Blockade of Platelet Membrane Glycoprotein Ib Receptors Delays Intracoronary Thrombogenesis, Enhances Thrombolysis, and Delays Coronary Artery Reocclusion in Dogs

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Abstract Von Willebrand factor and platelet membrane glycoprotein Ib receptors interact to mediate platelet adhesion and thrombogenesis in stenosed and endothelium-injured arteries. We wished to determine whether blocking glycoprotein Ib receptors with a recombinant von Willebrand factor binding domain (VCL) increases the time required for thrombus formation after injury to the coronary arteries. We also wished to determine whether, after thrombolysis with tissue plasminogen activator (TPA), VCL delays or protects against coronary artery reocclusion. Twenty-seven dogs were treated with either saline, VCL, or aspirin before thrombosis was induced in their coronary arteries by electrical injury. The time from injury to the formation of occlusive thrombi was significantly greater with VCL (70±10 minutes) and aspirin (69±20 minutes) than with saline (18±3 minutes, P<.001 and P<.05). Thrombosis was induced in 30 other dogs that then received thrombolytic treatment in four groups. Our major finding was that coronary artery reocclusion occurred in 72±11 minutes after treatment with TPA (80 μg/kg+8 μg·kg⁻¹·min⁻¹) and heparin (200 U/kg) (n=7); in 142±24 minutes after TPA, heparin, and VCL (4 mg/kg+2 mg·kg⁻¹·h⁻¹) (n=7) (compared with TPA and heparin, P<.05); in 74±13 minutes after TPA, heparin, and aspirin (5 mg/kg) (n=8); and in 173±8 minutes after TPA, heparin, VCL, and aspirin (n=8) (compared with TPA and heparin, P<.001). Thus, VCL increases the length of time required for thrombus formation in coronary arteries, and, when given with TPA and heparin, delays coronary artery reocclusion more effectively than aspirin. (Circulation. 1994;89:2822-2828.)

Key Words • platelets • tissue plasminogen activator • von Willebrand factor

Studies have revealed that most acute transmural (Q-wave) myocardial infarctions are caused by thrombus formation in atherosclerotic coronary arteries.1-4 An interruption of the protective endothelium covering the vessel wall results in platelet adhesion and aggregation and thrombosis, which blocks the blood supply to the myocardium.5-8 Thrombolytic agents, such as tissue plasminogen activator (TPA) and streptokinase, have been effective in the treatment of thrombosis in patients with acute transmural myocardial infarction (Q-wave infarcts).9,10 but 15% to 20% of patients do not achieve reperfusion. In addition, reocclusion of coronary arteries has limited the efficacy of thrombolytic therapy in some patients.12,13

In both thrombosis and reocclusion of coronary arteries, platelet adhesion is the initiating event. In platelet adhesion, von Willebrand factor, a multivalent protein, forms a bridge between the subendothelial extracellular matrix and glycoprotein Ib on the surface of circulating platelets.14-16 The adhesion of platelets to subendothelium results in activation of platelets and the release of ADP, thromboxane, and serotonin. These in turn activate additional platelets.17-20 Ultimately, a platelet-initiated thrombus forms. Therefore, preventing platelet adhesion may be useful in preventing thrombosis and reocclusion in coronary arteries.21 Several fragments isolated from von Willebrand factor have been shown to bind to the platelet membrane glycoprotein Ib receptor and inhibit the interaction of von Willebrand factor with glycoprotein Ib receptors. This inhibition has led to the inhibition of platelet adhesion and aggregation.22,23 VCL (Bio-Technology General Inc) is a recombinant fragment of von Willebrand factor, Lε60-Lys72, with a single intrachain disulfide bond that links residues Cys69 and Cys69 (Reference 23). We used a canine model to determine the effect of VCL on (1) the formation of intracoronary thrombosis and (2) reocclusion of coronary arteries after thrombolysis with TPA. Our results suggest that VCL delays thrombus formation and reocclusion of coronary arteries.

