Effect of the Multimeric Structure of the Factor VIII/von Willebrand Factor Protein on Binding to Platelets

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The characteristics of the intact factor VIII/von Willebrand factor protein binding to human platelets was compared to 2-mercaptoethanol-treated factor VIII/von Willebrand factor protein and to fractions of plasma factor VIII/von Willebrand factor protein that elute after the void volume. These studies indicate that the factor VIII/von Willebrand factor protein larger size oligomers bind preferentially with high affinity to low capacity sites on human platelets. The intermediate and smaller size oligomers bind with intermediate or low affinity to sites with a much greater capacity. The results from binding analysis are also paralleled by the competitive inhibition of the intact factor VIII/von Willebrand factor protein by the various 2-mercaptoethanol-treated materials. These studies indicate that the two classes of binding sites seen in previous reports of factor VIII/von Willebrand factor binding reflect heterogeneity in the oligomer size of the factor VIII/von Willebrand factor protein used in these assays. This study provides a model for understanding some of the normal structure–function relationships of the normal factor VIII/von Willebrand factor protein and the defect(s) in a variant form of von Willebrand’s disease. In this form of the disease, decreased factor VIII/von Willebrand factor binding to platelets is reflected in decreased von Willebrand factor activity but coagulant and/or antigen levels are normal or only slightly decreased.

THE HUMAN FACTOR VIII/von Willebrand factor (f.VIII/vWF) protein has been characterized as a high molecular weight glycoprotein which, when purified, has two biologic activities: (1) procoagulant activity, i.e., the ability to correct the long clotting time of hemophilia A plasma, and (2) von Willebrand factor (vWF) activity, which in vivo is the ability to shorten the bleeding time and/or in vitro the ability to cause platelet agglutination of either normal human platelets or platelets from patients with von Willebrand’s disease (vWD) in the presence of ristocetin.1,4 The human f.VIII/vWF glycoprotein has been largely defined by these two criteria since its structural complexity and size has restricted detailed biochemical studies.

Studies from this and other laboratories have focused on the specic intermolecular interactions implicated in ristocetin-induced agglutination (vWF activity). Ristocetin, a positively charged molecule, is thought to decrease the negative charge on platelets reducing the electrostatic repulsion between platelets. This allows the f.VIII/vWF protein to act as an effective bridge to interconnect platelets.5,12 This interaction has been analyzed as the effect of two separate reactions: (1) f.VIII/vWF binding to platelets and (2) the platelet reaction of agglutination or aggregation in the presence of the f.VIII/vWF protein. Both reactions require ristocetin.

Through chemical and enzymatic modification of the intact protein, it has been possible to identify critical structural elements required for normal vWF function. It has previously been shown that an intact penultimate galactose moiety is necessary for f.VIII/vWF binding to platelets and for the retention of vWF activity.15,18 This specificity is likely due to a carbohydrate-mediated recognition by the platelet receptor.

Another approach to the question of f.VIII/vWF binding to platelets with resultant agglutination is found in recent reports describing marked size heterogeneity of the f.VIII/vWF protein in normals.19-24 In some patients with vWD, abnormalities of the size heterogeneity of the f.VIII/vWF protein have been described utilizing a variety of techniques.25-29 After complete disulfide bond reduction, the normal hemophilia A and vWD f.VIII/vWF protein is composed of a single subunit that appears homogeneous on sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis. Recent studies have investigated the effect of limited in vitro reduction of the normal f.VIII/vWF protein to form oligomers of varying sizes. These studies have reported that this reduction of the f.VIII/vWF protein results in loss of the ability to aggregate platelets but does not affect the factor VIII procoagulant activity.22,30 One study revealed no significant differences of platelet binding with the reduced compared to the intact f.VIII/vWF protein.36

These findings have significant implications in our understanding of the mechanism of the structure–function relationships of vWF as an interplatelet bridge as well as providing increased insight into the defects in vWD. In an attempt to examine the structure–function relationships of the molecular size of the human f.VIII/vWF protein to platelet binding and the ristocetin-induced platelet agglutination, we have compared the kinetics of binding of the intact and

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0006-4971/81/5802-0029$02.00/0
2-mercaptoethanol (2-ME) treated f.VIII/vWF protein to the platelet receptor. We report our findings on the ability of the different populations of the f.VIII/vWF oligomers to bind to platelets and induce ristocetin-dependent platelet aggregation.

MATERIALS AND METHODS

Factor VIII/von Willebrand Factor Protein

The f.VIII/vWF protein was purified as previously described. Intermediate purity factor VIII concentrates were obtained from the American National Red Cross (through the courtesy of Dr. M. Wickerhauser) and after sequential polyethylene glycol precipitation were subjected to gel chromatography on Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, N.J.). The void volume fraction comprised the ascending limb and peak of the Vo protein, while Vo fraction 2 was comprised of the descending limb of the Vo and the part of the plateau of protein following the Vo. These fractions were concentrated by ammonium sulfate precipitation and dialyzed against imidazole-saline buffer, 0.01 M imidazole, 0.015 M saline, pH 7.0.

