Epitope Mapping of the von Willebrand Factor Subunit Distinguishes Fragments Present in Normal and Type IIA von Willebrand Disease from Those Generated by Plasmin

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Abstract

A small but consistent proportion of the von Willebrand factor (vWF) in normal plasma is composed of 189, 176, and 140 kD fragments cleaved from the 225 kD subunit. A monoclonal antibody map of vWF, based on the reactivity of individual antibodies with cyanogen bromide and tryptic fragments of known carboxy and/or amino termini, showed that in normal and IIA von Willebrand disease (vWD) plasmas the 140 kD fragment was derived from the amino-terminal region, whereas the 176 kD fragment was derived from the carboxy-terminal region of the subunit. In type IIA vWD, however, the fragments comprised a greater proportion of circulating vWF. In contrast, plasmin cleaved a 176 kD fragment from the amino terminus and a 145 kD fragment from the carboxy terminus of the subunit. Species similar to these plasmin-cleaved fragments were demonstrated in plasmas from four patients treated with fibrinolytic agents, but not in IIA vWD.

Introduction

We have recently demonstrated that in vivo proteolysis of von Willebrand factor (vWF)[1] is a normal event by showing that a small but consistent proportion of the vWF 225 kD subunit is present in normal plasma as cleaved fragments with apparent molecular masses of 189, 176, and 140 kD (1). In type IIA von Willebrand disease (vWD) the relative proportion of the 176 and 140 kD fragments is increased, supporting the hypothesis that proteolysis is responsible, at least in part, for the absence of large multimers in that disorder (1, 2). Plasmin, the platelet calcium-activated protease (calpain), and one or more enzymes released from polymorphonuclear leukocytes proteolyze vWF in vitro with resultant loss of large multimers (2–6). Thus, all are candidates for producing the loss of large multimers seen in type IIA vWD.

In order to explore the possible contribution of these or other enzymes to vWF proteolysis in vivo, we have used monoclonal antibodies to construct a partial epitope map of the 2,050 amino-acid vWF subunit based on the reactivity of the antibodies with cyanogen bromide and tryptic fragments of known carboxy and/or amino termini (7). This map localizes vWF fragments to specific portions of the molecule, beginning amino-terminal to Met(288) and extending carboxy-terminal to Val(1927), and shows that the cleaved species present in IIA vWD derive from similar portions of the molecule as those present in normal plasma. Our results indicate that although plasmin-cleaved fragments may be present in deep venous thrombosis, plasmin cleavage does not account for the fragments seen in normal or IIA vWD plasmas. Although the enzyme(s) causing the fragments which are normally present is (are) unknown, evidence is presented that one or more cysteine proteinases may play a role in their generation, and the increased proteolysis seen in IIA vWD may be a heightened form of this process.

Methods

Reagents. Proteinase inhibitors included leupeptin (Chemicon, Los Angeles, CA), EDTA, *N*-ethylmaleimide (NEM), aprotinin, and phenylmethylsulfonyl fluoride (PMSF) (Sigma Chemical Co., St. Louis, MO). Trizma base was also obtained from Sigma Chemical Co. Electrophoresis-pure reagents including glycine, sodium dodecyl sulfate (SDS), acrylamide, *bis*-acrylamide, dithiothreitol (DTT), ammonium persulfate, and nitrocellulose paper were purchased from Bio-Rad Laboratories (Richmond, CA). Rabbit anti-mouse IgG was bought from Zymed (San Francisco, CA) and the ^{125}I from Amersham Corp. (Arlington Heights, IL).

Preparation of plasma samples. Blood from normal donors and patients with IIA vWD was drawn from antecubital veins into polypropylene syringes and transferred immediately into polypropylene tubes containing 0.1 final volume of 3.8% sodium citrate, pH 7.4, with or without proteinase inhibitors. Inhibitors were used in the following final concentrations in blood: leupeptin, 1 mM; NEM, 5, 6, 15, 30, or 60 mM; EDTA, 5 mM; and aprotinin 200–600 kallikrein inhibitory units (KIU)/ml of blood. Two combinations were utilized: leupeptin, EDTA, NEM, and aprotinin together or the latter two only.

Cell-deficient plasmas from normal individuals or patients with type IIA vWD (obtained by Dr. Carol Kasper, Orthopedic Hospital, Los Angeles, CA) were prepared by centrifugation of citrated blood at 1,700 *g* for 15 min at 23°C. Platelet counts after this procedure were 3,000–9,000/μl. In addition, normal plasmas were sometimes subjected to immediate ultracentrifugation at 100,000 *g* for 30 min at 19°C. Platelet counts after ultracentrifugation were 1,000–1,500/μl. These plasmas were used immediately or stored at −70°C. Plasmas from four patients with deep venous thrombosis without evidence of pulmonary emboli were collected in citrate without inhibitors in three patients and in citrate with the inhibitors mentioned above in one other patient (by Dr. Augusto Federici, Hemophilia and Thrombosis Center, University of Milan, Italy), and stored at −70°C until used.

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1. *Abbreviations used in this paper:* KIU, kallikrein inhibitory units; NEM, *N*-ethylmaleimide; vWD, von Willebrand disease; vWF, von Willebrand factor.

