Synergistic Inhibition of Platelet Aggregation by Fibrinogen-Related Peptides

Burt Adelman, Chris Gennings, John Strony, and Elizabeth Hanners

We have evaluated the ability of the fibrinogen-related peptides Gly-Arg-Gly-Asp-Ser (GRGDS), Gly-Gln-Gln-His-His-Leu-Gly-Gly-Ala-Lys-Gln-Ala-Gly-Asp-Val (γ-chain peptide), and Gly-Pro-Arg-Pro (GPRP) to inhibit platelet aggregation in platelet-rich plasma individually and in combination. When used alone, GRGDS totally inhibited ADP-induced aggregation of human platelets in platelet-rich plasma; however, the maximum inhibitory effect of the other peptides was less than 80%. The concentrations necessary to inhibit platelet aggregation in plasma by 50% were 100 μmol/l and 1 and 3.2 mmol/l for GRGDS, γ-chain peptide, and GPRP, respectively. When evaluating the effect of peptide mixtures, we discovered that the combination GPRP+GRGDS worked together synergistically (p<0.001, analysis by surface response methodology), whereas GPRP+γ-chain peptide did not. For example, our analysis indicated that a mixture of 50 μmol/l GRGDS plus 180 μmol/l GPRP would produce 50% inhibition of platelet aggregation. This is an effect twofold greater than that produced by 50 μmol/l GRGDS alone, and one that would require an 18-fold greater concentration of GPRP if used alone. These data indicate that the combination GPRP+GRGDS inhibited platelet aggregation in plasma in a synergistic fashion and suggest the potential value of their combined use in antithrombotic therapy. (Circulation Research 1990;67:941–947)

Platelet aggregation is an essential component of normal hemostasis and is dependent on the interaction of the membrane glycoprotein IIb/IIIa (GPIIb/IIIa) complex with plasma adhesive glycoproteins, including fibrinogen, von Willebrand factor, and fibronectin.1–7 The role of GPIIb/IIIa as an activation-dependent receptor for these adhesive proteins has been well established by both in vitro and in vivo studies.1,3,5–8,11 The relative importance of each of the plasma proteins in this process is less clear, and recent studies have suggested that local factors occurring at the site of platelet activation may determine which protein is most important. For example, von Willebrand factor interaction with GPIIb/IIIa may predominate under conditions of high shear stress.12–14

Studies of fibrinogen binding to GPIIb/IIIa have identified two distinct amino acid sequences within the fibrinogen molecule that mediate its attachment to the GPIIb/IIIa receptor. These sequences are Arg-Gly-Asp (RGD), which occurs twice in the fibrinogen Aα chain, and Gly-Gln-Gln-His-His-Leu-Gly-Gly-Ala-Lys-Gln-Ala-Gly-Asp-Val, which is the carboxy-terminus of the fibrinogen γ chain (γ-chain peptide).15–20 The sequence RGD is also present in fibronectin and von Willebrand factor and appears to mediate the binding of each to GPIIb/IIIa.2,21,22

Synthetic peptides containing the RGD sequence or the γ-chain peptide inhibit fibrinogen-dependent platelet aggregation via direct interaction with GPIIb/IIIa.8,15,20–22 Interestingly, peptides that include either sequence can also block the binding of fibronectin or von Willebrand factor to activated platelets despite the fact that neither of these proteins contains the γ-chain sequence.1,15 This later observation suggests that RGD and γ-chain peptides interact with the same or a mutually exclusive site on the GPIIb/IIIa complex.23

The fibrinogen-related peptide Gly-Pro-Arg-Pro (GPRP) has also been identified as an inhibitor of platelet aggregation.24 This sequence, partly analogous to the amino-terminus of the Aα chain (Gly-Pro-Arg-Val-Val) and Bβ chain (Gly-His-Arg-Pro) of fibrin, was first described by Laudano and Doolittle25 as an inhibitor of fibrin gel formation. Plow and Marguerie24 observed that GPRP could inhibit platelet aggregation and determined that this effect was mediated via its direct binding to the fibrinogen D domain. Thus, at least three distinct
Amino acid sequences have been identified that can inhibit platelet aggregation via interference directly or indirectly with GPIIb/IIIa–platelet glycoprotein interactions.