Reduction of the Factor VIII/von Willebrand Factor Protein

Labeled or unlabeled f.VIII/vWF was diluted in 0.025 M imidazole, 0.015 M saline (pH 7.0) buffer to a concentration of approximately 2 mg/ml. Reduction was achieved by the addition of 0.1 volume of 2-ME at 10 times the desired final concentration. The material was incubated for 1 hr at 37°C, and the reaction was terminated by the addition of 0.1 volume of 50 mM iodoacetamide (Sigma Chemical Co., St. Louis, Mo.) at room temperature for 10 min. Although several final concentrations of 2-ME were used for reduction, the two concentrations that were found to be most useful in our studies were 0.01% and 0.1% 2-ME. Iodoacetamide was added to intact f.VIII/vWF protein in some studies and in others the 2-ME and iodoacetamide-treated f.VIII/vWF protein was dialyzed prior to the binding studies and compared to material that had not been dialyzed. For the binding studies of reduced f.VIII/vWF protein, labeled intact f.VIII/vWF was reduced with the appropriate concentration of 2-ME.

Labeling of the Factor VIII/von Willebrand Factor Protein

The f.VIII/vWF protein at 3-5 mg/ml was radiolabeled by reduction with tritiated potassium borohydride (Amersham Searle Corp., Arlington Heights, Ill. or New England Nuclear Boston, Mass.) after modification of terminal galactose by protease-free galactose oxidase (Sigma Chemical Co. or Worthington Biochemical Corp., Freehold, N.J.) as previously described. The modification of the f.VIII/vWF protein was achieved by incubation with galactose oxidase at a final concentration of 4 U/mg of f.VIII/vWF protein and horseradish peroxidase at 10 µg/ml of protein (final concentration). These were incubated together at 37°C for 16 hr. Then, 1 µCi of potassium borohydride was added per milligram of the f.VIII/vWF protein and incubated at room temperature for 30 min. After 30 min at room temperature, the material was dialyzed for 16-24 hr extensively against Tris-saline buffer (Tris 0.05, sodium chloride 0.1 M) pH 7.35 at 4°C. After dialysis, the labeled f.VIII/vWF protein was kept at 4°C and used for experiments within 72 hr. The final product had a specific activity of 0.1-0.2 µCi/µg f.VIII/vWF protein. The reduced materials had almost identical specific activity as the intact f.VIII/vWF protein.

Assays

Factor VIII coagulant activity was assayed using a modification of a one-stage partial thromboplastin time with a celite cephalin mixture as activator (Plateletin, General Diagnostics, Morristown, N.J.). Factor VIII related antigen was measured either by the Laurell electroimmunoassay utilizing goat anti-human f.VIII/vWF protein or by counterimmunoelectrophoresis utilizing the same antibody. Ristocetin cofactor assay was performed using either a washed normal platelet system in the aggregometer (Chronolog and/or Peyton Dual Channel) or the formalinized platelet assay. The washed platelets were prepared from whole blood collected in sodium citrate (final concentration 129 mM). Platelet-rich plasma (PRP) was obtained by centrifugation at 25°C for 3 min at 600 g. PRP was removed and an equal volume of buffer (0.05 M Tris, 0.15 M NaCl, 0.02 M EDTA, pH 7.35) was added to the remaining red cells and plasma to replace the PRP. Red cells and buffer were mixed and spun at 600 g for 3 min at 25°C. The wash was removed and added to the original PRP. The PRP and wash were spun at 2000 g for 8 min at 25°C; the platelet pellet was then washed 4 times with 5.7 ml of the wash buffer. The final platelet pellet was dissolved in the final diluting buffer of 0.05 M Tris, 0.15 M sodium chloride, pH 7.35, and heated to 37°C for approximately 15 min. The platelets were counted and adjusted to the appropriate count. All procedures involving platelets were performed with plastic pipettes and plastic tubes.

The ristocetin-induced platelets agglutination assay consisted of 400 µl platelets (200,000/µl), 50 µl of sample, and 5 µl of ristocetin at a concentration of 50 mg/ml (final concentration 0.55 mg/ml). Two standard curves were performed on all studies: (1) a standard curve with a pooled normal plasma and (2) a standard curve with purified f.VIII/vWF protein that had not been reduced. When any reduced f.VIII/vWF protein was tested for vWF activity, the purified intact f.VIII/vWF used for the standard curve was always from the same preparation. All reduced f.VIII/vWF protein activities were expressed in relationship to the intact protein. Protein concentration was determined by the method of Lowry using bovine serum albumin as the standard.

