Pharmacokinetics and Pharmacodynamics of AJW200, a Humanized Monoclonal Antibody to von Willebrand Factor, in Monkeys

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Abstract—The interaction between platelet glycoprotein Ib and von Willebrand factor (vWF) plays a crucial role in platelet-mediated thrombus formation under high-shear-stress conditions. The aim of this study was to investigate the antiplatelet profile of a humanized anti-vWF monoclonal antibody, AJW200. In vitro studies were performed with a modified cone-and-plate viscometer and human platelets. AJW200 inhibited high-shear-stress–induced platelet adhesion, aggregation, and thrombin generation, but it did not have such effects under low-shear-stress conditions. Although abciximab inhibited platelet aggregation under both shear stress conditions, it did not inhibit platelet adhesion and thrombin generation. In addition, the pharmacokinetics and pharmacodynamics of AJW200 were evaluated in cynomolgus monkeys. Sustained inhibition of ristocetin-induced platelet aggregation was observed over 24 hours, 6 days, and 2 weeks after a single bolus injection of 0.3, 1, and 3 mg/kg, respectively. Moderate prolongation of the bleeding time was observed at the doses of 1 and 3 mg/kg. Abciximab markedly prolonged the bleeding time at 0.4 mg/kg, at which concentration complete inhibition of ADP-induced platelet aggregation was observed. These results suggest that glycoprotein Ib–vWF blockade with AJW200 results in a sustained antiplatelet effect without extensive prolongation of the bleeding time, probably due to a shear-stress–dependent inhibitory action. (Arterioscler Thromb Vasc Biol. 2002;22:187-192.)

Key Words: von Willebrand factor ■ platelets ■ shear stress ■ bleeding time ■ AJW200

Platelets play a crucial role in the pathophysiological progression of acute coronary syndromes (ACSs). Because the interaction of platelet glycoprotein (GP) Ib/IIa with adhesive protein (ie, fibrinogen and von Willebrand factor [vWF]) is the final common pathway of platelet aggregation, various GPIIb/IIa blockers have been developed. Abciximab is the Fab fragment of a chimeric monoclonal antibody (mAb) to platelet GPIIb/IIa that has shown pronounced clinical benefit in the setting of percutaneous coronary interventions in patients with coronary heart diseases.1–3 Especially under high-shear-stress conditions, as observed in stenosed coronary arteries, for example, the interaction between platelet GPIb and vWF is involved in platelet-mediated thrombus formation.4,5 Although some GPIIb-vWF blockers have been reported to be effective against thrombus formation in various animal models,6,7 none of the tested agents has yet been proved effective in clinical applications. Previously, we reported that the murine IgG1 against human vWF, AJvW-2, is a specific blocker of the GPIb-vWF interaction and inhibits arterial thrombus formation in various animal models.8–11 These reports suggest that AJvW-2 may represent a new therapeutic agent for the treatment of patients with ACSs. However, “humanization” of AJvW-2 would be needed for clinical practice because murine IgG1 is thought to have shown immunogenicity when administered to humans. Recently, we have succeeded in the humanization of AJvW-2 by grafting the mouse hypervariable regions onto a human IgG4 framework, which is known to have minimal Fc functions.

The purpose of this study therefore was to characterize the in vitro antiplatelet profile of a humanized AJvW-2 (named AJW200) and to investigate the pharmacokinetics and pharmacodynamics of AJW200 after its bolus injection into cynomolgus monkeys.

Methods

Reagents
AJW200 (a humanized AJvW-2) is an IgG4 humanized mAb to human vWF and derived from Sp2/0 mouse myeloma cells. Abciximab (c7E3 Fab, ReoPro™) was purchased from Eli Lilly Co.

Platelet Aggregation
Citrated blood was obtained from healthy volunteers by venipuncture, and platelet-rich and platelet-poor plasmas (PRP and PPP,
respectively) were prepared by centrifugation. The platelet counts in PRP were adjusted to ~250,000 per microliter by dilution with PPP. Platelet aggregation was measured with an aggregometer, the MCM Hematracer801 (MC Medical). After PRP was incubated with AJW200, abciximab, or vehicle at 37°C for 3 minutes, various agonists were added and platelet aggregation was monitored for 10 minutes as percent light transmission (with PPP set at 100%).

