

# Aggregate Formation Is More Strongly Inhibited at High Shear Rates by dRGDW, a Synthetic RGD-Containing Peptide

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**The effect of D-Arg-Gly-Asp-Trp (dRGDW), a synthetic RGD-containing peptide, on platelet adhesion and aggregate formation on various purified adhesive proteins and the extracellular matrix of endothelial cells was investigated with anticoagulated blood recirculating through a parallel-plate perfusion chamber. Aggregate formation on the extracellular matrix of phorbol myristate acetate (PMA)-stimulated endothelial cells and on collagen type I was more strongly inhibited by dRGDW at higher shear rates than at a low shear rate. Platelet adhesion to the extracellular matrix of nonactivated and PMA-stimulated endothelial cells was inhibited by dRGDW, especially at high shear rates, probably as a consequence of the inhibition of platelet spreading. Inhibition by dRGDW of platelet adhesion to von Willebrand factor, fibronectin, and fibrinogen was almost complete, indicating that platelet adhesion to these substrates is mediated through RGD-directed receptors. Platelet adhesion to laminin was not inhibited by the peptide, whereas platelet adhesion to collagen was increased as a consequence of the inhibition of aggregate formation. Our results show that dRGDW is a strong inhibitor of platelet adhesion and aggregate formation, especially at high shear rates. (*Arteriosclerosis and Thrombosis* 1993;13:1164-1170)**

**KEY WORDS • platelet adhesion • Arg-Gly-Asp peptides • glycoprotein IIb/IIIa**

**T**he interaction of platelets with components present in the vessel wall is a first and essential step in the hemostatic response. A large number of glycoproteins present in the vessel wall, including von Willebrand factor (vWF), fibronectin, various types of collagen (types I, III, and IV), laminin, thrombospondin, and fibrinogen, are involved in adhesion of platelets, their subsequent spreading, and the induction of aggregate formation.<sup>1-4</sup>

A number of studies have focused on molecular mechanisms that mediate platelet-vessel-wall interaction, and functional domains on participating glycoproteins have been characterized (for a review see Reference 3). An important breakthrough has been the identification of the Arg-Gly-Asp (RGD) sequence within the cell-binding domain of fibronectin<sup>5</sup> as the adhesive recognition sequence for platelet receptor glycoprotein (GP) IIb/IIIa, the integrin  $\alpha_{IIb}\beta_3$ .<sup>6-9</sup> The RGD sequence has been found in many other adhesive glycoproteins, among them vWF, collagen, laminin, fibrinogen, vitronectin, and thrombospondin.<sup>10-12</sup>

Synthetic peptides containing the RGD sequence have been shown to inhibit the interaction of platelet GPIIb/IIIa with fibronectin, vWF, fibrinogen, fibrin, thrombo-

spodin, and vitronectin under static conditions.<sup>13,14</sup> Small changes in and around the RGD sequence modify affinity for GPIIb/IIIa. The amino acid position after the aspartic acid residue has been shown to play an important role in modulating affinity and specificity.<sup>15</sup> When a tryptophan residue (W) is present in this position, a high-affinity peptide for GPIIb/IIIa is created.

The potency of RGD-containing peptides in inhibiting cell/substratum interactions is also determined by the stability of the peptides. Especially when used in whole-blood systems, plasma endopeptidases rapidly degrade the peptides. To overcome this problem, the N-terminal amino acid can be replaced by its D-isomer. In this way, a potent and stable peptide, dRGDW, has been created.

Recent studies with RGD-containing peptides under well-defined flow conditions have shown that these peptides are able to inhibit platelet adhesion to the subendothelium and aggregate formation induced by a collagen-containing surface.<sup>16-18</sup>

Here, we have extended these investigations with the more potent dRGDW and compared platelet adhesion to endothelial cell extracellular matrix (ECM) with adhesion to a number of purified adhesive glycoproteins. We found that (1) lower concentrations of dRGDW are necessary to inhibit aggregate formation at higher shear rates and (2) dRGDW completely inhibits platelet adhesion to purified vWF, fibronectin, and fibrinogen.

## Methods

### Synthetic Peptide

Synthetic D-arginyl-glycyl-L-aspartyl-L-tryptophan (dRGDW) peptide was kindly provided by Dr J.

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Bouchaudon, Rhône-Poulenc-Rorer, Chemistry Department, Centre de Recherches de Vitry, France. Details of its synthesis will be published elsewhere. Concentrations of dRGDW were determined by dry weight, using a peptide molecular mass of 530 g/mol. The median inhibitory concentration ( $IC_{50}$ ) of dRGDW for platelet aggregation induced by 2.5  $\mu\text{mol/L}$  ADP was  $2.1 \pm 0.3 \mu\text{mol/L}$ .

When tested in perfusion experiments, the peptide was added 5 minutes before perfusion to the whole-blood perfusate and incubated at 37°C. In a series of pilot experiments, Gly-Arg-Gly-Glu-Ser-Pro was used as a control peptide to measure nonspecific effects of adding peptides. There was never a difference in platelet adhesion and aggregate formation compared with no addition (see also Reference 19).