Immunoelectrophoresis was performed with 1% agarose in a pH 8.6 barbital buffer as previously described. Crossed antigen-antibody electrophoresis was performed as previously described, using goat anti-human f.VIII/vWF antibody. In all immunologic studies, the concentration of the intact and reduced forms of the f.VIII/vWF protein were identical.

Pollyacrylamide gel electrophoresis was performed in the presence of SDS with and without dithioerythritol (5 mM) as previously described. Two percent polyacrylamide-0.5% agarose gel electrophoresis was modified after the method of Perret et al. Electrophoresis was carried out at 8 mamps/gel (gel length 120 mm) so that the bromphenyl blue marker dye usually reached the bottom of the gel within 6 hr (± 15 min).

Binding Assay

All binding assays were performed under identical conditions, i.e., 400 µl platelets (platelet count of 200,000/µl), 50 µl of f.VIII/vWF protein, and 5 µl of ristocetin concentration 50 mg/ml (final concentration 0.55 mg/ml) were added together and incubated for 60 min at room temperature. After incubation, the mixture was centrifuged at 2500 g for 3 min, and the supernatant was removed and counted. Phase microscopy examination of the supernatant did not reveal any platelets to be present. The platelets were separated
by centrifugation (see below) and the radioactivity of the supernatant and of the platelet pellet was determined.

Binding assays were done at room temperature in a 12 x 75 mm polypropylene tube at a final volume of 0.455 ml. Nonspecific binding was tested by two techniques. The specific binding of 3H-f.VIII/vWF to platelets was determined by incubating the labeled f.VIII/vWF protein in the absence and presence of excess amounts of unlabeled protein (10-100-fold excess). The bound f.VIII/vWF protein not displaced by the presence of excess amounts of cold f.VIII/vWF protein represented nonspecific binding. The nonspecific binding subtracted from total binding was defined as specific binding.

The second method involved the incubation of 400 µl buffer, radiolabeled f.VIII/vWF protein 50 µl, and ristocetin 5 µl. At the designated time, the mixture was centrifuged at 2500 g for 3 min, the platelets and supernatant were removed and counted. The specific binding was considered that one molecule of the f.VIII/vWF protein bound to one platelet receptor. These studies were performed at an incubation period of 60 min and the cells and supernatant removed and counted. Determination of nonspecific binding was performed as described above.

For the Scatchard analysis of binding of the f.VIII/vWF protein to platelets, an identical incubation mixture was used except the f.VIII/vWF protein concentration was varied. In these studies we considered that one molecule of the f.VIII/vWF protein bound to one platelet receptor. These studies were performed at an incubation period of 60 min and the cells and supernatant from the centrifuged incubation mixture were counted in a scintillation counter (Nuclear Chicago, Des Plaines, Ill.).

Inhibition of binding of intact f.VIII/vWF protein to platelets was performed utilizing the radiolabeled intact material, 400 µl platelets (200,000/µl) and ristocetin 50 µl (final concentration 0.55 mg/ml). The incubation of platelets with f.VIII/vWF protein and ristocetin was allowed to proceed for 120 min. The incubation mixture was spun at 2500 g for 3 min and the supernatant removed and counted. Determination of nonspecific binding was performed as described above.

The time course of binding was performed by the addition of the intact or reduced species of f.VIII/vWF protein to 400 µl platelets (200,000/µl) and ristocetin 50 µl (final concentration 0.55 mg/ml). The incubation of platelets with f.VIII/vWF protein and ristocetin was allowed to proceed for 120 min. The incubation mixture was spun at 2500 g for 3 min and the supernatant removed and counted. Determination of nonspecific binding was performed as described above.

For the Scatchard analysis of binding of the f.VIII/vWF protein to platelets, an identical incubation mixture was used except the f.VIII/vWF protein concentration was varied. In these studies we considered that one molecule of the f.VIII/vWF protein bound to one platelet receptor. These studies were performed at an incubation period of 60 min and the cells and supernatant from the centrifuged incubation mixture were counted in a scintillation counter (Nuclear Chicago, Des Plaines, Ill.).

Inhibition of binding of intact f.VIII/vWF protein to platelets was performed utilizing the radiolabeled intact material, 400 µl platelets (200,000/µl), 5 µl radiolabeled intact f.VIII/vWF protein (0.8-1.0 µg final concentration), 25 µl unlabeled intact f.VIII/vWF protein (0.5-75 µg final concentration), and 5 µl of ristocetin 50 µg (0.55 mg/ml final concentration). This mixture was incubated for 60 min at room temperature. Reduced f.VIII/vWF proteins, Vo fraction 2 revealed single protein band (230,000 daltons) was present. This stained positive with both Coomassie blue and periodic acid Schiff stains. When Vo fraction 2 was studied on polyacrylamide gel electrophoresis, several bands entered the 5% acrylamide gel in the presence of SDS. After reduction, a single protein band (230,000 daltons) was seen, which also stained positive with both periodic acid Schiff and Coomassie blue.

