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Abnormality of the N-Terminal Portion of von Willebrand Factor in Type IIA and IIC von Willebrand Disease

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Key words

von Willebrand factor (vWF) – von Willebrand disease (vWD) – S aureus V8 protease (V8-protease)

Summary

We have established a new analytical method which allows the characterization of von Willebrand factor (vWF) degradation fragments in minute amounts (10 µl) of plasma, without the need for immunopurification of vWF. Plasma vWF was hydrolysed by S aureus V-8 protease (V-8 protease) and the cleaved fragments separated by SDS-agarose gel electrophoresis followed by staining with ^{125}I-labeled polyclonal or monoclonal antibodies against vWF and autoradiography. Quantification of the amount of each product was estimated by counting the incorporated radioactivity following excision. V-8 protease limitedly hydrolysed vWF in normal as well as type I von Willebrand disease (vWD) plasma and produced two distinct fragments with identical electrophoretic and antigenic characteristics to those produced from purified vWF, i.e. a C-terminal SpII and a series of N-terminal SpIII fragments (SpIIIa, b and c). The method was applied to further characterize the molecular abnormalities of vWF in eighteen patients with type II vWD. In seven individuals with type IIA and five patients with type IIC, SpIII appeared significantly modified as compared to normal. In type IIA, there was a marked decrease or absence of SpIIIa and an increase of SpIIIb and c. In type IIC, SpIIIb was lacking. In three patients with type IIB and in three patients with type IID, there was no significant modification of SpIII. In all cases, SpII was apparently not modified. In conclusion, the molecular abnormality of vWF in type IIA and IIC vWD appears to reside in SpIII, the N-terminal portion of the vWF-subunit (residues 1 to 1,365).

Introduction

Human von Willebrand factor (vWF) is a large glycoprotein which plays an important role in the initial attachment of platelets to vascular subendothelium at high wall shear rates (1, 2). Using agarose gel electrophoresis of low resolution (low agarose concentrations) (3, 4) normal plasma vWF appears as a set of multimers with a molecular weight (M_r) ranging from 0.5 to over 15 million daltons. By high resolution gel electrophoresis (high agarose concentrations) (4), each individual oligomer may be resolved into at least three bands (triplet), a major one and two satellite subbands. Other studies also established that each oligomer of plasma vWF is composed of predominant identical 270 kDa subunits of 2,050 amino acids (5, 6) and of various proportions of two components with a M_r of 140 and 120 kDa (5) corresponding respectively to the C- and N-terminal parts of the 270 kDa subunit (5, 7). More recently Zimmerman et al. (8) confirmed the existence of the latter species described as 176 kDa (C-terminal) and 140 kDa (N-terminal) (9), in addition to a C-terminal 189 kDa entity and established that the three species resulted from an apparently normal partial proteolytic cleavage of vWF in plasma.

Von Willebrand disease (vWD) results from quantitative or qualitative alterations of vWF leading to a prolonged bleeding time and defective platelet adhesion in affected patients. In type II vWD, vWF is characterized by a lack of high M_r multimers as compared to normal and several subtypes are distinguished (IIA to IIF) according to subtle alterations of the subband structure of multimers in plasma and platelets when analysed by highly resolutive agarose gel electrophoresis (10). In addition, the relative proportion of the 270 kDa subunit and of its derived fragments suggests that each subtype of type II vWD demonstrates a specific reactivity of abnormal vWF to proteases (8).

In order to further characterize vWF in the major subtypes of type II vWD (type IIA, IIB, IIC and IID), we have compared the pattern of digestion of normal and abnormal vWF in minute amounts of plasma with S aureus V-8 protease (V-8 protease). By SDS-agarose gel electrophoresis followed by staining with ^{125}I-labeled polyclonal or monoclonal antibodies against vWF (11), we first established that V-8 protease limitedly hydrolysed normal vWF in plasma, leading to two distinct fragments, SpII and SpIII, with the same characteristics as those produced from purified vWF (7). SpII is a dimer of the C-terminal end of the vWF subunit which has a M_r of 220 kDa. SpIII, the complementary N-terminal fragment appears as a triplet with M_r's ranging between 320 and 210 kDa. We then tested plasma of patients with type IIA, IIB, IIC and IID vWD and found clear evidence that the molecular abnormality in type IIA and IIC vWD led to an abnormal digestion pattern with the production of a modified SpIII, the N-terminal portion of vWF subunit.

Materials and Methods

Reagents

V-8 protease was obtained from Miles Laboratories Inc, Naperville, IL, USA; reagents for electrophoresis were from Bio-Rad Laboratories, Richmond, CA, USA; Seakem agarose HGT and Gel Bond Film were from Marine Colloids, Rockland, ME, USA; diisopropylfluorophosphate (DFP) from Fluka AG Chem., Buchs, Switzerland; all other reagents were of analytical grade from Merck, Darmstadt, FRG, or Prolabo, Paris, France.

Antibodies to vWF

Five polyclonal (rabbit) antibodies monospecific for vWF were used in this study. One of them was kindly donated by Diagnostica Stago, Franconville, France. Monoclonal antibodies to human vWF (MAbs) were produced and characterized as previously described (12, 13). Their

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reactivity towards proteolytic fragments of vWF produced by V-8 protease, SpII or SpIII, has already been reported (11).

Preparation of Plasma

Nine parts of blood were collected in one part of 3.8% trisodium citrate, pH 7.4, with or without 5 mM ethylenediaminetetraacetic acid disodium salt (EDTA). Platelet-poor plasma (PPP) was separated by centrifugation at 3,000 × g for 20 min at 4° C. PPP was immediately used or stored in aliquots at −80° C. Normal plasma samples were obtained from nine unrelated healthy subjects. Three different plasma pools from twenty normal donors each were used as reference. Supernatants of cryoprecipitate were prepared from aliquots of normal pool plasma as previously described (3) and tested in this study as samples of low M_r forms of normal plasma vWF. Twenty-four patients with vWD were selected for the study. Seven patients with type IIA vWD belonged to four families. Among those, there were three related cases originally reported by Hill et al. (14), two other related patients and two unrelated ones previously characterized in our laboratory (15). Plasma from three patients with type IIB were studied. Two patients were originally reported by Federici et al. (16) and by Holmberg et al. (17). The plasma from the third patient with type IIB was kindly provided by Dr. M. Dreyfus (Clamart, France). Four unrelated patients with type IIC vWD were studied, previously described by Armitage and Rizza (18), Mannucci et al. (19), Mazurier et al. (20) and Ruggeri et al. (21). Plasma from the heterogenous mother of one type IIC patient (18) was also tested for comparison. Three patients with type IID vWD belonged to two families. The two related cases were reported by Hill et al. (22). Plasma samples from the third patient were kindly provided by Dr I. M. Nilsson. Three patients with type I vWD previously described (15) were also tested in this study. Plasma from three patients with severe vWD (type III) were used as controls. Plasma from all patients was stored in aliquots at −80° C.