Because aggregated platelets are an important constituent of thrombi that form in atherosclerotic arteries, blockade of the platelet GPIIb/IIIa complex has been proposed as a potential antithrombotic therapy. Recent reports have suggested that RGD-related peptides and antibodies that block glycoprotein binding to GPIIb/IIIa can prevent platelet-dependent thrombus formation in experimental models of coronary artery thrombosis. Use of these agents alone or in combination with other anticoagulants may add an important new dimension to current antithrombotic and thrombolytic treatment strategies. In this study we have examined individually and in combination the activity of peptide inhibitors of platelet aggregation and have tested the combinations Gly-Arg-Gly-Asp-Ser (GRGDS)+GPRP and γ chain+GPRP for synergistic interactions.

Materials and Methods

Materials

The peptides GRGDS, GPRP, and γ-chain peptide used in this study were purchased from Bachem Inc., Torrance, Calif., and Peninsula Laboratories, Inc., Belmont, Calif. The identity and purity of each peptide were documented by the supplier. Purity of each peptide (greater than 98%) was determined by high-performance liquid chromatography with a C18 column eluted with 0.1% trifluoroacetic acid followed by 0.1% trifluoroacetic acid in 50% acetonitrile. The composition of each peptide was confirmed by amino acid analysis. Other chemicals were of reagent grade or better.

Platelet Aggregation and Thrombin Time Studies

Platelet-rich plasma was prepared by centrifugation of blood from normal donors anticoagulated in 0.38% sodium citrate (final concentration). The platelet count was adjusted to 200,000/μl by the addition of autologous plasma. Platelet aggregation studies were conducted using a standard turbidimetric technique in an aggregometer (Sienco Morrison, Colo.). The agonist used was ADP (final concentration, 5 μmol/l). The effect of individual peptides or combinations of peptides on ADP-induced platelet aggregation was studied. Each peptide or combination of peptides dissolved in phosphate buffered saline (PBS; sodium phosphate 0.01 mol/l, sodium chloride 0.15 mol/l, pH 7.4) was added to 200 μl platelet-rich plasma that was stirred at 37°C in an aggregation cuvette. In control experiments, PBS alone was added. In all experiments the volume of material added was held constant. The inhibitory effect of each peptide on platelet aggregation was determined by comparing the maximal change in light transmission after ADP-induced activation of control (only PBS added, maximal response) or peptide-containing platelet-rich plasma (experimental response). This was accomplished by directly measuring (in millimeters) the height of the aggregation tracing at its peak. Results were expressed as percent inhibition of aggregation, calculated using the formula % Inhibition=100 (experimental response/maximal response). Thrombin time determinations were done using the method of Clausen.

Statistical Analysis

The data generated in these studies were analyzed by response surface methods. This methodology has been previously used to describe multi-dimensional dose-response relations and to test for synergy. For the experiments described in this paper, the following logistic model was used to relate the percent inhibition of aggregation produced by individual peptides and combinations:

$$\log [Y/(Y-1)] = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{12} X_1 X_2$$

where Y is the mean response (percent inhibition of aggregation in this study), X1 is the dose of peptide 1, X2 is the dose of peptide 2, β0 is the unknown parameter associated with the background response rate, β1 is the unknown parameter associated with the effect of peptide 1, β2 is the unknown parameter associated with the effect of peptide 2, and β12 is the unknown parameter associated with the interaction between peptides 1 and 2. The method of least squares was used to estimate the model parameters. For the experiments described in this report the model parameters and associated p values are given in Table 1. After the parameters were estimated, χ2 goodness-of-fit test was used to verify the adequacy of the model by comparing the predicted response curves with the observed data.