### Blood

Whole blood was obtained from healthy volunteer donors who had taken no aspirin or other platelet-function inhibitors in the preceding week and was anticoagulated with 1/10 vol 110 mmol/L trisodium citrate (citrated blood) or with 1/10 vol 200 U/mL low-molecular-weight heparin in 0.15 mol/L NaCl (LMWH blood; Fragmin, Kabi Pharmacia, Stockholm, Sweden).

### Surfaces

The purified adhesive proteins collagen, fibronectin, fibrinogen, and fibrin were sprayed on glass or Thermanox (Miles, Naperville, Ill) coverslips with a retouching airbrush (Badger model 100, Badger Brush Co, Franklin Park, Ill).<sup>20,21</sup> Collagen (Horm collagen, equine type I) was purchased from Hormon Chemie (Munich, FRG) and sprayed on glass or Thermanox coverslips. Calf skin collagen (type I) was obtained from Sigma Chemical Co (St Louis, Mo). The final density of these proteins on the coverslip was 30  $\mu\text{g}/\text{cm}^2$ .<sup>20</sup> After being sprayed, the coverslips were incubated with 1% human albumin in phosphate-buffered saline (PBS; 10 mmol/L sodium phosphate, 150 mmol/L NaCl, pH 7.4) for 1 hour.

Fibronectin was isolated from human plasma by affinity chromatography on gelatin-Sepharose as described.<sup>22</sup> Fibronectin was sprayed on the coverslips with a final density of 20  $\mu\text{g}/\text{cm}^2$ . After they were coated, the coverslips were incubated with 1% human albumin in PBS for 1 hour.

vWF was isolated from cryoprecipitates by gel filtration on Sepharose CL-4B as described.<sup>23</sup> No contamination with fibronectin or fibrinogen could be detected. vWF was coated on a glass coverslip by incubating the coverslip with 10  $\mu\text{g}/\text{mL}$  vWF for 1 hour. A final density of 70  $\text{ng}/\text{cm}^2$  coverslip was reached as checked with <sup>125</sup>I-labeled vWF (H. van Breugel, PhD, et al, unpublished observations). After they were coated, the coverslips were incubated for 1 hour with 1% human albumin in PBS.

Human fibrinogen was purchased from IMCO (Stockholm, Sweden). Before use, fibrinogen was passed over a gelatin-Sepharose column to remove fibronectin impurities and over an anti-vWF immune-affinity column to remove traces of vWF. Fibrinogen was sprayed on glass coverslips with a final density of 30  $\mu\text{g}/\text{cm}^2$ . Fibrin was formed on the glass coverslips by

spraying fibrinogen to which 0.4 U/mL thrombin was added, just before the procedure was started.<sup>24</sup> Control experiments have shown that blocking of the coverslips with human albumin was not required.<sup>25</sup>

Mouse laminin was purchased from GIBCO (Grand Island, NY) and coated on a glass coverslip by incubating the coverslip with 1 mg/mL laminin in 50 mmol/L tris(hydroxymethyl)aminomethane HCl, pH 7.2, and 0.15 mol/L NaCl for 1 hour. A final density of 350  $\text{ng}/\text{cm}^2$  was reached.<sup>4</sup> The coverslips were blocked with 1% human albumin in PBS.

For the ECM, human umbilical vein endothelial cells were isolated and grown to confluence as described.<sup>26</sup> Cells of the second passage were seeded on glass coverslips precoated with gelatin. The ECM was isolated by exposing endothelial cells to 0.1 mol/L  $\text{NH}_4\text{OH}$  for 15 minutes at room temperature and subsequently washing three times with PBS.

For stimulated ECM, human umbilical vein endothelial cells were grown to confluence on Thermanox plastic coverslips. Endothelial cells were stimulated with 20  $\text{ng}/\text{mL}$  phorbol myristate acetate (PMA) for 4 hours, and the ECM (PMA-stimulated) was isolated as described.<sup>27</sup>

### Perfusions

Perfusions were performed in a parallel-plate rectangular perfusion chamber, the characteristics of which have been described by Sakariassen et al.<sup>21</sup> Duplicate coverslips were inserted into the chamber and washed with 15 mL HEPES-buffered saline (HBS; 10 mmol/L *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, 150 mmol/L NaCl, pH 7.35). Fifteen milliliters of whole blood was prewarmed at 37°C for 5 minutes and then recirculated through the chamber for 5 minutes at wall shear rates of 300/s, 1000/s, 1600/s, and 2250/s. After perfusion, the coverslips were removed, rinsed with HBS, fixed in 0.5% glutardialdehyde in PBS, dehydrated in methanol, and stained with May-Grünwald/Giemsa as previously described.<sup>27</sup>

To evaluate aggregate height, perfused Thermanox coverslips were embedded in Epon, and 1- $\mu\text{m}$ -thick cross sections were prepared for evaluation as described.<sup>26</sup> Before and after perfusion, samples of the perfusate were taken to measure single-platelet disappearance (SPD) as a check on platelet clumping caused by handling of blood, as described.<sup>28</sup> For perfusions without dRGDW, SPD values of 20% were found; in the presence of dRGDW, SPD values dropped to 0% because spontaneous platelet clumping was completely prevented.