Preparation of vWF-Deficient Plasma

Fresh PPP (50 ml) was depleted of vWF by immunoadsorption onto a MAb to vWF (10 mg of IgG) coupled to Sepharose 2B (10 ml) by recirculating for 15 h at 4° C. The residual level of vWF antigen (vWFAg) in PPP was less than 0.05 U/dl as estimated by immunoradiometric assay (23). It was not detectable by autoradiography when analysed by SDS-agarose gel electrophoresis followed by staining with ^{125}I-labeled polyclonal antibody against vWF. The concentration of protein in depleted plasma was 65 mg/ml. In this study vWF-deficient plasma was used for diluting normal or vWD plasma in order to adjust their vWFAg to similar levels.

Preparation of Fragments SpII and SpIII from Purified Human vWF

Human vWF was purified from lyophilized cryoprecipitate (Centre National de Transfusion Sanguine, Orsay, France), according to the method of Thorell and Blombäck (24). Fragments SpII and SpIII were prepared by digestion of purified vWF with V-8 protease coupled to Sepharose and isolated as previously decribed (7, 11) using ion exchange chromatography, gel filtration and immunoadsorption onto selected MAbs coupled to Sepharose.

Digestion of vWF with V-8 Protease in Plasma

Plasma (10 µl) was introduced into the tip of 1.5 ml polypropylene microcentrifuge test tubes and diluted with 20 µl of 0.1 M Tris-HCl, pH 7.8, 0.1 M NaCl, 10 mM EDTA. Hydrolysis was performed at 22° C by adding 3.6 µl of a V-8 protease solution prepared in the same buffer and stored in aliquots at −80° C. The optimal enzyme/protein ratio to digest vWF in normal plasma was determined using 6 h hydrolysis and serial concentrations of V-8 protease to obtain a final ratio of 1/3,200 to 1/100, assuming a concentration of 65 mg/ml of protein in plasma. Kinetics of digestion of vWF in normal plasma with V-8 protease were studied using a 1/200 enzyme/protein ratio. When the digestion of plasma with type II vWD was compared to that of normal individual subjects or plasma pool, control plasma was diluted in vWF-deficient plasma so that the level of vWFAg in both samples was identical by immunoradiometric assay (23). Hydrolysis of both samples was performed for 15 min to 6 h using an enzyme/protein ratio of 1/200. In some experiments hydrolysis was prolonged to 24 h. Digestion was stopped by adding 1.4 µl of 25 mM DFP in distilled water (final concentration 1 mM). After 1 h, 36 mg of urea, 10 µl of 10% SDS and 5 µl of 0.25% bromophenol blue tracking dye were added to the samples. Following heating for 15 min at 65° C, the final mixture was centrifuged (Beckman microcentrifuge M 12, Palo Alto, CA) at 12,000 × g for 3 min and submitted to gel electrophoresis.

Analysis of vWF, SpII and SpIII by Gel Electrophoresis

SDS-agarose-polyacrylamide gel electrophoresis (0.1% SDS, 6 M urea, 1.5% acrylamide, 1% agarose, 6 mM EDTA in 0.1 M Tris-phosphate buffer, pH 7) was performed as previously described (5) using a continuous buffer system (0.1% SDS, 0.1 M Tris-phosphate buffer, pH 7). The gels (115 × 240 × 2 mm) were poured on Gel Bond Film (Marine colloid, Rockland, ME). Electrophoresis was run horizontally using a LKB multiphor 2117 (LKB Instruments AB, Bromma, Sweden) cooled at 10° C. After the samples (80 µl) had completely entered the gel, wells (4 × 13 mm) were filled with 1% agarose in 0.1 M Tris-H_3PO_4, pH 7.0 containing 0.1% SDS and migration was carried out at constant voltage (50 mV/gel) until the tracking dye reached the anodal edge of the gel. To improve the resolution, longer vertical slab gels were used in some experiments. Following electrophoresis, gels were fixed for 2 h in 10% trichloroacetic acid, 50% methanol, extensively washed with distilled water and incubated for 2 h with 0.4% bovine serum albumin (BSA) in 25 mM Tris-HCl, pH 7.4, 0.15 M NaCl and reacted with ^{125}I-labeled polyclonal or monoclonal antibody overnight at 22° C. After extensive washing with 0.5 M NaCl under stirring, the gel was rinsed with water, dryed and autoradiographed using Kodak X-Omat films and Kodak X-Omatic cassettes (Eastman Kodak & Co., Rochester, NY). The radioactivity was quantitatively estimated following autoradiography by slicing individual lanes (15 mm wide) into strips of 2.5 mm sections and counting. Non-specifically bound radioactivity was estimated by counting strips using as control vWF-deficient plasma or plasma from patients with severe vWD. For each fragment results were expressed as percent of the total specific radioactivity of the lane. Comparison between patient sample and control plasma was expressed using as 100% the values determined for the control. The significance of the results was estimated by statistical analysis according to Student's t-test.

Electrophoresis on SDS-polyacrylamide vertical slab gels was performed in a discontinuous buffer system according to the method of Laemmli (25) using as running gel 5% or a linear gradient (3.5–16%) of polyacrylamide. Two-dimensional vertical slab gel electrophoresis combined in the first dimension a SDS-agarose-polyacrylamide gel and in the second dimension a SDS-3.5% polyacrylamide stacking gel, and a SDS-5% polyacrylamide running gel. Samples containing digested plasma were prepared with urea and SDS as described for SDS-agarose gel electrophoresis and 80 µl of heat-treated samples were loaded per well of 8 ×

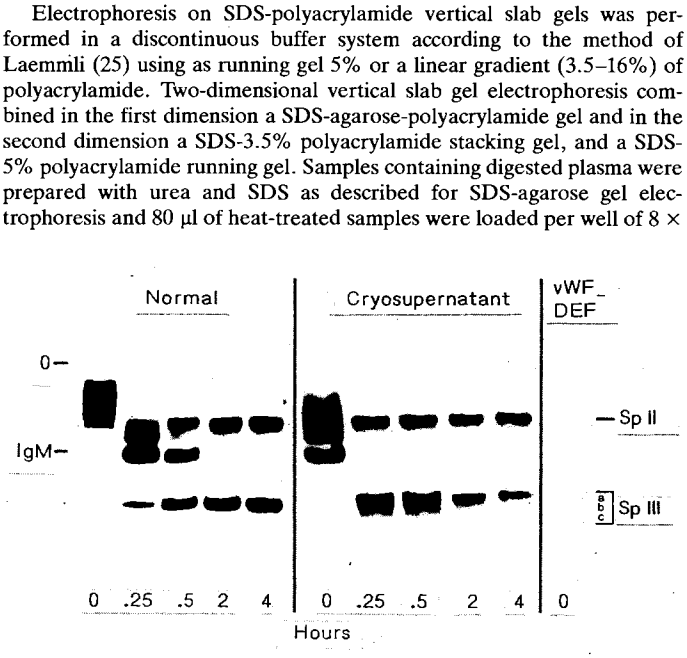

Fig. 1 Digestion by V-8 protease of vWF in normal plasma or cryosupernatant. Digestion (enzyme/protein ratio 1/200) was stopped with 1 mM DFP. Samples were subjected to SDS-agarose gel electrophoresis and vWF or vWF fragments were revealed by immunostaining using ^{125}I-polyclonal antibody to vWF and autoradiography. Time is indicated at the bottom of the gel. Sample of vWF-deficient plasma (right lane) was run to determine the specificity of the antibody. Markers (left) show the position of origin and IgM. On the right are the positions of purified SpII and SpIII triplet (a, b and c)