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Monoclonal antibodies. The monoclonal antibodies utilized in these experiments were prepared as previously described (8) using the following immunogens: native vWF, reduced and alkylated intact vWF, reduced and alkylated 52/48 kD and 22 kD tryptic fragments of vWF (8, 9), and a whole tryptic digest of vWF (9). All monoclonal antibodies used for immunoblotting were reactive with the intact 225 kD subunit. vWF fragments were initially identified with a pool of 55 antibodies including all of those used for epitope mapping. The antibody used for immunoisolation of vWF (5.5.72) was raised against native vWF and did not react with reduced vWF.

Preparation of tryptic and cyanogen bromide fragments of vWF. vWF was purified by immunoadsorption and digested with trypsin as previously described (9). The digest was subjected to size exclusion chromatography and the resulting five fractions (A–E) were reduced and *S*-carboxymethylated. Chromatofocusing of fraction B yielded polypeptides of 52/48 kD and 22 kD (9). A 55 kD polypeptide was purified in a similar manner from fraction C.

Cyanogen bromide fragments of vWF were prepared by Dr. Koiti Titani as described (7). In brief, a complete set of 42 fragments was generated from the whole carboxymethylated protein by cleavage with cyanogen bromide at methionyl bonds. These fragments were grouped by their placement within two large segments obtained by limited proteolysis with *Staphylococcus aureus* V8 protease. The sequence of the methionyl fragments were aligned by using peptides isolated from two digests: one by cleavage at lysyl bonds with *Achromobacter* protease I, and the other by cleavage at arginyl bonds with trypsin after blocking ϵ-amino groups of the carboxymethylated protein with citraconylation.

Urokinase and plasmin. Human urinary urokinase (Behring Diagnostics, La Jolla, CA) was solubilized in phosphate-buffered saline, pH 7.3. Plasminogen was purified from normal donor plasma by affinity chromatography on lysine-Sepharose and eluted with ϵ-amino caproic acid as described (10). Plasmin at 1.8 mg/ml was generated with 11.52 Ploug units of urokinase and 54 μg of plasminogen.

Immunoisolation of plasma vWF. vWF was immunoisolated from plasma samples essentially as described (1), using anti-vWF monoclonal antibody 5.5.72 coupled to cyanogen bromide-activated Sepharose CL4B beads (Pharmacia Fine Chemicals, Piscataway, NJ) at a density of 4 mg of IgG per ml of beads. The beads were prewashed with 0.1 M Tris-HCl, 0.15 M NaCl, pH 7.3, but without SDS and then incubated with plasma (1.5 ml to 275 μl of beads) in a minicolumn (VWR Scientific Div., Univar, Los Angeles, CA or Isolab, Inc., Akron, OH) at 4°C for 1 h on a rotating wheel. ~15 μg of vWF was bound for each milliliter of beads used. The beads were subsequently washed with 10 bed volumes of 0.5 M LiCl, 0.1 M Tris-HCl, pH 8.0, followed by 10 bed volumes of 0.02 M NaCl, 0.05 M Tris-HCl, pH 8.0. Prewash and LiCl buffers contained 1 mM leupeptin, 5 or 6 mM EDTA, 6 mM NEM, and 200 KIU aprotinin per ml. In later experiments, prewash and LiCl buffers contained only 6 mM NEM and 200 KIU aprotinin per ml. The NaCl buffer contained only aprotinin 200 KIU/ml. The vWF was eluted from the beads with 135–150 μl of 2% SDS, 0.1 M Tris-HCl, pH 8.0, at 60°C for 20 min with the column capped at both ends. The beads and elution buffer were aspirated from the column, and 100–115 μl of the supernatant obtained after centrifugation was analyzed by 5% SDS-polyacrylamide gel electrophoresis (PAGE) after reduction as described below.

SDS-PAGE and immunoblotting. Immunoisolated or highly purified vWF was reduced in a final concentration of 65 mM DTT for 15 min at 60°C and electrophoresed in SDS-5% polyacrylamide gels as described by Laemmli (11). Cyanogen bromide or tryptic fragments were reduced with 65 mM DTT for 15 min at 60°C in 8 M urea, 25 mM Na_2HPO_4, 25 mM EDTA, and 1% SDS and electrophoresed in linear gradient gels (5%–15% or 5%–20%) according to the method of Scheele (12).

Transfer of proteins from the polyacryalmide gel onto nitrocellulose membrane was performed using a 25 mM Tris-HCl, 192 mM glycine, 20% (vol/vol) methanol buffer, pH 8.3, at 0.25 A, 3°C for 16 h. The nitrocellulose membranes were then reacted with anti-vWF monoclonal antibodies, either singly or in pools, followed by ^{125}I-labeled rabbit anti-mouse IgG as described (1). The membranes were then processed according to the method of Johnson and co-workers (13). Bands were visualized by autoradiography with Kodak XRP-1 film (Eastman Kodak Co., Rochester, NY) using a Cronex Quanta III intensifying screen (E. I. duPont de Nemours and Co., Wilmington, DE). In some cases the radioactivity associated with individual bands was quantified after excision from the nitrocellulose membrane using a gamma scintillation spectrometer (Packard Instrument Co., Inc., United Technologies, Downers Grove, IL).

