Evidence That Calpains and Elastase Do Not Produce the von Willebrand Factor Fragments Present in Normal Plasma and IIA von Willebrand Disease

By Scott D. Berkowitz, Hiroyuki Nozaki, Koiti Titani, Takashi Murachi, Edward F. Plow, and Theodore S. Zimmerman

Recent evidence suggests that proteolysis plays an important role in some forms of inherited and acquired von Willebrand disease (vWD). Because calpains and one or more enzymes released from polymorphonuclear leukocytes are known to proteolyze von Willebrand factor (vWF) in vitro with resultant loss of large multimers similar to that seen in IIA vWD, they have been suggested as being responsible for the proteolysis in vivo. Using monoclonal epitope mapping, we have examined the proteolysis of the vWF subunit by porcine calcium-activated neutral proteases (calpains) and human leukocyte elastase to determine whether they produce the vWF proteolytic cleavage products seen in normal individuals and IIA vWD. Purified vWF was digested with porcine calpains I and II. We found no difference in the size, location, and quantity of the fragments produced by calpain I or calpain II. New fragments were detected of approximately 200, 170, 150, and 125 Kd. There was no evidence for generation of the native 140 and 176 Kd fragments. Some loss of the native fragments was seen, which suggests that they were further cleaved. Epitope mapping of the 170- and 150-Kd calpain-cleaved fragments revealed them to be from different parts of the molecule than the regions from which the native 176- and 140-Kd fragments derived. This was further supported by determination of the amino-terminal sequence of the calpain-cleaved 170- and 150-Kd fragments. Digestion of vWF with human leukocyte elastase produced new fragments at 210/205, 190, 170/185, 145/140, and 130/125 Kd. No generation of native fragments was detected. Monoclonal epitope mapping of the 145/140-Kd elastase-cleaved band proved that it derived from the carboxyl-terminal portion of the vWF molecule, whereas the native 140-Kd fragment is derived from the amino-terminal end. Neither calpains nor human leukocyte elastase produced the proteolyzed fragments present in normal and IIA vWD and, therefore, probably do not cause the loss of large multimers that is seen in that disorder.

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solic inhibitor, calpastatin, lacks organ and species specificity.

Recently, it has also been shown that one or more enzymes released from polymorphonuclear (PMN) leukocytes proteolyze vWF in vitro with a resultant loss of large multimers. Elastase has been shown to be one of the major leukocyte granule fibrinolytic proteases active at neutral pH. In vivo proteolysis of fibrinogen by this enzyme has been demonstrated in proteolytic states.

We have now performed mapping of purified vWF after exposure to calpains or human leukocyte elastase and have found that fragments produced by these enzymes are different from those present in normal and IIa vWD. Therefore, these enzymes are probably not responsible for the proteolytic disaggregation of large multimers seen in vivo in IIa vWD.

MATERIALS AND METHODS

Reagents. Electrophoretically pure reagents including acrylamide, bis-acrylamide, dithiothreitol (DTT), ammonium persulfate, nitrocellulose paper, SDS, and N,N'N',N'-tetra methylethylenediamine (TEMED) were purchased from Bio-Rad Laboratories (Richmond, CA). Ultra-pure high gelling temperature (HGT)(P) agarose and GelBond film were obtained from FMC Corp (Rockland, ME). Para-amidinophenylmethylsulfonyl fluoride (p-APMSF) was purchased from Chemicon (El Segundo, CA). Acetic acid (aldehyde-free reagent) was obtained from Sigma Chemical Co (St Louis) or Mallincrodt, Inc (Paris, KY) and were of the best reagent grade.

Preparation of the calpains and elastase. Calpain I was isolated and purified from porcine kidney as described by Kitaara et al. Calpain activity was determined as described. In brief, casein was used as the substrate, and the increase in trichloroacetic acid–soluble products was measured at 750 nm by the method of Ross and Schatz. One unit of calpain was defined as the amount of enzyme that increased the absorbance by 1.0 at 750 nm after incubation at 30°C for 30 minutes.

