Effects of the Cell Adhesion Peptide, Arg-Gly-Asp-Ser, on Responses of Washed Platelets From Humans, Rabbits, and Rats

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Fibrinogen is a cofactor in the aggregation of human platelets, and is required for ADP-induced aggregation of washed platelets; however, exogenous fibrinogen is not required for ADP-induced aggregation of washed platelets from rabbits or rats. Because with human platelets the cell adhesion peptide, Arg-Gly-Asp-Ser (RGDS), inhibits aggregation and the binding of fibrinogen to ADP-stimulated platelets, its effects on rabbit and rat platelets were studied to investigate the differences in the fibrinogen requirements of platelets from the three species. RGDS (50 μmol/L) caused >80% inhibition of thrombin-induced or (ADP + fibrinogen)-induced aggregation of human platelets, but only 3% to 9% inhibition of the aggregation of rabbit or rat platelets, regardless of whether fibrinogen was added. RGDS inhibited the binding of fibrinogen to ADP-stimulated human platelets by 80% to 90%, but by only 15% to 27% in the case of rabbit or rat platelets. The differences were due to the species of platelets, since human and rabbit fibrinogens gave similar results. In addition, RGDS failed to displace fibrinogen from the surface of rabbit platelets that had been stimulated with ADP. Thus, there are species differences in the ability of the cell adhesion peptide, RGDS, to block the platelet fibrinogen receptor, even within the mammalian species.

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FIBRINOGEN is required for the aggregation of human platelets, and washed human platelets aggregate only very slightly upon stimulation with ADP in the absence of added fibrinogen under conditions in which there is little release of granule contents; washed platelets from rabbits or rats, on the other hand, are known to aggregate extensively in response to ADP in the absence of added fibrinogen. Both types of platelets, however, exhibit similar patterns of fibrinogen binding when stimulated with ADP. Fibrinogen is bound as the platelets aggregate and dissociates as they deaggregate. We previously reported that ADP, when added to rabbit platelets, causes more fibrinogen to be released into the suspending medium than is the case with human platelets, and recently we also observed that fibrinogen is expressed on the surface of rabbit platelets when they are stimulated with ADP (unpublished results). These observations may explain the lack of requirement for exogenous fibrinogen to support ADP-induced aggregation of washed rabbit platelets.

The sequence, Arg-Gly-Asp, first identified in the cell adhesion domain of human fibronectin, has been established as a widespread cell recognition sequence (for review, see ref. 15). This sequence is also present in rat and bovine fibronectin as well as in several other adhesive proteins, including fibrinogen and von Willebrand factor (vWF).

Synthetic peptides containing an Arg-Gly-Asp sequence inhibit the binding of such proteins to their cell surface receptors, and several investigators have reported that these peptides inhibit the aggregation of human platelets as well as the binding of fibrinogen to stimulated platelets.

This article describes experiments designed to compare the effects of the synthetic peptide Arg-Gly-Asp-Ser (RGDS) on reactions of human, rabbit and rat platelets, and to explore further the reason for the lack of requirement for exogenous fibrinogen for ADP-induced aggregation of rabbit or rat platelets.

MATERIALS AND METHODS

Human fibrinogen (grade L) was from AB Kabi, Stockholm, Sweden; rabbit fibrinogen was prepared as described by Regoeczi; it was 98% thrombin-clottable and showed three bands corresponding to the Aα, Bβ, and γ chains upon reduction followed by polyacrylamide gel electrophoresis in sodium dodecyl sulfate. Human α-thrombin (lot H-1) was kindly supplied by Dr. D. L. Aromson, Bureau of Biologics, FDA, Bethesda, MD. RGDS was from Peninsula Laboratories, Belmont, CA. Bovine albumin (Pentex, fraction V) was from Miles Laboratories, Elkhart, IN. ADP was from Sigma Chemical, St. Louis. F(ab')2 fragments of nonimmune and anti-rabbit fibrinogen IgG were prepared by standard methods from the corresponding IgG (sheep) obtained from Cooper Biomedical, (Cappel), Malvern, PA. Apyrase was prepared from potatoes by the method of Molnar and Lorand; it was dissolved in 0.15 mol/L NaCl at a concentration such that the addition of 3 μmol/L to ADP (9 μmol/L) caused 35% conversion to AMP in 10 minutes at 37°C. Na125I (carrier-free, NEZ 033L) was from New England Nuclear Canada, Lachine, Quebec; fibrinogen and F(ab')2; fragments were iodinated with 125I as described by McFarlane. 14C-Serotonin was obtained as 5-hydroxytryptamine-3H-C creatinine sulfate (54 mCi/mmol) from Amersham, Arlington Heights, IL. 125I-Albumin (0.1 mCi/mg) was from Charles E. Frosst, Montreal. All other chemicals were reagent grade. Reagents were dissolved in 0.15 mol/L NaCl before use, and concentrations are expressed as final values after all additions.