Shear-stress–induced platelet aggregation was measured with a modified cone-and-plate viscometer (Toray). Citrated PRP was incubated with AJW200, abciximab, or vehicle at room temperature for 10 minutes. The PRP sample was applied to the plate and exposed to shear stress at 25°C for 6 minutes. The cone was rotated at 1800 or 200 rpm, corresponding to shear stresses of 108 (high) or 12 (low) dyne/cm², respectively. Platelet aggregation was measured as percent light transmission.

**Platelet Adhesion**
Shear-stress–induced platelet adhesion was measured with a modified cone-and-plate viscometer (Tokii Sangyo). Coverglasses were coated with human type III collagen (Sigma Chemical Co) and left undisturbed overnight at 5°C. Anticoagulated blood with D-phenylalanyl-L-prolyl-L-arginine chloromethylketone (Calbiochem; final concentration, 100 µmol/L) obtained from healthy volunteers was preincubated with AJW200, abciximab, or vehicle at room temperature for 10 minutes. After the coverglass was mounted to the plate of a modified viscometer, the blood sample was applied to the plate and exposed to shear stress at room temperature for 5 minutes. The cone was rotated at 250 or 60 rpm, corresponding to a shear rate of 1500 s⁻¹ (high) or 360 s⁻¹ (low). The coverglass was rinsed, fixed with methanol, and stained with May-Grunwald-Giemsa. Platelet adhesion was quantified by light microscopy, and the surface area coverage with platelets (as a percentage) was measured and calculated with the use of a computerized image-generating graphic analyzer, Mac Scope. After analysis of 5 fields for each coverglass, the average value was calculated for each sample.

**Platelet Procoagulant Activity**
Platelet procoagulant activity was evaluated by measurement of thrombin amidolytic activity. Blood was obtained from healthy volunteers by venipuncture and anticoagulated with acid-citrate-dextrose (ACD; final concentration, 15%), and PRP was prepared by centrifugation. The platelet pellet was obtained by additional centrifugation of PRP and immediately suspended in ACD/HEPES buffer. The platelet suspension was centrifuged again, washed with ACD/HEPES buffer, and finally suspended in 1 mg/mL albumin/HEPES buffer. The platelet counts were adjusted to ~200,000 platelets/µL. The platelet suspension was incubated with AJW200, abciximab, or vehicle at room temperature for 10 minutes. Immediately after 1 U/mL human vWF and 2 mmol/L CaCl₂ were added, the platelet suspension (400 µL) was applied to the plate and exposed to shear stress at 25°C for 3 minutes with a modified cone-and-plate viscometer (Toray). The cone was rotated at 1800 or 200 rpm, corresponding to respective shear stresses of 108 (high) or 12 (low) dyne/cm². The sheared platelet sample was incubated with 3.9 mmol/L factor Xa and 1.2 mmol/L CaCl₂ at 37°C for 1 minute and further incubated with 0.82 µmol/L prothrombin and 4 µmol/L CaCl₂ at 37°C for 1 minute. Finally, after the sample (25 µL) was incubated with chromogenic thrombin substrate S-2238 (225 µL) at 37°C for 1 minute, the reaction was stopped by adding 6% citric acid. The optical density was measured at 405 nm, and the amount of thrombin generated was calculated by use of a standard curve with α-thrombin (Sigma Chemical Co).

**Ex Vivo Study in Monkeys**
All procedures were performed in accordance with the institutional Animal Care and Use Committee of the Pharmaceutical Research Laboratories of Ajinomoto Co, Inc. Forty-five adult cynomolgus monkeys weighing 4.2 to 6.8 kg were divided into 9 treatment groups (n=5 each). AJW200 (0.03, 0.1, 0.3, 1, and 3 mg/kg body weight), abciximab (0.1, 0.2, and 0.4 mg/kg), or phosphate-buffered saline (control) was intravenously administered by bolus injection via the cephalic vein in a forearm. Blood collection and measurement of bleeding times were performed before and 5 minutes; 3 and 24 hours; 2, 4, and 6 days; and 2 weeks after drug administration. Unless platelet aggregation had recovered at 2 weeks, an additional measurement was performed at 3 or 4 weeks after administration. Citrated blood was obtained from the femoral vein and used for the measurement of platelet aggregation as well as hematological and coagulant parameters. Residual plasma samples were frozen and stored at ~80°C.

