Inhibition of Platelet-von Willebrand Factor Binding to Platelets by Adhesion Site Peptides

By Robert I. Parker and Harvey R. Graalnick

Synthetic peptides containing the adhesion site recognition sequence present on the \( \alpha \) and \( \gamma \) chains of fibrinogen were studied for their effect on the binding of endogenous platelet-von Willebrand factor (vWF) to thrombin-stimulated platelets. In agreement with previously reported data, the tetrapeptide consisting of the RGDS sequence was a more potent inhibitor of plasma-vWF binding to platelets than was the pentadecapeptide of the carboxy terminus of the fibrinogen \( \gamma \)-chain (IC\(_{50} \) 10.6 \( \mu \)mol/L for the RGDS tetrapeptide vs 44.9 \( \mu \)mol/L for the \( \gamma \)-chain pentadecapeptide). No apparent synergy in the inhibition of plasma-vWF binding was noted when the RGDS and \( \gamma \)-chain peptides were used together (IC\(_{50} \) 15.2 \( \mu \)mol/L). In contrast, the \( \gamma \)-chain peptide was significantly more inhibitory than was the RGDS tetrapeptide on the binding of platelet-vWF to platelets (IC\(_{50} \) 1.4 \( \mu \)mol/L for the \( \gamma \)-chain pentadecapeptide vs 4.5 \( \mu \)mol/L for the RGDS tetrapeptide, \( P < .05 \)). And there was significant synergy in the inhibition of platelet-vWF binding noted when the \( \gamma \)-chain and RGDS peptides were used together (IC\(_{50} \) 0.04 \( \mu \)mol/L). These results indicate that the binding of platelet-vWF to its receptor on the platelet glycoprotein \( \text{Ib/IIa} \) complex involves both the RGDS and \( \gamma \)-chain recognition sites. In contrast to the results with plasma-vWF binding, the \( \gamma \)-chain recognition site appears to be more important than the RGDS recognition site in platelet-vWF binding to platelets.

This is a US government work. There are no restrictions on its use.

Platelets are known to contain a pool of endogenous von Willebrand factor (vWF) which upon platelet stimulation becomes expressed on the platelet surface. The great majority of this activation-dependent expression of platelet-vWF is mediated via binding of the secreted platelet-vWF to the platelet glycoprotein \( \text{Ib/IIa} \) complex (GP\( \text{Ib/IIa} \)), which also serves as the platelet fibrinogen receptor. Normal fibrinogen possesses two peptide recognition sequences for its platelet receptor: the arg-gly-asp tripeptide (RGD) located on the \( \alpha \) chains, and the dodecapeptide containing the 12 amino acids of the carboxy terminus of the \( \gamma \)-chain. Previous studies have demonstrated that synthetic peptides containing these amino acid sequences inhibit the binding of fibrinogen and plasma-vWF to platelets. When shorter incubations were used (30 to 45 minutes), the total amount of antibody bound was less than the antibody bound with the 90-minute incubations, but the degree of inhibition of platelet-vWF binding by the adhesion site peptides was identical. No significant platelet lysis occurred during the incubations as measured by serial electronic platelet counts (Cellozone Cell Counter, Particle Data Inc, Elmhurst, IL) and supernatant LDH content. The polyclonal affinity purified IgG rabbit antihuman vWF antibody used in these experiments has been extensively characterized and used in our laboratory. Specificity of the antibody has been demonstrated by immunoprecipitation studies with normal plasma, vWF-deficient plasma, and plasma purified from cryoprecipitate. This antibody does not react with whole platelets or platelet lysates from patients with severe von Willebrand disease (vWD) (<3% normal values of vWF), and under the experimental conditions employed does not significantly affect the thrombin-induced binding of vWF to platelets.

