Abolition of Cyclic Flow Variations in Stenosed, Endothelium-Injured Coronary Arteries in Nonhuman Primates With a Peptide Fragment (VCL) Derived From Human Plasma von Willebrand Factor–Glycoprotein Ib Binding Domain

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Background Platelets play an important role in the pathophysiology of acute coronary syndromes. The interaction between the platelet glycoprotein Ib receptor and von Willebrand factor is a critical event allowing platelet adhesion and aggregation and subsequent thrombus formation in vessels with high shear rates and damaged endothelium. Therefore, we tested the hypotheses that VCL, an antagonist of von Willebrand–glycoprotein Ib binding domain, (1) attenuates/abolishes cyclic flow variations in stenosed, endothelium-injured coronary arteries in nonhuman primates and (2) reduces botrocetin-induced platelet aggregation in vitro after intravenous in vivo administration.

Methods and Results Cyclic flow variations were established in anesthetized, open-chest baboons (n=18). The baboons were divided into three groups. One group (n=8) received a bolus of VCL (4 mg/kg IV) followed by an infusion (6 mg·kg\(^{-1}\)·h\(^{-1}\)) for 90 minutes (schedule A). Another group (n=6) received a 2-mg/kg bolus followed by an infusion of 3 mg·kg\(^{-1}\)·h\(^{-1}\) for 90 minutes (schedule B). The third group received a placebo infusion of normal saline. Under dosing schedule A, cyclic flow variations were abolished in 7 of 8 baboons after 33±18 minutes and markedly attenuated in 1. The frequency of cyclic flow variations fell from 18±9.4 per hour during the control period to 1±2.5 per hour after VCL infusion, P<.002. After cessation of infusion, cyclic flow variations remained abolished in 5 of 7 animals for >3 hours and returned in 2 of 7 after 2 to 2.5 hours. Under schedule B, cyclic flow variations were abolished in 3 of 6 baboons and markedly reduced in the remainder. The number of cyclic flow variations fell from 17±4.8 per hour during the control period to 5±4.9 per hour after the VCL infusion, P<.001. The cyclic flow variations returned spontaneously at 38±40 minutes under this dosing schedule. Placebo infusion of saline had no effect on cyclic flow frequency or severity. VCL administration was associated with slight prolongation in bleeding time and a reduction in botrocetin-induced platelet aggregation. The bleeding time increased from a control time of 88±32 to 276±204 seconds, P<.03, and from 142±28 to 176±36 seconds, P<.056, for schedules A and B, respectively.

Conclusions Therefore, administration of a peptide fragment corresponding to von Willebrand–glycoprotein Ib binding domain (1) is effective in abolishing cyclic flow variations in stenosed, endothelium-injured coronary arteries and (2) reduces platelet aggregation in vivo in response to botrocetin in nonhuman primates. (Circulation. 1994;90:2976-2981.)

Key Words • von Willebrand factor • glycoproteins • platelets • blood flow

In stenosed coronary arteries with endothelial damage, we and others have suggested that platelet-mediated obstruction is an important pathophysiological mechanism for the conversion of chronic, stable

to unstable coronary disease syndromes.\(^1\)-\(^4\) When there is loss of endothelial integrity, platelet adhesion is a critical step in the response of platelets, allowing interaction with exposed subendothelial components of the damaged vessel with circulating platelets.\(^5\)\(^6\)

The von Willebrand factor molecule plays a pivotal role in the process of platelet adhesion, by virtue of its ability to interact with both the subendothelial components and the glycoprotein Ib receptor on platelets. The formation of platelet aggregates is especially dependent on the glycoprotein Ib–von Willebrand factor interaction in blood vessels exposed to high shear stresses.\(^7\) In addition, there is increasing evidence that the interaction between von Willebrand factor and the glycoprotein Ib receptor results in activation of the glycoprotein IIb/IIIa receptor, leading to binding of fibrinogen and
ultimately to platelet aggregation.\(^8\)\(^{-10}\) Therefore, inhibition of the initial contact between the platelet glycoprotein Ib receptor and von Willebrand factor may be effective in preventing platelet activation and subsequent thrombus formation.