PRP and PPP were prepared by centrifugation of citrated whole blood (4.5 mL at room temperature at 700 rpm for 10 minutes and 3200 rpm for 10 minutes, respectively). The platelet counts in PRP were measured with an automated cell counter (model E-4000, Sysmex) and adjusted to ~300,000 platelets/µL by dilution with PPP. Platelet aggregation induced by ristocetin (final concentration, 2.5 mg/mL; Sigma Chemical Co) or ADP (final concentration, 20 µmol/L; Meiji Yakuhin) was measured by the change in light transmission for 8 minutes in an aggregometer (MC Matracer801, MC Medical). Ristocetin-induced platelet aggregation was performed in the control group and the AJW200-treated groups. On the contrary, ADP–induced platelet aggregation was performed in the abciximab–treated groups alone. Because frequent collection of large volumes of blood might have led to lethal anemia, both platelet aggregation assays were not performed for each animal. Plasma was obtained by centrifugation of the residual blood (0.5 mL) at 3000 rpm for 15 minutes, and prothrombin time and activated partial thromboplastin time were measured with an automated blood coagulation analyzer (Sysmex). Anticoagulated blood with EDTA–2K was obtained from the femoral vein, and hematological parameters were measured with an automated cell counter (Sysmex).

The template bleeding time was measured at the surface of the forearm with an automated spring-loaded device (Simplite R. Organon Teknika). Measurement was performed at serial intervals of 30 seconds up to a maximum time of 30 minutes, until the absorption of blood onto the filter paper had ceased, as determined by visual inspection.

**Plasma vWF Antigen Level**
A 96-well microtiter plate was coated with a rabbit anti-human vWF polyclonal antibody (10 µg/mL, Dako) and left at room temperature for 2 hours. The plates were blocked with 1% bovine serum albumin in phosphate-buffered saline at room temperature for 1 hour. Diluted plasma samples were added and incubated at room temperature for 2 hours. After being washed, the plates were incubated with a peroxidase-conjugated rabbit anti-human vWF polyclonal antibody (1:3000 dilution, Dako) for 1 hour. After the plates were washed again, the numbers of bound vWF molecules were quantified by measuring the optical density at 490 nm. The plasma vWF antigen level was calculated as the percentage of the value of predosing plasma sample in each monkey.

**Anti-AJW200 Antibody Formation**
A 96-well microtiter plate was coated with AJW200 (5 µg/mL) at 4°C overnight. The plates were blocked with 1% bovine serum albumin/phosphate-buffered saline at room temperature for 2 hours. Diluted plasma samples were added and incubated at room temperature for 2 hours. After being washed, the plates were incubated with AJW200–absorbed, biotin-conjugated rabbit anti–monkey immunoglobulins (Nordic Immunological Laboratories) for 1 hour. After being washed again, the plates were incubated with a streptavidin–horseradish peroxidase conjugate for 1 hour. After a third washing step, bound monkey immunoglobulins were quantified by measuring the optical density at 490 nm. The plasma level of anti-AJW200 antibody was expressed as the optical density value. A positive reaction was defined as more than twice the predosing optical density value in each monkey.