### Image Analysis

Platelet adhesion was evaluated en face with a light microscope (magnification  $\times 1000$ ) connected to an image analyzer (AMS 40-10, Saffron Walden, UK). Platelet adhesion was expressed as a percentage of total surface covered with platelets.

Aggregate formation was analyzed en face with the IBAS image analysis system (Zeiss/Kontron, Eching, FRG). Coverslips were enlarged with a  $\times 40$  objective and transilluminated with a green filter. The slides were scanned with a Panasonic charge coupled device camera (WV-CD50), digitized 10 times, and averaged to improve the signal-to-noise ratio (frame size,  $640 \times 512$

pixels; 256 gray levels). The system was calibrated geometrically. A pixel in the image memory corresponded to a  $0.042\text{-}\mu\text{m}^2$  area on the slide.

Each image was corrected for shading, and the overall contrast of the image and contrast transitions within the image were enhanced. Then the aggregates were selected by interactive discrimination of a range of gray levels. An automatic computer-assisted procedure was applied to detach aggregates connected to each other through thin pseudopods. The area covered with aggregates or platelets was measured and expressed as a percentage of the total area of the image. Total coverage values obtained with the IBAS image analysis system were in agreement with the values obtained with the AMS 40-10 analysis system described above, indicating that the applied calibration of the IBAS analysis system was comparable to the calibration of the AMS 40-10 analysis system. The IBAS image analysis system was used to evaluate aggregate formation on collagen and PMA-ECM because it allowed the measurement of the area covered by each aggregate or platelet, in contrast to the AMS 40-10 analysis system.

Platelet interaction was subdivided into percentages of surface covered with (1) contact and spread platelets with an area of 0 to  $8\ \mu\text{m}^2$ , (2) aggregates with an area between 8 and  $40\ \mu\text{m}^2$ , and (3) aggregates with an area  $>40\ \mu\text{m}^2$ .

Cross sections were evaluated as described for en face evaluation. Platelet interaction was subdivided into percentages of surface covered with (1) spread and contact platelets and aggregates with a minimum height of  $2\ \mu\text{m}$ , (2) aggregates between 2 and  $5\ \mu\text{m}$ , (3) aggregates between 5 and  $10\ \mu\text{m}$ , and (4) aggregates higher than  $10\ \mu\text{m}$ .

### Statistics

Student's *t* test<sup>29</sup> was used to test for the effect of peptide concentration on platelet coverage. Two-way analysis of variance (ANOVA) was used to test for the effects of peptide concentration and shear rate on the changes of "aggregate formation ratio" (ratio of percentage of aggregates [ $>8\ \mu\text{m}^2$ ] to the total coverage of platelets) and the "large aggregate ratio" (ratio of the large aggregates [ $>40\ \mu\text{m}^2$ ] to the percentage of small aggregates [ $8\ \text{to}\ 40\ \mu\text{m}^2$ ]). Values of  $P<.05$  were considered significant.

## Results

### dRGDW and ECM

**Nonstimulated ECM.** Whole blood anticoagulated with citrate was circulated for 5 minutes over ECM. Addition of dRGDW to whole blood inhibited platelet adhesion to the ECM in a dose-dependent manner (Table 1). When  $100\ \mu\text{mol/L}$  dRGDW was added to the perfusate, a maximum decrease of 30% to 50% in platelet adhesion was found, depending on the shear rate applied. The observed inhibition of platelet adhesion was statistically significant:  $P<.01$  at a shear rate of 300/s and  $P<.001$  at shear rates of 1600 to 2250/s. At shear rates of 1600 to 2250/s, the decrease in platelet adhesion is probably a result of the complete inhibition of platelet spreading on the ECM (Figure).

**PMA-stimulated ECM.** LMWH-anticoagulated whole blood was perfused for 5 minutes over the PMA-

TABLE 1. Influence of dRGDW on Platelet Adhesion to ECM

Shear rate	Control	12.5 $\mu\text{mol/L}$	50 $\mu\text{mol/L}$	100 $\mu\text{mol/L}$
300/s	100	93.7 $\pm$ 5.2§	79.7 $\pm$ 4.8‡	72.1 $\pm$ 0.8†
1600/s	100	84.6 $\pm$ 6.3§	81.4 $\pm$ 9.0§	52.9 $\pm$ 3.9*
2250/s	100	78.1 $\pm$ 3.4†	71.7 $\pm$ 1.4†	61.2 $\pm$ 3.8*

ECM, endothelial cell extracellular matrix.

Data are expressed as percent area covered by platelets normalized by control values in the absence of D-Arg-Gly-Asp-Trp (dRGDW). Values are mean $\pm$ SEM, n=6, and were obtained in three independent experiments. Actual control values of platelet coverage varied between 25.4% and 33.8% (300/s); 30.1% and 34.5% (1600/s); and 21.4% and 29.5% (2250/s). Significance of differences between means was calculated with Student's *t* test: \* $P<.001$ ; † $P<.01$ ; ‡ $P<.05$ ; § $P$ =NS.