The domain of the von Willebrand factor that interacts with the glycoprotein Ib receptor is composed of residues Val-449 to Lys-728.\(^{11}\) The same region contains the binding domains for heparin and collagen.\(^{12,13}\) A peptide fragment of the human von Willebrand factor, Leu-504 to Lys-728, named VCL, has been developed and produced by use of recombinant techniques.\(^{14,15}\) This domain represents approximately 10% of the sequence of mature von Willebrand factor. Previous in vitro experiments have shown that VCL is a potent inhibitor of the interaction between von Willebrand factor and the glycoprotein Ib receptor. VCL inhibited contact and spreading of platelets and also caused a marked decrease in thrombus formation. These results suggested that VCL may be an effective antithrombotic agent in partially stenosed coronary arteries that are exposed to high shear rates.

The aim of this study was to evaluate the in vivo effects of VCL, a peptide fragment of the human glycoprotein Ib binding domain, in a model of coronary artery stenosis with endothelial injury. By inhibiting the interaction between the platelet glycoprotein Ib receptor and von Willebrand factor, we anticipated that VCL would prevent platelet adhesion to the subendothelial components of an injured vessel and the subsequent activation of the platelet glycoprotein Ib/IIa receptor that ultimately leads to platelet aggregation and thrombus formation. Therefore, we set out to test the following hypotheses: (1) After in vivo administration, VCL, an antagonist of the von Willebrand–glycoprotein Ib binding domain, attenuates or abolishes cyclic flow variations in stenosed, endothelium-injured coronary arteries in nonhuman primates; and (2) in vivo administration of VCL reduces botrocetin-induced platelet aggregation in vitro.

**Methods**

**Surgical Preparation**

We studied 18 anesthetized open-chest male baboons. Catheters were inserted into a femoral artery and vein for measurement of aortic blood pressure and for drug and fluid administration, respectively. A left-sided thoracotomy was performed, and the heart was suspended in a pericardial cradle. Then, a 1- to 2-cm segment of the left anterior descending coronary artery was carefully isolated. An ultrasonic Doppler flow probe was placed around the proximal portion of the isolated segment of the left anterior descending coronary artery to measure coronary blood flow. Hemodynamics, including heart rate, systolic and diastolic arterial pressures, and mean and phasic coronary blood flow velocities, were continuously recorded on an eight-channel recorder. Cyclic flow variations were induced by gently squeezing the left anterior descending coronary artery with cushioned forceps to damage the endothelium and by placing a plastic constrictor around the injured portion of the artery. Once cyclic flow variations were induced, the animals were observed for a further 60 minutes to confirm the establishment of cyclic flow variations.

**Study Protocol**

The recombinant von Willebrand factor fragment (VCL), Leu-504 to Lys-728, was expressed in *Escherichia coli* and purified as previously described (Fig 1).\(^{14,15}\) The VCL was reconstituted from a lyophilized form in phosphate-buffered saline (pH 8) with ice-cold sterile water to a concentration of approximately 2 mg/mL. The solution was further diluted in normal (N)-saline to provide a suitable volume for infusion.

The baboons were divided into three groups. One group (n=4) received placebo (90-minute infusion of N-saline), and two groups received VCL. One group (n=8) received VCL as a bolus of 4 mg/kg followed by a 90-minute infusion of 6 mg·kg\(^{-1}\)·h\(^{-1}\) (schedule A), and the other received a bolus of 2 mg/kg followed by a 90-minute infusion of 3 mg·kg\(^{-1}\)·h\(^{-1}\) (schedule B). The baboons were observed for 3 hours after the end of the infusion.

Measurements of blood coagulation, hemostasis, hematocrit, and botrocetin-induced platelet aggregation were made before the administration of either VCL or placebo and again 90 minutes later at the end of the infusion. Activated clotting time (ACT) and partial thromboplastin times (pPTT) were measured with an automated blood coagulation timing device (HemoTec 2001370). Bleeding time was determined by the Ivy method using a Simplet device. Blood samples for ex vivo platelet aggregation in platelet-rich plasma were collected in plastic tubes containing 3.8% sodium citrate (9 vol blood to 1 vol sodium citrate). Platelet-rich plasma was obtained by centrifuging whole blood at 200g for 20 minutes at 37°C. An impedance method was used to determine platelet aggregation on a dual-channel aggregometer (Chrono Log Instruments). Platelet aggregation was reported as the increase in electrical resistance between two electrical fields placed in platelet-rich plasma. Plasma levels of VCL were determined with an ELISA technique developed by BioTechnology General.