To study the effect of the adhesion site peptides on platelet-vWF surface expression, various concentrations of synthetic peptides containing either the RGDS or \( \gamma \)-chain sequence were included in the incubations. The platelet incubations were performed in the following fashion: first, the radiolabeled anti-vWF antibody was placed into each polypropylene tube, and then the thrombin (final concentration 0.1 to 0.5 U/mL) was placed along the inside wall of the tube. Platelets were then added to the vial through the thrombin bubble. At the completion of the incubation, bound antibody was separated from free antibody by centrifugation (9,000 g for four minutes at 24°C). The supernatant was aspirated and the platelet pellet was counted for retained radioactivity. Nonspecific antibody binding to the platelet pellets was that observed with severe vWD platelets that contained <3% of normal plasma and platelet-vWF antigen. This value was similar to the antibody binding to the tube in the absence of platelets. Nonspecific binding was characterized by <20% of that bound to nonstimulated control platelets and <5% of previously described. The gradient was sliced at the interface, and the platelets were aspirated and diluted to a final concentration of 200,000/?L in Tyrode's buffer (290 mOsm, pH 7.1) containing 5 mmol/L CaCl\(_2\).

Surface-bound platelet-vWF was determined by measuring the binding of a \( ^{125} \)I-labeled polyclonal affinity-purified rabbit antihuman vWF antibody to thrombin-stimulated platelets as previously described. Briefly, 200 ?L aliquots of platelets were incubated, unstimred, with radiolabeled anti-vWF antibody (final concentration 3.75 \( \mu \)g/mL, 4 to 5 \( \times \) 10\(^4\) cpm/\( \mu \)g) at 37°C for 90 minutes. The platelets were incubated with the labeled anti-vWF antibody and thrombin for 90 minutes to allow binding to reach equilibrium. When shorter incubations were used (30 to 45 minutes), the total amount of antibody bound was less than the antibody bound with the 90-minute incubations, but the degree of inhibition of platelet-vWF binding by the adhesion site peptides was identical. No significant platelet lysis occurred during the incubations as measured by serial electronic platelet counts (Cellozone Cell Counter, Particle Data Inc, Elmhurst, IL) and supernatant LDH content. The polyclonal affinity purified IgG rabbit antihuman vWF antibody used in these experiments has been extensively characterized and used in our laboratory. Specificity of the antibody has been demonstrated by immunoprecipitation studies with normal plasma, vWF-deficient plasma, and vWF purified from cryoprecipitate. This antibody does not react with whole platelets or platelet lysates from patients with severe von Willebrand disease (vWD) (<3% normal values of vWF), and under the experimental conditions employed does not significantly affect the thrombin-induced binding of vWF to platelets.

Materials and Methods

Normal human platelets were prepared from sodium citrate/EDTA anticoagulated whole blood using an arabinogalactan (Stractan, Champion International, Tacoma, WA) gradient method as described. The gradient was sliced at the interface, and the platelets were aspirated and diluted to a final concentration of 200,000/?L in Tyrode's buffer (290 mOsm, pH 7.1) containing 5 mmol/L CaCl\(_2\).

This is a US government work. There are no restrictions on its use.

0006-4971/89/7404-0033$0.00/0

From the Hematology Service, Clinical Pathology Department, Clinical Center, National Institutes of Health, Bethesda, MD. Submitted March 31, 1989; accepted May 25, 1989. Address reprint requests to Robert I. Parker, MD, Hematology Service, CPD, Bldg 10, Room 2C-390, National Institutes of Health, Bethesda, MD 20892.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

This is a US government work. There are no restrictions on its use.
that bound to thrombin-stimulated control platelets. Specific binding was determined by subtracting the value for nonspecific binding from total binding to unstimulated or thrombin-stimulated normal platelets.

When the effect of a synthetic peptide was tested, the peptide was incubated with the platelet suspensions for 15 minutes (at 24°C) prior to the incubation of the platelets with the thrombin and radiolabeled antibody. For studies testing the combination of the γ-15 and RGDS peptides, the incubations contained equimolar amounts of each peptide and the IC50 and IC100 are expressed as the sum of the concentration of the peptides (eg, an IC50 of 0.04 μmol/L represents the results when both peptides are present at a concentration of 0.02 μmol/L).

