An association between the L1565 variant of von Willebrand factor and susceptibility to proteolysis by ADAMTS13

James Anthony Davies, Derrick John Bowen

From the Department of Hematology, School of Medicine, Cardiff University, Heath Park, Cardiff, CF14 4XN, UK.

Funding: this work was funded by the British Heart Foundation project grant PG/04/091/1746 and supported by NHS Research and Development through the University Hospital of Wales.

Acknowledgments: we are grateful to Mr. Steve J Bowley (SJB), MLSO2, for doing the reverse blood group analyses from plasma and for help in collecting and separating the samples and preparing DNA from them. Considerable thanks are also given to Mr. Richard Davies and Mrs Leigh. Hathaway for doing VWF:Ag assays.

Manuscript received August 9, 2006. Accepted January 10, 2007.

Correspondence:
Derrick J. Bowen, Department of Haematology, School of Medicine, Cardiff University, Heath Park, Cardiff, CF14 4XN, UK
E-mail: bowendj1@cf.ac.uk

ABSTRACT

The cysteine allele of the amino acid polymorphism (AAP) Y/C1584 in the A2 domain of von Willebrand factor (VWF) has been shown to correlate with enhanced VWF proteolysis by ADAMTS13. The frequencies and effect on VWF proteolysis of six reported AAP in VWF domains A1 and A2 were investigated. Only two AAP were variant: 4414 G/C (D/H1472) (allele frequency 0.86/0.14) and 4693 G/T (V/L1565) (allele frequency 0.92/0.08). D/H1472 had no apparent effect on VWF proteolysis. For V/L1565, a small but statistically significant increase in proteolysis was observed for V/L1565 VWF compared with V/V1565 VWF (p=0.0004).

Key words: von Willebrand factor, ADAMTS13, proteolysis, Y/C1584, V/L1565

Haematologica 2007; 92:240-243

The plasma glycoprotein von Willebrand factor (VWF) plays two essential roles in hemostasis: it carries (and protects) coagulation factor VIII in the circulation, and cross-links platelets with each other and with the exposed subendothelium during clot formation. VWF circulates as homopolymers (multimers) of varying lengths; the longest species are the most biologically active.[1,2] Multimer length is regulated, in part, by the metalloprotease ADAMTS13 (a disintegrin and metalloprotease with thrombospondin motifs).[3,4] Increased VWF proteolysis may predispose to bleeding,[5] whilst insufficient proteolysis may predispose to thrombotic thrombocytopenic purpura.[6,7] ABO blood group and the VWF Y/C1584 amino acid polymorphism (AAP) influence VWF proteolysis by ADAMTS13: proteolysis was greater for a purified, high molecular weight fraction of group O VWF than for that of non-O VWF,[8,9] and for heterozygous Y/C1584 VWF compared with homozygous Y/Y1584 VWF.[10] Blood group O[11] and C1584[12] are enriched in type 1 von Willebrand disease (VWD) cohorts and this may be explained, in part, by a deleterious effect of enhanced proteolysis on primary hemostasis.[13]

The present study addressed the hypothesis that AAP located near the ADAMTS13 site in VWF may influence proteolysis; six such polymorphisms were investigated.

Design and Methods

Subjects

The study cohort comprised 250 individuals (500 VWF genes) from the local population (South Wales, UK). The study was approved by the South East Wales Local Research Ethics Committee and the Cardiff and Vale NHS Trust Research and Development Office.

Genotype analysis

Six single nucleotide polymorphisms (SNP) were investigated: 4517C/T (encoding S/L1506), 4801C/A (P/T1601), 4414G/C (D/H1472), 4693G/T (V/L1565), 4713G/C (Q/H1571) and 4883T/C (I/T1628). These SNPs have been reported in a Caucasian pop-

ulation and they are the nearest polymorphisms to the ADAMTS13 proteolysis site. The polymorphisms were listed in the VWF Polymorphism Database (*http://www.vwf.group.shef.ac.uk/*) at the time of investigation. S/L1506 was concurrently listed in the VWF Mutation Database (*http://www.vwf.group.shef.ac.uk/*) as a type 2A VWD mutation.

Analysis of 4517C/T (S/L1506) and 4801C/A (P/T1601)

Polymerase chain reaction (PCR)-restriction digestion was employed: the SNPs respectively affect a *Sau3*AI (GATC) and *Hha*I (GCGC) restriction site. The primers used for the PCR were K2A and K1B.[10]

Analysis of 4414G/C (D/H1472), 4693G/T (V/L1565), 4713G/C (Q/H1571) and 4883T/C (I/T1628)

Heteroduplex analysis using heteroduplex generators (HG) was employed.[14] Details of the HG are available on request.