Suspensions of washed human platelets were prepared as previously described using blood drawn from consenting donors; the platelets were suspended at a count of 5 x 107/mL in Tyrode solution containing albumin at 3.5 mg/mL and apyrase at 1.5 μL/mL (Tyrode-albumin). Washed rabbit platelets were prepared...
Typical aggregation curves for rabbit and rat platelets are shown in Fig 1A; the extent of aggregation produced by ADP with ADP added at 0 minutes as indicated by arrows; stimuli used are shown next to aggregation curves. Human fibrinogen was used with human and rat platelets; rabbit fibrinogen was used with rabbit platelets. Final concentrations: thrombin, 0.9 U/mL; ADP, 9 μM/L; fibrinogen, 0.19 to 0.22 μM/L; platelet counts, 4.6 x 10^8/mL for human and rat platelets and 6.3 x 10^7/mL for rabbit platelets.

Fig 1. Typical aggregation responses of washed platelets stimulated with thrombin, ADP, or ADP in the presence of added fibrinogen (FBG). (A) Human platelets. (B) Rabbit platelets. (C) Rat platelets. Aggregating agents were added at 0 minutes as indicated by arrows; stimuli used are shown next to aggregation curves. Human fibrinogen was used with human and rat platelets; rabbit fibrinogen was used with rabbit platelets. Final concentrations: thrombin, 0.9 U/mL; ADP, 9 μM/L; fibrinogen, 0.19 to 0.22 μM/L; platelet counts, 4.6 x 10^8/mL for human and rat platelets and 6.3 x 10^7/mL for rabbit platelets.

Fig 2. Inhibition by RGDS of the extent of aggregation of human platelets. ADP-induced aggregation in the presence of added human fibrinogen (typical of three experiments) (O). Thrombin-induced aggregation (two experiments) (△). Concentrations used: ADP, 9 μM/L; thrombin, 0.9 U/mL; fibrinogen 0.19 μM/L (with ADP only); platelet count, 4.6 x 10^8/mL. In all cases, SD was ±4%.

RESULTS

Typical aggregation curves for thrombin-stimulated and ADP-stimulated human platelets are shown in Fig 1A: the extent of aggregation produced by ADP with ADP added at 0 minutes as indicated by arrows; stimuli used are shown next to aggregation curves. Human fibrinogen was used with human and rat platelets; rabbit fibrinogen was used with rabbit platelets. Final concentrations: thrombin, 0.9 U/mL; ADP, 9 μM/L; fibrinogen, 0.19 to 0.22 μM/L; platelet counts, 4.6 x 10^8/mL for human and rat platelets and 6.3 x 10^7/mL for rabbit platelets.

Typical aggregation curves for rabbit and rat platelets are shown in Figs 1B and C, respectively; in both cases, ADP caused extensive aggregation whether or not fibrinogen had been added to the platelet suspension.

Human platelets. The extent of aggregation of human platelets stimulated with ADP (9 μM/L) in the presence of added human fibrinogen (0.19 μM/L), as measured by the increase in light transmission through the platelet suspension, was inhibited in a dose-dependent manner by the addition of RGDS, as shown in Fig 2. At high concentrations of RGDS, 46 to 50 μM/L, inhibition was as high as 80% to 90%. When human fibrinogen was replaced by rabbit fibrinogen, the addition of RGDS at 24 μM/L caused a 50% decrease in aggregation peak height, similar to the 61% decrease observed in the presence of human fibrinogen. Aggregation in response to thrombin (0.9 U/mL) was inhibited to the same extent as that observed when ADP was the agonist (Fig 2), although release of 14C-serotonin (~80%) was not inhibited by the addition of RGDS even at 24 or 48 μM/L, concentrations that inhibited the extent of aggregation by up to 83%. When RGDS was added after the thrombin it did not cause deaggregation, but aggregation was partially inhibited, the degree of inhibition decreasing progressively as the interval between the additions of thrombin and RGDS was lengthened. Using RGDS at concentrations (24 to 35 μM/L) that caused ~50% inhibition of aggregation when added before thrombin, the extent of inhibition was decreased to 34% when RGDS was added 15 seconds after the thrombin and to 10% when added after 60 seconds. Under the same conditions, release of 14C-serotonin was essentially complete by 15 seconds. The small aggregation response to ADP (9 μM/L) in the absence of added fibrinogen (Fig 1A) was also inhibited by the prior addition of RGDS; this response was inhibited by 60% when RGDS was added at the low concentration of 12 μM/L and to the extent of 80% to 100% by the addition of RGDS at ≥17 μM/L, although precise values for inhibition were difficult to determine because of the very low aggregation peak height involved. The inhibition by RGDS of the binding of human fibrinogen to ADP-stimulated human platelets (Fig 3) was also dose dependent; the maximum inhibition
observed was between 80% and 90% at RGDS concentrations of ≥24 μmol/L.

