Further Specificity Characterization of von Willebrand Factor Inhibitors Developed in Two Patients With Severe von Willebrand Disease

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Circulating inhibitors against von Willebrand factor (vWF) that show the properties of heterologous IgG antibodies have been described in a few patients with severe von Willebrand disease (vWD). The present study provides further characterization of inhibitors from two patients with severe vWD. Inhibitors in both, like polyclonal rabbit antibody, detected all sizes of multimers and the complex structure of each multimer from platelets and plasma of normal individuals as well as from plasma of patients with type IIA, IIB, and IIC vWD. Both inhibitors and the rabbit antibody reacted mainly with the intact 225-Kd structure of each multimer from platelets and plasma of normal individuals as well as from plasma of patients with type IIA, IIB, and IIC vWD. This study was designed to further analyze the target specificity of inhibitors toward vWF that appear in two multitransfused patients with severe vWD.

MATERIALS AND METHODS

Patients

Two unrelated multitransfused patients (Hu 1 and Hu 2) with severe vWD who developed inhibitors against vWF were analyzed in this study. Patient no. 1 was a 24-year-old woman, with a history of epistaxis, bruising, gingivorrhagies, hemarthrosis, and menorrhagia, which on several occasions required treatment with human hemoderivatives. A bleeding time (Ivy method) was greater than 30 minutes. The patient's plasma had unmeasurable levels of FVIII:vWF activities, and a diagnosis of severe vWD was established. After multiple plasma and cryoprecipitate infusions, she developed a precipitating antibody to vWF. Patient no. 2 was the subject of earlier studies. Plasmas from normal adult volunteer subjects and from patients with type IIA, IIB, IIC, and severe vWD were also included.

Reagents

Proteinase inhibitors included leupeptin, EDTA, N-ethylmaleimide, aprotinin, and phenylmethysulfonylfluoride (Sigma Chemical Co, St Louis). Electrophoresis-pure reagents were acquired from Bio-Rad, Richmond, CA. Agarose HGT(P) from Seakem (Marine Colloids, Rockland, MD) and Sigma VII (Sigma) were used. Rabbit antismouse IgG was from Behning Diagnostics, Somerville, NJ. Rabbit antihuman IgG was from Pel-Freez Biologicals (Rogers, AR). Rabbit antihuman IgG from Zymed Laboratories (South San Francisco, CA). Rabbit antihuman IgG from Behring Diagnostics, Somerville, NJ. Sheep antisera against human IgG subclasses 1 and 4 and Staphylococcus aureus V-8 protease were from Miles Scientific, Naperville, IL. All other reagents were of the highest grade available.

Methods

The methods for blood collection and preparation of platelet-rich plasma (PRP) and platelet-poor plasma as well as for the determination of ristocetin-induced platelet agglutination (RIPA) in PRP were as previously described. Platelets were washed free of plasma.
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constituents and then lysed according to previously published procedures. The inhibition activity against vWF:Ag, vWF:RCo, VIII:C, and RIPA was assayed as described.

Sodium dodecyl sulfate–agarose electrophoresis. For analysis of vWF multimers, sodium dodecyl sulfate (SDS)-agarose electrophoresis was performed as described. When human inhibitor was used, the antibody was processed, affinity purified, and radiolabeled following the same procedure as for the rabbit antibody against vWF, as already published.

Digestion of vWF and purification of fragments II and III. vWF was immunoisolated from factor VIII concentrate (Armour Pharmaceutical Co, Terrytown, NY) as described elsewhere, digested with S aureus V-8 protease, and isolated as previously reported. By electrophoresis, reduced fragment II appeared mainly as a single band of 110 Kd; reduced fragment III, in addition to a band of 170 Kd, showed some other bands of smaller molecular weight (mol wt). However, fragment III retained RCo activity and inhibited the binding of 125I-vWF to platelets in the presence of ristocetin. Fragment II and III preparations were kindly supplied to us by Dr Z.M. Ruggeri and Dr T.S. Zimmerman (Scripps Clinic & Research Foundation, La Jolla, CA).

Subunit and fragment II and III analysis of vWF. For subunit analysis, immunoisolation of plasma vWF was carried out as already described.  SDS–polyacrylamide gel electrophoresis (PAGE) was done according to Laemmli and previously reported procedures. An outline of the immunoblotting procedure is as follows: after SDS–5% PAGE, reduced immunoisolated vWF (5 μg/lane) or reduced isolated fragments II and III (12 μg of each/lane) were transferred to a sheet of nitrocellulose. The membrane was cut into several strips, each containing reduced vWF and reduced fragment II and III lanes. Each strip with the immobilized vWF was then treated with the immunoglobulin-rich fraction of plasma samples from patients (at the same final inhibitory activity) or from a normal individual (twofold more concentrated than that used for Hu 2 inhibitor) that were obtained by three sequential saturated ammonium sulfate precipitations followed by extensive dialysis against 0.01 mol/L sodium phosphate–buffered saline, pH 7.2. The inhibitor fraction and protein blots were processed by using the bovine lactotransfer technique optimizer method. Inhibitor antibody bound to the vWF subunit or related fragments was detected by saturating quantities of 125I-labeled, affinity-purified rabbit antihuman IgG. As a control, immunoisolated and transferred vWF was also analyzed by using a pool of 55 anti-vWF monoclonal antibodies, all of which reacted with the reduced 225-Kd subunit, as already described, or with saturating amounts of 125I-radiolabeled, affinity-purified rabbit anti-vWF antibody (the same used for multimeric purposes). Finally, sheets of nitrocellulose were submitted to autoradiography at −70°C, and then each band was identified, excised, and counted in a gamma scintillation counter (Clinigamma, LKB, Bromma, Sweden) and its relative proportion calculated. To obtain the specific incorporation, the counts present in similar strips incubated with a normal immunoglobulin-rich fraction and the same amounts of second antibody were subtracted from the tests’ counts. For the analysis of fragments II and III, the relative proportion was calculated for the fragment II 110-Kd band and fragment III 170-Kd band.