**Plasma Concentration of AJW200**
A 96-well microtiter plate was coated with rabbit anti-human vWF polyclonal antibody (5 µg/mL, Dako) at room temperature for 2 hours. The plates were blocked with 1% bovine serum albumin/phosphate-buffered saline at room temperature for 1 hour. Diluted plasma samples and standard AJW200 solutions (0.0078 to 0.5
Platelet aggregation was not affected, even at a concentration of 0.03 mg/mL. Simultaneously, a human vWF concentrate (ConfactF) was added to each sample at a final concentration of 0.025 U/mL (as a factor VIII:C). After being washed, the plates were incubated with each sample containing 0.025 U/mL of vWF and 1:5000 dilution of peroxidase-conjugated mouse anti-human IgG4 mAb (1:5000 dilution, Southern Biotechnology Associates, Inc) at room temperature for 1 hour. After a third washing step, bound antibody conjugate activity was quantified by measuring the optical density at 490 nm, for 1 hour. After a third washing step, bound antibody conjugate activity was quantified by measuring the optical density at 490 nm, and the plasma AJW200 level was calculated from a standard curve. The lower limit of quantification was 39 ng/mL. For pharmacokinetics modeling, a biexponential fitting of the plasma concentrations versus time was performed by weighted nonlinear regression analysis. Parameters of the 2-compartment model were then calculated by using a curve-fitting program.

Statistics
Data are presented as mean±SEM. In the in vitro study of platelet procoagulant activity, 1-factor ANOVA followed by Scheffe’s and Dunnett’s tests was used for statistical analysis of the comparison between the 3 control groups and of the efficacy of mAb compared with control high-shear-stress conditions, respectively. In the ex vivo study, repeated-measures ANOVA, followed by Dunnett’s test, was used for statistical analysis of the efficacy of AJW200. Statistical analysis of the efficacy of abciximab was not performed. A value of *P<0.05 was considered significant.

Results
Platelet Aggregation
AJW200 specifically inhibited human platelet aggregation induced by ristocetin and botrocetin, although abciximab inhibited any type of platelet aggregation (Table I; please see http://atvb.ahajournals.org). Also, AJW200 inhibited the high-shear-stress–induced platelet aggregation in a concentration-dependent manner, with an IC50 value of 1.0±0.1 µg/mL (Figure 1A). Low-shear-stress–induced platelet aggregation was not affected, even at a concentration of 80 µg/mL. On the contrary, abciximab inhibited both platelet aggregations at the same efficacy, and the IC50 value was 1.1±0.03 µg/mL under high-shear- and 1.2±0.5 µg/mL under low-shear-stress conditions, respectively (Figure 1B).

Platelet Procoagulant Activity
The effects of AJW200 and abciximab on thrombin generation are shown in Figure 2. A significant increase in thrombin generation was observed under high shear stress compared with the low- or no-shear condition (4.0±0.5, 0.3±0.1, and 0.06±0.04 U · mL⁻¹ · min⁻¹, respectively). AJW200 inhibited high-shear-stress–induced thrombin generation in a concentration-dependent manner, and significant inhibition was observed at 4 µg/mL. On the contrary, abciximab did not affect thrombin generation, even at 16 µg/mL.

Pharmacokinetics in Monkeys
AJW200 showed approximately dose-proportional pharmacokinetics over the dose range 0.03 to 3 mg/kg (Figure 3). Plasma AJW200 concentrations 5 minutes after intravenous administration of 0.03, 0.1, 0.3, 1, and 3 mg/kg were 0.55±0.06, 1.93±0.16, 7.07±0.45, 23.08±0.88, and 67.83±2.53 µg/mL, respectively. A biphasic decline in plasma concentration was observed for the 2 highest doses (1 and 3 mg/kg), although a possible slow-clearance phase could not be detected owing to the sensitivity limit of the assay for the 3 lowest doses (0.03, 0.1, and 0.3 mg/kg). The dominant terminal disposition phase was characterized by a half-life of 25.1±5.5, 20.6±2.5, 19.1±1.0, 43.3±3.4, and 63.0±5.8 minutes.
hours for 0.03, 0.1, 0.3, 1, and 3 mg/kg, respectively (Table II; please see http://atvb.ahajournals.org).