**Rabbit platelets.** In contrast to human platelets, washed rabbit platelets aggregated in response to ADP to approximately the same extent regardless of whether fibrinogen had been added (Fig 1B); the same results were obtained with both human and rabbit fibrinogens. The addition of RGDS up to a final concentration of 48 μmol/L had only a small effect on the extent of aggregation of rabbit platelets stimulated with ADP at 9 μmol/L; with or without the addition of fibrinogen (0.19 μmol/L) the extent of inhibition was ~3%, whereas with human platelets inhibition was >80% at this concentration of RGDS. The aggregation of rabbit platelets in response to thrombin (0.9 U/mL) also was inhibited only slightly by RGDS at 48 μmol/L: 9% inhibition of aggregation peak height (compared with >80% with human platelets); RGDS had no effect on the release of 14C-serotonin (94%). Higher concentrations of RGDS caused slightly more inhibition of ADP-induced aggregation of rabbit platelets, to a maximum of only 31% when RGDS was added at 400 μmol/L. The inhibition of binding of 125I-fibrinogen to rabbit platelets stimulated with ADP at 9 μmol/L is shown in Fig 3. The binding of rabbit fibrinogen was inhibited to a slightly greater extent than that of human fibrinogen, but in neither case was the inhibition as high as observed with human platelets: a maximum inhibition of 27% with rabbit fibrinogen and 15% with human fibrinogen by RGDS at ~46 μmol/L as compared with 80% to 90% for human platelets.

We have observed, by measuring the binding to platelets of specific antifibrinogen antibodies, that ADP stimulation results in the expression of fibrinogen on the surface of rabbit platelets but not human platelets (unpublished observations). Because this fibrinogen might be available to support ADP-induced aggregation of rabbit platelets, it was of interest to determine whether RGDS blocked its expression on the platelet surface. Binding of 125I-F(ab')2, fragments of nonimmune and anti-rabbit fibrinogen IgG to formaldehyde-fixed rabbit platelets was measured with and without RGDS (50 μmol/L). The results (Table 1) indicate that there was nonspecific binding of both nonimmune and antifibrinogen F(ab')2 to resting platelets, and that the amount of nonimmune F(ab')2 that bound to platelets stimulated with ADP was not significantly different from the amount that bound to resting platelets. In agreement with our previous observations, however, more antifibrinogen F(ab')2 bound to platelets that had been stimulated with ADP than to resting platelets, an increase of 620 ng/10^9 platelets. This represents the expression of fibrinogen on the surface of rabbit platelets as a result of the ADP treatment. RGDS did not inhibit this surface expression of fibrinogen. When platelets were stimulated with ADP in the presence of RGDS (50 μmol/L) the increase in antifibrinogen F(ab')2 bound was 540 ng/10^9 platelets, not significantly different (p > 0.05) from the value observed without RGDS.

**Rat platelets.** Like rabbit platelets, washed rat platelets aggregated in response to ADP to approximately the same extent regardless of whether fibrinogen had been added (Fig 1C). As observed in the case of rabbit platelets, responses to ADP or thrombin were not extensively inhibited by RGDS. The extent of aggregation in response to ADP at 9 μmol/L, either with or without added human fibrinogen (0.19 μmol/L), was decreased by ~3% by RGDS at 48 μmol/L, and there was no inhibition of the aggregation or release of 14C-serotonin (72%) induced by thrombin (0.9 U/mL). Inhibition of binding of 125I-fibrinogen (human) to ADP-stimulated rat platelets is shown in Fig 3; the maximum inhibition by RGDS (48 μmol/L) was 27%, in the same range as that observed for rabbit platelets.

**DISCUSSION**

The sequence Arg-Gly-Asp represents a general cell attachment sequence, and synthetic peptides containing this

![Inhibition of Binding of 125I-F(ab')2 Fragments of Nonimmune and Anti-rabbit Fibrinogen IgG to Washed Rabbit Platelets Before and After Stimulation with ADP](image)

**Table 1.** Effect of RGDS on Binding of 125I-F(ab')2 Fragments of Nonimmune and Anti-rabbit Fibrinogen IgG to Washed Rabbit Platelets Before and After Stimulation with ADP

<table>
<thead>
<tr>
<th>RGDS</th>
<th>125I-F(ab')2 Bound (ng/10^9 Platelets)*</th>
<th>Nonimmune</th>
<th>Antifibrinogen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Resting Platelets</td>
<td>Stimulated Platelets</td>
<td>Increase Upon ADP Stimulation</td>
</tr>
<tr>
<td>Without RGDS</td>
<td>318 ± 82</td>
<td>304 ± 94</td>
<td>-14 ± 2</td>
</tr>
<tr>
<td>With RGDS (50 μmol/L)</td>
<td>435 ± 168</td>
<td>386 ± 191</td>
<td>-49 ± 6</td>
</tr>
</tbody>
</table>

Concentrations: ADP, 9 μmol/L; platelet count, 0.63 x 10^9/mL; 125I-F(ab')2 added at 66.7 μg to 10^9 platelets.