Identification of monoclonal antibodies by dot-blotting. Dot-blotting was performed as described (14). Cyanogen bromide fragments in 4 M urea were applied to nitrocellulose membranes. The membranes were then placed in a blocking solution (13) to block sites not occupied by the applied fragments and then reacted with specific anti-vWF monoclonal antibodies, followed by ^{125}I-labeled rabbit anti-mouse IgG. Reactivity was assessed by autoradiography as described above.

Highly purified vWF. Highly purified vWF was prepared from human cryoprecipitate by the method of Newman et al. (15) as modified by Switzer and McKee (16) with further purification by agarose gel filtration and radiolabeling as described (17).

Statistical analysis. A two-way analysis of variance was performed on the data in Table I (q.v.) to assess whether or not the proteinase inhibitors suppressed vWF cleavage equally (18). When statistically significant differences were found, Tukey's method of multiple comparisons was then invoked to compare the amount of each subunit or proteolyzed fragment in the control group and in each inhibitor group.

Results

Monoclonal antibody epitope map of the vWF subunit. The epitope map was constructed by determining the reactivity of individual antibodies with cyanogen bromide fragments of known amino and carboxy termini, or with tryptic fragments of known amino termini, using either linear gradient SDS-PAGE immunoblots or dot blots (Fig. 1). In the following discussions antibodies are designated by the name of the fragment with which they react, i.e., cyanogen bromide fragments M7, M9, M13, M19, M29–30, and M31 as well as the 52/48 kD, 55 kD, and 22 kD tryptic fragments (all depicted in Fig. 1). All antibodies reacted with the intact 225 kD subunit. After determining which cyanogen bromide and/or tryptic fragments the antibodies reacted with, they were then tested for reactivity with the native 189, 176, and 140 kD fragments present in normal plasma in the resting state. This allowed placement of these fragments within the intact subunit as shown in Fig. 1.

In this manner the native 189 kD fragment could be shown to extend in the amino-terminal direction at least as far as Met(843) and in the carboxy-terminal direction at least as far as Leu(1481). The amino terminus assignment was accomplished by demonstrating that this fragment reacted with a 55 kD antibody that also reacted with the native 140 kD species but not with cyanogen bromide fragment M19. The corresponding epitope, therefore, must lie between Asn(730) and Met(843) (Fig. 1). The 189 kD fragment did not, on the other hand, react with the more amino-terminal M13 antibodies. The fragment was shown to extend in the carboxy-terminal direction at least as far as Leu(1481) by reaction with M31 monoclonal antibodies. Reactivity with 22 kD monoclonal antibodies was weak, but extension of the 189 kD fragment into the 22 kD region was confirmed with polyclonal antibodies.

Placement of the native 176 and 140 kD fragments was accomplished in a similar fashion. The amino terminus of the 176 kD fragment was shown to lie between Val(844) and Met(947) because it reacted with M19 antibodies but not those reacting with more amino-terminal epitopes. The carboxy terminus was shown to extend beyond Val(1927) because of reactivity with 22 kD antibodies. The native 140 kD fragment was found to

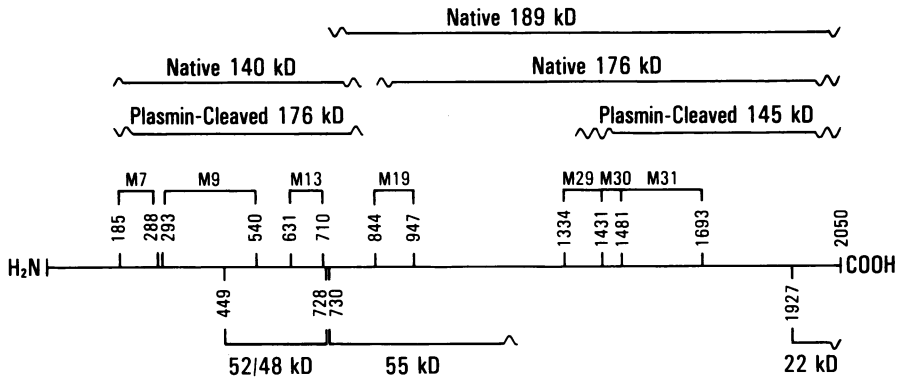

Figure 1. Monoclonal antibody epitope map of vWF showing differentiation of plasmin-cleaved fragments from native fragments. The 2,050-amino acid sequence of vWF is depicted with the amino (NH_2) terminus on the left and the carboxy (COOH) terminus on the right. Vertical tick marks indicate the amino- and carboxy-terminal residues of cyanogen bromide fragments (*brackets*). The designation given to each cyanogen bromide fragment (M7, M9, etc.) is that of K. Titani and co-workers (7). 52/48 kD (9), 55 kD, and 22 kD tryptic fragments are also shown. Monoclonal antibodies have been identified which react specifically in immunoblots or dot blots with each of these fragments. Cyanogen bromide fragments M29 and M30 were tested as a mixture. All monoclonal antibodies used react with the intact subunit. No monoclonal antibodies reacting with fragments amino-terminal to M7 were identified. Positions of all fragments were determined by monoclonal antibodies except for extension of 189 kD fragment carboxy-terminal into the 22 kD region, which was confirmed by a polyclonal antibody to the 22 kD fragment.

extend in the amino-terminal direction at least as far as Met(288), because it reacted with M7 antibodies (Figs. 2 and 3). Its carboxy terminus, on the other hand, lies between Asn(730) and Met(843) because it reacted with three 55 kD antibodies but not with any of the M19 antibodies. This observation also placed the epitopes of these 55 kD antibodies between Asn(730) and Met(843).