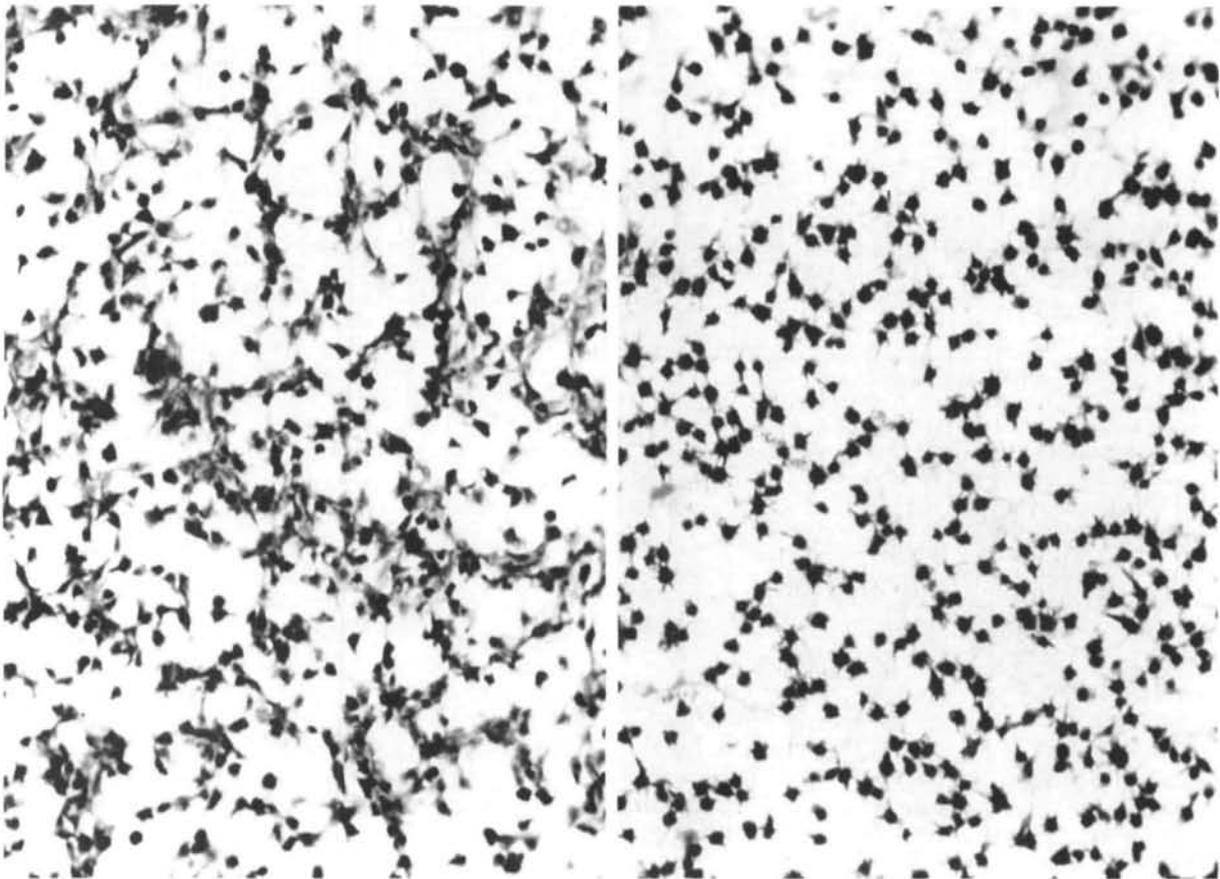
stimulated ECM. Addition of  $12.5\ \mu\text{mol/L}$  dRGDW peptide to the perfusate resulted in a significant increase of platelet coverage on PMA-stimulated ECM at a shear rate of 300/s ( $P<.05$ ). No significant change in platelet coverage was observed with higher concentrations of dRGDW (Table 2). At higher shear rates, platelet coverage was decreased by 60% at a concentration of  $50\ \mu\text{mol/L}$  dRGDW ( $P<.01$ ). Higher concentrations of dRGDW did not result in a further decrease in platelet coverage (Table 2), although platelet spreading was strongly inhibited, especially at 2250/s.

Table 3 shows the influence of dRGDW on aggregate formation on PMA-stimulated ECM. To evaluate the effects of dRGDW on aggregate area, en face analysis results are given as the ratio of the percentage of aggregates ( $>8\ \mu\text{m}^2$ ) to the total coverage of platelets (aggregate-formation ratio, Table 3, top). In Table 3, bottom, the inhibitory effects on aggregate size are expressed as the ratio of the percentage of large aggregates ( $>40\ \mu\text{m}^2$ ) to the percentage of small aggregates ( $8\ \text{to}\ 40\ \mu\text{m}^2$ ; large aggregate ratio). Two-way ANOVA was used to test for the effects of peptide concentration and shear rate on the changes in aggregate formation ratio and the large aggregate ratio. The aggregate formation ratio was significantly influenced by the peptide concentration at all shear rates ( $P<.001$ ). No influence of the shear rate alone on the aggregate formation ratio was found (Table 3, top). The peptide concentration had an effect primarily on the change in large aggregate ratio ( $P<.001$ , Table 3, bottom). A significant increase of this ratio with increasing shear rate was present only in the absence of peptide ( $P<.05$ ). Addition of peptide abolished the shear rate effect; this was caused by a stronger effect of the peptide at high shear rates ( $P<.05$ ).