Methods

All procedures used in this study were conducted according to the principles of the American Physiological Society and were approved by the Institutional Animal Care and Use Committee at the Texas Heart Institute, Houston, Tex.

Surgical Preparation

Mongrel dogs (n=63) weighing 25 to 35 kg were anesthetized with sodium pentobarbital (30 mg/kg IV), intubated, and

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connected to mechanical respirators (Harvard model 60). Plastic catheters were placed in a carotid artery for monitoring aortic pressures and in a jugular and a peripheral vein for drug and fluid administration. A left fifth intercostal space thoracotomy was performed, and the heart was suspended in a pericardial cradle. A 1- to 2-cm segment of left anterior descending coronary artery was carefully exposed and nearby branches ligated. An ultrasonic Doppler flow probe (Hartley Instruments) was placed around the proximal portion of the exposed left anterior descending coronary artery to measure the velocity of blood flow. Baseline hemodynamics, including heart rate, systolic and diastolic aortic pressures, and phasic and mean coronary blood flow velocities were recorded on an eight-channel recorder (Gould, model 3000). A needle electrode (the 8-mm tip of a 25-gauge needle crimped on the end of a 10-cm length of 30-gauge Teflon-insulated silver wire) was inserted obliquely approximately 4 mm into the lumen of the exposed left anterior descending coronary artery at a site distal to the Doppler flow probe. The needle was stabilized on the vessel with 6-0 silk suture. To prevent the electric current from injuring the surrounding tissue, heat-shrink tubing was applied to the needle/wire and soldered connection. A ground wire was connected to the subcutaneous tissue to complete the electrical circuit. To induce thrombosis, a current of 150 μA was applied through the electrode, which was connected in series with the positive terminal of a 9-V battery, a 50-kΩ potentiometer, a multimeter, and the ground wire. Thrombus formation was determined by the reduction of coronary blood flow velocity, which was monitored by the externally positioned Doppler flow probe. The electric current was maintained until 30 minutes after persistent thrombotic occlusion had occurred.

**Experimental Procedures**

Two separate protocols were used in this study of VCL; one examined its effect on coronary arterial thrombus formation (protocol 1), the other its effect on thrombolysis and reocclusion (protocol 2).

**Protocol 1**

To evaluate the effect of VCL on intracoronary thrombus formation, treatment was initiated on the dogs 30 minutes before electrical stimulation of their coronary arteries. Three different groups of animals were studied (Fig 1A). In the control group (1, n=12), saline was given intravenously at 1 mL/min. In one experimental group (group 2, n=7), VCL was given intravenously at 4 mg/kg body wt as a bolus dose and at 2 mg/(kg·h) as a continuous infusion until the end of study. This dose of VCL is enough to inhibit more than 50% of platelet aggregation induced by botrocatin and asialo-von Willebrand factor. In the other experimental group (group 3, n=8), aspirin was given intravenously at 5 mg/kg body wt as a bolus dose. The change in coronary blood flow before and after electrical stimulation was carefully monitored. The amount of time elapsed from the beginning of electrical stimulation to the total occlusion of the coronary artery was recorded. Three hours after the total occlusion of coronary arteries, all animals were given TPA (Genentech, Inc) intravenously at 80 μg/kg body wt as a bolus dose and at 8 μg/(kg·min) as a continuous infusion for 90 minutes. This treatment was intended to induce lysis of the thrombi formed in the coronary arteries.

A thrombus was considered to be lysed (and the artery reperfused) when the flow velocity of the coronary artery returned to at least 70% of the value that existed before thrombus formation. The amount of time elapsed from TPA administration to reperfusion was recorded as thrombolysis or reperfusion time. Dogs in whom reperfusion had not occurred after 90 minutes of TPA infusion were excluded from further study. Dogs in whom reperfusion occurred were further monitored until the coronary arteries reoccluded or 180 minutes had elapsed without reocclusion. The time from reperfusion to reocclusion was recorded as the reocclusion time. Dogs in whom coronary arteries had not reocluded after 180 minutes of reperfusion were considered not to have reoccurred. Dogs in whom reocclusion occurred were monitored for 30 minutes to verify persistent reocclusion.