In parallel experiments, the binding of 125I-labeled plasma-vWF to unstimulated and thrombin-stimulated platelets was tested. The plasma-vWF was purified from normal human cryoprecipitate, radiolabeled on lactoperoxidase beads, and incubated with Stractan purified platelets at a final concentration of 2 μg/mL. In these experiments, the only difference from the platelet-vWF binding experiments was that the rabbit antihuman vWF antibody was omitted and radiolabeled plasma-vWF was included.

The following peptides were tested: RGDS, RGRDSCP, RGDS PASSK/P (Peninsula Laboratories, Inc, Belmont, CA), and a peptide containing the 15 carboxyterminal amino acids of the fibrinogen γ-chain GQQHHLGGAKQAGDV (γ-15) (Bachem, Inc, Torrance, CA). Each peptide used in these studies was from one lot and the purity was determined, by the manufacturer, using high pressure liquid and thin-layer chromatography. By these methods, the peptides were demonstrated to be homogeneous. The amino acid analysis of the peptides was consistent with their predicted composition.

The inhibitory activity of the adhesion site peptides was expressed by measuring their effect on the binding of platelet-vWF to surface GPIIB/IIa on thrombin-stimulated platelets. The value of binding to GPIIB/IIa was determined by subtracting the amount of antivWF antibody binding to thrombin-stimulated platelets in which the GPIIB/IIa complex was dissociated by EDTA (10 mmol/L; 37°C for 30 minutes at pH 7.1) from the anti-vWF antibody binding to thrombin-stimulated platelets in Tyrode’s buffer in the presence or absence of the synthetic peptides. We previously demonstrated that approximately 70% of the thrombin-induced platelet-vWF surface expression occurred via binding of secreted platelet-vWF to the platelet surface GPIIB/IIa, and that this binding could be inhibited equally by prior incubation of the platelets in EDTA buffers, by fibrinogen, or by a monoclonal antibody directed against the fibrinogen binding site on GPIIB/IIa (monoclonal antibody 10E5).1 In order to test only the effect of the peptides on the platelet-vWF binding that is fibrinogen inhibitable (ie, that which occurs to surface GPIIB/IIa), our control platelets in this study were thrombin-stimulated. EDTA-treated platelets as described above. The inhibitory effect of the adhesion site peptides on plasma-vWF binding to platelets was assessed by comparing the binding of plasma-vWF with thrombin-stimulated normal platelets in the absence of the peptides to that which occurred in the presence of the peptides. For these studies, platelet GPIIB/IIa was not dissociated by EDTA.

**RESULTS**

The concentrations of the peptides required to effect a 50% and a 100% inhibition of thrombin-induced platelet-vWF surface expression on stimulated platelets (IC50 and IC100, respectively) are shown in Table 1, and the full binding curves for the RGDS and γ-15 peptides are presented in Fig 1. The pentadecapeptide containing the γ-chain sequence was the most effective peptide in inhibiting the binding of platelet-vWF to GPIIB/IIa with an IC50 of 1.4 μmol/L and an IC100 of 4.5 μmol/L. The RGDS tetrapeptide was somewhat less effective (P < .01 for both IC50 and IC100). t-test for

![Fig 1](https://example.com/fig1.png)

**Fig 1.** Inhibition curves for the effect of the γ-15 peptide (top); the RGDS peptide (middle); and the combination of both peptides (bottom) on the binding of platelet-vWF (○), and plasma-vWF (●) to thrombin-stimulated platelets. For studies using the combination of the RGDS + γ-15 peptides, the concentration represents the sum of equimolar amounts of the two peptides. Curves were fitted manually.
the comparison of the means of unpaired samples); the inhibitory activity of the RGDS peptide decreased as the number of amino acids in the peptide increased. The combination of the γ-chain peptide and the RGDS tetrapeptide was a significantly more potent inhibitor of platelet-vWF surface expression than was either peptide alone; \( P < .01 \) for both the IC\(_{50}\) and IC\(_{100}\) for either the RGDS or γ-15 peptide compared with the two peptides together.