Differentiation of plasmin cleavage products from native fragments of similar size. In order to evaluate the effect of activation of the fibrinolytic system on normal vWF, citrated plasma was treated with urokinase. This caused an apparent increase in the relative intensity of the 140 kD band when it was evaluated with the complete pool of 55 anti-vWF mono-

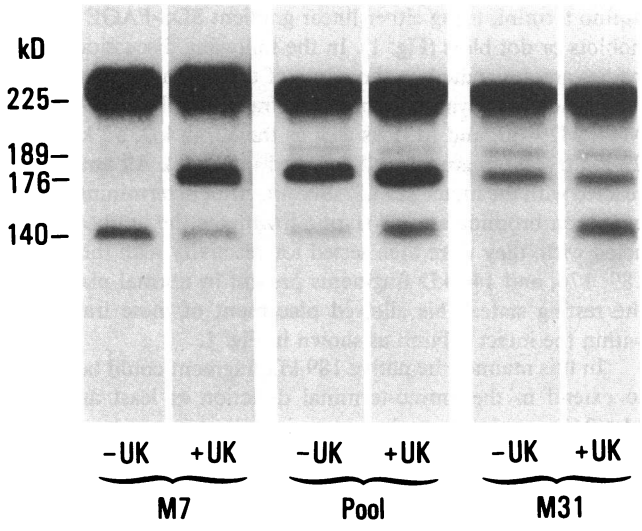

Figure 2. Plasmin-type vWF fragments created by treatment of plasma with urokinase differentiated from native vWF fragments. Normal plasma was drawn into citrate anticoagulant without proteinase inhibitors and incubated for 16 hours at 37°C in the absence (*−UK*) and presence (*+UK*) of urokinase (384 Ploug units/ml plasma). vWF was then immunoisolated, reduced, and analyzed by SDS-5% PAGE, immunoblotting, and autoradiography. ~1–2 μg of protein were applied per lane. A single monoclonal antibody (RG24) reactive with cyanogen bromide fragment M7 (*left*) showed an amino-terminal 176 kD fragment present in urokinase-treated plasma not found in untreated plasma. A single monoclonal antibody (RG5) to cyanogen bromide fragment M31 (*right*) showed a carboxy-terminal 145 kD fragment in urokinase-treated plasma not found in untreated plasma. The complete pool of 55 anti-vWF monoclonal antibodies revealed only a slight increase in bands with 140 and 176 kD mobility (*center*).

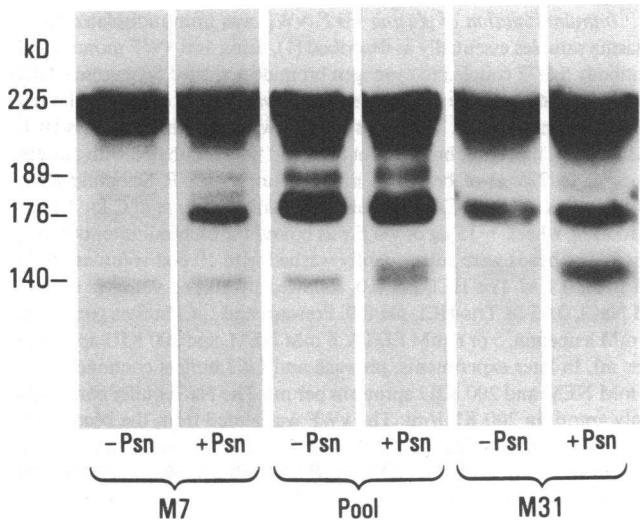

Figure 3. Fragments created by treating highly purified vWF with plasmin: differentiation from native vWF fragments. Highly purified vWF (0.81 mg/ml in Tris-buffered saline buffer) was incubated in the absence (*−Psn*) and presence (*+Psn*) of plasmin (10 μg/ml purified vWF) for 16 h at 37°C, reduced, subjected to SDS-5% PAGE, and immunoblotted. ~4.0 μg of protein was applied to each gel lane. The amino-terminal derivation of the plasmin-cleaved 176 kD fragment is shown by its reaction with an M7 monoclonal antibody (RG12, *left*) while the carboxy terminal derivation of the plasmin-cleaved 145 kD fragment was demonstrated by its reaction with an M31 monoclonal antibody (RG5, *right*). Reaction of the complete monoclonal antibody pool with plasmin-treated and untreated highly purified vWF is shown in the center.

