Contribution of von Willebrand Factor to Thrombus Formation on Neointima of Rabbit Stenotic Iliac Artery Under High Blood-Flow Velocity

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Objective—It has become clear that von Willebrand factor (vWF) plays important roles in platelet adhesion and aggregation under high blood-flow velocity conditions observed in stenotic atherosclerotic arteries. However, its roles in thrombus formation in vivo on diseased arteries have not been fully understood. We examined the contribution of vWF to thrombus formation and subsequent intimal growth by using a repeated balloon-injury model in rabbits.

Methods and Results—Rabbit iliac arteries 4 weeks after a first balloon injury showed 37% luminal stenosis by neointimal growth, and blood velocity increased by 2.1 times compared with that of uninjured arteries. The second balloon injury induced fibrin-rich thrombus formation on the injured neointima. Intravenous administration of a monoclonal antibody against vWF (AJW200, 1.0 mg/kg body weight) remarkably prevented botrocetin-induced platelet aggregation ex vivo for 2 days; moreover, thrombus formation, cell proliferation, and subsequent neointimal growth were significantly reduced at 30 minutes, 5 days, and 4 weeks, respectively, after the second balloon injury.

Conclusions—These results indicate that vWF plays a potent role in fibrin-rich thrombus formation on the neointima under high blood-flow velocity conditions. Inhibition of plasma vWF activity might be effective for the reduction of thrombus formation and/or subsequent neointimal development after coronary interventions. (Arterioscler Thromb Vasc Biol. 2003;23:1105-1110.)

Key Words: von Willebrand factor ■ thrombosis ■ blood flow ■ rabbits ■ AJW200

Thrombus formation on a disrupted atherosclerotic plaque is a threatening event that leads to acute coronary syndromes.1,2 Because platelet adhesion and aggregation are essential steps in hemostatic and thrombotic processes, the development of platelet-rich thrombi has been regarded as a trigger of acute coronary syndromes.2,3 On the other hand, autopsy studies have revealed that thrombi that have caused myocardial infarction contain not only platelets but also a large amount of fibrin, suggesting increased activation of the coagulation cascade at the time of the event.4,5 We and other investigators have demonstrated that tissue factor (TF) is expressed in atherosclerotic lesions,6–9 is an important determinant of thrombogenicity, and contributes to fibrin-rich thrombus formation after plaque disruption.7,10

Recent angiographic studies have revealed that coronary occlusions that induce myocardial infarction most frequently evolve in segments with stenoses that are <80%.11 On the basis of this evidence, fluid-dynamic forces (elevated shear rates) would be expected to have certain effects on thrombus formation in the stenotic arteries. Current ex vivo experiments have indicated a crucial role for von Willebrand factor (vWF) in platelet aggregation under rapid-flow conditions.12,13 In addition, inhibition of vWF activity prevents in vivo arterial platelet thrombus formation in the normal arteries of some species.14–16 However, the actual role of vWF in thrombus formation in stenotic, atherosclerotic arteries has not been elucidated.

We therefore examined whether or not vWF contributes to thrombus formation and the subsequent neointimal growth under high blood-flow velocity conditions by using a repeated balloon-injury model in rabbits and the humanized anti-vWF monoclonal antibody (AJW200), which reacts to the A1 domain of vWF.17,18

Methods

Repeated Balloon-Injury Model in Rabbits

In this study, 58 male Japanese White rabbits weighing 2 to 2.5 kg were used in research protocols approved by the Animal Care Committee of Miyazaki Medical College (No. 1998-025-6). All animals received humane care according to the Guide for the Care and Use of Laboratory Animals prepared by the Institute of Labo-
The animals were anesthetized with pentobarbital (25 mg/kg body weight IV), and the endothelium of the iliac arteries was denuded by insertion of a Fogarty 2F balloon catheter (Baxter Healthcare). For the first injury, the catheter was inserted via the anterior tibial artery into the iliac artery, inflated at 1.5 atm, and pulled down repeatedly three times for a distance of 5 cm. Four weeks after the first injury, the iliac arteries were imaged by magnetic resonance angiography (MRA), and the luminal diameter and blood-flow velocity were measured. Immediately after that, the catheter was inserted again via the femoral artery into the iliac artery, and the second injury was performed by using the same procedure as in the first. A bolus injection of the humanized anti-vWF monoclonal antibody (AJW200, 1.0 and 3.0 mg/kg IV) or saline was administered into the ear vein 30 minutes before the second injury. This antibody reacts to the A1 domain of vWF in some species, including humans and rabbits. Only ligation of the tibial and femoral arteries was performed on the contralateral uninjured side. The animals were killed 30 minutes, 5 days, or 4 weeks after the second injury for evaluation of thrombus formation, cell proliferation, or neointimal growth, respectively. All rabbits were killed with an overdose of pentobarbital (60 mg/kg body weight IV) 5 minutes after an injection of heparin was given (500 U/kg body weight IV). The animals were perfused with 50 mL of 0.01 mol/L phosphate-buffered saline and of heparin was given (500 U/kg IV). The animals were then perfusion-fixed with 4% paraformaldehyde. The iliac arteries were dissected out for subsequent studies.

