Recombinant von Willebrand Factor Fragment AR545C Inhibits Platelet Aggregation and Enhances Thrombolysis With rtPA in a Rabbit Thrombosis Model

Osnat Gurevitz, Abraham Goldfarb, Hanoch Hod, Monica Feldman, Boris Shenkman, David Varon, Michael Eldar, Aida Inbal

Abstract—Platelet adhesion to exposed subendothelium is mediated by platelet receptor glycoprotein Ib and polymeric von Willebrand factor (vWF). To improve the results of coronary arterial thrombolysis, fragments of vWF with enhanced glycoprotein Ib binding competitive with native vWF have been proposed as adjuvants to recombinant tissue-type plasminogen activator (rtPA). We designed a recombinant vWF fragment spanning Ala444 to Asp730 that contains the Arg545Cys mutation (named AR545C) and analyzed its antiplatelet properties in vitro and in vivo. AR545C-platelet interaction was assessed by ristocetin or botrocetin-induced platelet agglutination, or interaction with extracellular matrix under arterial flow conditions. AR545C showed enhanced reactivity with platelet glycoprotein Ib at low concentrations of ristocetin, and 60% bound spontaneously to platelets. AR545C inhibited ristocetin-induced platelet agglutination in a dose-dependent manner, with a concentration necessary to inhibit 50% of agglutination of 0.16±0.04 μmol/L. The inhibitory effect of AR545C on rabbit botrocetin-induced platelet agglutination was also dose dependent, with a concentration necessary to inhibit 50% of agglutination of 0.3 to 0.5 μmol/L. AR545C also completely inhibited aggregate formation and decreased the adhesion of platelets to extracellular matrix by 62.5%. The effect of AR545C on thrombolysis with rtPA was evaluated using a modified rabbit femoral thrombosis model. Local injection of AR545C into the thrombosed segment of rabbit femoral artery significantly shortened the time to reperfusion with rtPA (60±17.3 versus 103±15.2 minutes, P=.05) and significantly prolonged the total patency time (175 versus 21 minutes, P=.04). No significant difference was found in the reperfusion rate or time to reocclusion. AR545C is a potential antithrombotic agent that enhances the thrombolytic effect of rtPA in the rabbit model. (Arterioscler Thromb Vasc Biol. 1998;18:200-207.)

Key Words: von Willebrand factor ■ thrombolysis ■ antiplatelet agents ■ antithrombotic agents

During the past several years, significant progress has been made in understanding the molecular aspects of platelet function with regard to both their role in normal hemostasis and in the development of pathological vascular occlusion. It is clear today that the first event in normal primary hemostasis or development of arterial thrombosis is the binding (adhesion) of platelets to the subendothelium at sites of vascular injury. The first step of platelet adhesion occurs by binding of vWF to the platelet receptor GPIb after it binds to components of exposed subendothelium. Thus, vWF acts as a “bridging” molecule between platelets and the vessel wall. As a consequence of vWF binding to GPIb, glycoprotein Ib/IIa is activated through a complex signaling pathway leading to platelet aggregation mediated by fibrinogen or, under conditions of high shear stress, by vWF itself. 

vWF is a polymeric glycoprotein that circulates in the plasma as a series of multimers with molecular masses ranging from 0.25 to 20×10^7 Da. In addition to its role in platelet adhesion, it carries and stabilizes factor VIII in the circulation.

Progress has been made in identifying specific regions of vWF that are important for its function. The binding sites for GPIb have been located within amino acids 449 to 728 of the A1 domain of vWF. Because the first event in thrombogenesis is the recognition of vessel wall–bound vWF by platelets through the GPIb receptor, it is apparent that the selective inhibition of binding of endogenous vWF to GPIb may be an appropriate early intervention likely to result in a beneficial antithrombotic effect. It was previously shown that the tryptic fragment of vWF of 52/48 kDa composed of residues Val449-Lys728 inhibits the binding of native vWF to GPIb. Moreover, the GPIb-binding domain of vWF expressed in Escherichia coli has been shown to inhibit the binding of native vWF to GPIb, and unlike native vWF, this vWF domain binds to GPIb in the absence of any modulator.