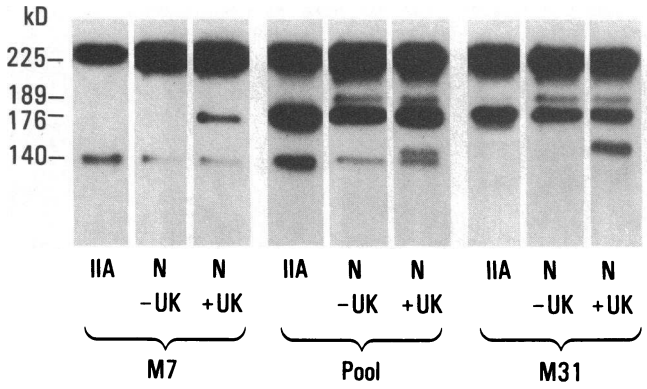

Figure 4. Comparison of native vWF fragments from type IIA and normal plasmas with plasmin-type vWF fragments. Normal and four IIA plasmas were drawn into anticoagulant with NEM, leupeptin, and EDTA and immediately frozen until immunopurification of the vWF. Normal plasma was also drawn without inhibitors and incubated at 37°C in the absence (*−UK*) and presence (*+UK*) of urokinase (384 Ploug units/ml of plasma). ~1–2 μg of protein were applied to each lane. Immunoblots were reacted with the complete pool of 55 anti-vWF monoclonal antibodies (*center*) or pools of the M7 (*left*) and M31 (*right*) specific monoclonal antibodies. One immunoblot, representative of the IIA patient plasmas, is shown. This analysis, as well as that with other specific antibodies (not shown), revealed that the vWF fragments in IIA vWD derive from the same portions of the vWF subunit as the analogous fragments in normal plasma, and were distinct from those fragments created by activation of plasminogen with urokinase.

clonal antibodies (Fig. 2, *pool*). A similar increase was seen in this band if highly purified vWF was treated with purified plasmin (Fig. 3, pool). However, a band at 145 kD was seen in some gel runs suggesting that this plasmin cleavage product was not, in fact, identical with the native 140 kD fragment (Figs. 2 and 3). That this fragment, and a new species with 176 kD mobility, were unique plasmin cleavage products was shown by mapping them to specific areas of the vWF subunit with monoclonal antibodies.

Placement of the plasmin-cleaved 145 kD band in the carboxy-terminal portion of the molecule was accomplished by showing that, in contradistinction to the native 140 kD species, it reacted with M29–30, M31, and 22 kD antibodies (Figs. 1–3) but not with M7, M9, M13, M19, or 55 kD antibodies. The plasmin-cleaved 176 kD fragment was shown to derive from the amino-terminal portion of the molecule by its reaction with M7, M9, M13, and 55 kD antibodies (Figs. 1–3). This contrasts with the carboxy-terminal placement of the native 176 kD fragment.

Similarity of the vWF fragments in type IIA vWD to those in normal plasma. We have previously reported that the vWF in IIA vWD shows an increased proportion of the 176 and 140 kD fragments, suggesting that in vivo proteolysis is increased in this variant (1). We have now obtained evidence to support the hypothesis that this increased cleavage results from an exaggeration of a process which is operative in normal individuals. In fact, the fragments seen in IIA vWD derive from the same sections of the 225 kD vWF subunits as those of similar size present in normal plasma (Fig. 4). Thus, the 140 kD fragment was shown to react with the same amino-terminal antibodies, and the 176 kD fragment with the same carboxy-terminal antibodies as did their counterparts in normal plasma. In contrast, there was no evidence for fragments of similar size being derived from the same portions of the subunit as those produced by plasmin (Fig. 4).

Identification of fragments similar to those produced by plasmin in plasmas from patients with deep venous thrombosis after urokinase or streptokinase therapy. The ability of this technique to identify plasmin-type vWF fragments in patients in whom activation of the fibrinolytic system had taken place was evaluated with plasmas from four patients with deep venous thrombosis after treatment with urokinase or streptokinase. Basal deep venous thrombosis plasma was available from two patients (pre-urokinase in one patient and pre-streptokinase in another). In the deep venous thrombosis plasmas after treatment with the fibrinolytic agent, fragments with the same size and epitope specificity as the plasmin-cleaved 176 and 145 kD species were detected (Fig. 5). No such species were ever identified in normal plasmas collected, processed, and stored in the identical manner as the deep venous thrombosis specimens.