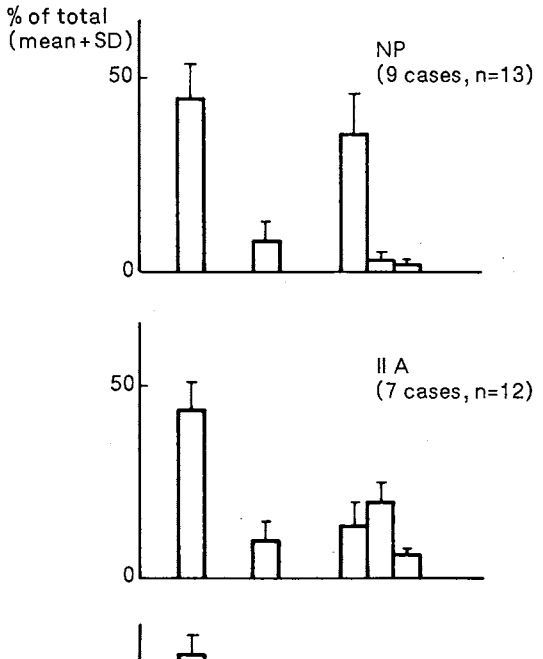

Fig. 2 Relative concentration of vWF fragments produced by V-8 protease digestion (4 h) of plasmas (enzyme/protein ratio 1/200) from normal individuals (NP) or from type IIA and IIC vWD. Following SDS-agarose gel electrophoresis and immunostaining using ^{125}I-polyclonal antibody, bound radioactivity was estimated by slicing the dry gel and counting

1.5 mm. Immunoblotting of the gel was performed according to the method of Towbin et al. (26) using nitrocellulose paper (Schleicher and Schüll, Dassel, FRG) followed by staining by ^{125}I-polyclonal or ^{125}I-monoclonal antibody and autoradiography. M_r markers included purified IgM (900 kDa), fibronectin (440 kDa), fibrinogen (340 kDa), IgG (160 kDa), a commercial M_r kit (Pharmacia Fine Chemicals AB, Uppsala, Sweden) and purified SpIII and SpII.

Radiolabeling of Antibody against vWF

IgG was labeled with Na^{125}I (Amersham International, Amersham, UK) using Iodo-Gen (Pierce Chemical Co, Rockford, IL, USA) as described by Fraker and Speck (27). Specific radioactivity was ~10 mCi/mg.

Results

Proteolysis of Human vWF in Normal Plasma by V-8 Protease

The kinetics of digestion of vWF in normal plasma pool by V-8 protease using a 1/200 enzyme/protein ratio was analysed by SDS-agarose gel electrophoresis followed by staining with a ^{125}I-polyclonal antibody (Fig. 1). With time there was a disappearance of the larger vWF multimers, a transient increase of the second and first multimers and a production of two main products with similar electrophoretic mobility as purified SpII and SpIII. SpII had an anomalous mobility with a band at the level of vWF multimers 4 or 5. SpIII migrated as a triplet with a major slow-moving band (SpIIIa) and two faint fast-moving bands (SpIIIb and SpIIIc) often barely visualized by simple inspection of the autoradiography. However these bands were consistently observed by counting the radioactivity after cutting the gels using either normal plasma pool or plasma from normal individuals (Fig. 2, top panel). The two fragments SpII and SpIII (Fig. 1) were already detected after 15 min of digestion and remained constant after 2 h. After 4 h of digestion a trace of radioactivity still remained at the level of the first oligomer of vWF. Results were confirmed by counting. The radioactivity incorporated in each lane rapidly decreased after 15 min and remained constant after 2 h (30% loss). Between 2 and 4 h of digestion no secondary degradation of SpII or SpIII was detected as indicated by the constant amount of total radioactivity and the constant proportion of fragments SpII and SpIII which together accounted for more than 80% (Fig. 2, top panel). In addition the trace of radioactivity present at the level of the first vWF-multimer (~8% of the total) was not modified.

The kinetics and pattern of the final products were similar in cryosupernatants lacking high M_r multimers of vWF (Fig. 1), to those of normal plasma and thus not dependent upon the degree of multimerization of the starting vWF.

Identification of the fragments produced from normal plasma with SpII and SpIII obtained by digestion of purified vWF was confirmed using ^{125}I-labeled MAbs to either SpII or SpIII (Fig. 3). When a MAb specific for SpII (MAb 9) was used instead of a polyclonal antibody, a single band appeared at the level of SpII using digested normal plasma. Similarly, when a MAb specific for SpIII (MAb 418) was used, only the bands with the same mobility as SpIII stained. Similar results were obtained using three distinct MAbs to SpII or to SpIII. Additional evidence was obtained by comparison of purified SpII, SpIII and fragments isolated from normal plasma by electrophoresis on SDS-polyacrylamide gels (not shown) or two-dimensional electrophoresis (Fig. 4). Immunostaining of electroblots with ^{125}I-polyclonal antibodies

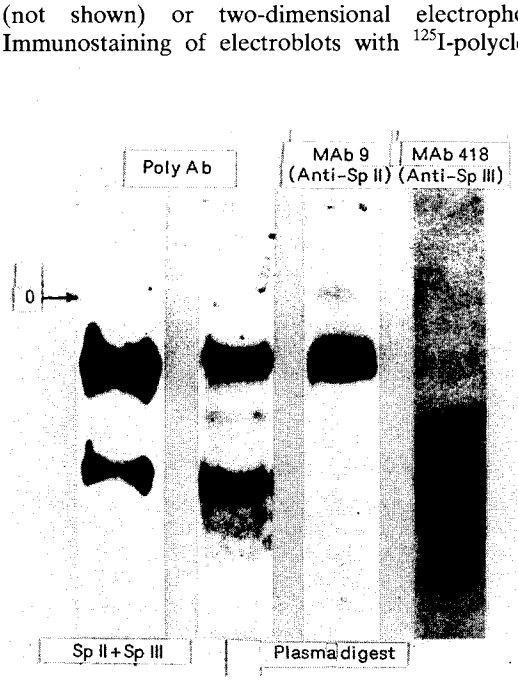

Fig. 3 Identification of the vWF fragments produced from normal plasma with purified SpII and SpIII obtained by V-8 protease digestion of purified vWF. Purified SpII and SpIII or 4h-digested plasma samples (plasma digest) were submitted to SDS-agarose gel electrophoresis. Following immunostaining using either ^{125}I-polyclonal antibody to vWF (two left lanes) or ^{125}I-MAb 9 to SpII or ^{125}I-MAb 418 to SpIII (two right lanes), the fragments were revealed by autoradiography

showed that the fragments from normal plasma had the same electrophoretic behavior as purified fragments SpII and SpIII with similar M_r of 220 kDa (SpII), 320 kDa (SpIIIa), 265 kDa (SpIIIb) and 210 kDa (SpIIIc) (Fig. 4).