These previous studies provided the rationale for the use of a recombinant vWF fragment as an antiplatelet/antithrombotic agent. Indeed, several fragments of vWF expressed in E coli containing the A1 domain have recently been shown to bind...
to the platelet membrane GPIb receptor and to inhibit the interaction of vWF with platelets.3,13,15 Experimental studies with VCL (Bio-Technology General, Inc.), another recombinant fragment of vWF that spans from leucine 504 to serine 708, showed delayed thrombus formation and reocclusion in dog coronary arteries.14 This fragment was also shown to have local antithrombotic effects on nitrogen laser–induced thrombus formation in guinea pig mesenteric arteries without compromising general hemostasis.15,16

Thrombolytic therapy has clearly revolutionized the outcome of patients sustaining acute myocardial infarction.16,17 Thrombolytic therapy reduces infarct size and improves survival of patients who have suffered a myocardial infarction.18,19 However, the benefit is limited by incomplete reperfusion, delayed recanalization time, and occurrence of thrombotic reocclusion in up to 15% of cases.20,21 Importantly, thrombolytic therapy is also accompanied by increased platelet activation by the direct action of plasmin on platelets, by the elaboration of thrombin through the action of plasmin, and by the exposure of subendothelial collagen after lysis of the thrombus.22 The prognosis of patients after acute myocardial infarction is related to early and sustained reperfusion of the infarct-related artery.23–27 During the last few years, large clinical trials have been conducted in an effort to find a combination of drugs that will achieve more complete and more early reperfusion with a lower rate of reocclusion.21,26–32

We have produced a recombinant fragment of vWF that encompasses alanine 444 to asparagine 730 and also contains the Arg545Cys mutation (hereafter termed AR545C). Arg545Cys, one of the most common type 2B von Willebrand disease mutations, results in an increased binding to platelet GPIb and also in a significant spontaneous binding of the mutant vWF to GPIb.33 We assumed that blocking the initial interaction between native vWF and platelet GPIb by AR545C vWF fragment would prevent any further process of platelet activation and would result in an antithrombotic effect. We report herein the platelet AR545C interaction and the effect of AR545C as an adjuvant on thrombolyisis with rtPA in a rabbit model.

Methods

Plasmid Constructs

Plasmid pSVH-WF1 contains a full-length cDNA for human vWF cloned into the expression vector pSV7D, as previously described.33 Plasmid pSVR545C was derived from the pSVH-WF1 by introducing a C to T transition at nucleotide 3922, which causes an arginine to cysteine substitution at amino acid residue 545 as previously reported from our laboratory.33 The plasmid pSVR545C underwent additional polymerase chain reaction–directed mutagenesis to produce a truncated cDNA vWF consisting of the following: (1) 230 nucleotides representing the 5′ untranslated region; (2) 75 nucleotides, starting with the methionine codon and followed by coding sequence for the remainder of the 22-residue vWF signal peptide; (3) a coding sequence for three amino acid residues from the amino terminus of the vWF propeptide; (4) 858 nucleotides coding for Ab 444–Asp 730 of the native vWF; and (5) translation termination codon and poly A tail at 3′. Two oligonucleotides that added an EcoRI restriction site at the very 5′ and 3′ ends of the construct were used for polymerase chain reaction.34 The amplified fragment and the wild-type plasmid pSVH vWF1 underwent EcoRI digestion followed by cloning within the EcoRI-digested expression vector pZEM229 (kindly provided by Dominic W. Chung, Department of Biochemistry, Seattle, Wash.). Thus, the recombinant-mutated plasmid pSVAR545C contains a fragment of vWF (AR545C), which comprises domains D3, A1, and part of A2. The DNA sequence of the mutated fragment was verified by sequencing.