Human leukocyte elastase was isolated from PMN leukocytes and purified by ion-exchange chromatography on CM-Sephadex followed by affinity chromatography on elastin-Sepharose as described. The concentration of the stock solution of human leukocyte elastase used was 0.32 mg/mL. The activity of the elastase preparation was determined by using the synthetic substrate N-t-BOC-L-alanine p-nitrophenyl ester (NBA), and the specific activity of the enzyme was approximately 1,000 NBA U/mg.

Purified vWF. Purified vWF was prepared from human cryoprecipitate by the method of Newman et al as modified by Switzer and McKee, with further purification by agarose gel filtration as described.

SDS-Agarose electrophoresis. SDS-agarose electrophoresis was performed as described previously, with 1.4% low-gelling temperature agarose cast in 9-cm gels.

SDS-PAGE and immunoblotting. Purified vWF was reduced in a final concentration of 65 mmol/L DTT and 2% SDS for 15 minutes at 70°C and subjected to electrophoresis in SDS-polyacrylamide gels (4% or 5% as noted) as described by Laemmli. Transfer of proteins from the polyacrylamide gel onto nitrocellulose membrane was performed by using a 25-mmol/L Tris-HCl, 192-mmol/L glycine, 20% (vol/vol) methanol buffer, pH 8.3, at 0.25 A, 3°C for 16 hours. The nitrocellulose membranes were then reacted with anti-vWF monoclonal antibodies (1:200 dilution) followed by labeled rabbit antimouse IgG. The membranes were then processed according to the method of Johnson et al. Bands were visualized by autoradiography with Kodak XRP-I film (Eastman Kodak Co, Rochester, NY) and a Cronex Quanta III intensifying screen (E.I. duPont de Nemours & Co., Wilmington, DE).

Electroelution and sequencing of amino-termini of calpain-cleaved fragments. Electroelution was performed with strict adherence to the described technique. In brief, 675 μg of calpain-cleaved vWF was subjected to electrophoresis through an SDS–5% PAGE gel after reduction in DTT at 60°C and addition of 0.1 mmol/L sodium thioglycolate to the cathode buffer reservoir. The gel was stained with 0.5% Coomassie blue and aldehyde-free acetic acid. The desired protein bands were sliced out of the gel and diced and the protein eluted and dialyzed by using an electrophoretic elution cell. The protein was stored at ~70°C until amino-terminal sequence analysis.

Amino-terminal sequence analysis was performed by using an Applied Biosystems (Foster City, CA) 470A Protein Sequencer with an on-line phenylthiohydantoin (PTH) analyzer.

Monoclonal antibodies to vWF. The monoclonal antibodies utilized in these experiments were prepared as previously described.

Preparation of plasma samples and immunosolation of plasma vWF. Plasma was prepared, and immunosolation of plasma vWF was performed as described previously.

Digestion of vWF with calpains. Purified vWF was digested with calpain I (0.52 mg/mL) or calpain II (0.26 mg/mL) in an imidazole buffer (pH 7.5) containing 50 mmol/L cysteine in the presence of a final concentration of 50 μmol/L CaCl₂ (for calpain I) or 6 mmol/L CaCl₂ (for calpain II). Digestions were performed at calpain-vWF ratios (wt/wt) of 1:10, 1:25, 1:50, and 1:100 for 30 minutes, 2 hours, 6 hours, and 24 hours at 30°C. The reactions were halted by the addition of EDTA and N-ethylmaleimide (NEM) at a ratio of 1:1.

RESULTS AND DISCUSSION

Effect of calpain digestion on multimeric and subunit structure of vWF. SDS–agarose gel electrophoresis of unreduced samples showed a pattern consistent with proteolysis in the calpain-digested samples: loss of large multimers and abnormalities of the triplet structure consisting of an increase in the middle (which is normally predominant) and fastest-migrating bands of the oligomers present (Fig 1).