Pharmacodynamics in Monkeys
The antiplatelet effect of AJW200 and bleeding time prolongation are shown in Figure 4. AJW200 significantly inhibited the ex vivo ristocetin-induced platelet aggregation at 0.03, 0.3, 1, and 3 mg/kg. Complete inhibition of aggregation was observed at 0.3 mg/kg and above and lasted >24 hours at 0.3 mg/kg, for 6 days at 1 mg/kg, and for 2 weeks at 3 mg/kg. Inhibition of the ristocetin-induced platelet aggregation at 3 mg/kg AJW200 disappeared by 3 weeks for 4 animals and by 4 weeks for 1 animal. AJW200 did not affect bleeding times up to a dose of 0.3 mg/kg. Although AJW200 significantly prolonged the bleeding time at 1 and 3 mg/kg, a lengthy prolongation (≥30 minutes) was never observed, even at 3 mg/kg. No changes in hematological parameters (leukocytes, erythrocytes, hemoglobin, hematocrit, and platelets), coagulation parameters (thrombin time and activated partial thromboplastin time), and plasma vWF antigen levels were observed in any of the groups (data not shown). In addition, antibody formation against AJW200 was not observed in any of the animals treated with AJW200 (data not shown).

The relationship between the antiplatelet effect and bleeding time 5 minutes after AJW200 administration is shown in online Figure II (please see http://atvb.ahajournals.org). A wide window between the effective dose (on ristocetin-induced platelet aggregation) and bleeding time was observed in monkeys treated with AJW200. On the contrary, complete inhibition of ADP-induced platelet aggregation by 0.4 mg/kg abciximab was associated with a lengthy prolongation (≥30 minutes) of bleeding time. Inhibition of platelet aggregation rapidly disappeared (within 24 hours) after a bolus injection of abciximab (data not shown).

Discussion
The major findings of this study are that (1) a humanized mAb to vWF (AJW200) shows high-shear-stress–dependent inhibitory action against platelet activation and that (2) a bolus injection of AJW200 results in sustained inhibition of ristocetin-induced platelet aggregation without extensive prolongation of the bleeding time in monkeys. Previous clinical trials showed that abciximab significantly reduced ischemic complications in patients with ACSs. However, it has also been reported that the high-shear-stress–induced platelet aggregation was augmented by the increase in plasma vWF levels in such patients. Although these reports indicated that vWF-mediated platelet aggregation might be associated with the incidence of ACSs, the GPIb-vWF blocker is not yet available for clinical practice.

We have reported that AJvW-2, a murine mAb to human vWF, is a specific blocker of the GPIb-vWF interaction and is capable of preventing thrombus formation in vivo. These studies suggest that AJvW-2 may be a new therapeutic agent for the treatment of patients with thrombotic disorders, including ACSs. To minimize the immunological responses against AJvW-2 when administered to humans, we humanized AJvW-2 by grafting the mouse hypervariable regions onto a human IgG4 framework. In this study, AJW200 specifically inhibited human platelet aggregation induced by ristocetin as well as by botrocetin. In addition, under high-shear-stress conditions, the GPIb-vWF interaction plays a crucial role in platelet adhesion and aggregation. AJW200 specifically inhibited high-shear-stress–induced platelet aggregation and adhesion; however, no effects were observed under low-shear-stress conditions.

Activated platelets can facilitate thrombin generation by providing a catalytic surface on which coagulation reactions occur and by releasing activated factor V, resulting in the formation of a secondary fibrin clot. A previous report indicated that tissue factor–induced thrombin generation in the presence of platelets was significantly inhibited by abciximab. However, these findings were observed under static
conditions, and no study investigating such an effect of abciximab under shear-stress conditions was reported. The current study indicated that an \( \sim 40 \) -fold increase in thrombin generation was observed under high-shear-stress conditions compared with low- or no-shear conditions, indicating the significance of high shear stress for platelet activation. AJW200 inhibited high-shear-stress–induced thrombin generation, although no effect of abciximab was observed even at 16 \( \mu \text{g/mL} \), at which concentration platelet aggregation was significantly inhibited. These in vitro findings suggest that abciximab therapy may result in the generation of many single platelets with a high procoagulant activity that are capable of adhering to collagen under high-shear-stress conditions, as occurs in stenotic coronary arteries, although platelet aggregation can be completely inhibited. Under these conditions, AJW200 inhibited platelet adhesion and the subsequent platelet aggregation, as well as activation due to inhibition of the GPIb-vWF interaction.