The specificity of the antibodies upon the estimation of the final products of digestion was analysed by comparing the results obtained with five distinct, freshly labeled, polyclonal antibodies, four normal plasma pools, nine individual normal plasmas and three cryosupernatants. Results of 3 h-digestion with an enzyme/protein ratio of 1/200 are summarized in Table 1. The relative distribution of radioactivity between SpII and SpIII appears characteristic of each antibody as demonstrated by the rather small variation from one experiment to the other using a given antibody and when comparing a normal pool to individual plasmas or cryosupernatants. However, changing the antibody resulted in significant differences of radioactive staining for the same products (Table 1) showing the broad variability of the anamnestic response against SpII or SpIII when rabbits are immunized using similar vWF preparations.

Effect of vWFAg Levels upon the Detection of vWF Fragments in Plasma by Immunostaining

Serial dilutions of normal plasma pool (vWFAg from 100 to 0.78 U/dl) in vWF-deficient plasma were digested for 3 h with V-8 protease using an enzyme/protein ratio of 1/200. Following gel electrophoresis, staining with ^{125}I-polyclonal antibodies was compared to that of vWF-deficient plasma (background) to determine the sensitivity of the method. Results showed that the background was approximately constant along the gel and reached ~200 cpm/2.5 mm slice. Assuming a detection limit of twice the background, SpII and SpIII could be detected when levels of vWFAg in plasma were 3.1 U/dl for SpII and 6.2 U/dl for SpIII.

Effect of Blood Collection and Storage of Plasma upon Digestion with V8-Protease

Aliquots of fresh normal plasma from one individual with or without 5 mM EDTA at the time of blood collection were either used immediately or frozen and thawed once to 8 times prior to digestion for 4 h with V-8 protease (1/200). Comparison of the results to those obtained using normal plasma pool stored at −80° C demonstrated no significant quantitative or qualitative change.

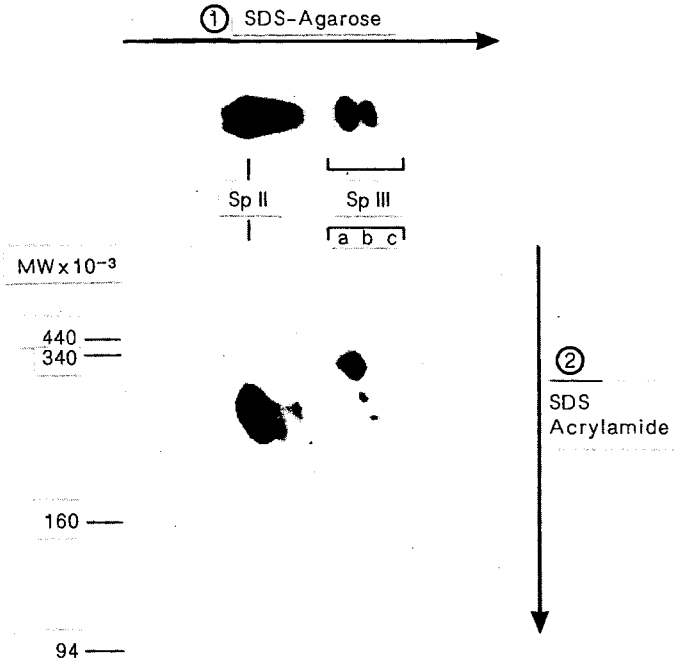

Fig. 4 Two-dimensional gel electrophoretic analysis of sample from normal pool plasma digested by V-8 protease for 4 h (enzyme/protein ratio 1/200) and revealed by autoradiography following immunostaining with ^{125}I-polyclonal antibody to vWF. The sample was electrophoresed in duplicate on a SDS-agarose slab gel (first dimension). One lane was cut and placed on the top of a SDS-3.5% polyacrylamide stacking gel/5% polyacrylamide running gel and electrophoresed (second dimension). The arrows indicate the direction of the migration. Position of the markers run in parallel in the first and second dimensions are indicated

Proteolysis of vWF in Plasma from Patients with Various Types of vWD

Analysis of vWF proteolysis by V-8 protease in twenty-four plasmas from patients with vWD was performed using kinetic studies by SDS-agarose gel electrophoresis followed by immunostaining, autoradiography and counting radioactivity in sliced gels. Products of digestion of plasmas from three patients with severe vWD (type III) were undetectable as were those obtained from vWF-deficient plasma.

Table 1 Percent of radioactivity incorporated into vWF fragments SpII and SpIII produced after 3h digestion by V-8 protease in normal plasma pool, 9 normal individual plasmas and 3 cryosupernatants immunostained by 5 distinct labeled polyclonal antibodies to vWF

Ab	Plasmas	Number of experiments	Percent of total			
			SpII	SpIII	SpIIIa	SpIIIb+SpIIIc
1	Pool	20	71.8 ± 0.8	13.5 ± 0.5	11.6 ± 0.4	2.1 ± 0.2
	NP	9	70.1 ± 1.1	13.4 ± 0.5	11.5 ± 0.4	1.9 ± 0.3
2	Pool	4	66.2 ± 0.3	26.4 ± 0.7	24.9 ± 0.9	1.4 ± 0.2
3	Pool	27	48.3 ± 2.1	48.7 ± 2.2	42.8 ± 2.8	5.8 ± 0.7
	NP	13	46.0 ± 2.2	43.0 ± 2.8	37.0 ± 3.1	6.0 ± 0.9
	SUP	3	42.8 ± 2.8	49.7 ± 3.7	36.8 ± 2.9	12.9 ± 1.2
4	Pool	10	31.9 ± 1.2	60.4 ± 1.6	50.4 ± 1.3	8.8 ± 0.8
5	Pool	5	55.0 ± 0.7	41.6 ± 0.6	35.7 ± 1.2	2.9 ± 0.7

Results expressed as % of total radioactivity of the lane are mean ± SEM. Pool: normal plasma pool; NP: normal individual plasma; SUP: supernatant of cryoprecipitate from normal plasma pool.