### Measurements of Luminal Diameter and Blood-Flow Velocity

Four weeks after the first injury, the iliac arteries were imaged by MRA, and the luminal diameter and blood-flow velocity were measured with a 1.5-T superconductive magnet unit (Visart/EX, version 4.01, Toshiba) with a 16-cm-diameter radiofrequency coil. We used a 2-dimensional spoiled gradient-echo sequence with the following parameters: repetition time, 25 ms; echo time, 9 ms; and flip angle, 60° with electrocardiographic gating. The original axial images were used to measure the luminal diameter. Three-dimensional reconstructions of MRA images were made with the maximum-intensity-projection technique as a postprocessing tool. To measure arterial blood-flow velocity, we used 2-dimensional phase-contrast acquisition with following scan parameters: repetition time, 24 ms; echo time, 10 ms; and flip angle, 20° with electrocardiographic gating.

### Light Microscopy and Immunohistochemical Studies

Six sequential cross sections were taken from each iliac artery. These sections were further fixed in 4% paraformaldehyde for 24 hours at 4°C and embedded in paraffin. Three-micron-thick sections were stained with hematoxylin and eosin/Victoria blue dye for morphological evaluation and were also examined immunohistochemically with the antibodies against α-smooth muscle actin (HHF35, DAKO Japan), rabbit macrophages (RAM11, DAKO Japan), TF (Chem鄄Sero-Therapeutic Research Institute), rabbit fibrin (a kind gift from Dr T. Kurokawa, Takeda Chemical Industries, Ltd., Osaka, Japan), vWF (Binding Site), and Ki-67 (MIB-1, DAKO Japan). To evaluate the thrombus volume and neointimal growth, the areas (in square microns) of thrombus, layers of neointima, and media were measured with an image-analyzing system (Axiolab Vision 2.05, Carl Zeiss) by two of the investigators (K.H. and K.M.) who were blinded to treatment assignment. In addition, to examine neointimal cell proliferation activity 5 days after the second injury, the Ki-67 labeling index (number of counts of Ki-67-positive nuclei for neointimal area) was calculated at 1000× magnification.

### Statistical Analysis

The data were expressed as mean±SE. ANOVA was used for multiple comparisons between groups. Differences for individual groups were tested with the unpaired Student’s t test. A value of P<0.05 was considered significant. Lower-case n indicates the number of animals used in the experiments.

### Results

#### Inhibition of Botrocetin-Induced Platelet Aggregation and No Significant Changes in PT and aPTT by AJW200 Injection

To study the possible effects of AJW200 administration on platelet function and systemic coagulation, ex vivo platelet aggregation, PT, and aPTT were measured. We used two doses (1.0 and 3.0 mg/kg) of AJW200, based on our data from previous studies. A bolus injection of AJW200 at a dose of 1.0 mg/kg significantly inhibited the botrocetin-induced platelet aggregation within 2 days; moreover, the aggregation was significantly reduced until 7 days after a bolus injection of 3.0 mg/kg AJW200 (Table). In contrast, AJW200 administration did not affect collagen-induced platelet aggregation and systemic coagulation (PT and aPTT).
On the basis of these data, we used a dose of 1.0 mg/kg AJW200 in the following study.

**Luminal Stenosis and High Blood-Follow Velocity in Iliac Arteries 4 Weeks After First Injury**

Representative MRA images are shown in Figure 1A. Histologically, the neointima was composed exclusively of smooth muscle cells (SMCs), and extracellular matrix was produced in the injured arteries (Figure 1C) but not in the uninjured ones (Figure 1B). The values for luminal diameter in injured and uninjured iliac arteries were 1.35±0.01 mm and 2.13±0.01 mm, respectively, and those for peak velocity in injured and uninjured iliac arteries were 33.7±3.1 cm/s and 16.1±2.1 cm/s, respectively. The luminal diameters of the injured arteries significantly decreased to 62.7±2.9% (Figure 2A), whereas the peak velocity of the arteries increased by 2.1±0.1 times compared with that of uninjured arteries (Figure 2B).