All procedures used in this study were conducted according to the principles of the American Physiological Society and were approved by the Animal Welfare Committee at the University of Texas Health Science Center at Houston, Texas.

**Statistical Analyses**

All values were expressed as mean±SD. Paired \(t\) tests were used to compare values before and after treatment within each group of animals. Statistical comparisons between groups were not performed. A significance value of \(P<.05\) was considered statistically significant.

**Results**

**Cyclic Flow Variations**

**Placebo**

Four baboons received a placebo infusion of N-saline. There was no significant change in the number of cyclic flow variations during the control period, 16±4.2, compared with 24±8.9 cycles per hour at the end of the infusion period, \(P=NS\) (Fig 2).

**Dosing Schedule A (4 mg/kg bolus plus 6 mg·kg\(^{-1}\)·h\(^{-1}\) 90-minute infusion)**

In 7 of 8 baboons that received this regimen of VCL infusion, cyclic flow variations were completely abolished after 33±18 minutes and became markedly attenuated in 1 animal. The frequency of cyclic flow varia-
tions was reduced from 18±9.4 cycles per hour during the control period to 1±2.5 cycle per hour after the 90-minute infusion period, \(P<.002\) (Fig 2). No statistically significant differences between the hematocrits, heart rates, or systolic or diastolic arterial pressures were observed. In 2 of the baboons, cyclic flows returned 2.0 to 2.5 hours after the infusion had ended. In 5 of 7 remaining baboons, cyclic flow variations remained abolished for >3 hours. An infusion of epinephrine was administered to 4 of 5 of these baboons and resulted in the restoration of cyclic flows in 3 animals.

Pharmacokinetic studies were performed in 3 baboons during dosing schedule A. Blood samples (5 mL) were drawn 1, 5, 10, 15, 20, 30, 40, 50, 70, and 90 minutes after the onset of infusion and at 2, 5, 10, 15, 20, 30, 40, 60, 90, 120, 160, and 180 minutes after cessation of the infusion. Plasma concentrations of VCL were determined by ELISA. A mean concentration of VCL during the infusion was 44±2.0 \(\mu\)g/mL VCL. After cessation of VCL infusion, the peptide was cleared rapidly, with a clearance half-time of the plasma of 29 minutes (Fig 3A).

**Dosing Schedule B (2 mg/kg bolus plus 3-mg \cdot kg^{-1} \cdot h^{-1} 90-minute infusion)**

In 3 of 6 baboons receiving this regimen, the cyclic flow variations were completely abolished after a mean of 20±17 minutes and were markedly attenuated in the remaining animals. The number of cyclic flow variations fell from 17±4.8 cycles during the control period to 5±4.9 cycles per hour after the period of VCL infusion, \(P<.001\) (Fig 2). No statistically significant differences between the hematocrits, heart rates, or systolic or diastolic arterial pressures were observed. In the 3 baboons in whom the cyclic flow variations were completely abolished, all returned spontaneously (mean, 38.0±40 minutes).

Blood samples were drawn in all baboons for determination of VCL levels during dosing schedule B. Samples were drawn at 5, 30, 60, and 90 minutes after the onset of infusion and at 10, 15, 20, 30, 40, 45, 60, 90, 120, 150, and 180 minutes after cessation of the infusion. The mean plasma concentration of 25±3.5 \(\mu\)g/mL VCL was measured during the infusion. After cessation of the infusion, the clearance half-time of VCL from the plasma was 22 minutes (Fig 3B).

**Blood Coagulation and Platelet Function**

Results of coagulation and bleeding times are summarized in the Table. Using dosing schedule A, VCL administration was associated with prolongation of the bleeding time from a control value of 88±32 seconds to 276±204 seconds, \(P<.03\). The lower-dose VCL regimen, schedule B, was also associated with a prolongation of the bleeding time from a control value of 142±28 seconds to 176±36 seconds, \(P=.056\). Despite the differences in the baseline values, the response to VCL appeared consistent. All the animals receiving dosing schedule A showed an increase in the bleeding time (mean increase, 175±158 seconds). In comparison, animals receiving the lower-dose schedule showed a smaller increase (mean increase, 33±33 seconds), including 2 animals with no change in the bleeding time.

