RGDV Peptide Selectively Inhibits Platelet-dependent Thrombus Formation In Vivo

Studies Using a Baboon Model

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Abstract

Since platelet hemostatic functions are mediated in part through the binding of adhesive proteins containing an RGD (Arg-Gly-Asp) recognition sequence, and since platelet reactions may be inhibited in vitro by RGD-containing peptides, we assessed in vivo the antithrombotic activity of RGDV (Arg-Gly-Asp-Val) tetrapeptide using a baboon thrombosis model. Thrombus formation was induced by a device consisting of a tubular segment coated with type I collagen, followed by two regions of expanded diameter exhibiting disturbed flow and stasis. The thrombogenic device was incorporated into femoral arteriovenous shunts under conditions of intermediate wall shear rate (100 s^{-1}). Thrombus formation was measured by scintillation camera imaging of ^{111}In-platelets and by counting of ^{125}I-fibrinogen/fibrin. Thrombus that formed on the collagen substrate was rich in platelets, while thrombus formed in the disturbed flow regions was rich in fibrin and red cells.

RGDV peptide was infused proximal to the thrombogenic device to maintain local plasma concentrations of 25, 50, and 100 μM. Infused RGDV decreased the accumulation of both platelets and fibrin on the collagen substrate in a dose-response manner. At the highest dose platelet and fibrin deposition after 40 min was reduced by > 80% ($P < 0.01$). In the region of disturbed flow, RGDV (100 μM) reduced platelet deposition by 85% ($P < 0.01$) but did not reduce the accumulation of fibrin ($P < 0.3$). Similarly, the peptide inhibited the release of granular proteins from platelets associated with thrombus (platelet factor 4, β-thromboglobulin; $P < 0.01$), but did not prevent the appearance of fibrinopeptide A in circulating blood ($P > 0.1$). No systemic alterations in blood pressure, bleeding time, or platelet aggregation ex vivo were produced by locally infused RGDV. The antithrombotic effects of RGDV peptide disappeared within 5 min after discontinuing the infusion. In control studies infused RGEV (Arg-Gly-Glu-Val, 100 μM) showed no antithrombotic activity. Thus, RGDV selectively blocks platelet-dependent thrombus formation in vivo.

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Introduction

After activation by physiologic agonists, platelets may bind adhesive glycoproteins, including fibrinogen, fibronectin, and vWf, through interactions with the platelet glycoprotein (GP)[1] IIb-IIIa receptor (1, 2). Fibrinogen binding mediates platelet aggregation (3, 4), which can also be supported by vWf binding to GP IIb-IIIa (5). Fibronectin and vWf are also present in subendothelial tissues, and may be involved in platelet GP IIb-IIIa–mediated adhesion and spreading at sites of vessel injury (6, 7). The importance of these mechanisms for normal hemostasis in man has been shown by the observation that hereditary abnormalities of GP IIb-IIIa, or the presence of autoantibodies against this receptor, may produce a severe bleeding tendency (8, 9). In addition, MAbs against GP IIb-IIIa have proven to be potent antithrombotic and antihemostatic agents in animal models of arterial disease (10–12), and have thus been proposed for clinical applications (13–15).

Because many adhesive proteins interact with platelet GP IIb-IIIa through a common RGD (Arg-Gly-Asp) peptide recognition sequence, which is also found in other adhesive molecules (e.g., collagen, laminin, thrombospondin, and vitronectin; 16–21), synthetic RGD-containing peptide analogues of the cell-binding domains of adhesive proteins may also represent an important therapeutic strategy. In vitro such peptides have been shown to block adhesive protein binding to platelets (18, 21–23) and inhibit both platelet adhesion and aggregation (18, 20–26). However, their effectiveness for antithrombotic therapy in vivo remains undefined. We have therefore evaluated the capacity of infused RGDV (Arg-Gly-Asp-Val) peptide to block both platelet and fibrin components of forming thrombus in vivo using a recently described baboon model (27). RGDV was evaluated because of its affinity for platelet GP IIb-IIIa (22). A baboon model was chosen because this species is hemostatically similar to man (28).