**Protocol 2**

To further explore the effect of VCL on thrombolysis and reocclusion, additional animals were studied and treated 3 hours after the occlusion of coronary arteries (Fig 1B). These animals were assigned to one of four additional groups: TPA and heparin (group 4, n=7); TPA, heparin, and VCL (group 5, n=7); TPA, heparin, and aspirin (group 6, n=8); and TPA, heparin, VCL, and aspirin (group 7, n=8). Heparin was given at 200 U/kg as an intravenous bolus. TPA, VCL, and aspirin were given at the same dose as in protocol 1. The follow-up for thrombolysis and reocclusion was also the same as in protocol 1.

**Pharmacokinetic Studies**

The pharmacokinetics of VCL were studied in 6 dogs: 3 received VCL at 4 mg/kg as an intravenous bolus; the other 3 received VCL as described for group 5 dogs. Blood samples were...
collected from an aortic catheter at 10, 20, 30, 40, 50, 60, 75, 90, 105, 120, 150, and 180 minutes after the administration of VCL. Sodium citrate at 3.8% was used as anticoagulant (9 vol blood to 1 vol sodium citrate). Plasma was obtained by centrifuging blood samples at 3000g for 15 minutes; it was stored at -20°C. An enzyme-linked immunosorbent assay was used to detect VCL in the plasma (Biotechnology General Ltd).

**Hematocrit, Coagulation, and Platelet Aggregation Studies**

Hematocrit was determined before and at the end of TPA administration in all dogs in protocol 2. Activated whole-blood clotting time was measured in these dogs before and 5, 60, 120, and 180 minutes after the administration of TPA on an automated blood coagulation timing device (HemoTec 2001370). Hemoglobin and platelet count were measured in 3 dogs before and after the treatments with TPA, heparin, and VCL.

Ex vivo platelet aggregation was analyzed before and 10 minutes after the administration of VCL and aspirin in dogs in protocol 1. Blood samples were collected in plastic tubes containing a 3.8% solution of sodium citrate (9 vol blood to 1 vol sodium citrate). Platelet-rich plasma was obtained by centrifuging blood samples at 200g for 20 minutes, and platelet-poor plasma was obtained by centrifuging the residual blood at 3000g for 10 minutes. The platelet count in platelet-rich plasma was adjusted to 300,000/mm³. A four-channel platelet aggregometer (Bio-Data, model PAP-4) was used for the assay. Agonists and their final concentrations were ADP (Sigma) at 5, 10, and 20 μmol/L; botrocetin (purified from Bothrops jararaca venom [Sigma] at Bio-Technology, Rehovot, Israel)²⁴ at 1.1, 2.2, and 4.4 μg/mL; and arachidonic acid (Sigma) at 12.5, 25, and 50 μg/mL. Botrocetin induces platelet aggregation by inducing von Willebrand factor to bind to the membrane glycoprotein Ib, an action resembling that of ristocetin.²⁴,²⁵ Because canine platelets do not aggregate in response to arachidonic acid, epinephrine was added to the platelet suspension at 10 μmol/L before arachidonic acid. The degree of platelet aggregation was reported as the maximal percentage that light transmission increased in platelet-rich plasma over light transmission in platelet-poor plasma.

**Statistical Analyses**

All values are expressed as mean±SEM. Fisher's exact test was used to compare the frequency of reperfusion and reocclusion in different groups of animals. A one-way ANOVA with repeated measurements was used to compare the hemodynamic values and activated clotting times obtained at different time periods and the duration of time to reperfusion and reocclusion in different groups of animals. The Student's t test was used to compare the percentage of platelet aggregation and hematocrit values before and after treatment in each group of animals. A value of P<.05 was considered significant.