Reduction of these samples and analysis by SDS–5% PAGE and immunoblotting revealed new fragments of approximately 200, 190, 170, 150, and 125 Kd (Fig 2, pool 85). No differences in the quantity of fragments and type of cleavages produced by the two calpains were observed when vWF was digested in calpain-vWF ratios (wt/wt) of 1:10, 1:25, 1:50, or 1:100 after incubation times of 30 minutes, 2 hours, 6 hours, or 24 hours (results not shown). By reacting the samples with pools of monoclonal antibodies specific for the amino-terminal cyanogen bromide fragment M7 (residues Lys85 through Met288) or the carboxyl-terminal cy-
CLEAVAGE OF vWF BY CALPAINS AND ELASTASE

Fig 1. SDS-agarose gel electrophoresis of purified vWF before (-CALP) and after digestion with calpain I (+CALP I) and calpain II (+CALP II) at a calpain-vWF ratio of 1:50 (wt/wt) as described in Materials and Methods. The direction of electrophoresis is from top to bottom. Evidence of proteolysis is noted on the right, with loss of large multimers and abnormalities of the triplet structure that consist of an increase in the middle (which is normally predominant) and fastest-migrating bands of the oligomers present.

Fig 2. Immunoblots of the vWF subunit and fragments present in control (-CALP) and calpains I and II (+CALP I and II)-digested purified vWF after reduction and SDS-5% PAGE. Purified vWF was digested as indicated in Materials and Methods. Five micrograms of protein were applied to each lane. The apparent molecular mass of the subunit and native fragments is indicated in kilodaltons (kD) on the left. A pool of monoclonal antibodies reactive with cyanogen bromide fragment M7 (M7, left) revealed fragments of similar quantity at molecular masses of approximately 200, 170, and 125 kD after digestion with calpains I or II. No increase in the native 140-kD fragment is seen. A pool of monoclonal antibodies reactive with cyanogen bromide fragment M31 (M31, right) showed fragments of similar quantity at molecular masses of approximately 200, 190, 170, and 150 kD after digestion with calpains I or II. No increase in the native 176-kD fragment was observed. Reaction of the undigested and digested protein with the complete pool of 85 monoclonal antibodies (pool 85) shows the native fragments in the control material and the new fragments produced after calpain digestion.
bly generated during vWF purification. However, in the calpain-treated vWF this fragment reacted with M7 antibodies. This 200-Kd calpain cleavage product may be the same as the 205-Kd fragment described by Kunicki et al.\(^{12}\) after the addition of platelet sonicates to \([^{25}I]\) vWF.

A comparison of the molecular mass and antibody reactivity of the calpain-cleaved fragments with that of the native vWF fragments can be found in Table I.

We attempted to cleave vWF in plasma with calpains I and II by using a 1:1 ratio (enzyme-vWF, wt/wt). No cleavage of vWF was detected (results not shown). Several plasma inhibitors are known to exist for the calpains. Eighty percent to 90% of the platelet calpain-inhibitory capacity of plasma is due to high-mol wt kininogen (HMWK).\(^{15}\) The addition of calpain II at a 1:1 (enzyme-vWF, wt/wt) ratio to HMWK-deficient plasma did not produce any detectable cleavages. α-Cysteine proteinase inhibitor (which is identical to low-mol wt kininogen) also inhibits calpains,\(^{15}\) as does α₂-macroglobulin.\(^{26}\) It is likely that these inhibitors prevented cleavage by calpains of vWF in HMWK-deficient plasma.