These results were confirmed by the analysis of a single cross-sectional experiment. Almost complete inhibition of aggregates  $>10\ \mu\text{m}$  was observed at a shear rate of 300/s with  $50\ \mu\text{mol/L}$  dRGDW, whereas at higher shear rates,  $12.5\ \mu\text{mol/L}$  dRGDW was sufficient for complete inhibition. An effect of shear rate on the inhibition by dRGDW of aggregate formation in general was also evident. The percentage of aggregates was reduced, eg, by 50%, after addition of  $12.5\ \mu\text{mol/L}$  dRGDW at 300/s, whereas it was reduced to about 10% at 2250/s (not shown).

### dRGDW and Purified Proteins

**Inhibition of platelet adhesion and aggregate formation on collagen.** LMWH-anticoagulated whole blood was



Photomicrographs showing influence of D-Arg-Gly-Asp-Trp (dRGDW) on platelet spreading on endothelial cell extracellular matrix (ECM). Left, Control: platelet adhesion to ECM at a shear rate of 2250/s. Right, Addition of 100  $\mu\text{mol/L}$  dRGDW to the perfusate. Original magnification  $\times 100$ .

perfused over Horm collagen (predominantly equine type I). Perfusion over both collagen surfaces led to the formation of aggregates. Addition of dRGDW to the perfusate led to a similar increase of platelet coverage on both collagens at all shear rates (Table 4). The increase in platelet coverage was caused by increased adhesion of platelets. At higher shear rates, inhibition of platelet spreading was observed. Comparable results were obtained with calf skin collagen.

Addition of dRGDW inhibited aggregate formation on Horm collagen type I in a dose-dependent and

shear-rate-dependent way. Table 5 shows the influence of dRGDW on aggregate formation on Horm collagen type I. To evaluate the effects of dRGDW on aggregate area, en face analysis results, expressed as the ratio of

TABLE 2. Influence of dRGDW on Platelet Coverage on PMA-Stimulated ECM

Shear rate	Control	12.5 $\mu\text{mol/L}$	50 $\mu\text{mol/L}$	100 $\mu\text{mol/L}$
300/s	100	130.5 $\pm$ 9.7§	114.8 $\pm$ 12.8	99.4 $\pm$ 5.6
1600/s	100	83.4 $\pm$ 10.5	53.4 $\pm$ 14.3§	26.8 $\pm$ 0.6*
2250/s	100	79.6 $\pm$ 10.5	40.2 $\pm$ 12.3†	30.9 $\pm$ 10.3†

PMA, phorbol myristate acetate; ECM, endothelial cell extracellular matrix.

Data are expressed as percent area covered by platelets normalized by control values in the absence of D-Arg-Gly-Asp-Trp (dRGDW). Values are mean $\pm$ SEM, n=8, and were obtained in four independent experiments. Actual control values of platelet coverage varied between 20.5% and 31.3% (300/s); 29.5% and 39.9% (1600/s); and 28.7% and 34.9% (2250/s). Significance of differences in means was calculated with Student's *t* test: \* $P < .001$ ; † $P < .01$ ; ‡ $P < .02$ ; § $P < .05$ ; || $P = \text{NS}$ .

TABLE 3. Aggregate Formation on PMA-Stimulated ECM: Influence of dRGDW on Aggregate Formation and Size

Shear rate	Control	12.5 $\mu\text{mol/L}$	50 $\mu\text{mol/L}$	100 $\mu\text{mol/L}$
Aggregate formation				
300/s	0.95 $\pm$ 0.01	0.89 $\pm$ 0.01	0.56 $\pm$ 0.02	0.50 $\pm$ 0.07
1600/s	0.95 $\pm$ 0.01	0.77 $\pm$ 0.07	0.38 $\pm$ 0.13	0.34 $\pm$ 0.08
2250/s	0.96 $\pm$ 0.01	0.77 $\pm$ 0.05	0.49 $\pm$ 0.01	0.48 $\pm$ 0.06
Aggregate size				
300/s	3.46 $\pm$ 0.48	2.18 $\pm$ 0.53	0.17 $\pm$ 0.01	0.22 $\pm$ 0.08
1600/s	8.19 $\pm$ 1.17	1.10 $\pm$ 0.27	0.14 $\pm$ 0.08	0.08 $\pm$ 0.03
2250/s	7.87 $\pm$ 1.65	1.41 $\pm$ 0.57	0.09 $\pm$ 0.04	0.15 $\pm$ 0.05

PMA, phorbol myristate acetate; ECM, endothelial cell matrix; dRGDW, D-Arg-Gly-Asp-Trp.

