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Anti-thrombotic effects and bleeding risk of AJvW-2, a monoclonal antibody against human von Willebrand factor

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1 A murine anti-human vWF monoclonal antibody, AJvW-2, was developed that inhibited the interaction between platelet glycoprotein Ib (GPIb) and von Willebrand factor (vWF) during the ristocetin- ($IC_{50} = 0.7 \pm 0.1$ $\mu g\ ml^{-1}$) and botrocetin- ($IC_{50} = 1.8 \pm 0.3$ $\mu g\ ml^{-1}$) induced aggregation of human platelets.

2 AJvW-2 inhibited the high shear stress (10.8 N m^{-2}) induced aggregation of human platelets dose-dependently with an $IC_{50} = 2.4 \pm 0.3$ $\mu g\ ml^{-1}$, but had no effect on low shear stress induced platelet aggregation (1.2 N m^{-2}) up to 100 $\mu g\ ml^{-1}$.

3 AJvW-2 also inhibited the high shear stress (5.0 N m^{-2}) induced adhesion of human platelets to collagen I with the same efficacy ($IC_{50} = 2.4 \pm 0.3$ $\mu g\ ml^{-1}$), but had no effect at low shear conditions (1.5 N m^{-2}).

4 AJvW-2 inhibited the botrocetin-induced aggregation of platelets from guinea-pig, rat, rabbit, dog and pig at the same concentration range as human platelets; it likewise also inhibited the high shear stress induced aggregation and adhesion to collagen I of guinea-pig platelets.

5 AJvW-2 prevented arterial thrombus formation in guinea-pigs at a dose of 100 $\mu g\ kg^{-1}$ without prolonging the template bleeding time, whereas the GPIIb/IIIa antagonist lamifiban mediated inhibition of thrombosis at 1000 $\mu g\ kg^{-1}$ was accompanied by a significant prolongation of the bleeding time.

6 These results suggest that AJvW-2 is a potent inhibitor of the GPIb-vWF interaction and a potential novel antithrombotic agent with lower bleeding risk than GPIIb/IIIa antagonists.

Keywords: AJvW-2; von Willebrand factor; GPIb; GPIIb/IIIa; shear stress; thrombosis; bleeding risk

Introduction

Anti-platelet agents, primarily aspirin, but more recently also ticlopidine, thromboxane A_2 (TXA_2) synthetase inhibitors, phosphodiesterase inhibitors and prostacyclin analogues have been investigated in great detail, both in animal models and during clinical trials of cardiovascular diseases (Ashby *et al*., 1990). Though proven, the clinical efficacy of these antiplatelet agents remains moderate (Peto *et al*., 1988; Balsano *et al*., 1990).

The recent introduction of platelet glycoprotein IIb/IIIa (GPIIb/IIIa) antagonists has attracted clinical attention, because these agents inhibit the final common pathways of platelet aggregation, which is the interaction between the platelet membrane GPIIb/IIIa receptor and serum fibrinogen (Philips *et al*., 1991). Many investigators have shown that GPIIb/IIIa antagonists are highly antithrombotic *in vivo* in various experimental models of thrombosis (Coller *et al*., 1986; Gold *et al*., 1988). However, the clinical application of GPIIb/IIIa antagonists may be limited because of their high bleeding risk in the case of excessive inhibition of platelet aggregation.

During the very initial process of thrombus formation, von Willebrand factor (vWF) plays a crucial role (Sakariassen *et al*., 1979; Baumgartner *et al*., 1980). After vascular injury, platelets adhere to the exposed subendothelium through an interaction between subendothelial vWF and platelet glycoprotein Ib (GPIb), which functions as a vWF receptor on the platelet. Thus, vWF bridges platelets and the vessel wall especially under high shear conditions, such as observed in stenosed arteries and microvessels and it mediates the formation of platelet aggregates in such blood vessels. As a consequence of vWF binding to GPIb, the resulting platelet activation will trigger the conformational activation of the platelet GPIIb/IIIa receptor, leading to fibrinogen binding and finally to platelet aggregation. Therefore, the GPIb-vWF axis is an attractive target to focus on for the prevention of thrombus formation in stenosed arteries and microvessels.

Previous studies have demonstrated antithrombotic effects for inhibitors of the GPIb-vWF interaction in arterial thrombosis. However, the administration of anti-GPIb antibodies or snake venom proteins reacting with GPIb has been shown to induce thrombocytopenia in animals (Becker & Miller, 1989; Johnson *et al*., 1991; Peng *et al*., 1993). In addition, the anti-vWF polymeric aurin tricarboxylic acid has been found to prevent coronary artery thrombosis (Strony *et al*., 1990). However, at the effective dose, it also prolonged the prothrombin time (PT) and activated partial thromboplastin time (APTT), as well as the bleeding time, during thrombosis studies in the dog (Kawasaki *et al*., 1994). Finally, the recombinant vWF A1 domain fragment designated as VCL, was shown to prevent thrombosis in several animal models (McGhie *et al*., 1994; Yao *et al*., 1994), even though the action of this peptide was of short duration following intravenous administration (Azzam *et al*., 1995).