Cell Culture and Expression of pSVARS545C and pSVH vWF1

A thymidine kinase–deficient BHK cell line, BHK-570 (ATCC No. CRL 10314, kindly provided by Dominic W. Chung), was used as the host cell for the transfection experiments.33 Cells were grown in Dulbecco’s modified Eagle’s medium with 5% fetal calf serum, 50 μg/mL of penicillin, 50 μg/mL of streptomycin, and 100 μg/mL of neomycin (Beit Ha’emek, Israel) in a 5% CO2 atmosphere at 37°C. For transfection, BHK-570 cells were plated overnight at 1:15 split ratios in 90-mm plates (Falcon) and transfected for 4 hours in 10 mL of medium with 30 μg of plasmid precipitated with calcium phosphate. After a 1-minute shock in 15% glycerol in Tris-buffered-saline (25 mmol/L of Tris-HCl, pH 7.4, 0.14 mol/L of NaCl, 5 mmol/L of KCl, 0.7 mmol/L of CaCl2, 0.5 mmol/L of MgCl2, and 0.6 mmol/L of Na2HPO4), the cells were grown for 24 hours in normal medium. The cells were then subjected to selective medium containing 1 μmol/L of methotrexate (Abic, Israel). The methotrexate concentration was increased gradually to 20 μmol/L and the clones were picked and propagated. At confluence, the cells were washed twice in PBS and cultured in 8 mL of serum-free Dulbecco’s modified Eagle’s medium per T-75 culture flask. The medium was collected after 24 hours, and EDTA, PMSF, leupeptin, and pepstatin were added to a final concentration of 100 μmol/L, 10 μg/mL, 1 μg/mL, and 1 μg/mL, respectively. Conditioned medium was concentrated by ultrafiltration, (Amicon Inc). Because the heparin binding site remained within the AR545C vWF fragment, for in vivo experiments the media was further purified on heparin affinity chromatography (Econo-PAC Heparin cartridge, BioRad). The purity of the vWF fragment was verified by gel electrophoresis. The amount of vWF in conditioned medium was quantified by a sandwich ELISA using 1:100 rabbit anti-human vWF (Dakopatts A082) as the coating antibody and 1:1000 peroxidase-conjugated anti-vWF antibody (Dakopatts P226) as the detecting antibody. The standard was a human pool of platelet-poor plasma (30 volunteers), which was assumed to contain ~10 μg/mL of vWF. ELISAs were developed with o-phenylenediamine as the colorimetric substrate and quantified at A490 on an ELISA reader (Molecular Devices).

By Western blot analysis, the AR545C fragment was subjected to 8% SDS-PAGE under nonreducing or reducing conditions (65 mmol/L of DTT, 55°C, for 20 minutes). After transfer onto nitrocellulose, the membrane was incubated with peroxidase-conjugated anti-vWF antibody (Dakopatts P226) and visualized by an ECL kit (Amersham). In some of the experiments, the membrane was first incubated with 1 μg/mL of monoclonal antibody 36 C4 followed by incubation with peroxidase-labeled anti-mouse antibody (Amersham). The 36 C4 antibody (a gift from Dr Claudeine Mazurier, Lille, France) recognizes the A1 domain of nonreduced vWF.

The purity of AR545C was verified by reverse-phase-high-performance liquid chromatography on a Vydac C8 column using a gradient of 4% to 90% acetonitrile in 0.1% trifluoroacetic acid. The N-terminal sequence was performed on a peptide sequencer ABI 494 (Applied Biosystem Division, Perkin Elmer) at the Protein Research Center, Technion, Haifa, Israel. The presence of a full range of multimers of

**Selected Abbreviations and Acronyms**

AR545C = Arg 545Cys mutation
CAP = cone and plate
ECM = extracellular matrix
GPIb = glycoprotein Ib
rtPA = recombinant tissue-type plasminogen activator
TBS = Tris phosphate-buffered saline
vWF = von Willebrand factor
the wild-type vWF was confirmed by SDS agarose multimer gel electrophoresis performed as previously described.53

**vWF-Platelet Binding**

Ristocetin-induced binding was performed as previously described.53 AR545C or wild-type vWF from conditioned medium at a concentration of 1 μg/mL was incubated with formalin-fixed platelets (8×10⁶/mL) in TBS (50 mmol/L of Tris, pH 7.35, 150 mmol/L of NaCl) containing 3% BSA and increasing concentrations of ristocetin (from 0 to 1.2 mg/mL) for 30 minutes at room temperature. The samples were then centrifuged for 5 minutes at 12,500 g. Quantification of the unbound vWF that remained in the supernatant was performed by ELISA as in the previous section.