Evidence that one or more cysteine proteinases plays a role in the generation of the vWF fragments present in normal plasma. In order to evaluate the effect of NEM (5–60 mM), leupeptin (1 mM), and EDTA (5 mM) on the formation of the normal native vWF fragments in vitro, blood from four normal donors was drawn into citrate anticoagulant alone as a control or with a single inhibitor and studied with the pool of 55 anti-vWF monoclonal antibodies (Fig. 6). A two-way analysis of variance was performed to assess whether or not suppression of proteolysis by each proteinase inhibitor was equal. Overall differences were determined between the percentage of counts per minute of the intact 225 kD subunits from the control sample and those samples drawn into different inhibitors. Overall differences were also determined between the proteolyzed fragments. Similarly treated samples from each of four normal donors were so evaluated (Table I). Significant differences existed at the $P < 0.01$ level, except for the 189 kD fragments, which were significant at the $P < 0.025$ level. Because there were statistically significant differences, analysis to determine to what the differences were due within each group is permitted. Using Tukey's method of multiple comparisons, no statistically significant differences at the $P < 0.05$ level were noted between the groups containing leupeptin (1 mM) and EDTA (5 mM) and the group containing no inhibitors. However, there was a statistically significant difference between the NEM groups and the other groups at an overall level of significance of $P < 0.05$. There were no statistically significant differences at the $P < 0.05$ level between the two different concentrations of NEM (15 and 30 mM).

In a second experiment the inhibitors were used in combination. Three monoclonal antibodies, each one chosen by its specificity for the 189, 176, or 140 kD fragment, were used to detect changes in quantity of each. In no instance was any combination significantly more effective than 5 mM NEM alone (Table II). In other experiments increasing the NEM concentration to 60 mM or adding aprotinin (200–600 KIU/ml) did not reduce the proportion of the fragments further.

Highly purified vWF, which had been isolated from plasma by cryoprecipitation and agarose gel filtration, displayed the same bands as immunopurified vWF (Fig. 3). This suggested that the 189, 176, and 140 kD fragments were not simply an artifact of

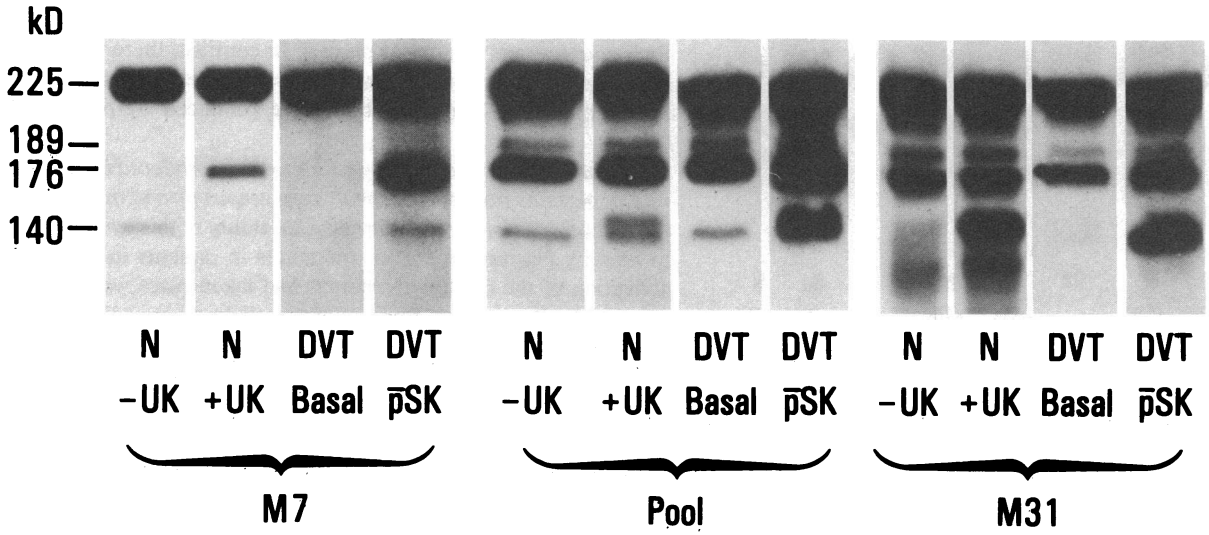

Figure 5. vWF from a streptokinase-treated patient with deep venous thrombosis (*DVT*) compared with vWF from normal plasma (*N*) in the absence (*−UK*) or presence (*+UK*) of urokinase treatment. Basal and serial post-streptokinase (*p̄SK*) plasma samples drawn in inhibitors were examined by monoclonal antibody mapping. The p̄SK plasma vWF shown is from 24 h after the start of SK infusion. On the left, a pool of monoclonal antibodies reactive with cyanogen bromide fragment M7 were used, demonstrating that a 176 kD fragment present in plasma from a streptokinase-treated DVT patient had the same amino-terminal derivation as that created in normal plasma in vitro with urokinase. The 140 kD native fragment is present in the control and DVT basal lanes, although it is only faintly visible in this reproduction. In the center, the pool of 55 monoclonal antibodies was used and showed an increase in the 140 and 176 kD fragments in DVT. On the right, a pool of monoclonal antibodies reactive with cyanogen bromide fragment M31 was used showing that a 145 kD fragment present in DVT plasma had the same carboxy-terminal derivation as that created in normal plasma in vitro with urokinase. Also, an increase in the native 176 kD band is noted after streptokinase. The same alterations were found in three patients treated for DVT with urokinase.