In the present study, we have developed and characterized a murine anti-human vWF monoclonal antibody, AJvW-2. This antibody inhibits GPIb-vWF interactions specifically in various animal species. We have investigated its effects on the aggregation and the adhesion of platelets to collagen under high shear conditions. Finally, we have evaluated the compromise between antithrombotic efficiency and bleeding risk for AJvW-2 in a guinea-pig model and compared it with a selective non-peptide GPIIb/IIIa antagonist. These studies have shown that thrombosis can be effectively prevented by inhibition of the GPIb-vWF axis, without an enhanced risk of bleeding.

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Methods

Preparation of monoclonal antibody

A murine anti-human vWF monoclonal antibody named AJvW-2 was prepared according to the methods described by Koehler and Milstein (1975). BALB/c mice were immunized by subcutaneous injection of human vWF. Three days after the second immunization, mouse splenocytes were fused with Sp2/0-Ag14 mouse myeloma cells and cultured in HAT selection medium. Hybridomas producing anti-vWF antibodies were screened by the binding assay for vWF and further cloned by a limiting dilution method. Monoclonal antibody, AJvW-2 was produced in mouse ascites fluid and the IgG was purified from the fluid and stored at -20°C. The concentration of IgG was calculated from u.v. absorbance at 280 nm with an extinction coefficient of E1%280 = 14.3.

In vitro *platelet aggregation studies*

Blood samples were collected on 10% of 0.13 M trisodium citrate. Platelet rich plasma (PRP) and platelet poor plasma (PPP) were prepared by centrifugation of citrated blood at 900 r.p.m. for 10 min and 3000 r.p.m. for 10 min, respectively. The platelet count in PRP was measured by a Sysmex E-2000 (Toa Medical Electronics, Japan) cell counter and adjusted to 300,000 μl^{-1} by dilution with PPP.

Platelet aggregation was measured with an aggregometer, NBS HEMA TRACER 801 (Niko Bioscience, Inc., Japan). To this end, 225 μl of PRP was equilibrated at 37°C under constant stirring at 1000 r.p.m. Then 2 μl of AJvW-2 were added at a final concentration of 0.1 – 10 μg ml^{-1}, 3 min before the addition of 25 μl of the aggregating inducers, such as ristocetin (1.25 mg ml^{-1} final concentration) or botrocetin (0.50 μg ml^{-1} final concentration). IC_{50} values were determined from the dose-response curves.

Shear stress-induced platelet aggregation

Shear stress-induced platelet aggregation was measured by the modified cone and plate viscometer (Toray, Inc., Japan) developed by Ikeda *et al.* (Fukuyama *et al.*, 1989). Briefly, citrated PRP was incubated with AJvW-2 at a final concentration of 1 – 100 μg ml^{-1}, at room temperature for 10 min. Then, 400 μl of these PRP samples were applied onto the surface of a polymethylmethacrylate plate and exposed to shear stress at 25°C for 6 min. The rotation rate of the cone was increased to 200 r.p.m. or 1800 r.p.m. for the initial 15 s, which corresponds to shear stresses of 1.2 or 10.8 N m^{-2}, respectively, and then was kept constant for the following 345 s. Aggregation was monitored continuously by recording the intensity of light transmitted through the platelet suspension. Data are expressed as the mean ± s.e.mean (n = 3).

Shear stress-induced platelet adhesion

Shear stress-induced platelet adhesion was measured by a modified cone and plate viscometer developed in our laboratory. Cover glasses, 0.22 mm thick (Micro Cover Glass, Matsunami, Japan), were coated with porcine skin collagen I (Cellmatrix Type LA, 20 μg ml^{-1}) at 4°C overnight. Then, 1 ml of citrated blood was preincubated with 100 μl AJvW-2 at a final concentration of 1 – 64 μg ml^{-1} at room temperature for 10 min. After the collagen coated coverglass had been mounted, 1 ml of blood sample was applied onto the plate and exposed to shear stress at room temperature for 10 min. The cone was rotated at 60 r.p.m. or 250 r.p.m. which corresponded to shear stresses of 1.5 N m^{-2} for 5.0 N m^{-2}, respectively. These shear stresses corresponded to the shear rates of 360 s^{-1} and 1500 s^{-1}. The cover glass was then removed from the plate, rinsed with 10 mM HEPES buffer (pH 7.4) containing 150 mM NaCl, fixed in 95% ethanol and stained with basic fuchsin. Platelet adhesion to collagen was quantitated via light microscopy: the area occupied by platelets was measured and calculated by a personal image analysis system (LA-555, PIAS, Inc., Japan). Platelet adhesion was expressed as the percentage of the surface covered by platelets and was evaluated by analysing 5 fields for each coverglass (magnification × 400). Data are expressed as the mean ± s.e.mean (n = 3).