Methods

Animals studies. 11 normal male baboons (*Papio anubis/cynocephalus*) were used. The animals received either isotonic saline (controls), RGDV peptide, or RGEV peptide, all administered by continuous infusion as described below. Platelet counts (mean, 347±15 × $10^3/\mu$l), hematocrits (32.6±0.7%), and fibrinogen levels (3.7±0.2 mg/ml) were determined as reported elsewhere (27), and were equivalent between the different study groups. All animals had a chronic exteriorized silicone rubber arteriovenous shunt surgically placed between the femoral artery and vein (12, 27). These shunts do not shorten platelet survival detectably or produce measurable platelet activation

1. *Abbreviations used in this paper:* FPA, fibrinopeptide A; GP, glycoprotein; PF4, platelet factor 4; βTG, β-thromboglobulin.

(12). In each experiment a thrombogenic device was inserted into the shunt system as an extension segment and exposed to non-anticoagulated blood for 40 min. The device consisted of a tubing segment (2 cm length, 3.2 mm i.d.) coated with covalently bound type I collagen, followed by two regions of expanded diameter (2 cm length, 9.3 mm i.d.) exhibiting flow recirculation and stasis. Blood flow was maintained at 20 ml/min using a roller pump (Cole-Parmer Instrument Co., Chicago, IL). The wall shear rate in the collagen tubing segment was 100 s^{-1}. As discussed elsewhere (27), this device generates a complex thrombus in vivo having a proximal region rich in platelets (collagen segment) simulating arterial thrombus formation, and a distal component (disturbed flow region) enriched in fibrin and red cells as observed in venous thrombosis.

Autologous baboon blood platelets were labeled with 1 mCi ^{111}In-oxine according to a method described previously (27). After allowing at least 1 h for the reinfused cells to distribute within the vasculature, the thrombogenic device was incorporated into the shunt system and exposed to native blood for 40 min. The accumulation of ^{111}In-platelets within each device region was measured in real time using a gamma scintillation camera (model 4/11; Picker Corp., Highland Heights, OH). Data were analyzed using a computer-assisted image processing system (Medical Data Systems A^3; Medtronic Inc., Minneapolis, MN) interfaced with the camera (27). Images were acquired at 5-min intervals. The total number of deposited platelets (labeled plus unlabeled cells) was calculated by dividing the deposited platelet activity (counts per minute) by the whole blood platelet ^{111}In-activity (counts per minute/milliliter) and multiplying by the circulating platelet count (platelets/milliliter) (12, 27).

Homologous baboon fibrinogen was purified and labeled with ^{125}I as described (27). The labeled fibrinogen preparation was > 90% clottable by thrombin. 10 min before initiating thrombus formation 5 μCi of ^{125}I-fibrinogen was injected intravenously. After blood exposure for 40 min the device was thoroughly rinsed with isotonic saline and cut into regions corresponding to the collagen segment and expanded flow regions (27). After allowing at least 30 d for the ^{111}In to decay ($t_{1/2}$, 2.8 d) the device components were counted for ^{125}I-fibrinogen radioactivity using a gamma counter. Total fibrin accumulation was calculated by dividing the deposited ^{125}I-radioactivity (counts per minute) by the clottable fibrinogen radioactivity (counts per minute/milliliter) and multiplying by the plasma fibrinogen concentration (milligrams/milliliter) as measured in each experiment.

Blood pressure was continuously measured using a monitor (model 2101; Honeywell Inc., Denver, CO) and pressure transducer connected directly to the arterial inlet of the arteriovenous shunt. Bleeding times were performed on the shaved volar surface of the forearm of the baboon using the standard template method (29). All procedures were approved by the Institutional Animal Care and Use Committee in accordance with federal guidelines (Guide for the Care and Use of Laboratory Animals, 1985).

Peptide preparations. Peptides were prepared by the method of simultaneous multiple peptide synthesis, and characterized by HPLC as described (30). Both RGDV and RGEV peptides were diluted in saline before use. RGDV peptide was infused proximal to the device at rates sufficient to maintain local plasma concentrations of 25, 50, and 100 μM. The infusion rates were 0.35±0.01, 0.68±0.02, and 1.31±0.06 μmol/min at the three plasma concentrations, respectively, and were calculated on the basis of a controlled blood flow rate (20 ml/min) and hematocrit measurements in each animal. RGEV peptide was infused at a rate of 1.39±0.03 μmol/min to achieve a local concentration of 100 μM within the extracorporeal circuit. These doses were chosen because in vitro studies showed that 100 μM RGDV peptide was the minimum concentration required to effectively abolish platelet aggregation in response to ADP and collagen. Under the same conditions, RGEV peptide (up to 1 mM) had no effect on platelet aggregation.