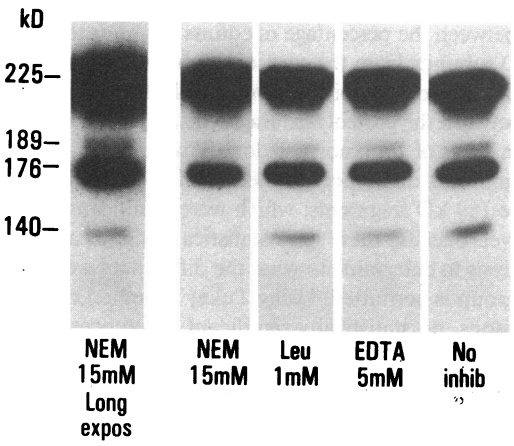

Figure 6. The effects of specific inhibitors on in vitro generation of native vWF fragments. Blood from four normal donors was collected without inhibitors or with *N*-ethylmaleimide (*NEM*) (15 mM), leupeptin (*Leu*) (1 mM), or EDTA (5 mM) followed by immunoisolation of vWF, reduction, SDS-5% PAGE and immunoblotting. The immunoblots were reacted with the complete pool of 55 anti-vWF monoclonal antibodies. The results from individual no. 4 are shown here with a 24-h exposure. There was a marked suppression in production of the 140 kD fragment in the presence of NEM as compared with Leu, EDTA, or no inhibitors. Although EDTA modestly suppressed the 140 kD production in this donor and donor no. 1 on visual inspection, this was not apparent in the two other donors, and it was shown by excising individual bands and determining counts per minute in a gamma scintillation counter that these differences were not statistically significant (Table I). Leupeptin appeared to have no significant effect. A long exposure lane (41 h) to the far left shows that some 140 kD fragment was present in the 15 mM NEM sample.

the immunopurification technique. Direct evidence that immunopurification did not alter the relative proportions of these fragments was obtained in the following manner: highly purified vWF was labeled with ^{125}I, added to plasma in the presence or absence of 5 mM NEM, immunopurified, and analyzed by SDS-5% PAGE followed by electrophoretic transfer to nitrocellulose. Autoradiography was then performed without immunoblotting. The relative concentrations of the 225 kD subunit and the fragments were compared with those of ^{125}I-labeled vWF which was subjected to SDS-5% PAGE and transfered to nitrocellulose without being added to plasma and immunoisolated. No differences were discernable.

Discussion

Several recent publications have provided evidence that proteolysis plays an important role in the pathogenesis of IIA vWF. Gralnick and co-workers (4) and Batlle et al. (19) have shown that inclusion of NEM, leupeptin, and EDTA in the anticoagulant into which blood is collected blocks in vitro loss of intermediate-sized multimers in some patients. We have demonstrated that a small but consistent proportion of vWF exists in normal plasma as proteolyzed fragments of 189, 176, and 140 kD (1). In addition, we have shown that the 176 and 140 kD vWF fragments are proportionately increased in type IIA vWF, even in blood collected in these inhibitors, thus suggesting that in vivo proteolysis of vWF is enhanced in this subtype.

Both plasmin (2, 3) and calpain (4, 5) have been shown to cause loss of large vWF multimers in vitro, and both enzymes may be inhibited by one or more of the agents in the NEM,

Table I. Effect of Proteinase Inhibitors on the Percentage of Total Counts per Minute per Polypeptide Determined After Immunoblotting and Excision from Nitrocellulose

M_r of bands	Inhibitor	Donor* 1	2	3	4	Mean	F statistic‡	P value‡
kD	*mM*							
225							16.34	<0.01
	None	66.7	78.7	75.8	72.1	73.3 §		
	Leu 1	74.7	77.9	80.1	70.2	75.7		
	EDTA 5	77.0	79.5	82.8	73.6	78.2		
	NEM 30	81.9	85.7	84.7	84.1	84.1		
	NEM 15	81.7	85.0	85.8	80.8	83.3		
189							5.01	<0.025
	None	3.0	3.4	2.6	3.0	3.0		
	Leu 1	2.7	3.0	2.4	3.0	2.8		
	EDTA 5	2.9	2.8	2.8	2.5	2.8		
	NEM 30	1.5	2.8	2.5	1.8	2.2		
	NEM 15	2.4	2.4	2.1	2.2	2.3		
176							9.225	<0.01
	None	23.6	14.5	17.4	18.9	18.6		
	Leu 1	18.1	15.5	13.9	21.0	17.1		
	EDTA 5	15.9	14.8	11.4	19.1	15.3		
	NEM 30	13.5	8.7	9.5	11.2	10.7		
	NEM 15	12.6	9.5	9.2	13.8	11.3		
140							5.923	<0.01
	None	6.7	3.4	4.2	6.0	5.1		
	Leu 1	4.5	3.6	3.6	5.8	4.4		
	EDTA 5	4.2	2.9	3.0	4.8	3.7		
	NEM 30	3.1	2.8	3.3	2.9	3.0		
	NEM 15	3.3	3.1	2.9	3.2	3.1		

Anticoagulant = sodium citrate 0.38% final. Abbreviation: Leu, Leupeptin. * Samples were obtained from four different normal individuals whose vWF was processed separately. ‡ With 4,12 degrees of freedom, F statistic derived from analysis of variance for testing equality of suppression of cleavage by proteinase inhibitors. P values for corresponding F statistics were obtained from standard tables (18). § Each group of the same M_r polypeptide bands from plasmas collected in different inhibitors was analyzed by Tukey's method of multiple comparisons to determine differences within the group once an overall statistical difference (F statistic and P value shown) was found (18). Brackets denote that no statistically significant differences at the $P < 0.05$ level were noted among the Leu 1 mM, EDTA 5 mM, and no inhibitors groups, and between the NEM 30 and 15 mM groups, but that there was a statistically significant difference between the NEM groups and the others.