Treatment of the intact f.VIII/vWF with 2-ME for periods ranging from 30 min to 2 hr did not affect procoagulant activity. However, different concentrations of 2-ME reduced the vWF activity in direct relationship to their final concentration (Table 1). We found that incubation for 1 hr was optimum, and all incubations of f.VIII/vWF protein were performed for this period of time. After the 1-hr incubation, alkylation was achieved by addition of iodoacetamide for 10 min. Comparison of the procoagulant and vWF activity of the labeled and unlabeled reduced material revealed no loss of activity.

In initial studies of the affect of 2-ME and alkylation with iodoacetamide, the treated material was assayed in the ristocetin cofactor assay and in binding studies either without dialysis to remove excess 2-ME and iodoacetamide or after dialysis. The protein concentrations were comparable, and the nondialyzed

### Table 1. Characteristics of the Reduced f.VIII/vWF Protein

<table>
<thead>
<tr>
<th>Sample</th>
<th>vWF Percent</th>
<th>Kd (± 95 M)</th>
<th>Number of Molecules per Platelet, 10^6</th>
<th>Largest Size Oligomer, 10^6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact Vo</td>
<td>100</td>
<td>0.39 ± 0.07</td>
<td>2.8 ± 0.3</td>
<td>10.2</td>
</tr>
<tr>
<td>2-ME 0.01%</td>
<td>41.7 ± 4.0</td>
<td>1.92 ± 0.38</td>
<td>3.4 ± 0.8</td>
<td>6.9</td>
</tr>
<tr>
<td>(27-66)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intact Vo</td>
<td>39.4 ± 4.4</td>
<td>2.76 ± 0.41</td>
<td>4.0 ± 0.8</td>
<td>5.6</td>
</tr>
<tr>
<td>(19-63)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-ME 0.1%</td>
<td>13.1 ± 3.3</td>
<td>22.1 ± 5.0</td>
<td>25.2 ± 5.0</td>
<td>1.6</td>
</tr>
<tr>
<td>(8-25)</td>
<td></td>
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</tbody>
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* vWF activity is expressed in mean percent of the intact ± 1 SD, n = 6; numbers in parentheses indicate range of observation.
† Values are mean ± 1 SD, n = 6, for the highest affinity binding for each preparation and for the capacity of those sites.
sample had almost identical vWF activity and binding to platelets in the presence of ristocetin, as did the dialyzed sample. Thus, dialysis was not performed prior to testing the f.VIII/vWF protein in the subsequent assays.

We monitored the affect of the change in molecular size of the f.VIII/vWF protein after incubation with 2-ME by four techniques: (1) immunoelectrophoresis against goat anti-human f.VIII/vWF protein, (2) crossed antigen–antibody electrophoresis against the same antibody, (3) SDS polyacrylamide gel electrophoresis without reducing agent, and (4) agarose acrylamide gel electrophoresis in the presence of SDS. We could detect significant changes in the migration of the f.VIII/vWF protein after treatment with 0.01% or 0.1% 2-ME in immunoelectrophoresis. The 2-ME-treated f.VIII/vWF had increased anodal migration. The f.VIII/vWF protein in the agarose acrylamide gel electrophoresis was helpful in showing altered migration of the f.VIII/vWF protein after 2-ME treatment. In general, the greater the concentration of 2-ME incubated with the f.VIII/vWF protein, the more anodal the migration of the protein in the crossed-antigen–antibody electrophoresis (Fig. 1). In fact, at 0.01%, there was a significant loss of the more cathodal, i.e., slower moving, forms of f.VIII/vWF protein and an increase in the faster, i.e., more anodal, forms of the f.VIII/vWF protein. This change was even more pronounced with the 0.1% 2-ME-treated material (Fig. 1).

Use of the third technique, the polyacrylamide gel electrophoresis in the presence of SDS, was helpful in that after 2-ME treatment, the protein band of the intact material that did not enter the gel then entered the gel and multiple protein bands were seen. However, the technique was not sensitive enough to distinguish the molecular weights of the different protein bands at higher concentrations of 2-ME, although the protein bands did enter the gel to a greater distance than those of the lower 2-ME concentrations.