The duration of the antithrombotic effects of infused RGDV peptide was determined in a separate group of three animals. In this study RGDV peptide was infused proximal to the thrombogenic device (1.33±0.03 μmol/min) to provide a local peptide concentration of 100 μM. After 40 min the RGDV infusion was discontinued and saline alone was infused for an additional 40-min interval. ^{111}In-Platelet imaging was performed throughout the 80-min exposure period. In separate control studies performed in the same animals, saline alone was infused for a period of 40 min.

Laboratory procedures. RIAs for β-thromboglobulin (βTG), platelet factor 4 (PF4), and fibrinopeptide A (FPA) were performed as previously described (27) on blood samples drawn before and at the end of each experiment. The latter samples were drawn immediately distal to the device and therefore reflected the local, rather than systemic, concentrations of the markers assayed.

Platelet aggregation studies were performed using an aggregometer (Chrono-Log Corp., Havertown, PA) as reported elsewhere (29). The concentration of platelets in platelet-rich plasma was adjusted to 250,000/μl. The agonists used were ADP (Sigma Chemical Co., St. Louis, MO) and collagen (Hormon Chemie, Munich, FRG). Results were expressed as the concentration of agonist required to produce 50% of the maximum achievable increase in light transmission (ED_{50}) through the stirred suspensions of platelet-rich plasma (29). At the end of each experiment blood was drawn from sites proximal and distal to the site of thrombus formation to evaluate both local and systemic effects of the infused peptides.

Data analysis. The two-tailed t test for paired or unpaired groups was used when the data were normally distributed (Wilk-Shapiro test). Otherwise, the Wilcoxon sign rank test was chosen. All data are given as mean±1 SE.

Results

In four animals studied before device exposure the baseline agonist concentrations required to produce a half-maximal platelet aggregation response (ED_{50}) were 1.7±0.3 μM for ADP and 1.5±0.5 μg/ml for collagen. After device exposure for 40 min in control studies (saline infusion only), the ED_{50} values determined with blood samples taken distal to the device were equivalent to control values (ADP, 1.7±0.5 μM; collagen, 1.4±0.5 μg/ml; $P > 0.5$), indicating that platelet aggregation was unaffected by device placement over the study interval.

Similarly, the aggregation response in effluent blood was unchanged in four animals having infused RGDV peptide at plasma concentrations of 25 and 50 μM ($P > 0.5$ vs. control results in both cases). In animals infused with RGDV peptide at a local concentration of 100 μM, platelet aggregation was inhibited in response to both ADP (ED_{50}, 3.4±0.5 μM; $P < 0.05$) and collagen (ED_{50}, 3.0±0.5 μg/ml; $P < 0.01$). In four animals given the RGEV peptide (100 μM) the ED_{50} results were unchanged vs. control values ($P > 0.5$ for both agonists).

The peptide infusions were without systemic effects. Thus, for all doses of RGDV and RGEV peptides, the ED_{50} values measured in arterial blood sampled proximal to the site of peptide infusion were equivalent to baseline values measured in the same animals ($P > 0.5$ in each case). In addition, infusion of RGDV peptide at the highest dose caused no change from baseline values in either the bleeding time, measured after 15 min of peptide infusion (5.1±0.9 vs. 4.0±0.5 min; $P > 0.2$), or the systolic blood pressure, monitored throughout the study interval (137±16 vs. 135±16 mmHg; $P > 0.5$).

The effects of peptide infusion on thrombus formation associated with the collagen substrate are shown in Fig. 1. RGDV peptide (25–100 μM) reduced platelet accumulation in a dose-dependent manner (Fig. 1 *A*). After 40 min of blood exposure the highest dose reduced platelet deposition by > 80% vs. control values ($3.0\pm0.6 \times 10^8$ vs. $15.6\pm2.6 \times 10^8$ platelets; $P < 0.01$). Similarly, fibrinogen/fibrin deposition