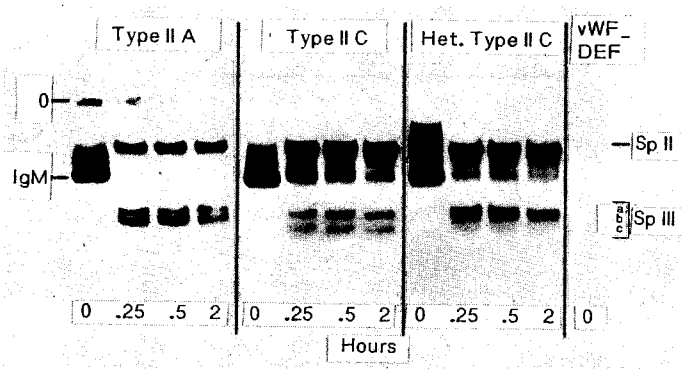

Fig. 5 Digestion by V-8 protease of vWF in plasma from type IIA, IIC and heterozygous type IIC vWD. Samples were submitted to SDS-agarose gel electrophoresis and vWF or fragments were displayed by autoradiography after immunostaining using ^{125}I-polyclonal antibody to vWF. Control sample of vWF-deficient plasma was run on the right lane. Markers show the position of origin and IgM (left) and of purified SpII and SpIII triplet (right). Time of digestion is indicated at the bottom of the gel

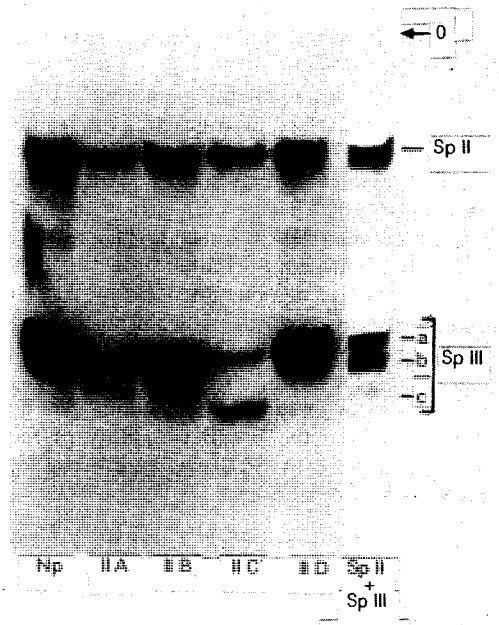

Fig. 6 SDS-agarose gel electrophoretic analysis of the products of digestion of vWF in normal plasma (Np) or plasma with type IIA, IIB, IIC and IID vWD by V-8 protease. Digestion was performed for 4 h using an enzyme/protein ratio of 1/200. Fragments were stained using ^{125}I-polyclonal antibody to vWF and displayed by autoradiography. The right lane shows purified SpII and SpIII run on the same gel but revealed by Coomassie blue staining. The arrow shows the origin of the gel

In plasma from three patients with type I vWD, there was no significant difference of the digestion pattern as compared to normal plasma pools or cryosupernatants.

When eighteen plasmas with type II vWD (IIA, B, C and D) were tested, the most striking finding was an aberrant distribution of the 3 subbands of SpIII triplet in type IIA and IIC as compared to normal plasma or cryosupernatant. In these two subtypes (Fig. 5) the kinetics progressed through a similar mechanism to that of control plasma with a rapid disappearance of the higher M_r multimers (0 to 30 min) to the first multimer of vWF. The latter appeared as the immediate precursor of the two stable terminal fragments. These fragments were already detected in the early time of digestion (0–15 min) and their pattern was not modified by prolonging the digestion. They were identified as SpII and SpIII triplet by their mobility and staining with specific labeled MAbs (not shown). However, while SpII consistently showed the same mobility and a similar staining pattern to that produced in normal plasma, the proportion of SpIIIa, b, and c in SpIII triplet varied and appeared characteristic of the variant subtype tested. SpIII appeared diffuse in type IIA and as a doublet lacking SpIIIb in type IIC. Using plasma from a patient with heterozygous type IIC vWD, SpIII also appeared as a doublet but with a decrease intensity of the fast moving SpIII band as compared to homozygous type IIC (Fig. 5).

The structure of SpIII triplets in variant vWD was further analysed by agarose electrophoresis using long gels (Fig. 6). In type IIA, there was an apparent decrease of SpIIIa and the presence of a distinct subband having a mobility in-between normal SpIIIb and SpIIIc. By contrast no band could be distinguished at the level of SpIIIb and SpIIIc when compared to normal plasma. In type IIC, there was a significant decrease of SpIIIa, a total lack of the SpIIIb species and a striking increase of the band at the level of SpIIIc. However, when analysed unreduced by SDS-polyacrylamide gel electrophoresis (Fig. 7) the latter band had a M_r of 170 kDa and was clearly distinct from SpIIIc produced from normal plasma (M_r 210 kDa) which migrated in that electrophoretic system at the top of SpII. As shown in Fig. 6, the pattern of type IIB and type IID did not significantly differ from that of control.

These results were verified quantitatively by slicing the gels and counting the radioactivity. Results obtained for type IIA and IIC vWD after staining with one of the polyclonal antibodies (No. 3, Table 1) are shown in Fig. 2. When the group of type IIA

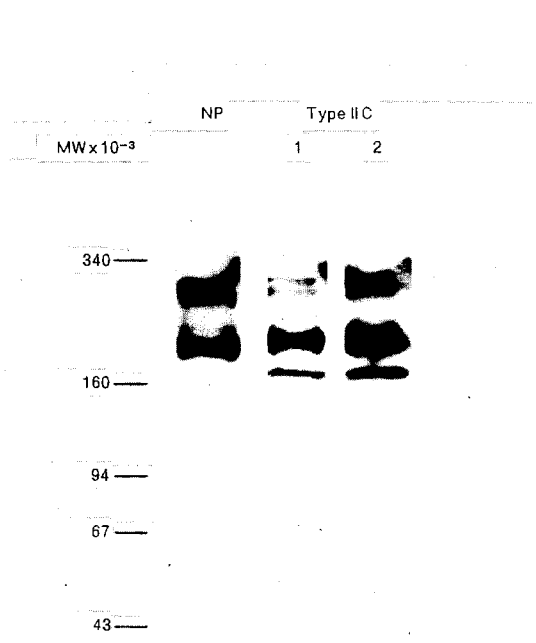

Fig. 7 SDS-5% polyacrylamide gel electrophoretic analysis of products of digestion (4h) of vWF in normal plasma pool (Np) or two unrelated plasmas with type IIC vWD by V-8 protease (enzyme/protein ratio 1/200). Following electroblotting on nitrocellulose paper the fragments were stained with ^{125}I-polyclonal antibody to vWF and displayed by autoradiography. Position of ^{125}I-markers is indicated on the left

Table 2 Comparison of radioactive staining of vWF degradation fragments by V-8 protease from plasma of patients with vWD and normal plasma pools using 5 distinct ^{125}I-polyclonal antibodies to wWF

