Increased Vascular Wall Thrombogenicity Combined With Reduced Blood Flow Promotes Occlusive Thrombus Formation in Rabbit Femoral Artery

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Objective—Plaque disruption does not always result in complete thrombotic occlusion. The mechanism of arterial thrombus propagation remains unclear.

Methods and Results—We studied how vascular wall thrombogenicity and blood flow reduction affect thrombus propagation using a rabbit model of single and repeated balloon injury. After balloon injury of the normal femoral artery, the blood flow was reduced to 50%, 25%, or 10% (n = 5). Small mural thrombi composed of aggregated platelets were produced, but no occlusive thrombi developed in any flow reduction. Three weeks after the first balloon injury, neointima with tissue factor expression and increased procoagulant activity was developed. Balloon injury of the neointima with the same blood flow reduction (n = 5) induced fibrin-rich thrombus formation. Additionally, injury with flow reduced to 25% and 10% promoted thrombus propagation resulting in vessel occlusion within 160 ± 18 and 71 ± 17 seconds, respectively. An injection of anti-von Willebrand factor (vWF) monoclonal antibody (AJW200; 1.0 mg/kg) prevented occlusive thrombus formation.

Conclusions—Increased vascular wall thrombogenicity together with a substantial blood flow reduction is crucial for occlusive thrombus formation, and vWF plays an important role in thrombus propagation. Reduced blood flow at plaque disruption sites might contribute to thrombus propagation leading to acute coronary syndromes. (Arterioscler Thromb Vasc Biol. 2004;24:2420-2424.)

Key Words: thrombus propagation ■ blood flow ■ von Willebrand factor ■ tissue factor

The rapid closure of coronary arteries caused by occlusive thrombi is the major cause of acute myocardial infarction. Disruption of coronary atherosclerotic plaques is recognized as one trigger of coronary thrombosis.1,2 However, this process does not always result in complete thrombotic occlusion with subsequent acute myocardial infarction. Because microscopic coronary thrombi are frequently detected during autopsies of noncardiac death,3,4 plaque disruption is considered a common complication and a high proportion of such events would be clinically silent.5 Therefore, whether thrombus on plaque disruption is occlusive or nonocclusive is critical to the onset of clinical events. Although the mechanisms of plaque rupture and thrombus formation in lesions have been intensively investigated, how arterial thrombi are propagated remains unclear.

The thrombotic response to plaque disruption is probably regulated by the thrombogenicity of exposed plaque constituents, local hemorheology, systemic thrombogenicity, and fibrinolytic activity.6 Many thrombotic factors are involved in acute thrombus formation. In particular, von Willebrand factor (vWF) binding to glycoprotein (platelet glycoprotein [GP]) Ibα and GP IIb-IIIa that plays an important role in platelet aggregation under conditions of rapid flow, might arise in atherosclerotic stenotic arteries.6–8 Tissue factor (TF) is a trigger of the extrinsic coagulation cascade. When simultaneously released from atheromatous plaques, TF potentially activates the coagulation cascade leading to the formation of fibrin9 that contributes to the stabilization of initial and loosely packed platelet aggregates and to thrombus propagation during high blood flow. We demonstrated that thrombi on injured neointima are fibrin-rich in a model of repeated balloon injury even when blood flow is high.10 However, these thrombi remained exclusively mural and did not become occlusive.

Blood flow is a key modulator of thrombus propagation. After plaque disruption, coronary blood flow is frequently...
impaired, and microembolism together with microvascular constriction are considered to be contributing factors. Subsequent events would elevate distal vascular resistance, resulting in reduced blood flow at sites of plaque disruption. However, the effect of reduced blood flow on thrombus propagation in diseased arteries remains obscure.

We therefore examined whether reduced distal blood flow contributes to thrombus propagation and to subsequent occlusive thrombus formation in diseased arteries. We also examined the role of vWF in this process using a rabbit model of repeated balloon injury.

Materials and Methods

Rabbit Model of Single and Repeated Balloon Injury of Femoral Artery

We used 55 male Japanese white rabbits (Kyudo Corp, Kumamoto, Japan) weighing 2.5 to 3.0 kg in research protocols that were approved by the Animal Care Committee of Miyazaki Medical College (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 Laboratory Animal Resources and published by the National Institutes of Health (NIH Publication 85-23, revised 1985). Aseptic surgery proceeded under general anesthesia induced by an intravenous injection of pentobarbital (25 mg/kg, body weight).