Ex vivo *function analysis*

Ex vivo platelet aggregation studies were done on adult male Hartley guinea-pigs, weighing 300 to 500 g, anaesthetized with diethyl ether. AJvW-2 was administered intravenously via the ear vein at doses of 30, 100 or 300 μg kg^{-1}. Five min after administration, blood samples were collected from the aorta and citrated. Platelet aggregation was performed as described above. Coagulation assays were equally carried out on adult male Hartley guinea-pigs of 450 – 600 g, anaesthetized with diethyl ether. AJvW-2 was administered intravenously via the ear vein at doses of 100, 300 or 1000 μg kg^{-1}. Each group was composed of 4 animals. After 5 min, blood samples were collected from the aorta and plasma samples were prepared by centrifugation. Prothrombin time (PT) and activated partial thromboplastin time (APTT) were measured with a Sysmex CA-3000 (Toa Medical Electronics, Inc., Japan) analyser.

Plasma vWF antigen levels

Rabbit anti-human vWF polyclonal antibodies (DAKO) were coated in microtitre plates at 10 μg ml^{-1} at 4°C overnight. After being blocked by 1% BSA, 1/40 diluted guinea-pig plasma samples were added and incubated at room temperature for 2 h. Bound vWF was detected by peroxidase conjugated-rabbit anti-human vWF polyclonal antibody (DAKO). The relative plasma vWF concentration in each group was calculated as a percentage of the average value in a control (vehicle) group.

Photochemically-induced thrombosis model

Transluminal thrombosis was induced by photochemical injury to the endothelium according to the method described by Nakashima (Takiguchi *et al.*, 1992). Briefly, adult female Hartley guinea-pigs, weighing 300 to 500 g, were anaesthetized by i.p. administration of urethane (2 g kg^{-1}). A polyethylene cathether was inserted into the femoral vein for the administration of rose bengal (10 mg kg^{-1}), AJvW-2 or lamifiban. Then, the carotid arteries were exposed and a pulse doppler flow probe (DBF-10, Crystal Biotech America, U.S.A.) was positioned for monitoring blood flow with a pulse doppler flow meter (PDV-20, Crystal Biotech America, U.S.A.). Flow was recorded continuously on a thermal recorder (WT-645G, Nihon Kohden, Japan). Transillumination of green light (540 nm) was achieved by using a xenon lamp with a heat-absorbing filter and a green filter (Hamamatsu Photonics, Japan). The irradiation was directed with an optic fibre positioned 5 mm away from a section of intact carotid artery proximal to the flow probe. Irradiation was started after baseline blood flow had stabilized. AJvW-2 or lamifiban were administered intravenously 5 min before injection of rose bengal and blood flow was monitored for 30 min, or until occlusion of the vessel.

Bleeding time

Adult female Hartley guinea-pigs, weighing 300 to 500 g, were anaesthetized by i.p. administration of pentobarbitone (50 mg kg^{-1}). AJvW-2 or lamifiban were administered intravenously via the ear vein. The medial plantar artery was incised with the automated spring-loaded device (Simplate R, Organon Teknika Co., U.S.A.) 5 min after the administration and template bleeding times were measured.

Reagents

Ristocetin sulphate, U46619 (9,11-dideoxy-9α,11α-methanoepoxy prostaglandin $F_{2\alpha}$) and Bothrop jararaca venom were obtained from Sigma Chemical Co (St. Louis, MO, U.S.A.). Botrocetin was purified with this venom as described previously (Fujimura *et al*., 1991). Adenosine diphosphate (ADP) was from Boehringer Mannheim GMBH (Germany). Collagen reagent Horm was from Nycomed Arzneimittel GMBH (Germany). Porcine skin collagen type I (Cellmatrix Type LA) was purchased from Nitta Gelatin, Inc. (Japan). Rabbit anti-human vWF polyclonal antibodies (A082) and peroxidase conjugated-rabbit anti-human vWF polyclonal antibodies (P226) were purchased from DAKO (Denmark). Rose bengal was from Wako (Japan). Lamifiban (Ro44-9883; [[1-[N-(p-amidinobenzoyl)-L-tyrosyl]-4-piperidinyl]oxy]trifluoacetate) was synthesized in the Central Research Laboratories, Ajinomoto Co., Inc. (Japan).

Statistical analysis

All values are expressed as a mean ± s.e.mean. For the *ex vivo* studies, AJvW-2-treated groups were compared with a control group by one-way factorial ANOVA, followed by Dunnett's (two-tailed) *post-hoc* test. For the *in vivo* studies, the AJvW-2- or lamifiban-treated groups were compared with a control group by a nonparametric Kruskal-Wallis test, followed by Dunnett's test. A probability value, $P<0.05$ was considered significant.