*Expressed as means ± SD of values from three experiments.

†These values are not significantly different (P > 0.05).
RGDS AND HUMAN, RABBIT, AND RAT PLATELETS

sequence inhibit many reactions of cells mediated by the binding of adhesive proteins such as fibronectin, fibrinogen, or vWF, all of which contain the Arg-Gly-Asp sequence. Such peptides prevent the binding of these proteins to activated human platelets and inhibit platelet aggregation by ADP or thrombin. Because the same sequence has been identified in rat and bovine as well as human fibronectins, and since Arg-Gly-Asp-containing peptides inhibit the adhesion of cells in a broad range of vertebrate species including human, rat, mouse, chicken, and bovine as well as in several invertebrates, the inhibitory effects of the synthetic peptide, RGDS, on fibrinogen-dependent reactions of rabbit and rat platelets were expected to be similar to those already observed with human platelets.

The present results confirm the findings of previous investigators that, in the case of human platelets, RGDS inhibits thrombin-induced and ADP-induced platelet aggregation, as well as fibrinogen binding to ADP-stimulated platelets, in a dose-dependent manner. RGDS inhibits thrombin-induced aggregation of human platelets without preventing the release of granule contents, and thus it clearly causes inhibition of aggregation mediated by secreted fibrinogen as well as by exogenous fibrinogen. Because RGDS has been reported to cause detachment of cells from their substrate, it might have been expected to cause deaggregation of thrombin-stimulated platelets, and its failure to do so is probably a reflection of the nonreversible stabilization of platelet aggregates upon release of granule contents. Because it caused partial inhibition of aggregation even when added after release had taken place, however, it may have displaced some of the fibrinogen bound to the platelet surface but not yet irreversibly bound. With rabbit and rat platelets, on the other hand, RGDS was a much less effective inhibitor of aggregation and fibrinogen binding. Thus, with human platelets, RGDS at a concentration of ≥40 μmol/L produced 80% to 90% inhibition of both ADP-induced and thrombin-induced aggregation as well as of fibrinogen binding to ADP-stimulated platelets, but at 48 μmol/L it caused <10% inhibition of aggregation of rabbit or rat platelets and the maximum inhibition of fibrinogen binding was 27%. Even at a much higher concentration of RGDS, the inhibition of aggregation of rabbit platelets did not approach that observed with human platelets. The small aggregation response of human platelets to ADP in the absence of added fibrinogen was 80% to 90% inhibited by RGDS at the low concentration of 17 μmol/L, whereas a concentration three times as great caused only 3% inhibition of the much more extensive aggregation of rabbit or rat platelets without added fibrinogen. That these differences were due to the platelet species rather than the species of fibrinogen used, was shown in two ways: (a) with human platelets, RGDS inhibited ADP-induced aggregation in the presence of either human or rabbit fibrinogen, and (b) with rabbit platelets, RGDS failed to inhibit the binding of both human and rabbit fibrinogens upon ADP stimulation. Thus, RGDS caused extensive inhibition with human platelets, but not with rabbit platelets, regardless of the species of fibrinogen. Although rat fibrinogen does not contain the RGDS sequence in the position corresponding to Arg(572 through 575), Plow and colleagues recently showed that the binding of rat fibrinogen to human platelets is inhibited by RGDS and other Arg-Gly-Asp-containing peptides, confirming that the differences we have observed in RGDS inhibition are associated with the species of platelets rather than the species of fibrinogen. Fibrinogen was detected on the surface of rabbit platelets that had been activated with ADP, perhaps explaining the lack of requirement for exogenous fibrinogen to support ADP-induced aggregation. This surface-expressed fibrinogen was not displaced by RGDS, however, nor was ADP-induced aggregation inhibited as would have been expected by analogy with the inhibition of thrombin-induced aggregation of human platelets. Thus, there are differences in the ability of RGDS to block the fibrinogen receptor on platelets from different species, even when they are as closely related as human and rabbit or rat. This finding was unexpected in view of the broad range of adhesive reactions mediated by proteins or peptides containing the Arg-Gly-Asp sequence and serves as a warning to exercise caution in generalizing from experiments in which animal models of different species have been used.

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REFERENCES


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