**Fibrin-Rich Thrombus Formation in the Second-Injury Iliac Arteries**

Thirty minutes after the second injury, the injured neointima were covered with fresh thrombi (Figure 3A), which showed a diffuse and strongly positive immunoreactivity for fibrin (Figure 3B). The thrombi were also positive for vWF, but the neointima showed only a weak immunoreactivity for vWF (Figure 3C). Furthermore, the thrombi and neointimal SMCs showed positive immunoreactivity for TF (Figure 3D). These findings indicate that thrombi produced on the neointima of the stenotic arteries are fibrin rich and that a high content of vWF is intermingled, probably with platelets.

**AJW200 Suppresses Fibrin-Rich Thrombus Formation, Cell Proliferation, and Neointimal Growth After the Second Injury**

A bolus injection of AJW200 significantly reduced fibrin-rich thrombus formation 30 minutes after the second injury (Figure 4). The medial area did not vary significantly among the arteries examined (data not shown). The Ki-67 labeling index of neointimal SMCs was significantly smaller in AJW200-treated vessels (11.8±0.7 per 10 000 μm²) than in
control vessels (14.8±1.1 per 10 000 μm²; n=5, P<0.05). The two-layered neointima was observed in the iliac arteries 4 weeks after the second injury (Figure 5A). Areas of the inner neointima and media and the ratio of the inner neointima to media are shown in Figure 5B and 5C. The antibody significantly suppressed development of the inner neointima after the second injury.

Discussion

The main findings of this study were that in the rabbit stenotic iliac arteries with high blood-flow velocity, balloon injury to the neointima (the second injury) induced fibrin-rich thrombus formation. In addition, administration of an anti-vWF monoclonal antibody reduced this thrombus formation and the subsequent neointimal growth after the second injury.

Clinical and experimental studies have established the role of plaque disruption and acute thrombus formation in the onset of acute coronary events and the progression of atherosclerosis. The most widely accepted hypothesis is that plaque disruption activates circulating platelets by exposure of the subendothelium to the blood circulation, and subsequent platelet adhesion and aggregation would result in obstructive thrombus formation. Based on this hypothesis, antagonists for the platelet glycoprotein (GP) IIb/IIIa receptor, which is the final common pathway of platelet aggregation, have recently been tested in randomized, placebo-controlled trials of acute coronary syndromes and percutaneous coronary interventions. For patients undergoing percutaneous revascularization, these agents have demonstrated efficacy in reducing death, myocardial infarction, or urgent reintervention. However, more modest or less apparent benefits have been seen in trials of GPIIb/IIIa receptor antagonists for patients with acute coronary syndromes. The latter evidence raises the possibility of a different mechanism of thrombogenesis on plaque disruption. Actually, autopsy studies after acute myocardial infarction have revealed that only 15% of the occlusion consisted of pure platelet thrombus, but 85% of occlusive thrombi were composed of a large amount of fibrin as well as platelets. The evidence indicates that blood coagulation as well as platelets plays a crucial role in the process of occlusive thrombus formation. We and other investigators have already demonstrated that macrophages and SMCs in the plaques consistently express abundant TF; consequently, atherosclerotic lesions possess high procoagulant activity. Moreover, plasma levels of TF were significantly elevated in patients with ischemic heart diseases compared with those in control subjects. These lines of evidence suggest that increased activity of the TF-mediated coagulation cascade strongly contributes to thrombus formation on plaque disruption sites. In fact, we recently reported that adenovirus-mediated local expression of a TF pathway inhibitor reduces thrombus and neointimal formation in injured rabbit carotid arteries. Therefore, to elucidate the mechanism of thrombus formation in diseased arteries, we used the repeated balloon-injury model of rabbit iliac arteries in this study, because neointimal SMCs continuously express active TF protein similar to human atherosclerotic lesions. Recent coronary angiographic studies have revealed that plaque disruption would more frequently occur in atherosclerotic coronary arteries with <50% diameter stenosis. The injured iliac arteries in this study showed ∼40% stenosis by neointimal formation and high blood-flow velocity. On the basis of these findings, this animal model closely mimics diseased human arteries.

Figure 4. Area of thrombus (A) and thrombus/media (B) ratio in iliac arteries 30 minutes after the second injury. (n=9, *P<0.05).