Type	Number of patients	Number of experiments	Percent of control normal plasma pool (1)			
			SpII	SpIII	SpIIIa	SpIIIb+SPIIIc
IIA	7	23	105 ± 3.3 p <0.05	90.6 ± 5.8 p <0.1	33.6 ± 4.1 p <0.0005	454 ± 57.0 p <0.0005
IIB	3	10	92.2 ± 3.0 p <0.05	110 ± 3.9 p <0.05	98.3 ± 3.5 NS	222 ± 39.0 p <0.01
IIC	4	17	132 ± 5.8 p <0.0005	53.2 ± 4.0 p <0.0005	33.4 ± 2.4 p <0.0005	268 ± 49.0 p <0.0025
HET IIC	1	4	108 ± 6.2 NS	88.0 ± 10.3 NS	77.0 ± 8.8 p <0.1	201 ± 10.0 p <0.0025
IID	3	4	97.1 ± 2.3 NS	105 ± 6.0 NS	114 ± 5.2 NS	121 ± 69.0 NS

[1] The percent of radioactivity on each fragment from normal plasma pool was taken as 100%. Results are expressed as mean ± SEM. p-values ⩾0.1 were not significant. NS = not significant.

vWD was compared with the group of normals, the total amount of SpIII was not significantly different ($p < 0.1$ by the Student's test). By contrast the decrease of SpIIIa and the increase of SpIIIb and SpIIIc were highly significant ($p < 0.0005$).

Similarly, comparison of the group with type IIC vWD with that of normals showed that both the decrease of the total amount of SpIII, SpIIIa and SpIIIb and the relative increase of SpIIIc were highly significant ($p < 0.0005$). The use of other polyclonal antibodies with a poor reactivity for SpIII (Nos. 1, 2 and 5, Table 1) reduced the accuracy of the estimation based on direct inspection of the autoradiograph but did not modify the quantitative estimation. This is clearly established (Table 2) by showing that the ratio of staining for one species derived from type II vWD or normal plasma pool remained essentially constant from one experiment to the other and independently of the antibody used or patient tested.

Discussion

Variants of vWD (type II), which represent a heterogeneous set of disorders with a variable mode of inheritance and distinct abnormalities of vWF multimeric structure and function, are classified into four main subtypes IIA, IIB, IIC and IID, as well as rare subtypes IIE (8), IIF (28).

The qualitative abnormality of vWF in plasma of patients with type II vWD has first been analysed by low and high resolution discontinuous SDS-agarose gel electrophoresis (10). It has been established that in type II vWD vWF lacks the larger multimers as compared to normal or type I vWD. In addition, using high resolution electrophoresis, each multimer from normal vWF appears to be composed of three or more bands with a predominant central one. Consistent specific abnormalities of this banding pattern characterize each subtype of type II vWD (19–22, 28–30). It has been suggested that in normal vWF this banding pattern results from the presence in each vWF multimer of degraded forms of the 270 kDa subunit, namely a N-terminal 120 kDa and a C-terminal 140 kDa species produced during proteolysis of native vWF (5, 7, 31). This was recently confirmed and extended by Zimmerman and coworkers who have reported an analytical method that allows the assessment of the vWF subunit composition by immunoisolation of plasma vWF, reduction and, following SDS-polyacrylamide gel electrophoresis, immunoblotting with a pool of monoclonal antibodies (8). These authors established that vWF is partially proteolysed in normal plasma and consistently contains low proportions of degraded species with a slightly different apparent M_r than that found for our degraded forms (5), probably due to the electrophoretic system used. Zimmerman et al. (8, 9) found in addition to the intact 225 kDa subunit a N-terminal 140 kDa fragment, a major C-terminal 176 kDa and a minor C-terminal 189 kDa species. In plasma of patients with type IIA and IIB vWD, there was an increased proportion of the 176 and 140 kDa fragments with a lack of the 189 kDa species. In type IIC and IID vWD, the three vWF fragments were undetectable or present in only trace amounts (8).

We present here a new method to further characterize the subunit abnormality of vWF in variants of vWD. Plasma was submitted to digestion by V-8 protease and fragmentation of vWF was followed by gel electrophoresis and immunostaining by labeled antibodies to vWF. V-8 protease cleaved vWF in plasma milieu into two distinct fragments. These species were clearly identified as SpII and SpIII, the two complementary non overlapping terminal fragments of digestion of purified vWF by V-8 protease (7, 11). SpII, which has a M_r of 220 kDa, is a dimer of the C-terminal end of the vWF subunit, lying between residues 1,366 and 2,050 (M_r 110 kDa). SpIII, the complementary fragment, appears as a triplet composed of a predominant band of 320 kDa and two additional species of 265 and 210 kDa. The 320 kDa fragment is homodimeric and contains the N-terminal end of vWF subunit extending between amino acid residues 1 and 1,365. The two minor species with M_r 265 and 210 kDa derive from the main SpIII fragment by secondary proteolysis by V-8 between residues 910 and 911. This releases low amounts of a monomeric fragment, SpI (M_r 55 kDa) extending between residues 911 and 1,365 and of the corresponding amount of SpIII shortened either on one (M_r 265kDa) or on both of its chains (M_r 210 kDa). Although SpII and SpIII migrate close to each other by SDS-polyacrylamide gel electrophoresis, they can easily be separated by SDS-agarose gel electrophoresis as SpII shows an anomalous mobility corresponding to that of the fourth or fifth vWF multimer.

Under our experimental conditions, hydrolysis of vWF in normal plasma appears highly reproducible. This is demonstrated by the limited variation of the quantitative results from one experiment to the other when performed with the same polyclonal antibody to vWF. Results are independent of the plasmas tested. They are also clearly independent of the size of the vWF multimers as similar results were observed by immunostaining using either normal plasma or supernatant of cryoprecipitates which only contained normal low M_r multimers. However data obtained with the same plasma varied from one antibody to the other suggesting that eventhough the qualitative data are independent of the antibody specificity, the accuracy of the quantita-

tive estimation is strongly dependent upon the relative proportion of the N-terminal and C-terminal epitopes recognized by the antibody. For this reason our experiments on plasmas from patients with vWD were performed using antibodies reacting as well with SpII or SpIII.