**Results**

**Intracoronary Thrombus Formation**

Insertion of the electrode needle into the coronary artery caused some stenosis in the arteries of all animals, as determined by a reduction of blood flow velocity to approximately 65% of the baseline level. After electrical stimulation, all animals developed total occlusion of the affected coronary arteries. In protocol 1, the elapsed time from electrical stimulation to total occlusion of the coronary arteries was significantly longer in dogs treated with VCL (P<.001) and aspirin (P<.05) than in dogs treated with saline (Fig 2). In all animals, aortic blood pressures and heart rates changed slightly after the occlusion of coronary arteries.

**Thrombolysis**

In protocol 1, 3 hours after the occlusion of coronary arteries, only TPA was given to the animals. The administration of TPA resulted in thrombolysis in 4 of 12 saline-treated dogs (33%), 5 of 7 VCL-treated dogs (71%), and 4 of 8 aspirin-treated dogs (50%). The average elapsed time from TPA treatment to thrombolysis (thrombolysis time) was significantly shorter in VCL-treated than in saline-treated dogs (47±12 versus 81±4 minutes, P<.05, Fig 3).

In protocol 2, dogs were not pretreated before their coronary arteries were occluded, and 3 hours after the occlusion of coronary arteries, they received thrombolytic treatments: TPA and heparin induced thrombolysis in 5 of 7 dogs (71%); TPA, heparin, and VCL induced thrombolysis in 6 of 7 dogs (86%); TPA, heparin, and aspirin induced thrombolysis in 7 of 8 dogs (85%); and TPA, heparin, VCL, and aspirin induced thrombolysis in 8 of 8 dogs (100%). The average thrombolysis time in dogs treated with TPA, heparin, VCL, and aspirin was slightly more than half that in dogs treated only with
TPA and heparin (23.5±4 versus 45±12 minutes). The difference, however, was not statistically significant (Fig 4). Mean aortic pressure had decreased by approximately 20 mm Hg at 3 hours after thrombolysis in dogs treated with TPA, heparin, and VCL or TPA, heparin, VCL, and aspirin.

Reocclusion

After thrombolysis, many animals developed reocclusion of the coronary arteries during the 3-hour monitoring period. In protocol 1, the frequency of reocclusion was not significantly different among dogs pretreated with saline (4 of 4), aspirin (4 of 4), or VCL (4 of 5). However, the average time from thrombolysis to reocclusion (reocclusion time) was significantly longer in dogs pretreated with VCL (114±18 minutes) than in dogs pretreated with saline (42±4 minutes) or aspirin (55±14 minutes) (P<.05, Fig 5).

In protocol 2, coronary artery reocclusion developed in 5 of 5 dogs treated with TPA and heparin, and the addition of aspirin did not change the frequency of reocclusion (7 of 7). The addition of VCL to TPA and heparin significantly reduced the frequency of reocclusion in the reperfused coronary arteries of dogs (2 of 6; P<.05 compared with the TPA and heparin group). The addition of VCL and aspirin also significantly reduced the frequency of reocclusion (1 of 8; P<.01 compared with the TPA, heparin, VCL group). The average reocclusion time was also significantly longer in VCL-treated dogs than in dogs who did not receive VCL (Fig 6).

Pharmacokinetic Studies

A plasma concentration of VCL at 20.6±0.6 μg/mL was detected at 10 minutes after VCL was injected as an intravenous bolus at 4 mg/kg. Thereafter, the VCL level decreased constantly (Fig 7). The half-life of VCL was 24 minutes. After the injection and continuous infusion of VCL (4 mg/kg+2 mg·kg⁻¹·h⁻¹), the level of VCL reached a plateau in 60 minutes at approximately 10 μg/mL (Fig 8).