In the top section, the results of en face analysis of aggregate formation are shown. Data are expressed as the ratio of percent area covered by aggregates divided by percent of total coverage. Values are mean $\pm$ SEM, n=3, and were obtained in three independent experiments. In the bottom section, the results of en face analysis of aggregate size distribution are shown. Data are expressed as the ratio of percent area covered by large aggregates (>40  $\mu\text{m}^2$ ) divided by percent area covered by small aggregates (8 to 40  $\mu\text{m}^2$ ). Values are mean $\pm$ SEM, n=3.

**TABLE 4. Influence of dRGDW on Platelet Coverage on Collagen**

Shear rate	Control	12.5 $\mu\text{mol/L}$	50 $\mu\text{mol/L}$	100 $\mu\text{mol/L}$
<b>Horm collagen</b>				
300/s	100	184.4 $\pm$ 14.1†	220.9 $\pm$ 16.1*	188.6 $\pm$ 24.0§
1600/s	100	168.1 $\pm$ 3.8†	198.6 $\pm$ 26.0§	190.6 $\pm$ 22.9§
2250/s	100	173.4 $\pm$ 14.5‡	198.2 $\pm$ 31.3	194.0 $\pm$ 30.3
<b>Calf skin collagen</b>				
300/s	100	128.6 $\pm$ 19.0	365.1 $\pm$ 27.0	417.5 $\pm$ 9.5
1600/s	100	236.8 $\pm$ 8.5	223.1 $\pm$ 0.9	283.8 $\pm$ 4.3
2250/s	100	107.9 $\pm$ 0	205.3 $\pm$ 18.4	135.1 $\pm$ 7.0

Data are expressed as percent area covered by platelets normalized to control values in the absence of D-Arg-Gly-Asp-Trp (dRGDW). Values are mean $\pm$ SEM, n=6, and were obtained in three independent experiments. Actual control values of platelet coverage on Horm collagen type I varied between 6.9% and 14.4% (300/s); 11.0% and 13.3% (1600/s); and 11.6% and 14.1% (2250/s). Significance of differences in means was calculated with Student's *t* test: \**P*<.001; †*P*<.01; ‡*P*<.02; §*P*<.05; ||*P*=NS.

Actual control values of platelet coverage on calf skin collagen type I varied between 4.5% and 8.8% (300/s); 10.3% and 13.1% (1600/s); and 11.1% and 11.7% (2250/s). Values are mean $\pm$ SEM, n=2.

the percentage of aggregates (>8  $\mu\text{m}^2$ ) to the total coverage of platelets, are given in Table 5, top. In Table 5, bottom, the ratio of the percentage of large aggregates (area, >40  $\mu\text{m}^2$ ) to the percentage of small aggregates (area, 8 to 40  $\mu\text{m}^2$ ) is given as a measure of the effect of dRGDW on aggregate size. The overall effect is that dRGDW inhibited aggregate formation dose dependently (Table 5, top) and induced a shift from large aggregates (>40  $\mu\text{m}^2$ ) to small aggregates (8 to 40  $\mu\text{m}^2$ ).

As in the analysis of aggregate formation on PMA-stimulated ECM, two-way ANOVA was used to test for the effects of peptide concentration and shear rate on the changes of the aggregate formation ratio and the large aggregate ratio. The aggregate formation ratio was significantly influenced by the peptide concentration at

**TABLE 5. Aggregate Formation on Horm Collagen: Influence of dRGDW on Aggregate Formation and Size**

Shear rate	Control	12.5 $\mu\text{mol/L}$	50 $\mu\text{mol/L}$	100 $\mu\text{mol/L}$
<b>Aggregate formation</b>				
300/s	0.85 $\pm$ 0.01	0.81 $\pm$ 0.01	0.60 $\pm$ 0.01	0.54 $\pm$ 0.04
1600/s	0.89 $\pm$ 0	0.81 $\pm$ 0.01	0.59 $\pm$ 0.02	0.60 $\pm$ 0.02
2250/s	0.92 $\pm$ 0	0.77 $\pm$ 0.01	0.54 $\pm$ 0.08	0.41 $\pm$ 0.01
<b>Aggregate size</b>				
300/s	0.89 $\pm$ 0.02	0.84 $\pm$ 0.02	0.25 $\pm$ 0.04	0.30 $\pm$ 0.01
1600/s	1.31 $\pm$ 0.19	1.33 $\pm$ 0.17	0.29 $\pm$ 0.06	0.28 $\pm$ 0.06
2250/s	2.62 $\pm$ 0.19	0.75 $\pm$ 0.09	0.23 $\pm$ 0.13	0.06 $\pm$ 0.01

dRGDW, D-Arg-Gly-Asp-Trp.

In the top section, the results of en face analysis of aggregate formation are shown. Data are expressed as the ratio of percent area covered by aggregates divided by percent of total coverage. Values are mean $\pm$ SEM, n=3, and were obtained in three independent experiments. In the bottom section, the results of en face analysis of aggregate size distribution are shown. Data are expressed as the ratio of percent area covered by large aggregates (>40  $\mu\text{m}^2$ ) divided by percent area covered by small aggregates (8 to 40  $\mu\text{m}^2$ ). Values are mean $\pm$ SEM, n=3.