The rabbits were divided into single injury group (SI group) and repeated injury group (RI group). Blood flow rate (mL/min) in the femoral artery of each group was continuously measured by placing a Doppler flow probe over the femoral artery and with a transit time blood flowmeter (T106; Transonic Systems Inc). Data were analyzed using a digital recording system (PowerLab system; ADInstruments Pty Ltd). The sampling rate was 1000/sec. In SI group, a 2-French balloon catheter (Baxter Healthcare) was inserted via the anterior tibial arteries into the femoral artery and inflated at 1.4 atm, and then pulled down 2 times until 3 cm long. Immediately thereafter, the blood flow rate (mL/min) of the injured femoral artery was maintained or reduced to 50%, 25%, or 10% (n=5 each) by incomplete ligation of the distal femoral artery. The ligation was quantified by tightening a screw connected with the ligatures, and the blood flow was adjusted to each reduction level under continuous measurement (T106; Transonic Systems Inc). The RI group underwent repeated injury by inserting a 2.5-mm-diameter, 9-mm-long angioplasty balloon catheter (Boston Scientific Japan) via the carotid artery into the femoral artery. Briefly, the right common carotid artery was cannulated, and an angioplasty wire was fluoroscopically guided into the femoral artery. Thereafter, the angioplasty balloon was also fluoroscopically guided into the artery. The catheter was then inflated at 1.5 atm, and pulled back repeatedly 3 times until 5 cm.

Three weeks after initial injury, the femoral arteries were imaged by angiography and the luminal diameter was measured. The arteries were subsequently injured in the same areas with a 2-French balloon catheter using the same procedure as used for the SI group. Immediately after the second injury, the blood flow rate of the injured artery was reduced to the same levels as those of the SI group (n=5 each). The role of vWF in arterial thrombus formation was evaluated by injecting an intravenous bolus of human anti-vWF monoclonal antibody (AJW200; at doses of 1.0 mg/kg) or saline (as control) via the ear vein 30 minutes before the second balloon injury. This antibody reacts with the A1 domain of vWF in some species, including humans and rabbits. The antibody dosage used in this study significantly inhibited botrocetin-induced platelet aggregation over the next 24 hours, and did not affect collagen-induced platelet aggregation and systemic coagulation as described. Thirty minutes after the single or second injury, the rabbits were injected intravenously with heparin (500 U/kg) and euthanized 5 minutes later with an overdose of pentobarbital (60 mg/kg, intravenous) to evaluate thrombus formation. The animals were perfused with 50 mL of 0.01 mol/L phosphate-buffered saline, followed by perfusion fixation with 100 mL of 4% paraformaldehyde for immunohistochemical evaluation or with 4% neutralized formaldehyde and 1% glutaraldehyde in 0.1 mol/L phosphate buffer for electron microscopy. The femoral arteries were dissected out for subsequent studies.

Light Microscopy and Immunohistochemistry

The femoral arteries were fixed in 4% paraformaldehyde for 24 hours at 4°C and embedded in paraffin. Sections (3-μm-thick) were stained with hematoxylin and eosin/Victoria blue dye and immunohistochemically examined using antibodies against α-smooth muscle actin (HHF35; DAKO Japan), rabbit macrophages (RAM11; DAKO Japan), GP IbIIIa (Affinity Biologicals Inc), rabbit fibrin (a gift from Dr T. Kurokawa, Takeda Chemical Industries, Ltd), vWF (The Binding Site), and tissue factor (The Chemo-Sero-Therapeutic Research Institute).

Transmission Electron Microscopy

Small pieces of injured arteries were immersion-fixed with the same fixative for 12 hours at 4°C, post-fixed with 2% OsO4 in phosphate buffer for 90 minutes at room temperature, and embedded in Epon 812. Ultrathin sections were stained with uranyl acetate and lead citrate and examined using a JEOL 1200EX (Nihon Denshi, Japan).

Tissue Factor Activity of Femoral Artery

The intima and media of the femoral arteries without perfusion fixation were carefully separated from the adventitia. The activity of TF was measured and expressed in arbitrary units (AU) as described. The protein concentration was determined using the Bradford reagent (Bio-Rad Laboratories).

Statistical Analysis

Data are expressed as means±standard error. The unpaired Student t test or Fisher exact test evaluated differences between individual groups. P<0.05 was considered significant (n indicates the number of animals studied).

Results

Luminal Stenosis and Neointimal Formation in Femoral Arteries After Initial Injury of RI

Angiographs and microphotographs of rabbit femoral arteries obtained 3 weeks after the initial balloon injury revealed luminal stenosis and neointimal formation at injured (Figure IA and IC) sites and not at uninjured (Figure IA and IB) sites. The luminal diameters in the injured and uninjured arteries were 0.98±0.06 mm and 1.62±0.07 mm (n=5, P<0.01). Immunohistochemistry demonstrated that the neointima was composed...
Blood flow was also preserved without or with 50% reduction to 50%, 25%, and 10% (Figure 2A through 2D; preserved for at least 30 minutes after injury, even when injured femoral artery. Blood flow in the SI group was significantly increased in the injured neointima (1496 ± 180 AU/mg protein) and media than in normal intima and media reduction to 25% and 10% and disappeared within 160 seconds, respectively (Figure 2F and 2H). Repeated injury with 75% blood flow reduced to 75% promoted occlusive thrombus formation in rabbit femoral arteries. Either condition alone induced mural, but not occlusive, thrombi. In addition, anti-vWF monoclonal antibody prevented the formation of occlusive thrombi.