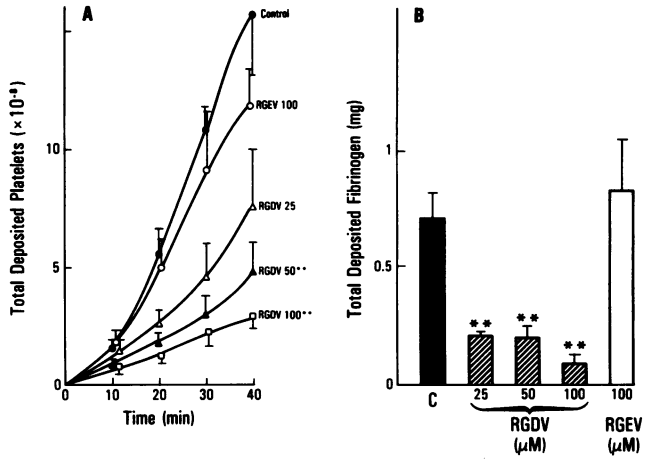

Figure 1. Effect of RGDV and RGEV peptides on thrombus formation on collagen. (*A*) The accumulation of platelets over 40 min of blood exposure was reduced vs. control values (saline infusion) by RGDV peptide (25–100 μM) in a dose-response manner. RGEV peptide (100 μM) was ineffective. (*B*) The accumulation of fibrinogen/fibrin after 40 min of blood exposure was reduced vs. control values (*C*) by RGDV peptide (25–100 μM), but not by RGEV peptide (100 μM). $^{**}P < 0.01$.

onto collagen was reduced significantly by RGDV peptide at all doses tested (Fig. 1 *B*). At the highest dose the collagen substrate accumulated only 0.09±0.04 mg of fibrinogen (vs. 0.71±0.11 mg in the control studies; $P < 0.01$). The RGEV peptide (100 μM) had no effect on the accumulation of either platelets or fibrinogen (Fig. 1, *A* and *B*).

Measurements of thrombus formation in the annular regions of low shear flow are given in Fig. 2. The dose-response effects of infused RGDV peptide on platelet accumulation in the low shear regions (Fig. 2 *A*) were similar to those observed with the proximal collagen segment; i.e., at the highest dose of peptide the deposition of platelets after 40 min was reduced vs.

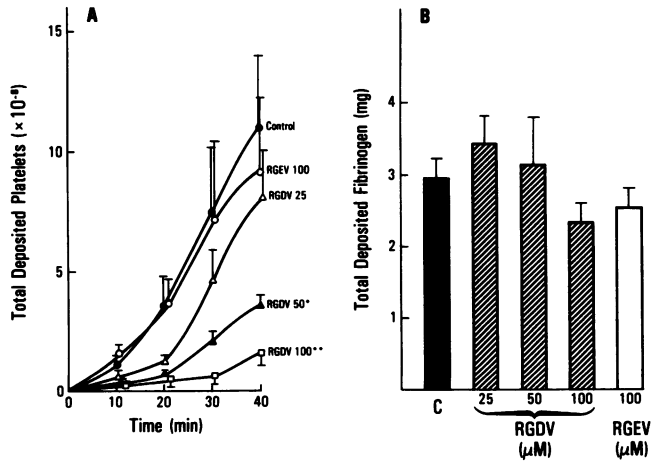

Figure 2. Effects of RGDV and RGEV peptides on thrombus formation within annular regions of low fluid shear. (*A*) The accumulation of platelets was reduced vs. control values (saline infusion) by RGDV peptide (25–100 μM) in a dose-response manner. RGEV peptide (100 μM) was ineffective. (*B*) The accumulation of fibrinogen/fibrin was not reduced vs. control values (*C*) by RGDV (25–100 μM) or RGEV peptides (100 μM). $^{*}P < 0.05$; $^{**}P < 0.01$.

control values by ~ 85% ($1.7\pm0.5 \times 10^8$ vs. $11.1\pm2.9 \times 10^8$ platelets; $P < 0.01$). However, in contrast to the results observed with the collagen surface, RGDV peptide (25–100 μM) had no effect on the accumulation of fibrinogen/fibrin in the regions of low shear flow ($P > 0.3$ vs. control values; Fig. 2 *B*). Infused RGEV peptide (100 μM) was without effect on the deposition of either platelets or fibrinogen (Fig. 2, *A* and *B*).

Markers of platelet activation (βTG and PF4) and fibrinogen cleavage by thrombin (FPA) were assayed in effluent blood sampled distal to the site of thrombus formation (Fig. 3). Infused RGDV peptide (25–100 μM) reduced the plasma levels of βTG and PF4 in a dose-response manner ($P < 0.01$ vs. control values at 100 μM RGDV), consistent with the observed reduction in platelet thrombus formation (Figs. 1 *A* and 2 *A*). However, FPA levels were not reduced significantly by RGDV peptide (25–100 μM; $P > 0.1$), and this result was in accord with the inability of the peptide to reduce the accumulation of fibrin in the regions of low shear flow (Fig. 2 *B*). Infusion of RGEV peptide (100 μM) had no effect on either the markers of platelet activation or thrombin formation (Fig. 3).