Phenotype analysis

VWF antigen (VWF:Ag)[15] and VWF collagen binding activity (VWF:CB)[16] were measured using an enzyme-linked immunosorbent assay, ABO blood group was determined by reverse analysis of plasma using *Reagent Red Cells* (National Blood Service, Birmingham, UK). Multimers were analyzed as previously described.[17]

Proteolysis studies

Cryoprecipitate (the source of plasma VWF) was prepared from 500 μL of plasma[10] and was incubated with group O cryodepleted plasma (the source of ADAMTS13) in a final volume of 50 μL in the presence of urea and barium ions.[8,10,16] In a given experiment, all incubations contained an equal quantity of VWF:Ag and an equal volume of group O cryodepleted plasma. The VWF content of the cryodepleted plasma, and the ADAMTS13 content of the cryoprecipitate were not significant relative to the added sources of VWF and ADAMTS13. Multimer analysis confirmed that the residual VWF content of the cryodepleted plasma was predominantly dimer (*not shown*).

Proteolysis was assessed using multimer analysis and/or VWF:CB applied to samples taken at the start (T=0h) and end (T=3h or 6h) of incubations. Proteolysis was expressed as the *percent loss of VWF:CB* (decrease in VWF:CB between T=0h and T=3h or 6h, expressed as a percentage of the VWF:CB at T=0h).

In all proteolysis studies the samples used were homozygous Y/Y1584.

Comparison of proteolysis for VWF of phenotype D/D1472, D/H1472 and H/H1472

Multimer analysis was used to assess proteolysis of plasma VWF from two samples of each phenotype. All samples were blood group O and homozgyous V/V1565.

Preliminary comparison of proteolysis for VWF of phenotype V/V1565, V/L1565, L/L1565

Initially, proteolysis was assessed using multimer analysis (two samples of phenotype V/V1565 and V/L1565, one sample of phenotype L/L1565). Subsequently, proteolysis was assessed using VWF:CB (triplicate analyses on one plasma sample of each phenotype, reactions sampled at T=0h and T=6h). All samples were blood group O and homozygous D/D1472.

Formal comparison of proteolysis for VWF of phenotype V/V1565 and V/L1565

Plasma VWF from 31 samples of each phenotype was subjected to proteolysis. Reactions were sampled in duplicate for VWF:CB at T=0h and T=3h.

Interspecies protein sequence comparisons

Vertebrate VWF sequences were aligned using Lasergene Megalign software (DNAStar Inc, Wisconsin, USA) (GenBank accession numbers: human NP_000543, mouse AAP41950, dog NP_001002932, pig AAC06229, horse AAB51549).

Statistical analysis

Statistical analyses were done using Microsoft Excel; Student's t-test was used for all comparisons. Averages are presented as mean±1 SD.

Results and Discussion

Genotype analysis

The genotype analyses demonstrated that, of the six SNP investigated, two were variant within the 500 VWF genes screened: 4414G/C (D/H1472) and 4693G/T (V/L1565), and four were invariant: 4517C (S1506), 4801C (P1601), 4713G (Q1571) and 4883T (I1628). Additionally, two rare sequence variations were detected: 4431T/C (silent T/T1477) and 4435G/A (encoding G/S1479) (Table 1).

Proteolysis of plasma VWF

Comparison of proteolysis of VWF of phenotypes D/D1472, D/H1472, H/H1472, G/G1479 and G/S1479

There was no detectable difference in the extent of proteolysis between VWF of each phenotype (*data not shown*).

Preliminary comparison of proteolysis of VWF of phenotypes V/V1565, V/L1565, L/L1565

Multimer analysis indicated that L1565 may be associated with a small increase in proteolysis (Figure 1) and this was further suggested by subsequent analysis using VWF:CB: V/V1565=38.6±4.8%, V/L1565=45.6±1.1%, L/L1565=49.5±4.8%.

The proteolysis of V/L1565 and V/V1565 VWF was

Table 1. Allele and genotype frequencies for variants detected among 500 VWF genes from the local population (South Wales, UK).