We determined the amino-terminal sequence of the calpain-cleaved fragments that were close in molecular mass to the native fragments. The amino-terminal sequence of a 170-Kd fragment cleaved by calpain II is SLSCRPMPMVK, which is identical to the first ten amino acid residues of the native vWF subunit. This fragment must be the one seen by the M7 antibody pool. We could not detect any sequence consistent with the presence of a 170-Kd fragment with a more carboxyl-terminal origin, which implies that only one calpain-cleaved 170-Kd fragment exists. Moreover, this finding further supports the conclusion that the calpain-cleaved 170-Kd and native 176-Kd fragments are different.

The amino-terminal sequence of the 150-Kd fragment cleaved by calpain II is SHRVNCDRGL and begins with the 1151st amino acid. This latter cleavage site agrees with the subsite specificity reported for the calpains: cleavage usually occurs immediately after a Tyr, Met, Arg, or Lys residue in the P₁ position preceded by a Leu or Val residue in the P₂ position.\(^{22}\) The location of the amino-terminal sequence supports the conclusion obtained with antibodies that the calpain-cleaved 150-Kd fragment and the native 140-Kd fragment are from opposite ends of the vWF molecule.

<table>
<thead>
<tr>
<th>Terminus From Which Fragments Originate</th>
<th>Amino (M7)*</th>
<th>Carboxyl (M31)*</th>
</tr>
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<tbody>
<tr>
<td>Native†</td>
<td>140</td>
<td>176</td>
</tr>
<tr>
<td>Calpain-Cleaved†</td>
<td>125</td>
<td>150</td>
</tr>
<tr>
<td>Elastase-Cleaved†</td>
<td>130/125</td>
<td>145/140</td>
</tr>
<tr>
<td>(Residue 1→)</td>
<td>170</td>
<td>1151→</td>
</tr>
<tr>
<td></td>
<td>165</td>
<td>165</td>
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</tbody>
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*Detection by monoclonal antibodies to this cyanogen bromide fragment epitope.
†Values are in kilodaltons.
‡Amino acid residue numbers of N-termini.

While porcine calpains were used in these experiments, the substrate specificity of porcine and human calpains is nearly identical. Both calpains I and II isolated from human skeletal muscle hydrolyze neurotensin at Tyr₁-Gln₂, dynorphin at Arg₂-Arg₃, and luteinizing hormone-releasing hormone (LH-RH) at Gly₁-Leu₂.\(^{37}\) These results are in perfect agreement with those reported by Sasaki et al.\(^{22}\) who used porcine calpains I and II. Both human\(^{37}\) and porcine\(^{22}\) calpains rapidly cause deamination of substance P at Met₁-NH₂. Porcine calpains I and II catalyze the hydrolysis of succinyl-Leu-Met-MCA (4-methylcoumaryl-7-amide) and tert-butoxycarbonyl-Val-Leu-Lys-MCA at comparable rates\(^{22}\) with no action on benzoyloxycarbonyl-Arg-Arg-MCA. Human calpains I and II have been found to perform these reactions in the same way (S. Kishi and T. Murachi, unpublished observations).

Calpain I isolated from human erythrocytes is known to preferentially cleave bands 4.1 and 3 when incubated with human erythrocyte membrane preparations in the presence of calcium and analyzed by SDS PAGE.\(^{29,40}\) Calpain II isolated from human placenta\(^{41}\) was found to give results almost indistinguishable from those obtained with human calpain I, and nearly identical results were recorded when using porcine calpains I and II in place of human enzymes (T. Hamakubo and T. Murachi, unpublished observations). In view of these data supporting the close similarity of the substrate specificities of human and porcine calpains and the fact that porcine enzymes are more accessible, we conducted the present experiments using porcine calpains I and II.

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**Table 1. Molecular Mass of Fragments on SDS-PAGE Gel**

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*Detection by monoclonal antibodies to this cyanogen bromide fragment epitope.
†Values are in kilodaltons.
‡Amino acid residue numbers of N-termini.