To determine the duration of antithrombotic effects after RGDV administration, platelet deposition on collagen was measured in three animals infused with RGDV peptide (100 μM) for 40 min, then with saline for a subsequent 40-min period (Fig. 4). After 40 min of RGDV peptide infusion, total platelet accumulation on the collagen substrate plateaued at a level that was reduced by > 85% vs. the number of platelets deposited in control studies. Within 5 min after discontinuing the RGDV infusion, platelet deposition onto collagen rapidly increased. Over the 40–80-min period both the rate and extent of platelet deposition on the collagen substrate were equivalent to results obtained in the control studies (Fig. 4).

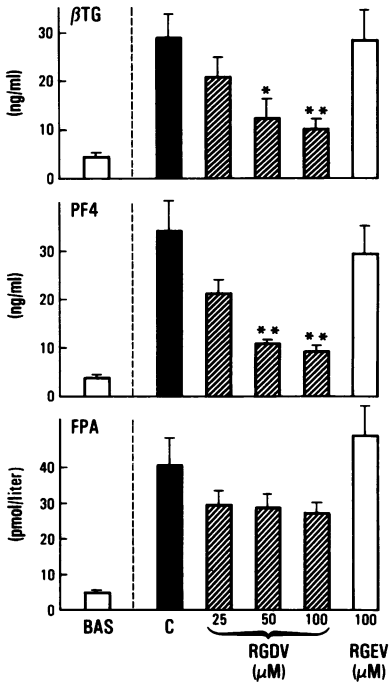

Figure 3. Effects of RGDV and RGEV peptides on the release into plasma of platelet-specific proteins (β-TG, PF4) and FPA. All samples were taken distal to the site of thrombus formation after 40 min exposure to native blood. Control values (*C*) for each measurement were elevated significantly ($P < 0.01$) above baseline values (*BAS*) taken before device placement. Increased levels of β-TG and PF4 observed after device placement were reduced by RGDV peptide (25–100 μM) in a dose-response manner, but not by RGEV peptide (100 μM). FPA levels were not reduced significantly by either peptide at these doses. $^{*}P < 0.05$; $^{**}P < 0.01$.

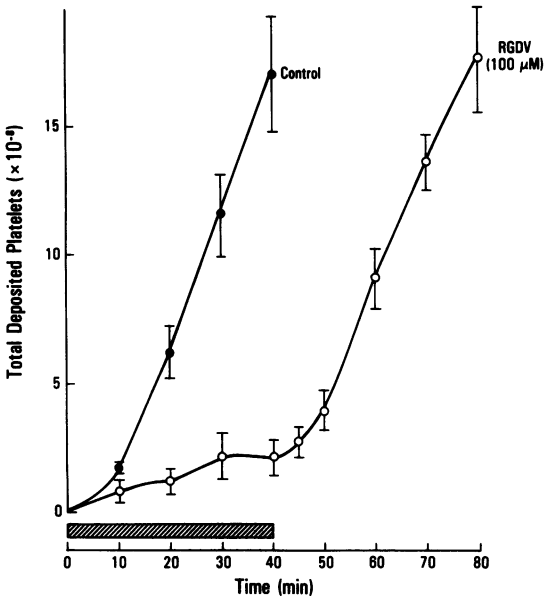

Figure 4. Duration of antithrombotic effects of infused RGDV peptide. Platelet accumulation on collagen was measured in three animals infused for 40 min with RGDV peptide to achieve a local concentration of 100 μM, then infused for the next 40-min interval with saline only (*open circles*). In three control studies the same animals were also infused with saline alone for 40 min (*closed circles*). Platelet deposition on the collagen surface was reduced by > 85% vs. control studies after the first 40 min of RGDV infusion. After discontinuing the peptide infusion, both the rate and extent of platelet accumulation over the 40–80-min period were equivalent to the results obtained in control experiments.

Discussion

This study demonstrates that the RGDV peptide is a potent antithrombotic agent in vivo, as shown by its ability to block both the platelet and fibrin components of thrombus formed on a collagen substrate under arterial flow conditions. Inhibition of the capacity of platelets to participate in thrombus formation was rapidly reversible; no antithrombotic effects could be demonstrated within 5 min after discontinuing the infusion of peptide. Interestingly, the peptide also blocked the accumulation of platelets, but not fibrin, in thrombus formed under conditions of low shear flow recirculation and stasis. Specificity was documented by control studies with RGEV peptide, which exhibited no antithrombotic effects.