Results

Inhibition of in vitro *platelet aggregation*

The inhibitory potency of AJvW-2 on platelet aggregation was compared for platelet-rich plasma derived from both man and guinea-pig (Table 1). AJvW-2 (0.1 – 10 μg ml^{-1}) inhibited the ristocetin- and botrocetin-induced platelet aggregation dose-dependently and complete inhibition was observed at 4 μg ml^{-1} and 8 μg ml^{-1} in human PRP and guinea-pig PRP, respectively. On the other hand, AJvW-2 did not affect the ADP-, collagen- and U46619-induced platelet aggregation up to 100 μg ml^{-1} in either type of PRP (data not shown).

AJvW-2 is a murine monoclonal antibody (IgG_1,κ) raised by human von Willebrand factor (vWF). Interestingly, this antibody inhibited botrocetin-induced platelet aggregations in various species including rat, rabbit, dog and pig (Table 1), in agreement with the cross reactivity of AJvW-2 with the vWF of these diverse species. Ristocetin failed to induce platelet aggregation in these species.

Table 1 Effects of AJvW-2 on *in vitro* platelet aggregation induced by ristocetin and botrocetin in various species

	IC_{50} (μg ml^{-1})	
Agonist	*Ristocetin*	*Botrocetin*
Human PRP (4)	0.7 ± 0.1	1.8 ± 0.3
Guinea-pig PRP (3)	1.4 ± 0.6	4.0 ± 0.5
Rat PRP (1)	NT	7.5
Rabbit PRP (3)	NT	3.0 ± 0.1
Dog PRP (6)	NT	4.3 ± 0.5
Pig PRP (3)	NT	2.9 ± 0.3

Platelet aggregations were carried out in an aggregometer: 2 μl of various concentrations of AJvW-2 were added to 225 μl of PRP, 3 min before adding agonists (25 μl). Ristocetin and botrocetin were used at a concentration of 1.25 mg ml^{-1} and 0.50 μg ml^{-1}, respectively. The number of animals used in each experiment is indicated in parentheses. Each value represents the mean ± s.e.mean of the concentration causing 50% inhibition of the control response. NT: not tested.

Inhibition of shear stress-induced platelet aggregation

The inhibitory effect of AJvW-2 on shear stress-induced platelet aggregation was investigated at low (1.2 N m^{-2}) and high (10.8 N m^{-2}) shear conditions both for human and guinea-pig PRP. AJvW-2 completely inhibited the high shear stress-induced platelet aggregation in human PRP at 4.0 μg ml^{-1} (IC_{50} = 1.4 ± 0.1 μg ml^{-1}). At low shear stress conditions, AJvW-2 did not influence platelet aggregation up to 100 μg ml^{-1} (Figure 1a). In guinea-pig PRP, platelet aggregation was not induced by low shear stress. High shear stress-induced guinea-pig platelet aggregation was inhibited by AJvW-2 (IC_{50} = 7.5 ± 1.7 μg ml^{-1}) with comparable efficiency (Figure 1b).

Inhibition of shear stress-induced platelet adhesion

The inhibitory effect of AJvW-2 on human and guinea-pig platelet adhesion to porcine skin collagen type I was investigated at low (1.5 N m^{-2}) and high (5.0 N m^{-2}) shear conditions. When exposed to shear stress for 10 min, the percentage of surface coverage equalled 7.3 ± 0.6% at 1.5 N m^{-2} and 23.3 ± 2.5% at 5.0 N m^{-2} for experiments performed with human blood. With guinea-pig blood, surface coverages ranged from 10.8 ± 0.2% at 1.5 N m^{-2} to 19.8 ± 1.6% at 5.0 N m^{-2}. At 5.0 N m^{-2}, AJvW-2 inhibited the adhesion of platelets to collagen dose-dependently, both in human and