The effect of the RGDS and γ-15 peptides on the binding of plasma-vWF to platelets is presented in Table 2 and Fig 1. These data demonstrate a different pattern of inhibitory potency for the adhesion site peptides tested when compared with their effect on platelet-vWF binding. In contrast to the effect on platelet-vWF binding, the RGDS peptide is a more potent inhibitor of plasma-vWF binding than is the γ-chain pentadecapeptide (γ-15). When the two peptides are used together to inhibit plasma-vWF binding, significant synergistic inhibition is not observed.

**DISCUSSION**

Previous studies have demonstrated that the platelet GPIIb/IIIa complex serves as an important receptor binding the adhesive glycoproteins fibrinogen and fibronectin. Fujimoto et al were the first to demonstrate that thrombin induced vWF binding to platelets, and others subsequently demonstrated that this binding is mediated by GPIIb/IIIa, not GPIIb. The binding of vWF to GPIIb/IIIa is thought to be important for the function of platelet spreading on subendothelium, and has been demonstrated to mediate platelet-platelet interactions in the absence of fibrinogen. While plasma vWF is necessary for normal platelet-mediated primary hemostasis, recent studies have shown that platelet-vWF may play an important role in hemostasis. We and Mannucci et al have shown that type 1 vWD with normal platelet-vWF levels is associated with a normal bleeding time and decreased clinical severity. In addition, Bowie et al performed a bone marrow transplant in a pig with severe vWD with the resultant normalization of platelet-vWF but no significant increase in plasma-vWF. When the pig's platelet count had normalized post-transplant, its bleeding time was noted to be significantly shortened and the degree of clinical bleeding was also decreased.

Although no data have been reported regarding the inhibition of the binding of platelet-vWF to GPIIb/IIIa by these adhesion site peptides, earlier studies have documented the inhibitory properties of synthetic peptides containing the RGD and fibronogen γ-chain adhesion site sequences on the binding of plasma-vWF to platelets. Our studies of the inhibition of plasma-vWF binding agree with these earlier results. The RGDS peptide is a more potent inhibitor of plasma-vWF binding to platelets than is the γ-15 peptide; the IC\(_{50}\)'s for these peptides determined in this study are similar to those reported in the literature. In contrast to the effects of these peptides on plasma-vWF binding, we have demonstrated that the γ-chain peptide is more potent than the RGDS peptide in the inhibition of platelet-vWF binding to GPIIb/IIIa. Additional data suggesting that the binding of platelet-vWF to platelets is qualitatively different from the binding of plasma-vWF to platelets is provided by the results of the studies using the γ-15 and RGDS peptides in combination. The results of these studies indicate that sites recognizing each sequence are involved in a synergistic fashion in the binding of platelet-vWF to the platelet GPIIb/IIIa complex, and that a similar interaction of the RGDS and γ-15 recognition sites is not observed in the binding of plasma-vWF to the platelet GPIIb/IIIa.

