CONCISE REPORT

Mediation of Platelet Adhesion to Fibrillar Collagen in Flowing Blood by a Proteolytic Fragment of Human von Willebrand Factor

By Kjell S. Sakariassen, Edith Fressinaud, Jean-Pierre Girma, Hans R. Baumgartner, and Dominique Meyer

The effect of purified von Willebrand Factor (vWF) fragments, SpII (dimer of 110 kd subunits) and SpIII (dimer of 170 kd subunits) obtained with Staphylococcus aureus V-8 protease was tested upon platelet adhesion to collagen. Purified fibrillar human collagen coated onto cover slips was incubated with SpII, SpIII, or undigested vWF and exposed to reconstituted human blood in a parallel-plate perfusion chamber at a high shear rate. Platelet-collagen interactions were estimated using 51Cr-platelets and quantitative morphometry. When blood was reconstituted with citrated autologous plasma, SpII and vWF strikingly enhanced platelet adhesion to collagen whereas SpIII had no effect. When blood was reconstituted with human albumin and divalent cations, SpII and vWF again promoted platelet adhesion to collagen. In conclusion, our data suggest that (1) SpII, the N-terminal portion of vWF which binds to platelet membrane glycoprotein Ib, functionally substitutes for vWF in supporting platelet adhesion to collagen; (2) SpIII, the C-terminal portion which binds to glycoprotein IIb/IIIa, has no such effect; (3) in addition to its platelet binding domain, SpIII contains another site for binding to collagen; and (4) the multimeric structure of vWF is not required for platelet adhesion to collagen.

© 1986 by Grune & Stratton, Inc.

From www.bloodjournal.org by guest on October 23, 2017. For personal use only.
thrombin III (CRTS, Lille, France) coupled to Sepharose 2B (Pharmacia Fine Chemicals, Uppsala, Sweden) (1.5 mg antithrombin III per mL Sepharose 2B). Perifusates were reconstituted immediately prior to perfusion by adding washed red cells to the resuspended platelets (hematocrit 40%, platelet count $1.5 \times 10^{11}$/L).

**Perfusion chamber, exposed surfaces, and perfusion procedures.** A modification of the original parallel-plate perfusion chamber was used. This chamber allows simultaneous exposure of the same perfusate to two collagen-coated cover slips positioned symmetrically on each side of the flow slit. Approximately $7 \mu g/cm^2$ fibrillar type III collagen was sprayed onto plastic cover slips (Thermanox, Miles Laboratories Inc, Naperville, III) by means of an air brush (Model 100 II, Badger Air-brush Co, Franklin Park, IL) at a nitrogen operating pressure of 1 atm.

The collagen-coated surfaces were incubated 20 minutes at $22^\circ$C with 0.4 mL of purified vWF, SpII and SpIII (15 $\mu g/mL$) dissolved in 25 mmol/L Tris-HCl, 150 mmol/L NaCl buffer, pH 7.4, containing 2.5 mmol/L CaCl$_2$ and 0.9 mmol/L MgCl$_2$. Collagen-coated cover slips incubated with buffer served as control. The various surfaces were preperfused at $37^\circ$C with 40 mL of modified Tyrode buffer containing 2.5 mmol/L CaCl$_2$, 0.9 mmol/L MgCl$_2$, and 5 mmol/L glucose, followed by perfusion with reconstituted blood perfusates at a flow rate of 41 mL/min corresponding to 2,600 seconds$^{-1}$ shear rate for 5 minutes.

**Quantitation of platelet-collagen interactions.** After perfusion, both cover slips were cut parallel to the flow axis in two equal halves. One half was used for $^{31}Cr$ counting, and platelet deposition was expressed as number of deposited platelets per cm$^2$. The other half served for morphometric quantitation of platelet-collagen interactions. The percentage surface covered with platelets and microthrombi higher than 2.5 $\mu m$ was estimated on semithin epon sections at $2 \pm 1$ mm downstream positions. Fixation, embedding, and removal of exposed surfaces from cover slips for production of semithin sections were recently described in detail.

**Statistical analysis.** Significance of grouped data was calculated with Student t-test, and P-values <0.05 were considered as significant.