Single nucleotide polymorphism	*Amino acid polymorphism*	*Frequency*	*95% CI*	*Genotype*	*Frequency*
4414**G**/C	**D**/H1472	**0.86**/0.14	±0.030	GG GC CC	0.74 0.24 0.02
4413**T**/C	**T**/T1477 (silent)	**0.998**/0.002	±0.004	TT TC CC	0.996 0.004 0
4435**G**/A	**G**/S1479	**0.996**/0.004	±0.006	GG GA AA	0.992 0.008 0
4693**G**/T	**V**/L1565	**0.92**/0.08	±0.024	GG GT TT	0.844 0.152 0.00

The allele frequencies for 4414G/C are consistent with those previously reported for a Caucasian population (http://www.vwf.group.shef.ac.uk).

then formally compared by analysis of 31 samples of each phenotype. Only one individual in the cohort was homozygous L/L1565, therefore it was not possible to undertake a multiple-sample analysis for this phenotype. The mean proteolysis was 13.5±4.5% and 17.5±3.9% for V/V1565 and V/L1565 VWF, respectively. The absolute difference between the V/V and V/L phenotypes was not considerable (4%), however it was statistically significant (p=0.0004). ABO blood group has been shown to influence VWF proteolysis by ADAMTS13[8,9] therefore the representation of ABO blood groups in the V/V1565 and V/L1565 sample sets was inspected: group O = 15 (V/V) and 12 (V/L); group A = 11 (V/V) and 16 (V/L); group B = 5 (V/V) and 3 (V/L). Group O is associated with elevated proteolysis and non-O with decreased proteolysis,[8,9] thus ABO blood group can not underlie the higher proteolysis observed for the V/L1565 sample set, which had fewer group O and more non-O samples.

The proteolysis of V/L1565 and V/V1565 group O VWF was 17.4±4.2% and 14.9±4.5% respectively (p=0.15), and that of the corresponding group A VWF 17.2±3.6% and 13.2±4.3%, respectively (p=0.02). These comparisons involve small numbers of samples, but are consistent with increased proteolysis for heterozygous V/L1565 VWF.

Phenotypic data

The following values were obtained for, respectively, 31 V/V1565 and 31 V/L1565 samples: mean VWF:Ag 103±40% and 117±40% (p=n.s.), mean VWF:CB 89±33% and 79±27% (p=n.s.), mean ratio of VWF:CB to VWF:Ag 0.91±0.26 and 0.73±0.30 (p=0.01).

Interspecies protein sequence comparisons

Residues 1472 and 1479 are not highly conserved and lie within a region showing minor homology (38% conservation over 39 residues, Figure 2A). In contrast, residue 1565 is highly conserved and lies within a region of considerable homology (62% conservation over 42 amino acids, Figure 2B).

Previously, the VWF C1584 variant was shown to correlate with enhanced proteolysis by ADAMTS13.[10] The present data indicate that the VWF L1565 variant is associated with a small increase in proteolysis by ADAMTS13. A preliminary comparison with Y/C1584 VWF indicated that the relative proteolysis was 1 (V/V1565):1.18 (V/L1565):1.28 (L/L1565):1.59 (Y/C1584) (*data not shown*). C1584 is enriched in cohorts of patients with type 1 von Willebrand disease;[12] it remains to be seen whether L1565 has a pathologic relevance.

L1565 may influence proteolysis directly or may be linked to a causative change elsewhere in VWF. A potential direct effect is supported by the fact that residue 1565 is highly conserved, occurs in a region of homology between species, and is near the ADAMTS13 proteolysis

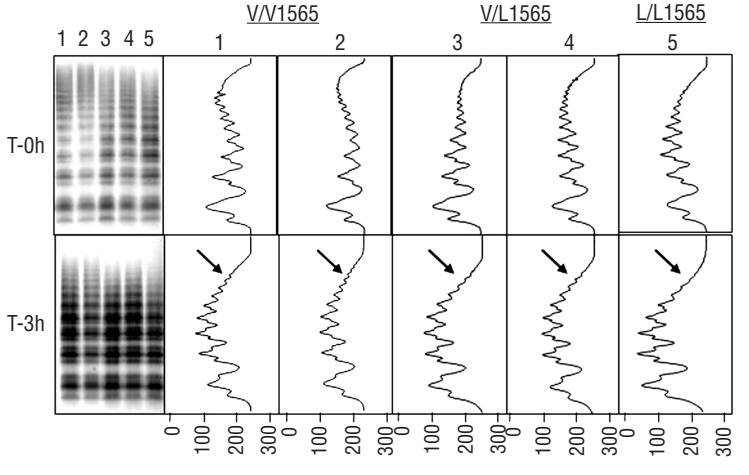

Figure 1. Proteolysis of V/V1565, V/L1565 and L/L1565 VWF assessed using multimer analysis. The multimer profiles at the start (T=0h) and end (T=3h) of the proteolysis reaction are shown on the left hand side, the densitometric scans of each lane are shown on the right hand side. Two independent samples of phenotype V/V1565 (lanes 1 and 2), V/L1565 (lanes 3 and 4) and one sample of phenotype L/L1565 (lane 5) were analyzed. Increased proteolysis associated with L1565 is indicated by the additional loss of high molecular weight multimers for V/L1565 VWF and, more so, L/L1565 VWF compared with V/V1565 VWF (compare regions arrowed), and by the greater increase in low molecular weight multimers relative to high molecular weight multimers. The scale at the base of each scan corresponds to color intensity: 0 represents black, 300 represents white.