Type IIA vWD is characterized by a lack of intermediate and large multimers of plasma vWF and decreased reactivity of platelet-rich plasma to ristocetin. Each vWF oligomer has an apparently normal triplet structure when analysed by high resolution SDS-agarose gel electrophoresis although the fastest moving satellite band of each oligomer is markedly increased as compared to normal. In addition it has been shown that abnormal type IIA vWF is more susceptible than normal to proteolytic degradation occurring in vivo (8, 32) and in vitro (33). Our results of digestion by V-8 protease showed that SpIII, the N-terminal portion of vWF subunit, was modified as compared to normal plasma, results being consistent in all cases. In both normal and type IIA plasma, SpIII staining accounted for a similar total amount but there was a striking difference of the distribution between the three subbands of SpIII triplet. In type IIA vWD, SpIIIa appeared significantly decreased together with an increased band at the level of SpIIIb and SpIIIc. This apparently modified SpIII fragment was already detected at the earliest times of digestion by V-8 protease and was not affected by prolonged hydrolysis. In addition, using plasmas from one family with type IIA vWD, we found that the SpIII pattern as well as the vWF multimeric structure did not vary when blood was collected in the presence of EDTA. Similar data were also observed in fresh plasma and in the corresponding plasma stored at −80° C for one month to five years (not shown). It is thus unlikely that the abnormality of SpIII observed in type IIA vWD results from a specific V-8 digestion of vWF. Our results are consistent with the occurrence of greater than normal in vivo proteolytic degradation of the N-terminal end of vWF subunit in type IIA, resulting in significantly increased amounts of the N-terminal 120 kDa species (7) together with a decreased proportion of the intact subunit. V-8 protease would then act at the same proteolytic sites as normally, i.e. at residues 1,365 and 910. This would produce a normal SpII (M_r 220 kDa) and, besides low amount of SpIIIa (M_r 320 kDa), SpIIIb (270 kDa) and SpIIIc (220 kDa) all composed of 160 and 110 kDa species, other degraded SpIII fragments associating a 160 kDa and a 120 kDa (M_r 280 kDa) or a 120 kDa and a 110 kDa chain (M_r 230 kDa). Thus, the similarity of M_r observed for SpIIIb and SpIIIc from normal and SpIII species from type IIA vWF is consistent with the diffuse pattern of SpIII in type IIA vWD by gel electrophoresis and confirms previous findings showing that the in vivo proteolysis as well as secondary cleavage of vWF by V-8 protease occurs in a common protease-sensitive region of the vWF subunit (7).

Type IIC vWF clearly differs from type IIA vWF in that each multimer essentially shows a single band when analysed by high resolution SDS-agarose gel electrophoresis (19–21, 29). This band corresponds to the major one exhibited in the triplet structure of normal vWF. In addition, when analysed by SDS-polyacrylamide gel electrophoresis under reducing conditions, type IIC vWF subunit shows a normal mobility with the absence or presence in only trace amounts of other degradation fragments (8). Thus, it appears that the in vivo proteolysis of vWF observed in type IIA vWD (68) and to a lesser extent in normal (7, 8) is almost undetectable in type IIC vWD. However, our data clearly show that besides a normal SpII, the proteolysis of type IIC vWF by V-8 protease produced two distinct species of SpIII. One corresponds to the main SpIIIa band produced from normal plasma. The second migrates as SpIIIc in agarose but with an apparent M_r of 170 kDa in polyacrylamide gel electrophoresis. This band, which was barely detectable or absent in digested normal plasma, did not appear to derive from the other (i.e. SpIIIa) by secondary degradation, since the intermediate SpIIIb species was never observed and both SpIIIa and the fast migrating band were produced simultaneously with a constant ratio and independently of the time of digestion. Thus the mechanism of proteolysis of vWF subunit in type IIC vWD by V-8 protease is not clear but appears characteristic of this subtype. It may be that, in contrast to type IIA vWF, a structural modification of the N-terminal part of vWF subunit decreases the susceptibility to in vivo proteolysis but allows the accessibility of V-8 protease to a specific site absent or hidden in the normal protein.

In the present study, digestion of type IIB and IID vWF by V-8 protease could not be clearly distinguished from that of the normal protein. In both cases, SpII fragment was normal while the banding pattern for SpIII triplet appeared at the limit of normal in type IIB and entirely normal in type IID. When analysed by high resolution SDS-agarose gel electrophoresis, type IIB vWF lacks the highest M_r multimers but to a lesser extent than in type IIA whereas in both types the structure of individual multimers is characterized by a similar triplet pattern and a similar subunit composition with increase of degradation fragments (8). Thus the structure of vWF in type IIB may result from an in vivo proteolysis with a similar mechanism to that previously identified for type IIA (8) as well as for normal plasma (7–8). The formation of normal SpIII triplet by V-8 protease from type IID vWF was more surprising since the vWF structure in this type appears different from that of other subtypes. Type IID vWF exhibits an aberrant multimeric structure by high resolution SDS-agarose gel electrophoresis (30) with multiple subbands with abnormal mobilities and composed as type IIC vWF by association of apparently non-proteolysed subunits (8). However our discrepant data using V-8 protease in type IIC and IID vWF strongly suggest that the structure of their respective subunits is different with the presence of normal V-8 protease sensitive sites in type IID and modified sites in type IIC.

We have thus developed a new analytical method allowing the evaluation of products of enzymatic degradation of vWF obtained from minute amounts of plasma. We found evidence that abnormality of vWF in type IIA and IIC vWD is associated with the production by V-8 protease of a modified SpIII, the N-terminal part of the protein. We do not know as yet if the apparent abnormality of variant SpIII results from normal cleavages by V-8 protease in specific fragments of subunits previously produced by an in vivo proteolysis or from unique cleavages by the enzyme in the type II subunit. Our results using type IIA plasma are rather consistent with the first hypothesis while they agree with the second using type IIC plasma. We previously established that SpIII contains the domain of vWF interacting with the platelet glycoprotein Ib in the presence of ristocetin while SpII is not involved in this mechanism (7, 11). Our results showing an abnormal SpIII fragment and an apparently normal SpII are thus consistent with previous data establishing the unability of type IIA and IIC vWF to interact with platelet in the presence of ristocetin.

In addition, considering the rather large size of the gene coding for vWF, it appears that this new method which allows the localization of the abnormality on specific parts of the vWF molecule should be a useful tool to seek potential modification along the DNA sequence.