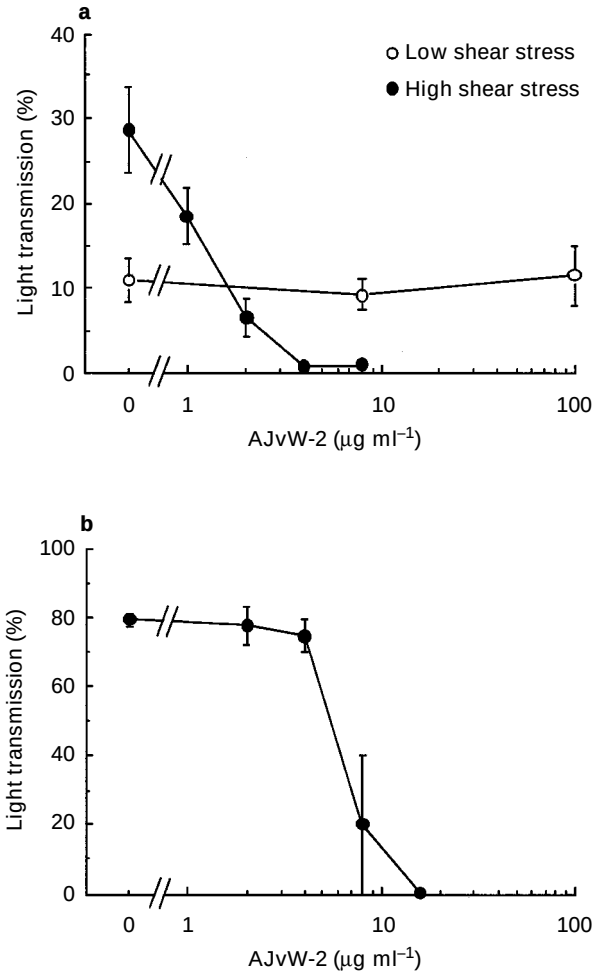

Figure 1 Effects of AJvW-2 on the *in vitro* aggregation of human (a) and guinea-pig (b) platelets induced by high and low shear stress. PRP samples were exposed to a constant high shear stress (10.8 N m^{-2}) or low shear stress (1.2 N m^{-2}) for 6 min. Data are presented as the mean and vertical lines show s.e.mean for three separate experiments.

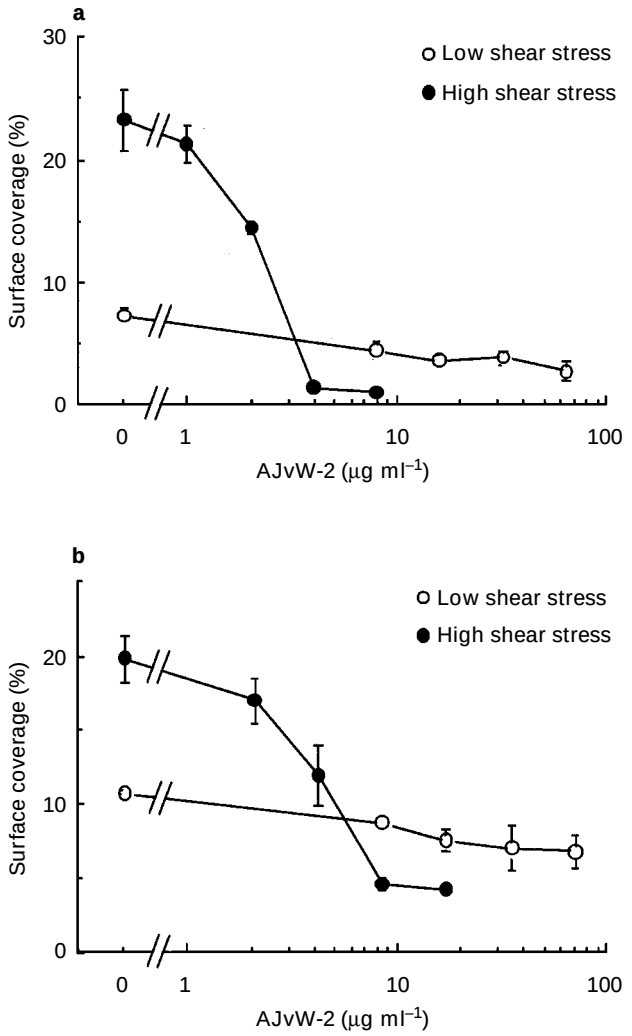

Figure 2 Effects of AJvW-2 on the *in vitro* adhesion of human (a) and guinea-pig (b) platelets to collagen-coated cover slips at high and low shear stress. Citrated blood was exposed to a constant high shear stress (5.0 N m^{-2}) or a low shear stress (1.5 N m^{-2}) for 10 min. Data are presented as the mean and vertical lines show s.e.mean for three separate experiments.