Table II. Relative Percentage of Counts per Minute of VWF Subunit and Fragments

Inhibitor	M_r of bands 225	189	176	140
	kD	*kD*	*kD*	*kD*
None	62.7	12.3	9.6	15.4
NEM	81.5	6.1	6.4	6.0
EDTA/NEM	81.1	5.9	7.6	5.4
Leu/NEM	82.8	4.9	6.2	6.1
EDTA/Leu/NEM	83.4	3.9	7.1	5.6
EDTA	69.8	9.8	9.4	11.0
Leu	61.9	12.0	10.8	15.3
EDTA/Leu	70.7	11.1	8.1	10.1

NEM 5 mM, EDTA 5 mM, Leupeptin 1 mM; results from one normal donor.

leupeptin, and EDTA mixture. More recently, one or more granulocyte proteinases, not inhibited by EDTA or leupeptin, has been shown to proteolytically disaggregate large vWF multimers (6). The studies described here show that although plasmin can cause in vivo proteolysis of vWF in certain disease states, plasmin is unlikely to be responsible for the increased cleavage of vWF observed in IIA vWD or for the basal cleavage of normal vWF.

The nature of the enzyme or enzymes giving rise to the native 189, 176, and 140 kD fragments is uncertain at present. The ability of NEM to suppress formation of these fragments in vitro suggests that a cysteine proteinase is involved (20). On the other hand, neither leupeptin nor EDTA were as effective as NEM, calling into question the identity of the enzyme(s) as a calcium-dependent neutral protease, such as platelet calpain.

The partial epitope map on which these observations were based was constructed using monoclonal antibodies that reacted with cyanogen bromide and tryptic fragments deriving from different regions of the vWF subunit. The antibodies used for this

purpose had been raised against reduced, reduced and alkylated, or trypsinized vWF. Almost without exception, antibodies raised against intact vWF were of no use, probably because they were directed toward conformational epitopes which did not survive the reduction and denaturation necessary for SDS-PAGE analysis.

Information provided by the map allows tentative conclusions as to the number of cleavages necessary to give rise to the different fragments described here. Placement of the carboxy terminus of the native 140 kD between residues Asn(730) and Met(843) and placement of the amino terminus of the native 176 kD fragment between residues Val(844) and Met(947) suggest that these two fragments could have arisen from a single cleavage between Met(843) and Val(844) or two or more cleavages between Asn(730) and Met(947). Because the fragments in IIA vWD map to similar portions of the subunit, a similar cleavage(s) is likely responsible for them. On the other hand, extension of the amino terminus of the 189 kD fragment to an epitope present on the carboxy-terminal portion of the 140 kD fragment demonstrates that it arises from one or more cleavages distinct from that responsible for generation of the 140 kD and 176 kD species.

Neither of the plasmin cleavage products reacts with M19 [Val(844) to Met(947)] antibodies, suggesting that at least one or more undetected plasmin-cleaved fragments from this region exists or that the M19 epitope is destroyed by proteolysis. Failure to detect such a fragment could have resulted from its being too small to be retained in the polyacrylamide gel or the nitrocellulose membrane used for immunoblotting, or from its failure to react with any of the monoclonal antibodies.

A small and relatively constant quantity of the 189, 176, and 140 kD fragments was always detected in vWF from normal plasma even if the concentration of NEM was increased to 60 mM and if EDTA and leupeptin, as well as aprotinin, were included in the anticoagulant into which blood was drawn. The experiments in which ^{125}I-labeled vWF was added to plasma and then immunopurified showed that these fragments did not arise during the immunopurification process. Because immunopurification did not cause increases in the fragments, and even 5 mM NEM alone could suppress their in vitro formation, they must have been present in plasma prior to addition of the inhibitors. Although these studies do not exclude in vitro generation of fragments, the addition of inhibitors at the earliest possible time makes it highly likely that the fragments were the result of in vivo proteolysis. This is also true of the fragments in the patients with IIA vWD from whom blood was collected in the presence of leupeptin and EDTA as well as NEM.

Only one of the samples from the patients treated with fibrinolytic agents was collected in the presence of inhibitors. However, blood from normal individuals which was similarly collected and stored did not show the plasmin-cleaved fragments that were so readily demonstrable in the treated patients. The possible usefulness of these fragments as markers of intravascular activation of the fibrinolytic system, and their implications for the disordered hemostasis in fibrinolytic states will require further evaluation. Nevertheless, the ease with which the epitope map distinguishes cleavages caused by different proteinases illustrates the potential utility of these techniques for investigating the role of proteolytic enzymes in the physiologic processing and pathologic modifications of vWF.

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