The last technique used to monitor the reduction of f.VIII/vWF protein was the agarose acrylamide gel electrophoresis. With this technique, we were able to delineate the size of the oligomers present in the intact preparation of the f.VIII/vWF protein as well as in the preparations that had been treated with 2-ME. The standard curve for molecular weight determinations included reduced and nonreduced fibronectin and crosslinked IgM. The curve was linear from 220,000 (reduced fibronectin) to 2.92 × 10⁶ (crosslinked IgM). The largest molecular weight oligomer seen in the intact f.VIII/vWF protein was 10.2 × 10⁶ daltons (Fig. 2). Eight distinct bands were seen in the intact material with calculated molecular weights of 1.6 × 10⁶, 2.8 × 10⁶, 4.2 × 10⁶, 5.6 × 10⁶, 6.8 × 10⁶, 8.0 × 10⁶, 9.1 × 10⁶, and 10.2 × 10⁶. The 6.8–10.2 × 10⁶ dalton oligomers represent 19% of the total, the 4.2 and 5.6 × 10⁶ 47%, and the 2.8 and 1.6 × 10⁶, 34%. The mean molecular weight derived from these data was 4.8 × 10⁶. The largest oligomer found in 0.01% reduced material was in the vicinity of 7.0 × 10⁶ daltons. Five distinct bands were seen, varying from 1.5 × 10⁶, 2.9 × 10⁶, 4.3 × 10⁶, 5.6 × 10⁶ to 6.9 × 10⁶ daltons. The 6.9 × 10⁶ molecular weight oligomers represent 3% of the total, the 4.2 and 5.6 × 10⁶ oligomers, 29%, and the 1.6 and 2.9 × 10⁶ oligomers, 67%. The average molecular weight derived from these data was 3.0 × 10⁶. In the 0.1% 2-ME-treated f.VIII/vWF protein, 4 bands were seen with molecular weights of 0.26 × 10⁶, 0.51 × 10⁶, 1.1 × 10⁶, and 1.6 × 10⁶ daltons. The 1.6 × 10⁶ oligomers represent 37% of the total, the 1.1 × 10⁶, 27%, the 0.52 × 10⁶, 21% and

![Fig. 1. Crossed antigen–antibody electrophoresis of the intact f.VIII/vWF protein and 2-ME-treated material. The f.VIII/vWF protein and the 2-ME-treated material were run simultaneously on the same plate with the same power source and equivalent protein concentrations. After staining, the plates were superimposed on each other and demonstrate three separate precipitin arcs. The slowest migrating material (I) is the intact f.VIII/vWF protein. The intermediate precipitin arc is 0.01% 2-ME-treated f.VIII/vWF protein (0.01), and the most anodal, i.e., fastest moving material, is the 0.1% 2-ME-treated f.VIII/vWF protein (0.1). The anode is to the right, cathode is to the left. The antibody is goat anti-human f.VIII/vWF antibody.](image-url)
the 0.26 × 10^6, 15%. The mean molecular weight derived from these data was 10^6. The Vo fraction 2 contained oligomers of 5.6 and 4.2 × 10^6 (22%), 2.8 × 10^6 (32%), and 1.6 × 10^6 (46%) daltons. The average molecular weight derived from these data was 2.4 × 10^6.

The time course of binding of the intact and reduced f.VIII/vWF proteins revealed that the f.VIII/vWF protein exposed to higher concentrations of 2-ME had a decreased rate of binding of the f.VIII/vWF protein to the platelet surface in the presence of ristocetin. However, after 30 min, the percent binding was similar, and by 60 min, the difference was approximately 5%, which over the next 60 min decreased to 2%. In 5 different studies, the 0.1% 2-ME f.VIII/vWF protein always had a slower initial slope of binding to the platelet and the 0.01% had an intermediate position between the 0.1%-treated f.VIII/vWF protein and the intact protein (Fig. 3).

The binding of the f.VIII/vWF protein to platelets was analyzed by Scatchard analysis using a molecular weight of 1.2 × 10^6 for the ligand and assuming that one molecule of the f.VIII/vWF protein binds to one receptor. With the intact protein, 9.0 × 10^3 molecules bound per platelet (high affinity) with a $K_d$ of 1.8 × 10^{-6}.
Fig. 4  (A) Scatchard analysis of intact f.VIII/vWf protein to platelets revealed a hyperbolic curve, two classes of binding sites. The asymptote of the high affinity sites revealed a capacity per platelet of $2.8 \times 10^3$ sites with $K_d$ of $0.39 \times 10^{-9}\text{M}$. The asymptote of the lower affinity binding molecules revealed a $K_d$ of $15.9 \times 10^{-9}\text{M}$ and a capacity of $5.9 \times 10^3$. The results are the average of six determinations with different preparations of the f.VIII/vWf protein. (B) The 0.01% 2-ME-treated f.VIII/vWf protein showed intermediate affinity binding sites of $3.9 \times 10^3$ with a $K_d$ of $1.91 \times 10^{-9}\text{M}$ and $9.6 \times 10^3$ molecules with low affinity. $K_d$ of $19.3 \times 10^{-9}\text{M}$. The results are the average of six determinations with different preparations of the f.VIII/vWf protein. (C) Scatchard analysis of the 0.1% 2-ME-treated material revealed that $25.2 \times 10^3$ molecules bound only with low affinity: $K_d$ $22.1 \times 10^{-9}\text{M}$. The results are the average of six determinations with different preparations of the f.VIII/vWf protein.
10^9, and 1.5 x 10^9 molecules bound per platelet (low affinity) with a K_d of 27.4 x 10^9. The 0.01% material had 10 x 10^9 molecules bound per platelet with an intermediate affinity K_d of 4.5 x 10^9 and 2.5 x 10^9 molecules bound with low affinity K_d of 29.7 x 10^9. The 0.1% material did not bind with high or intermediate affinity; 26.1 x 10^9 molecules bound per platelet with a low affinity K_d of 27.8 x 10^9.