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References

1 Meyer D, Baumgartner H R. Role of von Willebrand factor in platelet adhesion to the subendothelium. Br J Haematol 1983; 54: 1–9.
2 Tshopp T B, Weiss H J, Baumgartner H R. Decreased adhesion of platelets to subendothelium in von Willebrand's disease. J Lab Clin Med 1974; 83: 296–300.
3 Meyer D, Obert B, Piétu G, Lavergne J M, Zimmerman T S. Multimeric structure of factor VIII/von Willebrand factor in von Willebrand's disease. J Lab Clin Med 1980; 95: 590–602.
4 Ruggeri Z M, Zimmerman T S. The complex multimeric composition of factor VIII/von Willebrand factor. Blood 1981; 57: 1140–3.
5 Chopek M W, Girma J P, Fujimura K, Davie E W, Titani K. Human von Willebrand factor: A multivalent protein composed of identical subunits. Biochemistry 1986; 25: 3146–55.
6 Titani K, Kumor S K, Takio K, Ericson L H, Wade R D, Ashida K, Walsh K A, Chopek M W, Sadler J E, Fujikawa K. Amino acid sequence of human von Willebrand factor. Biochemistry 1986; 25: 3171–84.
7 Girma J P, Chopek M W, Titani K, Davie E W. Limited proteolysis of human von Willebrand factor by Staphylococcus aureus V-8 protease. Isolation and partial characterization of a platelet binding domain. Biochemistry 1986; 25: 3156–63.
8 Zimmerman T S, Dent J A, Ruggeri Z M, Nannini L H. Subunit composition of plasma von Willebrand factor. Cleavage is present in normal individuals, increased in IIA and IIB von Willebrand disease, but minimal in variants with aberrant structure of individual oligomers (Type IIC, IID and IIE). J Clin Invest 1986; 77: 947–51.
9 Berkowitz S D, Dent J, Roberts J, Fujimura Y, Plow E F, Titani K, Ruggeri Z M, Zimmerman T S. Epitope mapping of the von Willebrand factor subunit distinguishes fragments present in normal and type IIA von Willebrand disease from those generated by plasmin. J Clin Invest 1987; 79: 524–31.
10 Zimmerman T S, Meyer D. Structure and function of factor VIII and von Willebrand factor. In: Haemostasis and Thrombosis. Bloom A L, Thomas D P (eds.). Churchill Livingstone, Edinburgh 1987; pp 131–47.
11 Girma J P, Kalafatis M, Piétu G, Lavergne J M, Chopek M W, Edgington T S, Meyer D. Mapping of distinct von Willebrand factor domains interacting with platelet GPIb and GPIIb/IIIa and with collagen using monoclonal antibodies. Blood 1986; 67: 1356–66.
12 Meyer D, Zimmerman T S, Obert B, Edgington T S. Hybridoma antibodies to human von Willebrand factor. I. Characterization of seven clones. Br J Haematol 1984; 57: 597–608.
13 Meyer D, Baumgartner H R, Edgington T S. Hybridoma antibodies to human von Willebrand factor. II. Relative role of intramolecular loci in mediation of platelet adhesion to the subendothelium. Br J Haematol 1984; 57: 609–20.
14 Hill F G H, Enayat M S, George A J. Investigation including VIIIR:Ag multimeric analysis of a large kindred with type IIA von Willebrand's disease showing a dominant inheritance and similar gene expression in four generations. Thromb Haemostas 1983; 50: 735–9.
15 Girma J P, Piétu G, Lavergne J M, Meyer D, Larrieu M J. Abnormal antigenic reactivity of factor VIII/von Willebrand factor subunit in variants of von Willebrand's disease. J Lab Clin Med 1982; 99: 481–4.
16 Federici A B, Mannucci P M, Bader R, Lombardi R, Lattuada A. Heterogeneity in type IIB von Willebrand disease: two unrelated cases with no family history and mild abnormalities of ristocetin-induced interaction between von Willebrand factor and platelets. Am J Hematol 1986; 23: 381–90.
17 Holmberg L, Nilsson I M, Borge L, Gunnarsson M, Sjorin E. Platelet aggregation induced by 1-desamino-8-D-arginine vasopressin (DDAVP) in type IIB von Willebrand's disease. N Engl J Med 1983; 309: 816–21.
18 Armitage H, Rizza C R. Two populations of factor VIII-related antigen in a family with von Willebrand's disease. Br J Haematol 1979; 41: 279–89.
19 Mannucci P M, Lombardi R, Pareti F I, Solinas S, Mazzucconi M G, Mariani G. A variant of von Willebrand's disease characterized by recessive inheritance and missing triplet structure of von Willebrand factor multimers. Blood 1983; 62: 1000–5.
20 Mazurier C, Mannucci P M, Parquet-Gernez A, Goudemand M, Meyer D. Investigation of a case of subtype IIC von Willebrand disease: characterization of the variability of this subtype. Am J Hematol 1986; 22: 301–11.
21 Ruggeri Z M, Nilsson I M, Lombardi R, Holmberg L, Zimmerman T S. Aberrant multimeric structure of von Willebrand factor in a new variant of von Willebrand's disease (Type IIC). J Clin Invest 1982; 70: 1124–7.
22 Hill F G H, Enayat M S, George A J. Investigation of a kindred with a new autosomal dominantly inherited variant type von Willebrand's disease (possible type IID). J Clin Pathol 1985; 38: 665–70.
23 Ardaillou N, Girma J P, Meyer D, Lavergne J M, Shoa'i I, Larrieu M J. Variants of von Willebrand's disease. Demonstration of a decreased antigenic reactivity by immunoradiometric assay. Thromb Res 1978; 12: 817–30.
24 Thorell L, Blombäck B. Purification of the factor VIII complex. Thromb Res 1984; 35: 431–50.
25 Laemmli U K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) 1970; 227: 680–5.
26 Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of protein from polyacrylamide gels to nitrocellulose sheets: Procedure and some application. Proc Natl Acad Sci USA 1979; 76: 4350–4.
27 Fraker P J, Speck J C. Protein and cell membrane iodination with a sparingly soluble chloroamide, 1,3,4,6-tetrachlor-3a,6a-diphenyl glycoluril. Biochem Biophys Res Commun 1978; 80: 849–57.
28 Mannucci P M, Lombardi R, Federici A B, Dent J A, Zimmerman T S, Ruggeri Z M. A new variant of type II von Willebrand disease with aberrant multimeric structure of plasma but not platelet von Willebrand factor (type IIF). Blood 1986; 68: 269–74.
29 Batlle J, Lopez-Fernandez M F, Lasierra J, Fernandez Villamor A, Lopez Berges C, Lopez Borrasca A, Ruggeri Z M, Zimmerman T S. von Willebrand's disease Type IIC with different abnormalities of von Willebrand factor in the same sibship. Am J Hematol 1986; 21: 177–88.
30 Kinoshita S, Harison J, Lazerson J, Abildgard C F. A new variant of dominant type II von Willebrand's disease with aberrant multimeric pattern of factor VIII-related antigen (Type IID). Blood 1984; 63: 1369–71.
31 Fowler W E, Fretto L J, Hamilton K K, Erickson H P, McKee P A. Substructure of human von Willebrand factor. J Clin Invest 1985; 76: 1491–500.
32 Batlle J, Lopez-Fernandez M F, Campos M, Justica B, Berges C, Navarro J L, Diaz Cremades J M, Kasper C K, Dent J A, Ruggeri Z M, Zimmerman T S. The heterogeneity of type IIA von Willebrand's disease: Studies with protease inhibitors. Blood 1986; 68: 1207–12.
33 Gralnick H R, Williams S B, McKeown L P, Maisonneuve P, Jenneau C, Sultan Y, Rick M E. In vitro correction of the abnormal multimeric structure of von Willebrand factor in type IIA von Willebrand's disease. Proc Natl Acad Sci USA 1985; 82: 5968–72.

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