In the model described above, β12X1X2 is an interaction term that accounts for a response that may be greater or less than additive. If the parameter β12=0 or is not significantly different from zero, the compounds have only an additive relation; if β12 is significantly greater than zero, the compounds act synergistically; and if β12 is significantly less than zero, the compounds combine in a less than additive way. In addition, based on this analysis, a multidimensional plot of the estimated dose-response surface and selected contours, called isobolograms, of constant response were developed for each set of peptide combinations. These isobols predict the single or combined peptide doses necessary to produce a predetermined percent inhibition of aggregation.

Peptide Synthesis

The peptides GPRPGRGDS and GRGDSGPRP were synthesized by solid-phase methods (model 9600 peptide synthesizer, Milligen/Biosearch, Burlington, Mass.) with the appropriately BOC-amino acid–substituted resin (Advanced ChemTech, Louisville, Ky.). Disopropylcarbodiimide was the coupling agent; methylene chloride and dimethylformamide were the reaction solvents. After synthesis, the pep-
tides were deprotected and cleaved from the resin in anhydrous hydrogen fluoride (0°C, 30 minutes) and were extracted from the resin-peptide mixture by successive washes of dimethylformamide, 50% (vol/vol) dimethylformamide in water, water, and 50% (vol/vol) acetic acid in water. The solvent was removed under conditions of reduced pressure and gentle heating (60°C), and the peptide was recovered as the acetate salt by lyophilization from water. The purity of each peptide (greater than 90%) was determined by high-performance liquid chromatography with a C18 column eluted with 0.1% trifluoroacetic acid followed by 0.1% trifluoroacetic acid in 50% acetonitrile. The composition of each peptide was confirmed by amino acid analysis.

Results

Comparative Effects of GRGDS, γ-Chain Peptide, and GPRP on Platelet Aggregation in Platelet-Rich Plasma

As seen in Figures 1A–1C, each of the fibrinogen-related peptides inhibited ADP-induced platelet aggregation in platelet-rich plasma. The order of relative inhibitory activity was GRGDS>γ-chain peptide>GPRP. The concentrations necessary to inhibit aggregation by 50% (IC50) were 100 μmol/l for GRGDS, γ-chain peptide, and GPRP, respectively. Both the potency, on a molar basis, and maximal inhibitory effect of GRGDS were greater than those of the other two peptides.

Inhibition of Platelet Aggregation by Peptide Combinations

We examined the ability of the peptide combinations GRGDS+GPRP and γ-chain peptide+GPRP to inhibit platelet aggregation. Representative results of these experiments are seen in Figures 2A and 2B. The concentrations of GRGDS and the γ-chain peptide used in these combination experiments caused between 10% and 50% inhibition of platelet aggregation when used alone. GPRP was used over a range of concentrations that alone caused less than 10% inhibition of aggregation.

The results of these experiments were analyzed by response surface methods to determine whether the peptide combinations acted in an additive or synergistic fashion. These analyses are displayed graphically in Figures 3A and 3B, and the corresponding regression parameters are listed in Table 1. Analysis of these data indicated that the combination GRGDS+GPRP acted synergistically (response parameter β2>0, p<0.001), while the combination γ-chain peptide+GPRP acted only in an additive fashion (response parameter β2 was not significantly different from zero, p>0.5). Figures 4A and 4B represent isobols developed from the three-dimensional plots. The concave shape of the isobols for the combination GRGDS+GPRP are consistent with a synergistic interaction, and the straight lines describing the combination γ-chain peptide+GPRP are consistent with an additive interaction. The data presented in Figure 3 correspond to the raw data presented in Figure 2. Identical results were obtained with other similar sets of raw data.