**TABLE 6. Influence of 50  $\mu\text{mol/L}$  dRGDW on Platelet Adhesion**

Protein	Shear rate	Control	+dRGDW
vWF	1000/s	28.1 $\pm$ 1.5	1.6 $\pm$ 0.4
Fibronectin	300/s	15.5 $\pm$ 1.1	1.1 $\pm$ 0.2
Fibrinogen	300/s	19.5 $\pm$ 2.0	2.3 $\pm$ 0.2
	1600/s	12.0 $\pm$ 0.5	1.0 $\pm$ 0.2
Fibrin	300/s	19.6 $\pm$ 2.4	6.7 $\pm$ 1.2
	1600/s	27.7 $\pm$ 0.3	2.6 $\pm$ 0.1
Laminin	300/s	28.3 $\pm$ 2.4	32.1 $\pm$ 0.5

vWF, von Willebrand factor; dRGDW, D-Arg-Gly-Asp-Trp.

Values are expressed as actual values of platelet coverage, mean $\pm$ SEM, n=4, and were obtained in two independent experiments.

all shear rates (*P*<.001) but not by the shear rate alone, and there was no interaction effect (Table 5, top). The peptide concentration had an effect primarily on the change in large aggregate ratio (*P*<.001; Table 5, bottom). A significant effect of the shear rate on this ratio was present only in the absence of peptide (*P*<.05). Addition of peptide abolished the shear-rate effect; this was caused by a stronger effect of the peptide at high shear rates (*P*<.05). A comparable effect was observed after analysis of the results from cross-sectional evaluation. At high shear rate, large aggregate formation (height, >10  $\mu\text{m}$ ) was inhibited more effectively with 12.5  $\mu\text{mol/L}$  dRGDW than at low shear rate. Small aggregate formation (height, 5 to 10  $\mu\text{m}$ ) was poorly inhibited at low shear rate (not shown).

*Inhibition of platelet adhesion to purified adhesive proteins.* Platelet adhesion to various adhesive proteins was studied at shear rates at which adhesion is optimal. Citrate-anticoagulated whole blood was used in perfusions over vWF, fibronectin, fibrinogen, and fibrin, whereas LMWH-anticoagulated blood was used in perfusions over laminin, since this adhesion is cation dependent.<sup>4</sup>

Platelet adhesion to vWF, fibronectin, fibrinogen, and fibrin was effectively inhibited by 50  $\mu\text{mol/L}$  dRGDW, except for platelet adhesion to fibrin at low shear rate, which was only partially inhibited. Platelet adhesion to laminin was not affected by dRGDW. The results of these experiments are summarized in Table 6.

## Discussion

The purpose of the study presented here was to investigate the effectiveness of dRGDW in inhibiting platelet adhesion to and aggregate formation on various adhesive proteins and cellular matrices under flow conditions.

### Adhesion to the ECM

The addition of 50  $\mu\text{mol/L}$  dRGDW to the perfusate inhibited platelet adhesion to the ECM at all shear rates tested by 40% to 50%, although inhibition at high shear rates was slightly stronger. The spreading of platelets on the ECM was completely inhibited by dRGDW. Platelet adhesion is expressed as the total surface covered with platelets, and the contribution of platelet spreading to the total surface coverage is considerable. Therefore, the inhibition by dRGDW of platelet adhesion to the ECM was probably caused by the complete abolition of platelet spreading. Increasing the concentration of

dRGDW (to 200  $\mu\text{mol/L}$ ) or increasing the shear rate to 2600/s did not lead to further inhibition (data not shown).

The pattern of inhibition of adhesion to the ECM by dRGDW is the same as seen by Weiss et al<sup>16</sup> and Lawrence et al<sup>17</sup> with Arg-Gly-Asp-Ser peptides on the subendothelium. At low shear rate, sufficient receptors on the platelet membrane, such as GPIb, GPIa/IIa, and very late-acting antigen-6 (VLA-6),<sup>4,30-32</sup> are present to ensure optimal platelet adhesion. At higher shear rates, because of the increased shear stress, the interaction with RGD-directed receptors is evidently necessary to support platelet adhesion and spreading, in agreement with results obtained with blood of patients with Glanzmann's thrombasthenia.<sup>33</sup>