Human inhibitor class and subclass determination.  125I-labeled, affinity-purified human inhibitors were diluted to 1/5 by using their corresponding plasmas. Double dilutions of the mixture were tested by double-diffusion techniques against rabbit antisera specific for each human immunoglobulin class and also against sheep antisera specific for human IgG, subclasses I to 4 (the latter only with human inhibitor Hu 1). After washing in normal saline, the gels were dried, stained with Coomassie blue R-250, and then subjected to autoradiography.

RESULTS

Both human inhibitors (Hu 1 and Hu 2) were IgGs showing α and λ chains. Inhibitor Hu 1 was IgG4. VWF:RCo was inhibited when normal plasma or fragment III, both with the same activity, were previously incubated with plasma Hu 1 and Hu 2 at a dilution of 1/64 and 1/3, respectively. VWF:Ag was inhibited by Hu 1 and Hu 2 at 1/120 and 1/20, respectively. Some inhibition against VIII:C was observed with both (Hu 1, 1/32; and Hu 2, 1/3). The two antibodies blocked RIPA (induced by 1.2 mg/mL of ristocetin) at 1/80 and 1/6 dilutions, respectively.

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**Fig 1.** SDS-agarose (2%) electrophoresis of vWF in (from left to right) normal platelet lysate (pt), normal plasma (N), and plasmas of patients with type IIB, IIC, IIA, and severe vWF (IIB, IIC, IIA, and S, respectively). Left panel, polyclonal rabbit antibody (R); right panel, human inhibitor from patient Hu 1 (Hu1). A very similar result is obtained with both kinds of antibodies.
**Multimeric Analysis of vWF**

When vWF was analyzed by using high-resolution SDS-2% agarose gels, the $^{125}$I-labeled, affinity-purified Hu 1 and Hu 2 antibodies detected multimers of all sizes present in normal plasma and platelets. In a way similar to the rabbit antibody, these antibodies also detected multimers in plasma vWF from patients with type IIA, IIB, and IIC vWD. Some fast-moving material from plasma of individuals with severe vWD was detected by both inhibitors, and it was also observed when using the heterologous antibody. Both inhibitors reacted with the five bands of each multimer present in normal, IIA, and IIB vWF and also the heavy and faint bands of IIC vWF in a fashion similar to the rabbit antibody (Fig 1).

**Subunit Analysis of vWF**

When vWF from normal plasma was immunoisolated, reduced, subjected to SDS-PAGE, blotted, and reacted with the immunoglobulin-rich fraction from Hu 1 and Hu 2 plasmas, a higher relative proportion of the intact 225-Kd subunit (80% and 82% of the total counts, respectively) as well as a lower relative proportion of the 176-Kd fragment (5% and 4%, respectively) were observed. This is in contrast to the pool of monoclonals that showed 73% and 14% for 225 Kd and 176 Kd, respectively (Fig 3). No significant differences were observed when the relative proportions of the 189-Kd and 140-Kd fragments were analyzed. At this level the rabbit antibody showed relative proportions similar to those obtained with human inhibitors (82% and 4% for 225 Kd and 176 Kd, respectively). None of these bands were detected by the immunoglobulin-rich fraction obtained from normal plasma.

**Analysis of vWF Fragments II and III**

For a comparison of the reactivity of the human vWF inhibitors as well as rabbit and the pool of monoclonal antibodies toward vWF, fragment II and III, the relative proportion of the total radioactivity associated with each fragment was calculated for each kind of antibody following two different approaches. First of all, since reduced fragment III has been shown to be composed primarily of a 170-Kd band and our fragment III preparation showed some other bands of lower mol wt (Fig 3), initially the total counts (total counts per minute) were obtained for each antibody by addition of the radioactive present only in the 110-Kd band of fragment II and in the 170-Kd band of fragment III. In a second approach, the relative proportion was calculated by using the total counts obtained by addition of the radioactivity present in all the bands. Similar differences were observed between the different antibodies analyzed when using both approaches; accordingly, we will refer to the results calculated with the first one. When the immunoglobulin-rich fraction from Hu 1 and Hu 2 plasmas or rabbit polyclonal antibody were used, a lower relative proportion of fragment II was observed (28%, 4%, and 37% of the total counts, respectively) as compared with the pool of monoclonal antibodies, which showed a higher relative proportion of this fragment (50%) (Fig 3).