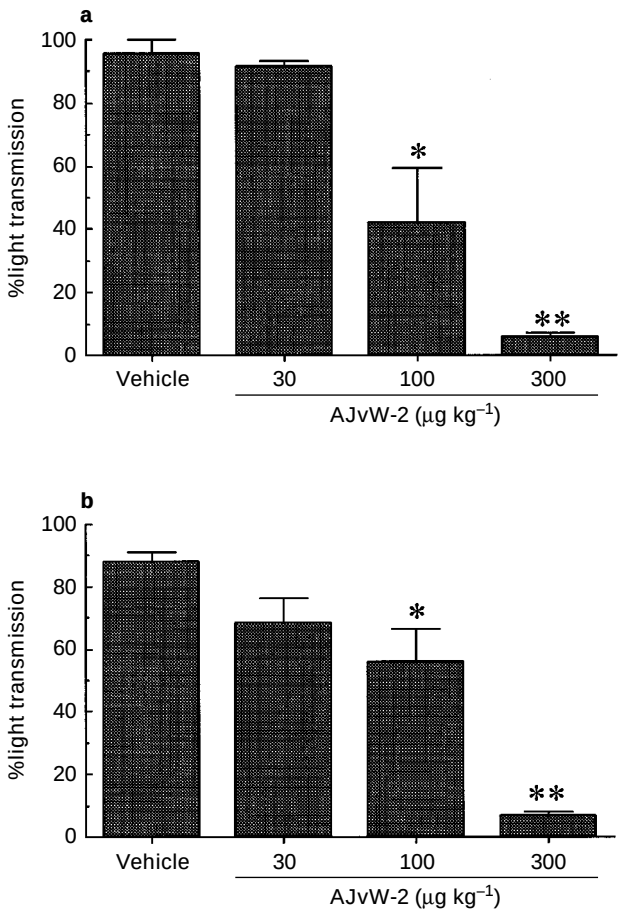

Figure 3 Effects of AJvW-2 on the *ex vivo* platelet aggregation induced by ristocetin (a) or botrocetin (b) in guinea-pig PRP. Data are presented as the mean ± s.e.mean of 3 experiments. Five minutes after the i.v. administration of AJvW-2 or saline (vehicle), citrated blood was collected and the PRP separated. $*P<0.05$, $**P<0.01$ as compared with control (vehicle) by one-way factorial ANOVA, followed by Dunnett's (two-tailed) *post-hoc* test.

in guinea-pig blood ($IC_{50}=2.4\pm0.3$ μg ml^{-1} and 5.2 ± 0.8 μg ml^{-1}, respectively). At 1.5 N m^{-2}, the platelet surface coverage was not inhibited by AJvW-2 in any species investigated (Figure 2).

Inhibition of ex vivo *platelet aggregation in guinea-pig*

AJvW-2 inhibited the *ex vivo* ristocetin- and botrocetin-induced platelet aggregation in a dose-dependent manner when administered intravenously to guinea-pigs (Figure 3). Maximal aggregation induced by 2.0 mg ml^{-1} ristocetin was reduced from 95% to 92%, 42% and 6% by injection of 30 μg kg^{-1}, 100 μg kg^{-1} and 300 μg kg^{-1} AJvW-2, respectively. Maximal aggregation induced by 0.5 μg ml^{-1} botrocetin was reduced from 88% aggregation to 68%, 56% and 7% by injection of 30 μg kg^{-1}, 100 μg kg^{-1} and 300 μg kg^{-1} AJvW-2, respectively. Statistically significant inhibition of aggregation induced by both agents was observed at the dose of 100 μg kg^{-1}.

Effect on coagulation parameters, platelet count and plasma vWF antigen level in the guinea-pig

AJvW-2 did not prolong the prothrombin time (PT) and the activated partial thromboplastin time (APTT) in guinea-pigs following injection of up to 1000 μg kg^{-1}. The PT in the control group averaged 33.5 ± 1.0 s. In AJvW-2 treated groups, the PTs were 33.3 ± 3.8, 29.1 ± 0.6 and 29.4 ± 0.7 s at doses of 100 μg kg^{-1}, 300 μg kg^{-1} and 1000 μg kg^{-1}, respectively. The APTT in the control group was 18.1 ± 0.4 s. In the AJvW-2 treated groups, the APTTs were 18.3 ± 0.6, 20.5 ± 3.7 and 16.9 ± 0.2 s at doses of 100 μg kg^{-1}, 300 μg kg^{-1} and 1000 μg kg^{-1}, respectively.

The platelet count and plasma vWF antigen levels were not decreased by injection of AJvW-2 up to 1000 μg kg^{-1} (data not shown).

Antithrombotic effect in a photochemically-induced thrombosis model

The antithrombotic effect of AJvW-2 was evaluated in a photochemically-induced thrombosis model in the guinea-pig. In the control group, carotid arteries occluded within 10 min after initiation of the photochemical reaction. AJvW-2 significantly prolonged the occlusion time at a dose of 100 μg kg^{-1}, which corresponded to the effective dose during the *ex vivo* studies. In 4 out of 5 animals, given 300 μg kg^{-1}, occlusion was not observed even 30 min after the onset of the green light irradiation (Figure 4a).

The synthetic non-peptide glycoprotein IIb/IIIa (GPIIb/IIIa) antagonist, lamifiban prolonged the occlusion time significantly at a dose of 1000 μg kg^{-1} (Figure 4b).