No changes in the ACT or aPTT were detected after VCL under dosing schedule A. However, under schedule B, there was minimal but statistically significant prolongation of both the ACT and aPTT, by 8±6 seconds and 4±2 seconds, respectively, \(P<.05\) and \(P<.01\), respectively.

Both VCL dosing schedules resulted in reduction of botrocetin-induced platelet aggregation (Fig 4). With botrocetin at a concentration of 20 \(\mu\)g/mL, platelet aggregation was reduced from a control value of 66±30.2 to 33±31.3 \(\Omega\), \(P=.04\), and from 64±23.5 to 46±15.8 \(\Omega\), \(P=.006\), under dosing schedules A and B, respectively. With botrocetin at a concentration of 40 \(\mu\)g/mL, platelet aggregation was reduced from 48±27.5 to 40±13.2 \(\Omega\), \(P=NS\), and 76±17.7 to 52±13.2 \(\Omega\), \(P<.005\), for dosing schedules A and B, respectively. A small but statistically significant fall in the platelet count was observed after VCL administration. The platelet count fell from 219±56 to 161±33×10^3/mm^3, \(P<.01\), and 236±151 to 176±126×10^3/mm^3, \(P<.01\), for dosing schedules A and B, respectively.

**Discussion**

**Main Findings**

The principal findings of this study are, first, that VCL, a recombinant peptide fragment corresponding to the von Willebrand–glycoprotein Iib binding domain, is effective in abolishing cyclic flow variations in stenosed and endothelium-injured coronary arteries in nonhuman primates and, second, that VCL administration decreases botrocetin-induced platelet aggregation.

**Effects of VCL on Cyclic Flow Variations**

In the canine heart, severe coronary artery stenoses associated with endothelial injury promote cyclic reductions in coronary flow, manifested by marked reductions in coronary blood flow followed by spontaneous increases in coronary flow.16–20 Similar cyclic flow variations have also been observed in some patients with unstable coronary syndromes.3,22 This phenomenon results from platelet adhesion and aggregation at the site of endothelial damage and the associated increase in proaggregatory and vasoconstrictor substances and decrease in antiaggregatory and vasodilator factors.1–3,16–20
In this study, we have shown that by administration of a peptide fragment of the von Willebrand–glycoprotein Ia binding domain, cyclic variations can be abolished or greatly diminished. The presumed mechanism by which this occurs is the inhibition of the interaction of the platelet glycoprotein Ib receptor and the surface-bound von Willebrand factor. This prevents platelet activation and aggregation and also the release of proaggregatory and vasoconstrictor substances responsible for cyclic flow variations.

The pharmacokinetic studies demonstrated that a steady state was achieved rapidly. VCL was cleared rapidly from the plasma, with a half-time of 20 to 30 minutes. When it was given by intravenous bolus followed by infusion, therapeutic effects of VCL occurred within 20 to 30 minutes after administration. Under dosing schedule A, the duration of VCL effects persisted for >2 hours, with cyclic flow variations returning only after infusion of epinephrine in some of the baboons. Under the lower-dose schedule B, the effects of VCL were less pronounced and their duration shorter.

**Effect of VCL on Platelet Function**

In a previous in vitro study, VCL inhibited ristocetin- and botrocetin-induced platelet aggregation in a static
system. In addition, VCL also inhibited contact and spread of platelets on the subendothelial surface and decreased thrombus formation in umbilical arteries at high shear rates in a flowing system. Sugimoto et al. also showed that a recombinant peptide fragment of von Willebrand–glycoprotein Ib domain is an effective competitive inhibitor of ristocetin- and botrocetin-induced aggregation.

Botrocetin, a venom coagglutinin, induces binding of von Willebrand factor to glycoprotein Ib. In vivo studies of peptide fragments of the von Willebrand–glycoprotein Ib binding domain inhibit binding of von Willebrand factor to platelets. Our data confirm that the in vivo administration of VCL results in decreased botrocetin-induced platelet aggregation and that this is the probable mechanism by which VCL abolishes/attenuates cyclic flow variations in this model.