The Scatchard analysis was performed identically for the 3 preparations of f.VIII/vWF protein, except that the molecular weight of the intact f.VIII/vWF protein was estimated to be 4.8 x 10^6 daltons, the molecular weight of the 0.01% 2-ME material 3.0 x 10^6 daltons, and 10^6 daltons for the 0.1% 2-ME material (Fig. 4 A–C). The mean molecular weight for the Vo fraction 2 was 2.4 x 10^6. The intact material had a total number of 8.7 x 10^3 molecules bound per platelet; of these, 2.8 x 10^3 molecules bound with high affinity (K_d of 3.9 x 10^9 M) and 5.9 x 10^3 with intermediate affinity (K_d of 1.59 x 10^9 M). The 0.01% 2-ME-treated material had 13.5 x 10^3 molecules bound per platelet, 3.9 x 10^3 molecules per platelet bound with intermediate-high affinity (K_d of 1.92 x 10^9 M), while 9.6 x 10^2 molecules per platelet bound with low affinity (K_d of 19.3 x 10^9 M).

The Scatchard analysis of the 0.1% 2-ME-treated material revealed a total number of 25.2 x 10^3 molecules bound per platelet, and all of these molecules bound with a low affinity (K_d of 22.1 x 10^9 M). The Vo pool 2, prepared from the Sepharose 4B column, was compared to the Vo pool in relation to its binding affinity to platelets. The Vo fraction 2 was similar to the 0.01% 2-ME-treated material. The molecules with intermediate affinity had a K_d of 2.76 x 10^9 M and a capacity of 4.0 x 10^9 per platelet, while molecules binding with low affinity had a capacity of 9.7 x 10^9 with a K_d of 25.6 x 10^9 M.

Thus, there was a progressive decrease in the affinity of the f.VIII/vWF protein binding, with greater reduction in the f.VIII/vWF protein; this was seen either when all preparations were designated at a single molecular weight (1.2 x 10^6) or when average molecular weights were used. As the molecular weight decreased, the affinity of the f.VIII/vWF multimer for the platelet receptor decreased while the capacity of binding increased. vWF activity correlated very well with high affinity binding of the f.VIII/vWF multimers to the platelet; as the K_d increased, the molecular weight decreased, and as the affinity for the binding sites changed to intermediate and low affinities, vWF activity decreased (Table 1).

Studies of the competitive inhibition of the intact radiolabeled f.VIII/vWF protein binding to platelets by each of the various modified f.VIII/vWF proteins as well as Vo fraction 2 were performed over a wide range of concentrations (Fig. 5). These studies revealed that it required 5.1 µg/ml final concentration (94 µg/ml) of the cold intact f.VIII/vWF protein to displace 50% of the radiolabeled intact protein (0.5–0.7 µg/ml final concentration). A 22.3 µg/ml final concentration (405 µg/ml) of the 0.01% 2-ME-treated material and 66.9 µg/ml final concentration of 0.1% 2-ME-treated material was required to displace 50% of the radiolabeled f.VIII/vWF protein. The Vo fraction 2, similar to the 0.01% 2-ME, required 28.4 µg/ml final concentration (516 µg/ml) to displace 50% of the intact f.VIII/vWF protein.

The competitive inhibition of the intact radiolabeled f.VIII/vWF by the various forms of f.VIII/vWF protein correlated very well with the K_d for each material compared to the intact material. The intact f.VIII/vWF was considered the control material with which to compare the other forms of the f.VIII/vWF protein. The K_d of the high affinity binding of the intact was 0.39 nM, while the K_d of the 0.01% 2-ME material was 1.92 nM (5 times greater). The K_d of the Vo 2 was 2.76 nM (7.1 times greater) and the K_d of the 0.1% 2-ME material was 22.1 nM (57 times greater). The µg/ml required for 50% inhibition were converted to nanomoles, and this showed that it required 43 nM of intact material, 300 nM for 0.01% 2-ME (7.0 times
greater), 470 nM for the 0.01% 2-ME material (62.8 times greater) to achieve 50% inhibition.