Inhibition by dRGDW of platelet adhesion to the matrix of PMA-stimulated endothelial cells was shear-rate dependent. In contrast to unstimulated endothelial cell matrix, no inhibition was found at a shear rate of 300/s, whereas at shear rates of 1600 to 2250/s, 75% inhibition of adhesion was found. The absence of inhibition of platelet adhesion at 300/s can be explained as a consequence of the inhibition of aggregate formation by dRGDW. Because of this inhibition, platelets are not incorporated into aggregates, and more platelets are thus available for platelet adhesion. Also, the accessibility of the surface for platelets is increased because no (large) aggregates are formed, which may have a lee effect.<sup>34</sup> The strong inhibition by dRGDW of adhesion to PMA-stimulated ECM observed at higher shear rates indicates that GPIIb/IIIa is important for the interaction of platelets with a surface that is able to generate a considerable amount of thrombin. This is emphasized by the almost complete inhibition of platelet adhesion to PMA-stimulated ECM at a shear rate of 2600/s and the similar strong inhibition by dRGDW of platelet adhesion to smooth muscle cell matrix and to fibroblast matrix that we also observed (data not shown). Both of these matrices generate considerable amounts of thrombin on their surface when subjected to flowing LMWH-anticoagulated blood.<sup>35</sup>

The strong inhibition of platelet adhesion to PMA-stimulated ECM at high shear rates in comparison with unstimulated ECM or collagen implies not only a more important role for GPIIb/IIIa but also a diminished role for other non-RGD-directed receptors that support platelet adhesion to unstimulated ECM and collagen, like GPIa/IIa<sup>31,32</sup> and/or VLA-6.<sup>4</sup> The role of another non-RGD-directed receptor, GPIb, in platelet adhesion to PMA-ECM is not diminished. Inhibition experiments with a monoclonal antibody directed against GPIb have shown that adhesion to PMA-stimulated ECM is completely dependent on GPIb.<sup>27</sup>

#### *Adhesion to Purified Proteins*

Platelet adhesion to GPIIb/IIIa-dependent surfaces, like vWF, fibronectin, and fibrinogen,<sup>25,30,36,37</sup> was inhibited almost completely by dRGDW. When platelet adhesion was independent of GPIIb/IIIa, like adhesion to laminin,<sup>4</sup> no inhibition was found with dRGDW. The observation that platelet adhesion to vWF is completely inhibited by dRGDW, although platelet interaction with vWF is completely dependent on the non-RGD-directed receptor GPIb,<sup>30</sup> agrees with the recent results of Savage et al,<sup>38</sup> who demonstrated that platelet adhe-

sion to vWF under static conditions is dependent on both GPIb and GPIIb/IIIa. Obviously, platelet adhesion under flow conditions needs an interaction with at least two different platelet receptors to resist the shear forces. It is striking that platelet adhesion to purified proteins is almost completely inhibited, whereas an ECM inhibition is, at the very best, 30%. Platelet adhesion to the ECM is mediated by the same proteins tested in purified form. This suggests that platelet adhesion to purified proteins is mediated by a different mechanism than adhesion to the ECM. What these differences are is not known at present.

The influence of dRGDW on platelet adhesion to collagen is twofold: at low shear rates, platelet coverage is strongly increased after addition of dRGDW; at high shear rates, this increased platelet coverage is partially inhibited, probably as a consequence of strong inhibition of platelet spreading. The explanation for the increased platelet coverage on collagen is the same as for PMA-stimulated ECM: An increased number of platelets can adhere to a more accessible surface as a result of inhibited platelet/platelet interaction and the increased surface expression of GPIIb/IIIa.

At higher shear rates, the increased platelet coverage is strongly diminished or even inhibited at a shear rate of 2600/s (data not shown), and platelet adhesion consists primarily of contact platelets, consistent with the observations of Fressinaud et al.<sup>18</sup> Especially at high shear rates, platelet spreading, which is strongly dependent on GPIIb/IIIa,<sup>33</sup> is important to resist shear forces. The observed platelet adhesion is determined by the opposing effects of an increased number of platelets available for adhesion and decreasing affinity of platelets for collagen resulting from the inhibition of platelet spreading mediated through GPIIb/IIIa.

#### *Aggregate Formation*

Inhibition of aggregate height on the matrix of PMA-stimulated ECM and on collagen was shear-rate dependent: At low shear rates, the concentration necessary to inhibit aggregate formation completely was much higher than at high shear rates. At a shear rate of 300/s, >50  $\mu\text{mol/L}$  dRGDW was necessary to completely inhibit aggregate formation on PMA-stimulated ECM, and >100  $\mu\text{mol/L}$  dRGDW would be necessary to completely inhibit aggregate formation on collagen. A comparable inhibitory effect was seen on aggregate area: Large aggregates (area >40  $\mu\text{m}^2$ ) were inhibited more strongly at high shear rates. This increased inhibition at high shear rate may arise because aggregates must be stable to resist high shear rates. Stability is dependent on the number of GPIIb/IIIa molecules available for ligand interaction. dRGDW competes with the ligands and inhibits GPIIb/IIIa. If this explanation is correct, GPIIb/IIIa is not inhibited more strongly, but the effect of the inhibition is visible earlier because more GPIIb/IIIa molecules are required at high shear rates to guarantee aggregate integrity.

In conclusion, dRGDW is a strong inhibitor of platelet aggregate formation, independent of the agonist responsible for platelet activation. dRGDW is a more effective inhibitor of platelet adhesion and aggregate formation at high shear rates than at low shear rates, suggesting that dRGDW is a more efficient drug for the

prevention of arterial thrombosis than for venous thrombosis.

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