Effect on bleeding time

The effects of AJvW-2 and lamifiban on the haemostatic function in guinea-pigs are shown in Figure 5. The bleeding

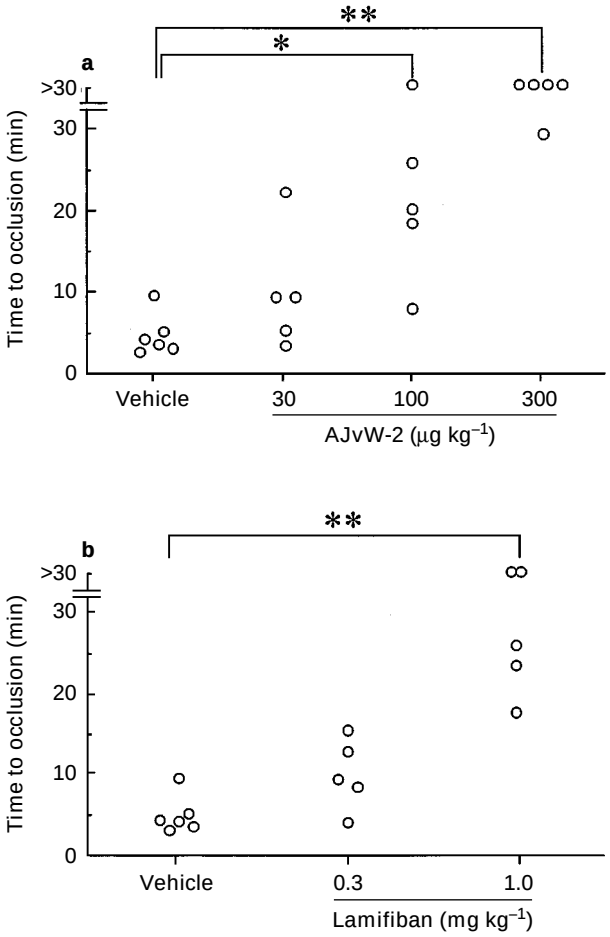

Figure 4 Antithrombotic effects of AJvW-2 (a) and lamifiban (b) during photochemically-induced thrombosis in the guinea-pig carotid artery. Five minutes after the i.v. administration of AJvW-2, lamifiban or saline (vehicle), rose bengal was injected and the time to occlusion was measured from the start of transillumination. $*P<0.05$, $**P<0.01$ as compared with control (vehicle) by the non-parametric Kruskal-Wallis test, followed by Dunnett's test.

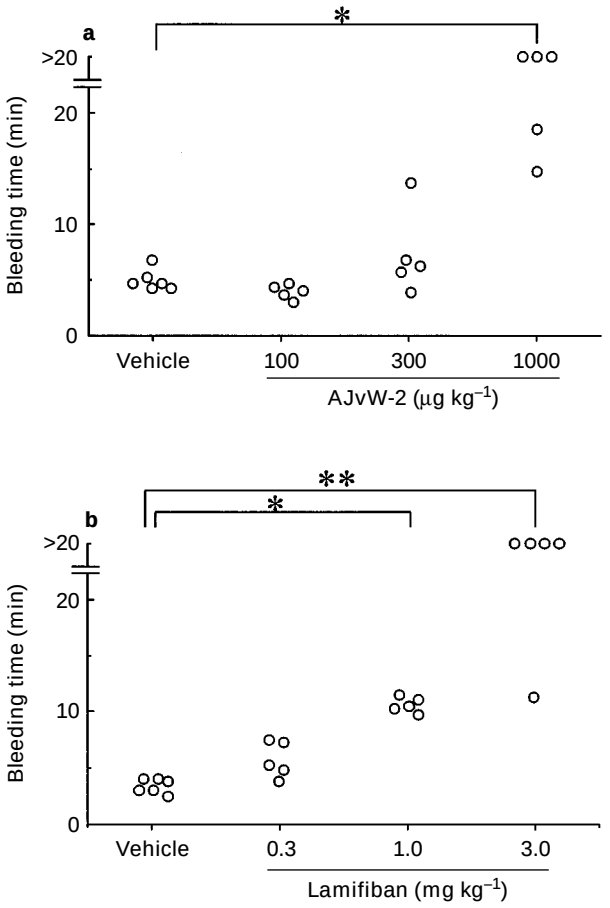

Figure 5 Effects of AJvW-2 (a) and lamifiban (b) on bleeding times in the guinea-pig. Five minutes after the i.v. administration of AJvW-2, lamifiban or saline (vehicle), the medial plantar artery was incised and the bleeding time was measured with the automated spring-loaded device Simplate R. $*P<0.05$, $**P<0.01$ as compared with control (vehicle) by the nonparametric Kruskal-Wallis test, followed by Dunnett's test.

time was not significantly prolonged by injection of 100 μg kg^{-1} and 300 μg kg^{-1} AJvW-2. Statistically significant prolongation was only observed at 1000 μg kg^{-1}, which was a 10 fold higher dose than that which was effective in the photochemically-induced thrombosis model.