We have shown that AJvW-2 prevents arterial thrombosis in various animals.\(^8\)–\(^11\) However, the effective dose and especially the duration of efficacy cannot be extrapolated to humans, because murine mAb was an exogenous protein for these animals and therefore susceptible to rapid clearance. Also, whether administration of a humanized mAb to the GPIb-binding domain of vWF to nonhuman primates results in severe von Willebrand disease has not yet been investigated. Therefore, we investigated the pharmacokinetics and pharmacodynamics (especially the hematological functions) of AJW200 in cynomolgus monkeys. In this study, no antibody formation against AJW200 was observed in any monkey, probably owing to the lower immunogenicity of a humanized mAb. Also, no decrease in plasma vWF level was observed, probably because of the minimal Fc functions of IgG. Additionally, no changes in hematological and coagulation parameters were observed. These results indicate that the inhibitory effect of AJW200 on ristocetin-induced platelet aggregation might be due to specific inhibition of the GPIb-vWF interaction in monkeys.

Multimeric forms of vWF are composed of 250-kDa polypeptide subunits (monomers) linked together by disulfide bridges, and each monomer has 1 binding site for GPIb. If it is assumed that the plasma vWF level in cynomolgus monkeys is identical to that in humans (\( \sim 10 \mu \text{g/mL} \) [40 nmol/L as a monomer]) and that the stoichiometry of binding of the vWF monomer to AJW200 is 2:1 or 1:1, then the AJW200 concentration needed for saturated binding to plasma vWF can be calculated at 3 or 6 \( \mu \text{g/mL} \). In this study, the mean plasma concentrations of AJW200 were 0.55, 1.93, 7.07, 23.08, and 67.83 \( \mu \text{g/mL} \) immediately after administration of 0.03, 0.1, 0.3, 1, and 3 mg/kg, respectively. These results suggest that at the 3 lowest doses tested, almost all of the AJW200 molecules in plasma may exist in the vWF-bound form immediately after administration and that AJW200 may be cleared at the rate to which it binds to vWF. Because excessive numbers of AJW200 molecules may also exist in the unbound form in the circulation at the 2 highest doses, it may persist for a time that is typical of IgG. In our preliminary experiment, binding of AJW200 to vWF in normal monkey plasma was saturated at \( \sim 2.7 \mu \text{g/mL} \) (data not shown). Furthermore, saturated vWF occupancy was observed at the 3 highest doses in this study (data not shown). These results tend to support our hypothesis described above.

A bolus injection of 0.3 mg/kg AJW200 completely inhibited ristocetin-induced platelet aggregation, and significant inhibition was sustained for 24 hours without prolongation of the bleeding time. Longer durations of inhibition (6 days and 2 weeks) after administration of 1 and 3 mg/kg AJW200, respectively, were accompanied by a moderate prolongation of the bleeding time. In contrast, complete inhibition of ADP-induced platelet aggregation after a bolus injection of abciximab was accompanied by extensive prolongation (\( \sim 30 \) minutes) of the bleeding time, which rapidly disappeared within 24 hours. Because abciximab is routinely administered as a bolus injection followed by a 12-hour infusion in clinical practice, a high risk of bleeding tendency may last for 12 hours. Although many clinicians have indicated that prolongation of the bleeding time is not predictive of bleeding events in patients,\(^16\) the low bleeding profile of AJW200 may be preferable in clinical practice. Furthermore, the plasma half-life of a humanized mAb is expected to be longer in humans than in cynomolgus monkeys, which is supported by a previous investigation that used a humanized mAb (IgG\(_4\)) to tumor necrosis factor-\(\alpha\).\(^17\) A long-term antithrombotic effect could be achieved by a single bolus injection of AJW200 in future clinical practice. In addition, easy bolus administration would be preferable in an emergency situation.

In conclusion, the specific inhibition of GPIb-vWF interactions by a humanized mAb to vWF (AJW200) results in sustained inhibition of ristocetin-induced platelet aggregation without an extensive prolongation of bleeding time, probably owing to the high-shear-stress–dependent inhibitory action. AJW200 may become a drug of choice for the treatment of patients with ACSs.

References


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