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**Fig 3. SDS-agarose gel electrophoresis of purified vWF before (-Elas) and after (+Elas) digestion with elastase at an elastase-vWF ratio of 1:16 as described in Materials and Methods.**

The direction of electrophoresis is from top to bottom. A loss of large multimers is noted after elastase digestion.
Effect of elastase digestion on the multimeric and subunit structure of vWF. Analysis of unreduced samples by SDS-agarose electrophoresis revealed a loss of large multimers as compared with the vWF control (Fig 3). No abnormality of triplet structure could be detected. SDS–4% PAGE of reduced vWF and epitope mapping of the fragments produced by elastase digestion is shown in Fig 4. Newly cleaved fragments, some appearing as doublets, were seen with approximate molecular mass of 210/205, 190, 170/165, 145/140, and 130/125 Kd. In no case did there appear to be generation of the native 140- and 176-Kd fragments. Rather, loss of the native 176- and 140-Kd fragments present in the untreated vWF occurred. The 145/140-Kd elastase-cleaved fragments (Fig 4, M31) were close in molecular mass to the native 140-Kd fragment (Fig 4, M7) but proved by epitope mapping to derive from the opposite end of the vWF subunit. The 170/165-Kd elastase-generated fragment reacted with both the M7 and M31 antibodies and was large enough to stretch between these two regions. Alternatively, this band may consist of two similar-sized fragments from opposite ends of the molecule. However, the elastase-cleaved band migrated more rapidly than did the native 176-Kd fragment. In addition, the native fragment reacted only with M31 antibodies.

A band migrating slightly slower than the native 189-Kd fragment can be seen in the elastase-treated vWF (Fig 4, pool 85). This elastase-cleaved 190-Kd fragment reacted with M31 antibodies, as does the native 189-Kd fragment.2 The native 189-Kd fragment is a minor fragment, representing only 3% of the vWF found in normal plasma,1,2 and it is not well visualized by immunoblotting (or by Coomassie blue staining). It could not be determined whether the elastase-cleaved 190-Kd fragment was due to an increase in the native 189-Kd fragment or whether it represented a new fragment.

A comparison of the molecular mass and location on the vWF subunit of the elastase-cleaved fragments with the known native vWF fragments is shown in Table 1.

Reaction of normal plasma with human leukocyte elastase at a 1:1 (enzyme-vWF, wt/wt) ratio produced no increase in native fragments or new cleavages (results not shown), probably due to inactivation of the added proteinase by naturally occurring inhibitors.

CONCLUSION

The studies described here show that digestion of purified vWF with calpains causes a loss of large multimers and alters the triplet structure similar to the type IIA pattern. Human leukocyte elastase similarly produces a loss of large multimers, although no significant alteration in the triplet pattern is apparent. These enzymes can therefore be included with plasmin as physiological proteinases that can proteolytically cleave vWF with the loss of large multimers. However, with the enzyme concentrations and incubation times used,
neither the calpains nor human leukocyte elastase produced an increase in the native fragments in plasma vWF or in the highly purified vWF used in vitro. Therefore, neither of these enzymes produced fragments with the characteristics of those found in normal or IIA plasma.

Because calpains are cellular enzymes residing in the cytosol or on the inner surface of the cell membrane, it may be that when used in vitro the enzymes are unable to align properly with the vWF molecule to produce the cleavage(s) that occur in vivo and therefore could not produce the expected fragments. Also, the compartment in which the proteolysis takes place is unknown. Proteolysis of vWF has been thought to occur in the plasma, but it is conceivable that cleavage may take place within the vascular endothelial cell or on secretion of vWF from the cells. Moreover, unproto-

lized human vWF is not available for study, and that form may be important in evaluating the role of any enzymes thought to cause the proteolysis of vWF in vivo.

Thus, the enzyme or enzymes responsible for the absence of large multimers in type IIA vWD remain(s) to be identified. Its description is pertinent because it would provide a better understanding of how normal vWF is processed and explain an important step in the pathogenesis of type IIA vWD.

ACKNOWLEDGMENT

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