**RESULTS**

**Perfusion experiments using blood reconstituted with autologous citrated plasma.** Preincubation of collagen with SpIII increased platelet deposition as effectively as purified vWF (Table 1). The number of deposited labeled platelets with SpII and vWF compared with buffer showed an enhanced platelet deposition of about 4.5-fold ($P < 0.025$). Morphometric evaluation of the percentage surface coverage with adherent platelets paralleled the data obtained by $^{51}$Cr counting (Table 1). The increased thrombus formation observed by preincubation of collagen with SpIII or vWF was not significant (Table 1).

Preincubation of the collagen-coated surface with SpII resulted in platelet-collagen interactions similar to those observed with buffer (Table 1).

**Perfusion experiments using blood reconstituted with a human albumin salt solution.** When citrated plasma was replaced by a purified albumin salt solution, preincubation of collagen-covered cover slips with SpIII again increased the platelet-collagen interactions. Similar results were obtained when using either SpIII or undigested vWF (Table 2). The percent surface coverage with platelets also paralleled the results obtained by $^{51}$Cr counting (Table 2).

As in citrated plasma, preincubation of collagen-coated surfaces with SpII did not influence platelet-collagen interactions (Table 2).

**DISCUSSION**

vWF mediates platelet adhesion to subendothelium and to purified collagen at high wall shear rates that prevail in small vessels. However, the respective role of plasma, platelet, and subendothelial vWF remains to be elucidated. Furthermore, the domains of vWF, a large multimeric protein with a complex structure that mediates platelet adhesion, are still unknown. We have shown in this study that a well characterized proteolytic fragment of vWF subunit, SpIII, mediates platelet adhesion to collagen at high shear rate whereas the complementary fragment SpII has no effect. The efficacy of SpIII in promoting platelet adhesion to fibrillar human collagen was established using both $^{31}$Cr-platelets and a morphometric method to evaluate platelet-collagen interactions. In our experimental conditions using blood reconstituted with citrated plasma and vWF or purified SpIII coated on collagen, plasmatic vWF present in the perfusate did not appear to compete with bound material.

**Table 1. Effect of von Willebrand Factor Fragments Upon Platelet-Collagen Interactions in a Flow System (Shear Rate:2,600 seconds$^{-1}$) Using Blood Reconstituted With Citrated Plasma**

<table>
<thead>
<tr>
<th></th>
<th>Number of Deposited Platelets ($x 10^5/cm^2$)</th>
<th>% Surface Coverage</th>
<th>With Thrombi $&gt;2.5 \mu m$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>With Platelets†</td>
<td>With Platelets†</td>
<td></td>
</tr>
<tr>
<td>Tris-HCl</td>
<td>26.8 ± 5.6</td>
<td>26.7 ± 5.3</td>
<td>4.2 ± 1.2</td>
</tr>
<tr>
<td>SpII</td>
<td>21.3 ± 2.4 (NS)</td>
<td>22.4 ± 2.6 (NS)</td>
<td>3.2 ± 1.0 (NS)</td>
</tr>
<tr>
<td>SpIII</td>
<td>122.3 ± 30.1†</td>
<td>52.8 ± 8.6§</td>
<td>20.7 ± 6.3 (NS)</td>
</tr>
<tr>
<td>vWF</td>
<td>121.8 ± 27.8†</td>
<td>78.0 ± 11.8§</td>
<td>13.5 ± 6.0 (NS)</td>
</tr>
</tbody>
</table>

*Type III collagen (7 $\mu g/cm^2$) was preincubated at $20^\circ$C for 20 minutes with purified proteins (15 $\mu g/mL$) or Tris-HCl buffer.
†Perfusions were performed with human blood reconstituted with citrated autologous plasma from three individuals and results represent mean ± SEM of three determinations in duplicate.
§P-values compared with Tris-HCl buffer: $P < 0.025$.

**Table 2. Effect of von Willebrand Factor Fragments Upon Platelet-Collagen Interactions in a Flow System (Shear Rate:2,600 seconds$^{-1}$) Using Blood Reconstituted With a Human Albumin Salt Solution**