In addition, our results demonstrate that both dosing schedules of VCL are associated with slight prolongation of the bleeding times. Presumably, this results from inhibition of von Willebrand–glycoprotein Ib interaction. No evidence of undue bleeding was noticed around the surgical wounds or at puncture sites in the experimental animals. Although it is unlikely that the degree of prolongation in these experiments would result in any significant hemostatic problems, the effect may be of importance when VCL is used in combination with antiplatelet and thrombolytic agents.

The fall in platelet count was unexpected, and the mechanism is not known. The decrease in platelet count was small and did not fall outside the lower limit of normal. It seems unlikely that it resulted from the experimental model, since there was no significant change in the platelet count in the placebo group. The decrease did not appear to be dose related, since the change was similar in both dosing schedules. Different production batches of VCL were used during this study, but no relation between the VCL batch used and the fall in platelet count was identified. It is possible that the fall in platelet count is immunologic in origin, with sequestration of platelets within the reticuloendothelial system. However, we have no evidence to support this hypothesis at this time. This finding requires further evaluation to determine whether it is a transient, self-limited phenomenon and to elucidate the underlying mechanism.

Study Limitations

Because of the expense and relative difficulty in obtaining the animals used in this study, the number of animals in each group is small. We used a paired t test design to help control for the extraneous variation that occurs in experimental models such as the one used in this study. For these reasons, the power of statistical analyses performed and the conclusions that can be drawn from these data are limited. Nevertheless, administration of VCL had a dramatic effect on the cyclic flow variations in this model of coronary stenosis and endothelial injury.

The clinical relevance of this experimental model has been questioned. However, there is increasing evidence that cyclic abnormalities in coronary flow also occur in at least some patients with severe coronary artery disease, which supports the likely pertinence of this model to acute coronary syndromes in man.

Summary of Hemodynamic and Laboratory Values in Placebo Group, Schedule A (4 mg/kg and 6 mg·kg⁻¹·h⁻¹), and Schedule B (2 mg/kg and 3 mg·kg⁻¹·h⁻¹)

<table>
<thead>
<tr>
<th></th>
<th>Placebo</th>
<th>Schedule A</th>
<th>Schedule B</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 min</td>
<td>150 min</td>
<td>0 min</td>
</tr>
<tr>
<td>HR, bpm</td>
<td>113 (14)</td>
<td>113 (13)</td>
<td>99 (19)</td>
</tr>
<tr>
<td>SBP, mm Hg</td>
<td>139 (9)</td>
<td>132 (11)</td>
<td>117 (27)</td>
</tr>
<tr>
<td>DBP, mm Hg</td>
<td>93 (9)</td>
<td>93 (9)</td>
<td>87 (17)</td>
</tr>
<tr>
<td>Hct</td>
<td>39 (2)</td>
<td>38 (2)</td>
<td>38 (3)</td>
</tr>
<tr>
<td>ACT, s</td>
<td>132 (9)</td>
<td>128 (9)</td>
<td>137 (27)</td>
</tr>
<tr>
<td>aPTT, s</td>
<td>28 (2)</td>
<td>32 (7)</td>
<td>29 (7)</td>
</tr>
<tr>
<td>BT, s</td>
<td>155 (31)</td>
<td>155 (48)</td>
<td>88 (32)</td>
</tr>
<tr>
<td>Platelets, 10³j/mm²</td>
<td>173 (26)</td>
<td>191 (12)</td>
<td>219 (56)</td>
</tr>
</tbody>
</table>

* HR indicates heart rate; bpm, beats per minute; SBP, DBP, systolic and diastolic blood pressures; Hct, hematocrit; ACT, activated coagulation time; aPTT, activated partial thromboplastin time; BT, bleeding time; and Platelets, platelet count. Values are mean (SD).

*p<.05; tP<.01; $P<.03; ¥P=.056; tP<.01; and ¥P<.01.
Conclusions
The role of platelet adhesion and aggregation in the conversion of chronic and stable to acute and unstable coronary artery syndromes is well documented. The interaction between the von Willebrand factor molecule of damaged endothelium and the glycoprotein Ib receptor of circulating platelets is an important one. It initiates the thrombogenic process in the presence of damaged vessels with high shear stress, as found in the coronary arterial system. Therefore, inhibition of this process is an attractive possibility for the prevention and treatment of acute coronary disease syndromes. This peptide fragment may provide an effective and relatively specific means of largely preventing or interrupting the thrombogenic process, and it may represent a new class of antithrombotic compounds.

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References
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