The selective inhibition of platelets by RGDV peptide is consistent with previous observations showing that adhesive proteins (e.g., fibrinogen, fibronectin, and vWf) mediate in part platelet cell–cell and cell–surface interactions after binding to the platelet GP IIb-IIIa receptor through a common RGD recognition sequence (16–21). Thus, synthetic analogues of specific adhesive protein binding domains, including the carboxy-terminal dodecapeptide of the fibrinogen γ-chain, have been shown to interfere with GP IIb-IIIa binding functions, and to inhibit platelet aggregation and static platelet adhesion in vitro (20–24). Peptide antagonists of GP IIb-IIIa have also been shown to inhibit platelet deposition onto collagen or subendothelium under flow conditions in vitro, especially at high shear rates (24–26, 31). Since these observations suggested an in vivo therapeutic potential for agents directed against platelet GP IIb-IIIa, we evaluated the RGDV tetrapeptide in a primate model involving exposure of a thrombogenic device to non-anticoagulated blood.

The device has been previously described (27), and consisted of a tubular segment coated with type I collagen, followed by two regions of expanded diameter exhibiting annular vortex formation and low fluid shear. Although this design is hemodynamically complex, it was chosen because the components of forming thrombus are both platelet dependent (collagen segment) and coagulation dependent (low shear regions). The device was placed in an extracorporeal circuit that was nonetheless subject to normal systemic dilution, filtration, and inactivation mechanisms in the host animal. Thus, while the collagen segment accumulated approximately the same number of platelets in control studies as the annular flow region (Figs. 1 and 2), the annular region accumulated nearly four times as much fibrin as the collagen substrate. Overall, the ratio of deposited platelets to fibrin was six times higher for thrombus formed on the collagen surface than for thrombus formed within the region of low shear flow. The importance of coagulation-dependent mechanisms in the low shear region was also shown previously by observations that heparin sensitively inhibits thrombus formation under these flow conditions (27).

In control studies this test system caused the rapid formation of large platelet thrombi. Thus, the exposure time to native blood was limited to 40 min, as embolization predictably occurs at later times (27). Previous morphological observations have also shown that forming thrombi quickly obscure the underlying collagen substrate surface (27). Further, the magnitude of cell deposition seen in the present study (~ 10^9 platelets/cm^2 of collagen surface; Fig. 1) also indicates that platelet thrombus consisted primarily of aggregated cells, with adherent platelets contributing relatively little to the total thrombus mass.

The concentrations of RGDV peptide achieved by local infusion in vivo were determined on the basis of in vitro measurements showing that platelet aggregation was abolished by 100 μM RGDV. This plasma concentration of infused peptide also markedly reduced platelet thrombus formation in vivo, but was without systemic effects on blood pressure or bleeding tendency. At the higher doses infused (50–100 μM) RGDV peptide blocked the accumulation of platelets on both the collagen substrate and within the regions of low shear flow. By comparison with results of a previous study (27) the RGDV peptide (100 μM) was more effective for blocking thrombus formation in this model than the potent platelet inhibitor prostacyclin, which was infused at a relatively high concentration (96 nM). While the reduction by RGDV peptide in the small amount of fibrin deposited on collagen may have been secondary to the reduction in platelet numbers (Fig. 1), the selectivity of the peptide for blocking platelet interactions was clearly shown by the observation that the overall accumulation of fibrin, which occurred predominantly within the regions of flow expansion, was unaffected by RGDV peptide infusion. Consistent with these findings were the observations that RGDV peptide also reduced the plasma levels of markers of platelet activation (βTG and PF4) but not fibrin formation (FPA).

Previous experimental animal studies with MAbs against platelet GP IIb-IIIa have shown that blocking this receptor may produce potent antithrombotic effects in vivo (10–14). However, such antibodies may also produce a dose-dependent bleeding tendency of variable duration and severity (12). Our data suggest that RGD-containing peptides, due to their actions and rapid clearance in vivo, may be administered in effective high concentrations locally, and without systemic effects. In addition, discontinuance of therapy would result in rapid normalization of hemostasis at sites of peptide administration. Based on these considerations, further efforts to develop peptides with greater affinity and specificity are warranted (22). Such agents might be therapeutically attractive in procedures requiring potent but transient inhibition of platelet function, such as extracorporeal circulation of the blood, coronary angioplasty, or following coronary reperfusion with thrombolytic therapy.

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