**Ristocetin-Induced Platelet Agglutination**

Ristocetin-induced platelet agglutination was performed using lyophilized formalin-fixed platelets (Bio Data) as described previously with slight modifications.8 Various concentrations of AR545C were incubated with the platelets (2×10⁹ platelets/mL) for 15 minutes in a platelet aggregometer PACKS-4 (Helena Laboratories) at 37°C before the addition of 25 μL of platelet-poor plasma as a source of vWF and 1.5 mg/mL of ristocetin (Sigma Chemical Co), and the percentage of agglutination was recorded.

**Platelet Interaction With ECM in the CPA Device**

Platelet adhesion and aggregation on ECM was tested as described recently.39 In brief, 0.25 mL of citrated whole blood was placed on an ECM-covered plate and subjected to arterial flow conditions (shear rate of 1300 seconds⁻¹) for 2 minutes, applying a CAP device specifically designed for this test.39 The sample was then washed and stained with May-Grunwald. The degree of adhesion was assessed by calculating the percentage of total area covered by platelets and expressed as a percentage of surface coverage. The extent of aggregation was estimated by measuring the average size of ECM-bound objects, expressed as the average size of the objects. Platelet adhesion and aggregation were determined using an image analysis system (Galai, Beit Ha‘emek, Israel). To evaluate the effect of AR545C on the parameters described, the blood samples were preincubated at room temperature for 15 minutes with various concentrations of the AR545C, and the extent of adhesion and aggregation was recorded. The normal values were defined as SC = 19.4%±5.9% and AS = 47.5%±15.2 μm², values that were determined based on results of testing 100 normol volunteers.76

**Botrocetin-Induced Agglutination of Rabbit Platelets**

Because rabbit plasma vWF does not respond to ristocetin,77 the rabbit vWF-platelet interaction was analyzed in the presence of botrocetin. Rabbit platelet-rich plasma was obtained after centrifugation of citrated whole blood (one part 3.2% trisodium citrate, eight parts blood) at 100g. The inhibitory effect of AR545C on rabbit platelet aggregation induced by botrocetin (1.0 μg/mL) was evaluated in an aggregometer (Helena Laboratories) as in the ristocetin-induced agglutination assay. Various concentrations of AR545C (or the appropriate volume of buffer in control mixtures) were added to platelet-rich plasma containing 5 mmol/L of EDTA at 37°C with stirring followed by botrocetin as described previously,77 and the percentage of agglutination was then recorded. Two-chain botrocetin was a gift from Dr. Y. Fujimura (Nara Medical College, Nara, Japan) and Dr. K. Titi (Fujita Health University, Toyoake, Japan).

