in such patients most likely depends on the elevated plasma concentrations of vWF. In addition, the ADP released by activated platelets accelerates the binding of vWF to platelet GPIIb/IIIa and enhances the high shear–induced SIPA mediated by larger vWF multimers. Although the precise mechanism for increased vWF-independent platelet aggregation in ACSs is not yet known, the pathological thrombus likely results from the shear-induced adhesion and aggregation arising from the stenosis and is further enhanced by the increasing shear imposed by the growing thrombus at the site of stenosis. Alternatively, the pathological thrombus in ACSs may be caused by local thrombus formation due to chemical activation. For example, elevated plasma levels of epinephrine or ADP during the onset of AMI may increase low shear–induced SIPA. Increased levels of epinephrine may also augment the SIPA induced by high shear in ACSs. Conditions of high and low shear may thus occur in close proximity to thrombotic deposits, and the sudden change from low to high shear around the growing thrombus may take place synergistically by reinforcing the binding to activated GPIIb/IIIa.

Although we observed a significant correlation between the plasma level of vWF or the amount of larger vWF multimers and the extent of high shear–induced SIPA, a direct relationship may not exist between these parameters. Rather, the elevation in plasma vWF and/or the extent of high shear–induced SIPA could lead to ACS onset. One drawback of the cone-and-plate viscometer method is that the high shear rotation rate of the cone in vitro may be too fast to estimate the vWF-dependent platelet activation in vivo. Although a parallel-plate flow chamber system in vitro may better mimic the in vivo conditions of active blood flow such as that in the coronary arteries, we believe that the cone-and-plate viscometer is the simplest method of quantitatively evaluating platelet aggregation under varying shear conditions.

AJvW-2 is a murine MoAb that recognizes a conformational epitope of the A1 domain of vWF involved in its binding to platelet GPIb. AjvW-2 has been shown to specifically inhibit platelet adhesion and aggregation under conditions of high shear stress by blocking the interaction between vWF and GPIb. Our finding that AJvW-2 completely inhibits the high shear–induced platelet aggregation in the blood from patients with a very acute phase of ACSs, as well as recent demonstrations that the vWF-GPIb interaction is essential during coronary occlusion and that inhibition of vWF binding to GPIb can prevent coronary thrombosis and further restenosis after vascular injury, suggests that AJvW-2, if administered after coronary interventions (eg, angioplasty, stenting, or atherectomy), may prevent new events in the coronary arteries and inhibit restenosis.

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