The dissociation of radiolabeled f.VIII/vWF protein from the platelet, after equilibrium had been reached, was tested by adding additional cold f.VIII/vWF protein and ristocetin (Fig. 6). Both the intact 0.01% 2-ME and the 0.1% 2-ME radiolabeled materials (1.1 μg/ml final concentration) showed minor amounts of displacement over time when cold f.VIII/vWF protein at equal concentration was added after equilibrium. More rapid displacement occurred with 2.2 μg/ml and at 22 μg/ml. All 3 materials showed an initial rapid displacement of the radiolabeled protein with a much slower second phase.

DISCUSSION

In these studies, we have examined the relationship between the size of purified f.VIII/vWF oligomers binding to the platelet surface and the correlation with the vWF activity as measured by the ristocetin cofactor assay. We have corroborated the findings of other laboratories that reduction and alkylation of the f.VIII/vWF protein decreases vWF activity but does not affect f.VIII coagulant activity, although we disagree that partially reduced f.VIII/vWF has normal binding to platelets. Similar structure-function relationship of vWF activity and size of the oligomer of the f.VIII/vWF protein has been described with porcine vWF.

We have found that the size of the oligomer of the f.VIII/vWF protein is important in binding to the platelet surface. The intact f.VIII/vWF binds to the platelet with high affinity, and as the size of the oligomers is decreased (0.01% and 0.1% 2-ME-treated material) the affinity of binding decreases. There is a direct correlation between the affinity of binding of the intact protein with high affinity to the platelet receptor and inducing vWF activity in the ristocetin cofactor assay. The 0.1% 2-ME-treated f.VIII/vWF protein, which has a smaller multimer size, binds with intermediate affinity and is almost unable to support vWF activity. The 0.01% 2-ME-treated f.VIII/vWF protein has an intermediate affinity for platelet binding and has moderately reduced vWF activity (Table I, Fig. 4). These studies clearly demonstrate that only the high affinity binding are of importance in supporting vWF activity. When the high affinity binding occurs there is platelet interaction causing agglutination or aggregation, and when low affinity binding occurs there is no significant interplatelet interaction. Thus, the low molecular weight oligomers of the f.VIII/vWF protein bind to the platelet surface, but by themselves do not appear to be of any importance in acting as an interplatelet bridge or possibly as a subendothelial platelet bridge to secure primary hemostasis. We hypothesize that the differences in binding characteristics are related to the size of the f.VIII/vWF oligomer and not to differences in the platelet receptor. The f.VIII/vWF of large multimer size binds with high affinity, possibly due to interaction with multiple receptors. The f.VIII/vWF of small or intermediate size multimers binds with low or intermediate affinity. Because of the reduction in molecular size, these smaller multimers bind to fewer or even to only one receptor.

These findings also suggest that the two classes of binding sites that have been previously reported by Schneider-Trip et al. and us are a reflection of the heterogeneity of the oligomer size of the f.VIII/vWF.
protein used in these assays.\textsuperscript{14,15} Although the intact purified f.VIII/vWF protein appears to be homogeneous by SDS polyacrylamide gel electrophoresis and/or crossed antigen–antibody electrophoresis, it can be proven to be heterogeneous by the use of the agarose acrylamide electrophoresis system. Several populations of oligomers are present. The different sized oligomers interact differently with the platelet f.VIII/vWF receptor and demonstrate that the larger molecular weight species are the most important in causing platelet-to-platelet interaction and result in the definition of high-affinity, low-capacity binding. The smaller oligomers appear to bind to a high-capacity binding site with low affinity, which does not result in platelet–platelet interaction. There is an inverse correlation between the binding studies and vWF activity as seen by comparing the affinity of binding to the vWF activity or the inhibition of the binding of the intact f.VIII/vWF by various preparations of the f.VIII/vWF protein of smaller oligomer size. The single class of binding sites described by Kao et al. may reflect a greater homogeneity of the f.VIII/vWF protein used in their studies.\textsuperscript{13}