**DISCUSSION**

The two human inhibitors included in this study were polyclonal IgGs thus showing $\kappa$ and $\lambda$ light chains, as usually seen in patients with severe vWD who have developed such inhibitors.

Regardless of their different titers, both human inhibitors reacted with multimers of all sizes present in normal individuals as well as in patients with variant-type vWD and also with the complex multimeric structure of vWF characteristic of normal and types IIA, IIB, and IIC vWD (Fig 1) in a similar fashion to the rabbit antibody. The two inhibitors detected some fast-moving material present in the plasma of a patient with severe vWD, also detected by rabbit antibody, that has been described in the plasmas from such patients (Fig 1).

An important difference was noticeable between the human inhibitors and the pool of monoclonal antibodies used.
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Fig 3. Upper panel, autoradiograph of nitrocellulose paper after SDS-PAGE and blotting of reduced immunoisolated fragments II and III (II and III, respectively) and then reacted with a pool of 66 anti-vWF monoclonals and then with 125I-labeled rabbit antihuman vWF (R) or with the immunoglobulin-rich fraction from inhibitor plasmas of patients Hu 1 and Hu 2 and then with 125I-labeled affinity-purified rabbit antihuman vWF (Hu 1 and Hu 2, respectively; + and −, longer and shorter exposure times, respectively, of the same nitrocellulose strip). The horizontal arrow indicates the anode, and the white arrow shows the almost complete absence of reactivity of Hu 2 toward the 110-Kd band of reduced fragment II. Lower panel, relative proportion of the counts associated with fragments II (110-Kd band) and III (170-Kd band) obtained after identification, excision, and counting of each of these bands present in the lanes shown in the upper panel. Means and ranges are from four different determinations. In this calculation the total counts were obtained by addition of the radioactivity present only in both of the aforementioned bands. A decreased reactivity toward fragment II proportional to that against the 170-Kd band of fragment III is observed for Hu 1 and rabbit polyclonal antibody as well as a very decreased or absent reactivity for Hu 2 inhibitors. This is in contrast to that observed for the monoclonal pool. Some minor differences in the reactivity toward the smaller bands present in the fragment III preparation are also noticeable when comparing the different antibodies used in this study.

to analyze the subunit composition of vWF as well as reduced vWF fragments II and III. Accordingly, the former reacted mainly with the intact 225-Kd subunit and 189- and 140-Kd related fragments, whereas the latter demonstrated a higher relative proportion of the 176-Kd peptide (Fig 2). At this level, polyclonal rabbit antibody showed a similar behavior to the human inhibitors. Furthermore, the pool of monoclonal antibodies reacted with both reduced vWF fragments II and III; however, the rabbit polyclonal antibody as well as Hu 1 inhibitor reacted poorly, and Hu 2 inhibitor did not react at all with reduced fragment II (Fig 3). None of these findings has been previously reported. The bands of mol wt of less than 170 Kd that are present in the fragment III preparation used in our study seem to be vWF since they are detected by the antibodies but not by the normal immunoglobulin-rich fraction. Most probably, they are the result of some proteolysis occurring during the preparation of the commercial FVIII concentrate that was used as the starting material for vWF purification.

Our findings suggest that human vWF inhibitors developed in patients with severe vWD, although behaving to some extent like the polyclonal heterologous antibodies against native vWF, show some striking differences in their target specificity. The reactivity of these inhibitors toward the 189- and 140-Kd fragments as well as fragment III, being decreased toward the 176-Kd and diminished (Hu 1) or absent (Hu 2) against fragment II, seem to indicate their ability to recognize the middle third and the region near the amino terminal portion of the vWF subunit.12,21 It explains the interference of the domain involved in the platelet GP Ib-vWF interaction and also the partial inhibition of FVIII as a consequence of steric hindrance rather than as a specific effect on the latter molecule.22,23 The lesser reactivity towards the carboxy terminal end of vWF could be due to a decreased antigenicity or accessibility of that region to the immune system.

A particularly striking feature of the vWF sequence is the presence of four distinct elements that are repeated from two to over four times each.24 The A elements are found in three imperfect copies. The GP Ib domain of vWF is located on one A element but is not repeated on the other A elements.22 Since the human inhibitors analyzed in our study react with GP Ib domain as well as recognize the 189-Kd fragment of vWF that does not possess a GP Ib domain,21 it is unlikely that just a single common epitope would be involved as a target for these antibodies. However, further analysis of more human inhibitors is needed to clarify this aspect.

Human inhibitors against vWF differ from those against...
FVIII developed in patients with severe hemophilia in that the latter show a much higher restricted specificity for their corresponding target, although the former show a certain restricted reactivity toward the subunit composition of vWF and also against vWF fragment II. This wider specificity of human vWF antibodies makes the small vWF-derived peptides useless as therapeutic agents in blocking their inhibitory reactivity in contrast to the strategy suggested for human FVIII inhibitors.

REFERENCES


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