Hematocrit, Coagulation, and Platelet Aggregation Studies

In dogs treated with TPA alone, bleeding around the surgical area was not significant. The addition of VCL or aspirin caused mild bleeding along the incisions. The combination of TPA, heparin, VCL, and aspirin resulted in moderate to severe bleeding around the surgical areas. However, the hematocrit did not change significantly in any group after TPA treatment, nor did the hemoglobin level change significantly after the treatment with TPA, heparin, and VCL (12.9±0.3% of control versus 14±0.9% at 3 hours after treatment). The platelet count decreased slightly from 293×1000/mm³ (baseline) to 262×1000/mm³ at 30 minutes, 265×1000/mm³ at 1 hour, 270×1000/mm³ at 2 hours, and...
269×1000/mm³ at 3 hours after treatment with TPA, heparin, and VCL (P>.05).

Activated clotting time was significantly prolonged immediately after TPA and heparin administration (to a peak of 6 times the baseline value in 10 minutes). It returned to 1.5 times the baseline value 1 hour after treatment and to just above the baseline value 3 hours after treatment. The addition of aspirin, VCL, or aspirin and VCL did not affect activated clotting time.

Ex vivo platelet aggregation induced by ADP was not affected by VCL infusion (Fig 9A), but it was slightly reduced by aspirin injection (Fig 10A). The botrocetin-induced platelet aggregation was completely inhibited by VCL treatment (Fig 9B), and arachidonic acid–induced platelet aggregation was completely inhibited by aspirin injection (Fig 10B).

Discussion

VCL, a fragment of the binding domain of von Willebrand factor, is an antagonist of the platelet glycoprotein Ib receptor. The data from our study demonstrate that VCL prolongs the time to the development of intracoronary thrombus, enhances TPA-induced thrombolysis, and delays coronary artery reocclusion.

Von Willebrand factor plays an important role in hemostasis, and its absence results in von Willebrand disease, an inherited bleeding disorder. Recent studies have found that von Willebrand factor is essential for the formation of platelet thrombi, especially under flow conditions characterized by high shear stress, such as occur in stenosed coronary arteries. Von Willebrand
factor interacts with glycoprotein Ib receptors on the platelet membrane to initiate platelet adhesion14-16 and to activate platelet release of ADP, thromboxone, and serotonin, which cause platelet aggregation and thrombus formation.17-20 Therefore, efforts have been made to prevent platelet adhesion and thrombus formation by blocking the platelet glycoprotein Ib receptors. Aur-intracarboxylic acid, for example, blocks the platelet glycoprotein Ib recognition site on von Willebrand factor and may be useful in antithrombotic therapy.27

The domain of von Willebrand factor that interacts with glycoprotein Ib is contained in a tryptic fragment of 52/48 kDa comprising residues Val1496-Lys1528 (References 28 and 29). Fragments of this domain have been expressed successfully in Escherichia coli by recombinant technique.22,23,30 VCL is a fragment that comprises residues Leu504-Lys728 (Reference 23), and it inhibits von Willebrand factor binding to platelet glycoprotein Ib receptors in vitro.23 In our study, intravenous administration of VCL before electrical injury to the coronary artery significantly increased the time required for formation of an occlusive thrombus in vivo. The treatment also completely inhibited ex vivo platelet aggregation induced by botrocetin. Because botrocetin causes platelet aggregation by inducing von Willebrand factor to bind to the membrane glycoprotein Ib receptors,24,25 the inhibition of botrocetin-induced platelet aggregation after VCL treatment indicates that VCL caused a blockade of platelet glycoprotein Ib receptors. These data suggest that blocking the interaction between von Willebrand factor and platelet glycoprotein Ib receptors diminishes both platelet aggregation in the stenosed and endothelium-injured area of coronary arteries and the formation of occlusive thrombi.

Although VCL increased the time to thrombus formation, it did not totally prevent the event despite a continuous infusion of VCL. The complete inhibition of botrocetin-induced platelet aggregation indicates that the dosage of VCL was sufficient to block platelet glycoprotein Ib receptors. The failure of VCL to prevent ultimate thrombus development may be due to factors other than von Willebrand factor and platelet glycoprotein Ib. This hypothesis is supported by the clinical observation that some patients with von Willebrand disease or Bernard-Soulier syndrome, a disease caused by a defect in platelet glycoprotein Ib receptors, still develop acute myocardial infarction.31-33 These data suggest that a blockade of platelet glycoprotein Ib receptors can diminish but cannot completely prevent arterial thrombus formation. A combination of VCL with other antithrombotic agents, such as aspirin, may be necessary to prevent arterial thrombosis.