**Animal Model of the Rabbit Femoral Artery Thrombosis**

All of the animal model procedures used in this study conformed to the position of the American Heart Association on “Research Animal Use,” adopted November 1984, and were approved by the Institutional Animal Care and Use Committee at the Neufeld Cardiac Research Institute, Sackler Faculty of Medicine, Tel-Aviv University, Tel-Aviv, Israel. We used a slightly modified model of Gold et al.59 Eighteen New Zealand White female rabbits, each weighing 3.0 to 4.0 kg, were anesthetized with intravenous sodium pentobarbital (Nembutal, 30 mg/kg of body weight) followed by 10 mg at 30- to 60-minute intervals, by means of the marginal ear vein. The auricular artery was cannulated for blood drawing later. The right femoral artery and vein were exposed, the side branches were ligated, and the right superficial epigastric artery was cannulated with a 24-gauge cannula for local thrombus induction and local drug administration. An electromagnetic flowmeter probe (Nihon Kohden, Inc) was positioned distally for arterial blood flow monitoring throughout the experiment. A stepwise stenosis was produced between the superficial epigastric artery and the flowmeter probe by constriciting the artery with a 6-0 silk suture to achieve a 50% reduction of baseline flow (called the stenotic flow). A 1-cm segment of the femoral artery was clamped distally and proximally to the superficial epigastric artery insertion, and the isolated segment was emptied by the side branch cannula. The isolated segment was traumatized by three compressions with blunt forceps to produce endothelial injury. Fifty units of bovine thrombin (Thrombinar R, Armour Pharmaceuticals Co) was mixed with 0.1 mL of freshly drawn blood from the cannulated auricular artery and injected into the isolated femoral artery segment through the 24-gauge cannula. Fifteen minutes later, the proximal and then the distal clamps were released. The absence of blood flow was monitored with the flow probe for 10 minutes after release of the clamps to verify a stable occlusion. Immediately after a stable occlusion was confirmed, the thrombosed segment was clamped again, and 0.1 mL of either 1 μg/μL of AR545C (experimental group, n=9) or mock transfection media (control group, n=9) was injected through the cannula in the superficial epigastric artery. Ten minutes later, the clamps were again removed, and four boluses of intravenous rtPA (Actilyse, Boehringer Ingelheim), 450 μg/kg each, were injected through the marginal ear vein every 15 minutes. Femoral flow was followed continuously for 120 minutes, beginning with the first injection of rtPA. At the end of the experiments, the animals were sacrificed by an overdose of pentobarbital.

**Definitions**

Reocclusion was defined as blood flow return of 50% or more of the stenotic value. Time to reperfusion was defined as the time interval between the first bolus of rtPA and occurrence of recanalization. Reocclusion was defined as flow deceleration to less than 15% of the stenotic flow after recanalization of the artery and persisting for at least 30 minutes or until the end of the experiment. Time to reocclusion was defined as the time from the beginning of reperfusion to the appearance of first reocclusion. Total patency time was defined as the sum of the patency time for each rabbit during the experiment.

**Statistical Analysis**

Statistical analysis was performed using BMDP Statistical Software (1990), edited by W.J. Dixon, University of California Press. Reperfusion rate was analyzed using Fisher’s exact response test. Time to reperfusion and time to reocclusion were analyzed using product-limit survival analysis with generalized Wilcoxon (Breslow) test statistics. Total patency time was compared using a nonparametric Kruskal-Wallis one-way ANOVA test using a X² test distribution with one degree of freedom. Probability values are two-tailed, and values of P<0.05 are considered significant.

**Results**

**Characterization of the Recombinant AR545C vWF Fragment**

RP-HPLC analysis of AR545C showed two peaks with retention time of 40.7 and 45.5 minutes, respectively. SDS-PAGE analysis of the peaks showed that the peak with the retention time of 40.7 minutes consists of a single band of ~48 kDa, and the peak with a retention time of 45.5 minutes consists of a single band of ~96 kDa. SDS-PAGE and Western
Figure 1. SDS-PAGE of the AR545C fragment. Concentrated recombinant AR545C from conditioned media was electrophoresed after reduction of disulfide bonds (lane 1) or in the absence of reducing agent on 8% SDS-PAGE (lanes 2 and 3). Fragments were transferred onto nitrocellulose, probed with peroxidase-conjugated anti-vWF antibody (lane 2) or monoclonal 36C4 antibody (lane 3), and visualized using the chemiluminescent substrate of the ECL kit as described in the “Methods” section. Lane HS represents percentage of SDS-PAGE gel stained with Coomassie blue of the nonreduced AR545C after heparin-Sepharose purification. Right, molecular masses are of prestained SDS-PAGE high-range standards (BioRad Laboratories).

In Vitro Studies

The platelet-AR545C interaction was studied using ristocetin- or botrocetin-induced binding or agglutination assays. AR545C had no effect on ADP or collagen-induced aggregation.