### Table 1. Estimates of the Model Parameters for the Interaction of GRGDS+GPRP and γ-Chain Peptide+GPRP

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Estimate</th>
<th>SE</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>GRGDS+GPRP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β0 (Intercept)</td>
<td>-2.524</td>
<td>3.049x10^-1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>β1 (GRGDS)</td>
<td>2.642x10^-2</td>
<td>4.367x10^-3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>β2 (GPRP)</td>
<td>9.734x10^-4</td>
<td>1.276x10^-3</td>
<td>&lt;0.446*</td>
</tr>
<tr>
<td>β12 (Interaction)</td>
<td>1.399x10^-4</td>
<td>3.599x10^-5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>γ-Chain peptide+GPRP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β0 (Intercept)</td>
<td>-2.306</td>
<td>4.136x10^-1</td>
<td>&lt;0.001</td>
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<tr>
<td>β1 (γ-Chain peptide)</td>
<td>3.041x10^-3</td>
<td>7.29x10^-4</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>β2 (GPRP)</td>
<td>2.088x10^-3</td>
<td>1.641x10^-3</td>
<td>&lt;0.203*</td>
</tr>
<tr>
<td>β12 (Interaction)</td>
<td>1.991x10^-6</td>
<td>3.446x10^-6</td>
<td>&lt;0.564*</td>
</tr>
</tbody>
</table>

*Not significant at the 5% significance level.
GPRP inhibits both platelet aggregation and fibrin gel formation. Importantly, the concentration range over which GPRP inhibits fibrin gel formation in plasma is lower than that over which it affects platelet aggregation. As can be seen in Figure 5, we found that thrombin time determinations were markedly prolonged by GPRP when added to plasma at concentrations that caused detectable synergy with GRGDS.

**Effect of the Novel Peptides GRGDGPRP and GPRPGRGDS on Platelet Aggregation**

We synthesized the fusion peptides GRGDGPRP and GPRPGRGDS and determined their effect on platelet aggregation. As seen in Figure 6, the inhibitory activity of each peptide was approximately equivalent to that of GRGDS alone. Neither of these peptides demonstrated enhanced antiplatelet activity.
Discussion

Platelets are a critical component of arterial thrombi, and effective pharmacological inhibition of platelet aggregation is a primary goal of antithrombotic therapy. Previous studies have identified three distinct fibrinogen-derived peptides that are inhibitors of platelet aggregation. The amino acid sequences of these peptides form RGD, γ-chain peptide, and GPRP.\textsuperscript{16–20} RGD-containing peptides and γ-chain–related peptides appear to inhibit platelet aggregation by binding to the platelet receptor complex GPIIb/IIIa and blocking adhesive glycoprotein attachment.\textsuperscript{8,15,20–22} GPRP binds directly to the fibrinogen D domain and inhibits the ability of fibrinogen to bind to GPIIb/IIIa.\textsuperscript{24,25} GPRP is also able to inhibit fibrin gel formation via binding to the D domain of fibrinogen.\textsuperscript{25}

The results of this study confirmed that peptides containing the sequences described above inhibited platelet aggregation in plasma. More importantly, these studies identified a previously unrecognized synergistic interaction between a peptide containing the critical RGD sequence and the peptide GPRP. In contrast, the combined inhibitory activity of the pentadecapeptide derived from the fibrinogen γ chain and GPRP was only additive. The synergistic interaction between the peptides GRGDS and GPRP was detected and statistically confirmed by use of response surface method analysis.\textsuperscript{31,32} This analysis facilitated examination of the platelet inhibitory effect of each peptide combination over broad dose ranges so that variability in the observed data could be properly considered.

Of the individual peptide types used in this study, the RGD-containing peptide GRGDS was the most active inhibitor of platelet aggregation in plasma followed by the γ-chain peptide and then GPRP. We found the IC\textsubscript{50}s in platelet-rich plasma to be 100 μmol/l and 1 and 3 mmol/l for GRGDS, the γ-chain peptide, and GPRP, respectively. It is difficult to compare these results with those of previously published reports. Our studies were conducted in platelet-rich plasma, whereas prior investigators have used only washed platelet systems containing limited amounts of fibrinogen (much less than that found in plasma) and no von Willebrand factor. We found the inhibitory activity of GRGDS in platelet-rich plasma to be about equivalent to its activity in washed platelet systems, whereas the γ-chain peptide and GPRP were less active (by factors of approximately 10–20 and 30, respectively).\textsuperscript{20}