Figure 5. A, Representative light photomicrograph of iliac artery 4 weeks after the second injury. Distinct two-layered neointima is observed I1 indicates inner neointima; I2, outer neointima, M, media. B, Areas of inner neointima (filled columns) and media (open columns) and (C) inner intima/media ratio. (n=10, *P<0.05).
Blood flow through stenotic atherosclerotic arteries results in a high shear rate. Under such hemodynamic conditions in ex vivo experiments, vWF and the two membrane receptors, GPIIb and GPIIb/IIIa, are reported to be crucial for platelet aggregation. In addition, epidemiological studies have shown a positive correlation between plasma vWF levels and the incidence of heart diseases caused by arterial thrombosis. These reports suggest that vWF could be involved in coronary thrombosis under rapid blood-flow conditions. We have already reported that AJW200 reduced platelet thrombus formation in animal experiments. However, these experiments were performed with animal models of endothelial denudation of normal arteries; therefore, these experiments and results are not sufficient to mimic the pathologic setting in diseased human arteries. In this study, the flow velocity in stenotic iliac arteries was two times higher than in nonstenotic arteries. We have assumed that the thrombi produced on the neointima under a high blood-flow conditions would be more platelet rich, but the present study demonstrates that, even under these condition, the thrombi on the neointima are fibrin rich. The histologic features of these thrombi are very similar to those of culprit lesions in patients with acute myocardial infarction. The thrombi were also rich in vWF, and a monoclonal antibody against vWF (AJW200) reduced thrombus growth. The results indicate the possibility of a considerable role for vWF, probably with platelets, in fibrin-rich thrombus formation under high blood-flow velocity.

Interaction between platelets and coagulation proteins is essential for hemostasis and thrombus formation. Platelets influence thrombin generation by providing a surface of phospholipid for the assembly of the prothrombinase complex, which consists of the zymogen, prothrombin, factor Xa, factor Va, and phospholipid. Prothrombin also interacts with GPIIb/IIIa on resting and activated platelets, which results in prothrombin activation. Taken together, inhibition of platelet adhesion and aggregation would suppress the coagulation process resulting in fibrin formation. Our previous in vitro study indicated that AJW200 inhibited not only high-shear-stress-induced platelet adhesion and aggregation but also thrombin generation. Moreover, vWF directly binds fibrin and also promotes platelet adhesion to fibrin in flowing blood. These lines of evidence suggest that vWF might play a role in anchoring platelets to fibrin.

Growth factors released from activated platelets and fibrin itself are well known to contribute to neointimal formation, and thrombi have long been implicated in neointimal growth and plaque progression. Much of the neointimal volume appears to be related to the initially produced fibrin-rich mural thrombus, because they provide the volume into which SMCs migrate and proliferate. It was reported that vWF deposits in the extracellular space of atherosclerotic plaques and intimal thickenings. The deposition may be the result of an increased release by endothelial cells, an influx from the plasma, or a decreased rate of removing vWF. The role of vWF in the neointima is still unclear. Giddings et al demonstrated that balloon angioplasty of the porcine carotid artery was associated with deposition and medial absorption of vWF over a time period that preceded and overlapped SMC proliferation and endothelial recovering. Moreover, certain in vivo studies have indicated that inhibition of vWF activity attenuated neointimal growth in animal models. These reports suggest that increased synthesis and/or deposition of vWF in the neointima might stimulate further neointimal development and plaque formation. On the other hand, a study of von Willebrand disease in pigs has indicated that vWF was not essential for neointimal development in normal carotid and femoral arteries. In this study, a bolus injection of monoclonal antibody against vWF reduced not only thrombus formation but also subsequent neointimal SMC proliferation and neointimal development after the second balloon injury. Our result supports an important role for vWF in neointimal growth.

In conclusion, the anti-vWF monoclonal antibody AJW200 reduced fibrin-rich thrombus formation on the neointima and subsequent further neointimal growth in the rabbit stenotic iliac arteries with high blood-flow velocity conditions. These results raise the possibility that a bolus injection of AJW200 in the treatment of unstable angina and after coronary interventions might reduce new events in the coronary arteries and inhibit restenosis. To confirm the efficacy of anti-vWF antibody AJW200 in the treatment of human arterial disorders, the inhibitory effects of the antibody should be evaluated in stenotic arteries with more complicated lesions in cholesterol-fed animals and in coronary arteries of large mammals, such as monkeys or pigs.

References

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