Binding of AR545C to Platelets

Direct GPIb binding to wild-type vWF or R545C was analyzed in the absence or presence of ristocetin as previously described. As shown in Fig 2, in the absence of ristocetin, 60% of the AR545C bound to platelets (spontaneous binding), and the binding increased to almost 80% at a ristocetin concentration of 0.8 mg/mL. This result is in contrast to those observed in the absence or presence of lower concentrations of ristocetin, which showed absent or very little binding of wild-type vWF to platelets, respectively (Fig 2).

Effect of AR545C on Ristocetin-Induced Human vWF-Platelet Agglutination

Preincubation of human platelets with AR545C resulted in inhibition of vWF-platelet agglutination in the presence of ristocetin as shown in Fig 3. AR545C inhibited ristocetin-induced platelet agglutination in a dose-dependent manner with a concentration necessary to inhibit 50% of agglutination of 0.16±0.04 μmol/L. The agglutination was completely abolished at 3 μmol/L of AR545C.

Effect of AR545C on Platelet Interaction With ECM

The normal whole-blood sample tested in the CAP system exhibited a typical adhesion and aggregation pattern with a surface coverage of 21.3% and an average size of 40.5 μm² (Fig 5A and 5B). The normal blood sample was then preincubated for 15 minutes at room temperature with various concentrations of the AR545C fragment. A representative picture is shown in Fig 5C and 5D. Preincubation of the normal sample with 0.2 μmol/L of AR545C for 15 minutes at room temperature resulted in complete inhibition of aggregate formation (average size of 21.8 μm²) and 62.5% decrease in adhesion (surface coverage of 8%).

In Vivo Studies

The effect of AR545C on thrombolysis with rtPA was studied in 18 rabbits using the modified thrombosis model of Gold et al. as described in the “Methods” section. Reperfusion was obtained in six of the nine experimental group animals, but only in two of the nine control group animals. However, the difference in the reperfusion rate between the two groups was not statistically significant (P=.15) (Table 1). Reperfusion occurred significantly earlier in the experimental than in the control group: 60.6±17.3 minutes versus 103.0±15.2 minutes, respectively (P=.05) (Table 1). In all but one animal (animal 2 in the experimental group), reperfusion was followed by reocclusion (Fig 6). The time to reocclusion was not
significantly different between the two groups: 27.7±11.9 minutes in the experimental group and 10.5±5.5 minutes in the control group (P=.62) (Table 1). Two animals in the experimental group reperfused for a second time (animals 4 and 6), whereas none from the control group showed second reperfusion (Fig 6). The total patency time was significantly prolonged in the experimental group: 175 minutes compared with 21 minutes in the control group (P=.04) (Table 1, Fig 6).

Discussion

Several agents were shown to block the interaction between vWF and platelet receptor GPIb. Some of these compounds, such as monoclonal antibodies, synthetic peptides, and recombinant vWF fragments, have been tested in various models of experimental thrombosis in animals. Aurintricarboxylic acid binds to the GPIb binding domain of vWF and acts as a competitive inhibitor of shear-induced platelet aggregation.40 Monoclonal antibodies to GPIb and to GPIb binding sites of vWF have been shown to inhibit thrombus formation in an experimental thrombosis procedure using a dye-pulsed laser in mesenteric arteries of guinea pigs.41 Synthetic peptides of vWF corresponding to GPIb binding domain (spanning from amino acids 474 to 488 and 692 to 708) inhibited thrombus formation in the same model.42 The recombinant T116 fragment (amino

Figure 3. Dose-dependent inhibition of human vWF-platelet agglutination with the AR543C. Fixed platelets (2×10⁶/mL) were incubated with AR545C for 15 minutes at the concentrations shown followed by addition of normal pool plasma and 1.5 mg/mL of ristocetin. The concentration necessary to inhibit 50% of agglutination (mean±SE) was determined from three independent experiments.