These studies may also have importance in the understanding of some of the abnormalities of the f.VIII/vWF protein in vWd. Several studies\textsuperscript{25–29,42} have indicated that f.VIII/vWF protein in vWd is of a smaller molecular weight than is the normal. This has been demonstrated by techniques such as agarose gel chromatography, crossed antigen–antibody electrophoresis, analysis of the multimeric structure of the f.VIII/vWF protein, and by functional assays showing that the ratio of vWF to antigen is decreased in some patients with vWd. Others have suggested that the defects in vWd are much like the supernatant from cryoprecipitate where there are significant amounts of coagulant and antigenic activities but reduced vWF activities.\textsuperscript{20,28} Specific changes in the size of the oligomers of the f.VIII/vWF protein have been described by Meyer et al.,\textsuperscript{27} Sixma et al.,\textsuperscript{28} and Ruggeri and coworkers.\textsuperscript{29} In each of these instances, there has been a suggestion that the oligomeric structure of the vWd f.VIII/vWF protein is smaller than that of normal and that only the larger oligomers of the plasma f.VIII/vWF protein bind to platelets. Recently, Ruggeri and Zimmerman\textsuperscript{31} reported a subset of vWd patients in whom there appeared to be enhanced binding of the smaller f.VIII/vWF protein to platelets.

From these studies it is clear that the size of the f.VIII/vWF protein is one determinant of binding to the platelets. There may be molecular alterations in these proteins that either enhance or interfere with their binding properties. In addition, there has not been a good correlation between the ability and affinity of these various oligomers to bind to the platelet surface and to induce agglutination or aggregation of platelets. Our studies indicate that f.VIII/vWF protein of various molecular sizes, i.e., oligomeric structures, which have no alteration other than the size of the oligomer, bind quite differently to the platelet surface. Thus, reduction of the oligomer size of the f.VIII/vWF protein would be, in itself, a defect that would result in decreased platelet binding and reduced platelet agglutination in the presence of ristocetin. Ruggeri and Zimmerman reported that the smaller multimers from some of their vWd patients did not bind to platelets.\textsuperscript{42} From our data, the smaller multimers derived from “normal” f.VIII/vWF protein do bind platelets with low affinity sites. Our small oligomers are formed from a “normal” intact molecule, while the oligomers from their patients are “native.” It is possible that the defect in these patients is not simply a defect of molecular size but may involve some other alteration of the molecule. Another explanation is that these multimers do bind to platelets but the assay measuring supernatant antigen indirectly is not as sensitive as our binding studies.

Additionally, the studies of the various sizes of the f.VIII/vWF protein may also help to clarify some of the questions involved in posttransfusion studies in patients with vWd. The smaller oligomers of the f.VIII/vWF protein are ineffective as molecular bridges between platelets compared to the larger oligomers. In the patient with vWd, transfusion of cryoprecipitate results in the infusion of large, intermediate, and small molecular weight oligomers, the patient with variant vWd may still have a significant amount of their own intermediate or small oligomers. At some point after transfusion, the ratio of the smaller molecular weight oligomers to the larger molecular weight oligomers may result in displacement of the larger oligomers from the platelet surface and interfere with their ability to act as intracellular bridges. Similar results were seen in the inhibition studies of the intact f.VIII/vWF protein by the 0.01%, 0.1% 2-ME reduced f.VIII/vWF protein and the Vo fraction 2. All three materials had significantly lower molecular size (and absence of the large oligomers) than did the intact material. This process may be aided by the observation in normals that the larger molecular weight species of the f.VIII/vWF protein are metabolized more rapidly than the smaller species;\textsuperscript{31} thus, the coagulant activity and the antigen levels may remain elevated for prolonged periods after transfusion, while the ristocetin cofactor activity, i.e., vWF activity, may decrease despite the persistence of normal or elevated levels of the two former activities.
A similar explanation may explain the discrepancy between bleeding time and the ristocetin cofactor activity or bleeding time and the antigen levels. There may be priorities in that the bleeding time may require the largest f.VIII/vWf protein for correction, while the ristocetin cofactor may require an intermediate size. The antigen and coagulant activity seem to be somewhat independent of the size of the f.VIII/vWf protein.

These studies support the concept that the size of the f.VIII/vWf protein is important in binding to the platelet surface and inducing platelet agglutination or aggregation, and they are in agreement with previous hypotheses from this laboratory and other laboratories.5,12,43,44 Two structure–function relationships of the vWf have been defined for the ability of the f.VIII/vWf protein to bind to the platelet surface with high affinity and to cause platelet agglutination: (1) the presence of penultimate galactose residues on the carbohydrate side chains of the intact f.VIII/vWf protein and (2) a minimum molecular size of the f.VIII/vWf oligomer.

ACKNOWLEDGMENT

The authors wish to thank Georgia Jackson and Eddie Cregger for excellent technical assistance, Dr. Craig Kessler for his gift of fibronectin; Dr. David Fass of the Mayo Clinic, Rochester, Minn. for his gift of crosslinked IgM; and Lynda Ray for secretarial assistance.

REFERENCES


Effect of multimeric structure of the factor VIII/von Willebrand factor protein on binding to platelets

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