It is possible that the platelet-vWF molecule recognizes two separate binding sites on the GPIIb/IIIa complex and that these are sites that bind near the RGDS and/or γ-15 peptide binding sites. Another possibility is that the binding of either or both of the peptides to the GPIIb/IIIa complex indirectly modulates the binding of platelet-vWF to the platelet in a manner analogous to the modulation of plasma-vWF binding to platelets. When either or both peptide binds to the platelet, it may induce a conformational change that results in the inaccessibility of the platelet-vWF binding site(s). These peptides have been shown to alter the conformation of purified GPIIb/IIIa, and occupancy of the adhesive glycoprotein receptor by the RGDS peptide has been shown to modify the expression of antigenic sites involved in cell adhesion. Studies by Bennett et al on the effect of α- and γ-chain peptides on the binding of fibrinogen to thrombin-stimulated platelets suggest that the binding of either peptide inhibits fibrinogen binding by inducing a conformational change in GPIIb/IIIa. Another possible explanation is that the binding of the peptides to GPIIb/IIIa may simply result in a steric hindrance that partially blocks the binding of platelet-vWF to its receptor. However, the data by Bennett et al indicate that the recognition sites on GPIIb/IIIa for these peptides are spatially separated making steric hindrance much less likely as the explanation for our results.

These studies measure the binding of platelet-vWF to thrombin-stimulated platelets. It is possible that secreted platelet fibrinogen can potentially affect the binding of both the platelet-vWF and the synthetic peptides to the platelet. However, this experimental constraint does not alter the interpretation of our findings since the amount of released platelet-fibrinogen would be similar in either the presence or absence of the synthetic peptides. Thus, the inhibition of platelet-vWF would be related to the presence of the adhesion-site peptides and not to the released fibrinogen. The present study and other studies measuring the inhibitory effect of these peptides on the binding of plasma-vWF to platelets have also used activated platelets in which one would expect secretion of platelet fibrinogen. Therefore, when compared with the results of the binding of plasma-vWF, the increased inhibitory potency of the γ-15 peptide is more potent than the RGDS peptide in the inhibition of platelet-vWF binding to GPIIb/IIIa.

| Table 2. Inhibitory Effect of Adhesion Peptides on Plasma-vWF Binding to Platelets |
|------------------------------|-----------------|-----------------|
| Peptide | IC\(_{50}\) (μmol/L) | IC\(_{100}\) (μmol/L) |
| γ-15 | 44.9 ± 6.8 | > 100 |
| RGDS | 10.6 ± 3.8 | 49.5 ± 1.5 |
| γ-15 + RGDS | 15.2 ± 5.3 | 50.8 ± 2.5 |

Results represent the mean ± SD of five to six determinations at peptide concentrations ranging from 0.01 to 100 μmol/L.
adhesion peptide, relative to that of the RGDS tetrapeptide, on the binding of platelet-vWF and the marked synergy in inhibitory activity noted for the combination of the γ-15 and RGDS peptides on platelet-vWF binding, are unlikely to be a consequence of platelet fibrinogen secretion.

While several reports demonstrate that RGDS peptides are more potent inhibitors of both fibrinogen and plasma-vWF binding to platelets than is the carboxy terminus γ-chain peptide,\textsuperscript{10,12,25,26,29} data reported by Hawiger et al\textsuperscript{20} suggest that the fibrinogen γ-chain recognition site is functionally more important than the α-chain (RGDS) site for the binding of fibrinogen to human platelets. Our peptide data suggest that the binding of platelet-vWF to GPIIb/IIIa is qualitatively different from that of plasma-vWF in a manner making it more susceptible to inhibition by the fibrinogen γ-chain sequence. This difference may be a consequence of the multimeric or other structural differences noted between plasma- and platelet-vWF,\textsuperscript{31,32} or these differences may be related to platelet-vWF binding in part to endogenous GPIIb/IIIa, and may represent a mechanism by which platelet-subendothelial and platelet-platelet interactions are modulated. Studies using purified platelet-vWF will be helpful in investigating the mechanism and unique elements of platelet-vWF binding.

REFERENCES

17. Gralnick HR, Williams SB, Coller BS: Fibrinogen competes with von Willebrand factor binding to the glycoprotein GPIIb/IIIa complex when platelets are stimulated with thrombin. Blood 64:797, 1984


Inhibition of platelet-von Willebrand factor binding to platelets by adhesion site peptides

RI Parker and HR Gralnick