Figure 4. Effect of AR545C on rabbit vWF-platelet agglutination induced by botrocetin. Rabbit platelet-rich plasma (2×10⁶ platelets/mL) containing 5 mmol/L EDTA was stirred in an aggregometer at 37°C and mixed with AR545C at the indicated concentrations followed by 1 μg/mL of botrocetin.
acids 449 to 728) has also been shown to be effective in the carotid artery stenosis model in pigs and monkeys. Similarly, Yao et al. have demonstrated that VCL (amino acids 504 to 728) delayed thrombogenesis and reocclusion and enhanced thrombolysis with tPA in a canine model of coronary artery thrombosis.

In the present study, we evaluated the antithrombotic properties of recombinant vWF fragment AR545C spanning Ala444 to Asp730, which contains the Arg545Cys mutation that was previously shown by us to result in spontaneous binding of mutant vWF to platelets. This point mutation is within the GPIb binding site, suggesting that the affinity for GPIb can be modulated by changes in this region. Indeed, Matsushita and Sadler recently performed charged-to-alanine mutagenesis of the vWF A1 domain to examine the roles of specific charged residues in the interaction of vWF with platelet GPIb. By this approach, alanine substitutions at Arg545 and the segments between Glu497-Arg511 and Arg687-Glu689 resulted in spontaneous binding of vWF to GPIb. The striking distribution of distinct positively and negatively charged regions of the A1 domain suggests that intramolecular electrostatic interactions among these sites play a major role in the regulation of vWF binding to GPIb. The antiplatelet/antithrombotic effect of the AR545C was evaluated in two systems, in vitro and in vivo. Recombinant AR545C, unlike native multimeric vWF, showed enhanced reactivity with platelet GPIb at low concentrations of ristocetin; and 60% of the fragment bound spontaneously to platelets (Fig. 2). Moreover, AR545C inhibited ristocetin-induced vWF-platelet agglutination in a dose-dependent way with a concentration necessary to inhibit 50% of agglutination of 0.16 ± 0.04 μmol/L. Similarly, AR545C inhibited botrocetin-induced agglutination of rabbit platelets. In addition, AR545C completely inhibited platelet aggregation and significantly decreased adhesion on ECM in the CAP model. The effect of AR545C on platelet-ECM interaction resembles the results obtained with plasma from patients with severe von Willebrand disease. Our findings are in agreement with those reported previously by other investigators who, using other recombinant vWF fragments, demonstrated that they interact directly with GPIb in the absence of any exogenous modulator and also that the binding of native vWF to platelets, whether mediated by ristocetin or botrocetin, has been inhibited.

### Measured Parameters in the Experimental and Control Groups

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<th>Parameter</th>
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<th>Control</th>
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<td>2/9 (22.2%)</td>
<td>.15</td>
</tr>
<tr>
<td>Mean Time to Reperfusion, Minutes</td>
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<td>.05</td>
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<tr>
<td>Mean Time to Reocclusion, Minutes</td>
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<td>10.5 ± 5.5</td>
<td>.62</td>
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<tr>
<td>Total Patency Time, Minutes</td>
<td>175</td>
<td>21</td>
<td>.04</td>
</tr>
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*Values are expressed as mean ± SE.
Consistent with our results that showed an inhibitory effect of AR545C on platelet-ECM interaction, Sixma et al.\(^\text{15}\) recently reported that another vWF fragment, VCL, inhibits platelet adhesion to ECM.

In the present study, the results of in vitro experiments showed that AR545C blocks the binding of the native vWF molecule to GP Ib, resulting in inhibition of platelet aggregation. Consequently, the AR545C was tested as an adjuvant antithrombotic agent in the animal femoral thrombosis model of Gold et al.\(^\text{39}\) The AR545C fragment injected into a thrombosed segment of the rabbit femoral artery significantly decreased the time interval to first reperfusion and increased the total patency time. It is tempting to assume that blocking the activity of von Willebrand factor corresponding to the platelet glycoprotein Ib, collagen and heparin binding domains. Biochem Biophy Res Commun. 1989;164:1339–1347.


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38. Deleted in proof.


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