The main findings of this study were that a combination of increased vascular thrombogenicity and a reduction in blood flow of >75% promoted occlusive thrombus formation in rabbit femoral arteries. Either condition alone induced mural, but not occlusive, thrombi. In addition, anti-vWF monoclonal antibody prevented the formation of occlusive thrombi. Atherosclerotic plaque disruption is a key event in the pathogenesis of acute coronary syndromes that does not always result in complete thrombotic occlusion of coronary arteries. Although the prevalence of silent plaque disruption in the clinical population is unknown, studies of autopsies have detected coronary thrombosis in up to 8% of individuals with coronary atheroma who died of noncardiac causes. Therefore, whether thrombi remain mural or become occlusive is crucial to the onset of cardiovascular events. Groves et al. reported that mural thrombi produced by repeated balloon injury of the rabbit aorta rapidly became nonreactive to further thrombus propagation and disappeared from the surface within a few days after injury. Our previous studies also demonstrated that the repeated balloon injuries of rabbit aortas and femoral arteries induce fibrin-rich mural thrombi, but do not become occlusive. These results suggest that not only increased vascular thrombogenicity but also other factors are required to propagate arterial occlusive thrombi.

Blood flow is one of the most important modulators of thrombus formation and propagation. Plaque disruption initiates thrombus formation at the disrupted site and frequently causes a reduction in coronary blood flow. This mechanism is considered to be largely responsible for the rapid elevation of distal vascular resistance because of microvascular embolism and vasoconstriction. Autopsy studies have identified microemboli in 54% to 79% of patients who died of ischemic heart disease. In addition to aggregated platelets and fibrin, several potent vasoactive substances are released from disrupted atheromatous plaques that can cause microvascular embolism and constriction. Such events would subsequently reduce coronary blood flow at disrupted sites. Some animal studies have revealed that the rupture of atherosclerotic lesions induces rapid and obvious increases in distal vascular resistance caused by severe microvascular constriction, and that coronary microembolism induces a transient decrease of coronary blood flow. Bonderman et al. have emphasized the importance of circulating TF released from disrupted plaques in this process. A clinical study has found that 5% to 9% reduction in coronary blood flow at sites with unstable angina. Many clinical reports have indicated that in spontaneous plaque rupture and ulceration
and also during coronary intervention, induced microembolization leads to a reduction in coronary flow reserve. Therefore, our animal model partly simulates the onset of cardiovascular events after plaque disruption.

The coagulation system contributes to the pathogenesis of acute coronary syndromes. This system is readily activated when blood flow is reduced. We therefore assumed that thrombi produced by a single balloon injury with blood flow reduction would be fibrin-rich. However, we found that the thrombi on normal vessels consisted exclusively of aggregated platelets, suggesting that increased thrombogenicity of the vascular wall is more crucial for fibrin-rich thrombus formation than blood flow reduction.

Recent studies have discovered that vWF plays a crucial role in platelet aggregation under rapid flow conditions. We and others have also demonstrated the contribution of vWF to the interaction between platelets and fibrin and also fibrin-rich thrombus formation. The occlusive thrombi in this study were composed of a large amount of fibrin and platelets and were rich in vWF. The presence of vWF in the occlusive thrombi suggests that vWF plays an important role in this process. A bolus injection of a monoclonal antibody against vWF (AJW200) obviously inhibited occlusive thrombus formation. Although the result seems to be unusual because crucial roles of vWF have been demonstrated under high shear stress, recent studies suggest that vWF plays a significant role under low-shear or disturbed flow conditions. The present results also indicate that vWF plays a considerable role in fibrin-rich thrombus propagation, even when blood flow is reduced or disturbed.

Occlusive thrombi were rich in TF. Himber et al have revealed that active TF is localized in human thrombi, where it was associated with platelets, fibrin, monocytes, and polymorphonuclear leukocytes. Our results support this observation. Whether intravascular TF originate from leukocytes, platelets, or other cells remains controversial. Rauch et al reported the transfer of TF from monocytes and polymorphonuclear leukocytes to platelets. Conversely, more recent studies have indicated that active TF are located in circulating microvesicles and in activated platelets. Although our results do not address this issue, the increased concentration and activity of plasma TF induced by blood flow alteration seems to accelerate thrombus propagation.

In conclusion, our results suggest that a combination of increased vascular wall thrombogenicity and blood flow reduction is crucial for occlusive thrombus formation in arteries, and that vWF plays an important role in this process. Considerable reduction of coronary blood flow at plaque disruption sites may contribute to thrombus propagation and lead to acute coronary events.

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