On the other hand, lamifiban prolonged the bleeding time significantly at 1000 μg kg^{-1}, which was the same as the effective dose in the photochemically-induced thrombosis model. These results suggest that the margin between the prevention of thrombus formation and prolongation of bleeding time is larger for AJvW-2 than for lamifiban.

Discussion

Platelet adhesion to an injured vessel wall is the initial step for thrombus formation (Roth, 1991). The platelet GPIb-vWF interaction is essential for platelet adhesion, especially under high shear stress conditions such as observed in stenosed arteries and microvessels (Sakariassen *et al*., 1979; Baumgartner *et al*., 1980). To investigate the role of the GPIb-vWF interaction in the process of arterial thrombus formation, we developed a murine monoclonal antibody against human vWF, AJvW-2 (immunoglobulin subtype IgG_1,κ), by standard hybridoma technology.

AJvW-2 inhibited both the ristocetin- and the botrocetin-induced aggregation of human and guinea-pig platelets. However, AJvW-2 had no effect on platelet aggregation induced by ADP, collagen and U46619, indicating that AJvW-2 specifically inhibited the vWF mediated platelet aggregation. AJvW-2 also inhibited the botrocetin-induced platelet aggregation in rat, rabbit, dog and pig PRP in the same concentration range, substantiating that it recognizes a very conserved epitope in the vWF molecule, which may be overlapping the interaction site with GPIb. For these reasons, AJvW-2 might be useful for investigating the physiological and patho-physiological role of the GPIb-vWF axis in various animal models.

We investigated the effect of AJvW-2 on the shear stress-induced aggregation of platelets. AJvW-2 inhibited the high shear stress-induced platelet aggregation dose-dependently both in human and guinea-pig PRP, whereas low shear stress-induced platelet aggregation was not inhibited. Ikeda *et al*. have shown that the high shear stress-induced platelet aggregation is triggered by the GPIb-vWF interactions, in contrast to the low shear stress-induced platelet aggregation which is mediated by GPIIb/IIa-fibrinogen interactions (Ikeda *et al*., 1991). Our data are compatible with a model in which AJvW-2 inhibits platelet aggregation under high shear stress conditions as a consequence of its blockade of the GPIb-vWF axis. AJvW-2 also inhibited the adhesion of platelets to porcine skin collagen at high shear conditions, whereas the adhesion observed at low shear stress was not inhibited by AJvW-2. Hence, AJvW-2 prevented the initiating step of thrombus formation in a shear stress-dependent manner.

Ex vivo antiplatelet efficiencies for AJvW-2 were evaluated in guinea-pigs. AJvW-2 inhibited the ristocetin- and botroce-

tin-induced platelet aggregation dose-dependently, a significant *ex vivo* inhibition of platelet aggregation even being observed following injection of 100 μg kg^{-1} AJvW-2. This effect was prolonged for more than 24 h (data not shown). However, AJvW-2 did not prolong coagulation parameters such as the PT and APTT. In view of the fact that vWF is a carrier protein for the coagulation factor VIII, these results imply that AJvW-2 did not influence the interaction between vWF and FVIII. Since in addition, platelet counts and plasma vWF antigen levels were not affected by AJvW-2, its *ex vivo* antiplatelet effects seem to be the result of a specific inhibition of the interaction between GPIb and vWF.

In the *in vivo* guinea-pig model, AJvW-2 significantly prevented thrombosis at doses of 100 μg kg^{-1} and 300 μg kg^{-1} without prolonging the bleeding time. On the other hand, the GPIIb/IIIa antagonist, lamifiban, prolonged the bleeding time at the same concentration as the effective dose in the thrombosis model. Because AJvW-2 exerts its effects in a shear stress-dependent manner, it does not influence haemostasis at low shear stress conditions, in contrast to GPIIb/IIIa antagonists, that by inhibiting the final common pathway of platelet activation, act independently of shear stress variations. Recently, the chimeric anti-GPIIb/IIIa monoclonal antibody c7E3 Fab (abciximab, ReoPro) has been found to be effective in conjugate therapy in patients undergoing high-risk coronary artery angioplasty and atherectomy (Topol *et al*., 1994). However, since GPIIb/IIIa antagonists have been shown to possess a high bleeding risk in clinical trials (Aguirre *et al*., 1995) as well as in experimental animal models of thrombosis (Cook *et al*., 1993; Rote *et al*., 1994), our results suggest that such therapy would further benefit from the application of GPIb-vWF inhibitors, as represented by AJvW-2, that have a lower bleeding risk.

In conclusion, AJvW-2 is a specific inhibitor of GPIb-vWF interactions, that cross-reacts with vWF of various animal species. AJvW-2 may be an effective antithrombotic drug with a lower bleeding risk than GPIIb/IIIa antagonists.

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