<table>
<thead>
<tr>
<th></th>
<th>Number of Deposited Platelets ($x 10^5/cm^2$)</th>
<th>% Surface Coverage</th>
<th>With Thrombi $&gt;2.5 \mu m$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>With Platelets†</td>
<td>With Platelets†</td>
<td></td>
</tr>
<tr>
<td>Tris-HCl</td>
<td>58.6 ± 8.6</td>
<td>33.7 ± 5.4</td>
<td>5.2 ± 2.5</td>
</tr>
<tr>
<td>SpII</td>
<td>62.7 ± 11.1 (NS)</td>
<td>21.8 ± 4.2 (NS)</td>
<td>6.3 ± 2.0 (NS)</td>
</tr>
<tr>
<td>SpIII</td>
<td>159.4 ± 12.0‡</td>
<td>92.9 ± 14.4‡</td>
<td>20.1 ± 10.3 (NS)</td>
</tr>
<tr>
<td>vWF</td>
<td>153.4 ± 8.1‡</td>
<td>95.0 ± 12.7‡</td>
<td>17.4 ± 10.3 (NS)</td>
</tr>
</tbody>
</table>

*Type III collagen (7 $\mu g/cm^2$) was preincubated at $20^\circ$C for 20 minutes with purified proteins (15 $\mu g/mL$) or Tris-HCl buffer.
†Perfusions were performed with human blood from three individuals reconstituted with 4% albumin solution in modified Tyrode buffer containing physiologic amounts of divalent cations. Results represent mean ± SEM of three determinations in duplicate.
‡P-values compared with Tris-HCl buffer: $P < 0.0025$.
§P-values compared with Tris-HCl buffer: $P < 0.0005$. From www.bloodjournal.org by guest on October 23, 2017. For personal use only.
SpIIl contains a binding domain for platelet membrane GPllb. Our study suggests that it also possesses a domain for binding to collagen, confirming results of other studies obtained with monoclonal antibodies (MoAb) to vWF. We showed that among 24 MoAb to vWF, six of them (B200 through B205) specifically inhibit binding of vWF to collagen while two other MoAB, MoAb H and MoAb 9, inhibit vWF binding to ristocetin- and to thrombin-stimulated platelets, respectively. Thus SpIIl, already known to contain a binding domain for GPllb, also possesses a distinct site for binding to collagen, and both domains appear to be required for platelet-collagen interactions at high shear rate. Contrary to SpIIl, SpII which binds to GPllb/IIla does not appear to be involved, suggesting that it contains no collagen-binding domain or no platelet-binding domain implicated in platelet adhesion. Alternatively, the GPllb/IIla binding sites for SpII may not be induced on the platelet membrane in our experimental conditions; at high shear rate, binding to GPllb may be a prerequisite for platelet activation leading to exposure of GPllb/IIla.

The finding that SpIIl supports platelet attachment to collagen is further substantiated by perfusion experiments using blood reconstituted with a salt solution containing albumin as the only exogenous protein. The enhanced effect of SpIIl or vWF as compared with experiments with blood reconstituted with autologous citrated plasma may be related to the presence of physiologic amounts of calcium added to the human albumin solution. Indeed, the importance of divalent cations in platelet interaction with subendothelium has been emphasized. Our results confirm previous findings that in vitro, at high shear rate, vWF appears to be the only protein required for mediating platelet interactions with fibrillar collagen and stress the relative importance of locally adsorbed vWF as compared with circulating vWF.

In conclusion, the N-terminal fragment SpIIl of vWF mediates platelet adhesion to collagen as well as undigested vWF. The multimeric structure of vWF thus does not appear to be required for platelet adhesion to fibrillar collagen in vitro. The possibility, however, that SpIIl aggregates on the collagen to provide a repetitive structure has not been excluded. Finally, our results do not stipulate that in vivo the large vWF multimers are unimportant for normal hemostasis but only indicate that in vitro a specific fragment may substitute for the fully polymerized vWF in promoting platelet adhesion to collagen.

ACKNOWLEDGMENT

We thank Dr H. Sage and Dr P. Bornstein, University of Washington, Seattle, for the generous gift of lyophilized pepsin-extracted collagens from human placenta. The expert technical assistance of C. Michael, Hoffmann-La Roche, Basle, Switzerland, is gratefully acknowledged. We thank V. Charles for excellent typing.

REFERENCES

17. Woessner JF: The determination of hydroxyproline in tissue and protein samples containing small proportions of this amino acid. Arch Biochem Biophys 93:440, 1961


Mediation of platelet adhesion to fibrillar collagen in flowing blood by a proteolytic fragment of human von Willebrand factor

KS Sakariassen, E Fressinaud, JP Girma, HR Baumgartner and D Meyer