Why the combination GRGDS+GPRP had a synergistic platelet inhibitory action and the combination γ-chain peptide+GPRP did not is not readily apparent. To assure comparability, all experiments were conducted under similar conditions. The mixing experiments included more than one concentration of GRGDS and γ-chain peptide, and the dosages chosen had approximately equivalent inhibitory activity (10–50%). The concentrations of GPRP added to either peptide mixture were well below its IC\textsubscript{50} preventing the possibility of attaining 100% inhibition simply on an additive basis. Laudano et al.\textsuperscript{43} have reported that GPRP binding to the D region of fibrinogen requires that the carboxy-terminus of the γ chain be intact, although direct binding of GPRP to the γ-chain peptide has not been demonstrated. Thus, it is possible that in the mixing experiments GPRP bound to the γ-chain peptide and inhibited its antiplatelet activity. A specific explanation for the observed difference in activity of the two
peptide combinations necessitates a clear understanding of the actual site and mechanism of action of the RGD and γ-chain peptides, information that is not yet available.

In addition to its inhibitory effect on platelet aggregation, the peptide GPRP is able to prevent fibrin gel polymerization.25 The range of concentrations at which GPRP exerts its antifibrin polymerization effect is significantly lower than that needed to inhibit platelet aggregation. As indicated in Figure 5, the concentrations of GPRP that produced a detectable synergistic interaction with GRGDS also caused prolongation of the plasma thrombin time. Thus, at the concentrations used, the combination GRGDS + GPRP acted as a potent inhibitor of both platelet aggregation and fibrin gel formation.

Platelets are an important component of clinically significant arterial thrombi, and many pharmacological strategies have been designed to inhibit platelet aggregation in vivo. Currently, the primary antiplatelet agents available for clinical use act as metabolic inhibitors of platelet activation. Drugs such as aspirin and ticlopidine have some ability to prevent arterial thrombi during chronic use but do not have a predictable effect when given during acute arterial thrombosis.34,35 Prostacyclin or its more stable analogues can be given acutely during active thrombus formation and will rapidly inhibit platelet activity; however, these agents have significant adverse effects, particularly hypotension, that may limit their usefulness in patients with cardiovascular instability.

Agents that interfere with fibrinogen and von Willebrand factor binding to GPIIb/IIIa or with von Willebrand factor binding to GPIb have already been proven effective antithrombotics in in vivo studies. Using models in which platelet-dependent thrombus formation occurred after coronary artery constriction, various investigators have demonstrated inhibition of thrombosis after infusion of a monoclonal antibody that binds to GPIIb/IIIa or by peptides that contained the RGD sequence.9,26–28 Antibody that bound to von Willebrand factor and prevented its attachment to activated platelets also inhibited arterial thrombus formation.36 Similarly, the agent auroincarboxylic acid, which binds to von Willebrand factor and prevents its attachment to GPIb, has been demonstrated in vivo to be a potent inhibitor of platelet-dependent thrombus formation within constricted arteries.37

Inhibition of platelet aggregation by direct blockade of platelet–adhesive glycoprotein interactions is a potentially effective means by which to prevent or arrest acute arterial thrombus formation. Agents that act in this manner will exert their effects rapidly and, because their activity is independent of the mechanism of platelet activation, should be able to inhibit platelet aggregation induced by any agonist. In this study we have identified a unique synergistic antiplatelet interaction between a peptide that contains the RGD sequence and the peptide GPRP. The results of this study may provide a model by which to develop new antithrombotic strategies.

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KEY WORDS • blood platelets • thrombosis • von Willebrand factor • fibrinogen • peptides
Synergistic inhibition of platelet aggregation by fibrinogen-related peptides.

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Circ Res. 1990;67:941-947
doi: 10.1161/01.RES.67.4.941

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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