After occlusive thrombi had formed in the coronary arteries, the infusion of TPA caused thrombolysis. The thrombolysis time was significantly shorter in VCL-pretreated animals than in control animals, which suggests that VCL pretreatment may enhance TPA-induced thrombolysis in coronary arteries. VCL given after the formation of thrombi, however, did not enhance thrombolysis by TPA as significantly. This difference may be due to the action of VCL. By blocking platelet glycoprotein Ib receptors, VCL mainly inhibits platelet adhesion. Pretreatment with VCL affects platelet adhesion and changes thrombus composition, possibly by involving fewer platelets, thereby enabling the thrombus to lyse more easily. If, instead, the thrombus has already matured and platelets have built up, treatment with VCL may not change the formed thrombus and may not affect thrombolysis. Acute thrombotic occlusion develops in 5% of the patients who undergo percutaneous transluminal coronary angioplasty (PTCA). Because pretreating with VCL enhances thrombolysis, this technique may be useful for patients who are undergoing PTCA. VCL given at the time of PTCA may (1) partly protect against thrombus formation or (2) promote thrombolysis, if thrombolytic therapy is needed. Pretreatment with aspirin was less effective than with VCL in promoting thrombolysis. This finding suggests that inhibition of platelet adhesion may be more important than inhibition of platelet aggregation for promotion of thrombolysis.

Coronary artery reocclusion has limited the efficacy of thrombolytic therapy in patients with acute myocardial infarction.12,13,34-36 Most clinical trials have used adjunctive treatment with antiplatelet agents (aspirin) to prevent reocclusion.37,38 In our study, when VCL was given with TPA or with TPA and heparin, the time from thrombolysis to coronary artery reocclusion was significantly prolonged, and in as many as two thirds of the animals, reocclusion was prevented. VCL was more effective than aspirin given under the same conditions. VCL mainly inhibits platelet adhesion. The success of VCL in preventing reocclusion in two thirds of the animals suggests that a complete or nearly complete dislodgment of aggregated and adhered platelets may have occurred during thrombolysis. The protection from VCL against further platelet adhesion prevented or delayed rthrombosis. Alternatively, the failure of VCL to prevent reocclusion in the other one third of the animals may be due to incomplete dislodgment of thrombi after thrombolysis, followed by further accumulation of platelet aggregates and complete reocclusion. To prevent this chain of events, an additional drug may be needed to inhibit platelet aggregation. Support for this hypothesis comes from our results: reocclusion was prevented in nearly 90% of animals treated with TPA, heparin, VCL, and aspirin. These data suggest that VCL may be useful as adjunctive therapy to TPA in future thrombolytic therapy for coronary artery reocclusion.

Bleeding is a common complication of thrombolytic therapy. In our study, treatment with VCL, TPA, and heparin caused mild to moderate bleeding around the surgical incisions, and the addition of aspirin to VCL, TPA, and heparin resulted in severe bleeding in 2 out of 8 cases. However, hematocrit and hemoglobin levels were not significantly changed. This lack of change may be due to the blood concentration that occurs immediately after bleeding. The decrease of aortic pressure in dogs treated with either TPA, heparin, and VCL or TPA, heparin, VCL, and aspirin may reflect a loss of blood volume. These data indicate that caution will be needed when administering such adjunctive thrombolytic therapy.

Conclusions

Blocking platelet glycoprotein Ib receptors with VCL may be effective in diminishing the formation of thrombi in injured coronary arteries. In delaying reocclusion of the coronary arteries after thrombolysis, VCL is comparable or superior to aspirin as an adjunctive treatment with TPA and heparin. Treatment with VCL and aspirin, in addition to TPA and heparin, either markedly
delays or prevents reoclusion in this experimental model.

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