Figure 2. Interspecies protein sequence alignments. Panel A. Sequence spanning D/H1472 and G/S1479. Panel B. Sequence spanning V/L1565 and Y/C1584. Numbering above the sequence represents amino acid residue positions in human pre-pro-VWF with the start methionine as residue 1. Conserved residues are boxed.

site. In contrast, D/H1472 and G/S1479, which do not appear to influence proteolysis, do not occur at conserved positions or in highly conserved regions. It has been hypothesized that VWF level and proteolyzis may be inversely related,[8,10] however VWF:Ag did not differ between V/V1565 and V/L1565 VWF. The increased proteolysis associated with L1565 is small and may require comparison of more individuals for an effect on VWF level to be detected. Alternatively, VWF proteolysis and level may not be directly related. No significant difference was observed in VWF:CB between V/V1565 and V/L1565 VWF, however the ratio of VWF:CB to VWF:Ag was significantly lower for V/L1565 VWF than for V/V1565 VWF (p=0.01). Because the ratio is a function of both VWF:Ag and VWF:CB, it may highlight differences that each parameter alone may not show. The lower ratio for V/L1565 VWF could reflect an effect of L1565 on average multimer length (through proteolysis) or on VWF:CB *per se*. These findings emphasize the need to ascertain the ethnic distribution of VWF AAP and identify those that influence proteolysis by ADAMTS13. This information may prove relevant to the understanding of VWD and natural variations in VWF biochemistry.

Authors' Contributions

JAD collected and analyzed the data and contributed to the preparation of the manuscript. DJB designed the research, collected and analyzed the data and prepared the manuscript.

Conflicts of Interest

The authors reported no potential conflicts of interest.

References

1. Sporn LA, Marder VJ, Wagner DD. Inducible secretion of large, biologically potent von Willebrand factor multimers. Cell 1986;46:185-90.
2. Sporn LA, Marder VJ, Wagner DD. von Willebrand factor released from Weibel-Palade bodies binds more avidly to extracellular matrix than that secreted constitutively. Blood 1987;69:1531-4.
3. Furlan M, Robles R, Lämmle B. Partial purification and characterization of a protease from human plasma cleaving von Willebrand factor to fragments produced by in vivo proteolysis. Blood 1996;87:4223-34.
4. Tsai HM. Physiologic cleavage of von Willebrand factor by a plasma protease is dependent on its conformation and requires calcium ion. Blood 1996;87:4235-44.
5. Dent JA, Berkowitz SD, Ware J, Kasper CK, Ruggeri Z. Identification of a cleavage site directing the immunochemical detection of molecular abnormalities in type IIA von Willebrand factor. Proc Natl Acad Sci USA 1990;87:6306-10.
6. Furlan M, Robles R, Galbusera M, Remuzzi G, Kyrle P, Brenner B, et al. Von Willebrand factor-cleaving protease in thrombotic thrombocytopenic purpura and the hemolytic-uremic syndrome. N Engl J Med 1998;339:1578-84.
7. Levy GG, Nichols WC, Lian EC, Foroud T, McClintick JN, McGee BM, et al. Mutations in a member of the ADAMTS gene family cause thrombotic thrombocytopenic purpura. Nature 2001;413:488-94.
8. Bowen DJ. An influence of ABO blood group on the rate of proteolysis of von Willebrand factor by ADAMTS 13. J Thromb Haemost 2003;1:33-40.
9. O'Donnell JS, McKinnon TA, Crawley JT, Lane DA, Laffan MA. Bombay phenotype is associated with reduced plasma-VWF levels and an increased susceptibility to ADAMTS13 proteolysis. Blood 2005; 106:1988-91.
10. Bowen DJ, Collins PW. An amino acid polymorphism in von Willebrand factor correlates with increased susceptibility to proteolysis by ADAMTS13. Blood 2004; 103:941-7.
11. Gill JC, Endres-Brooks J, Bauer PJ, Marks WJ, Montgomery RR. The effect of ABO blood group on the diagnosis of von Willebrand disease. Blood 1987;69:1691-5.
12. O'Brien LA, James PD, Othman M, Berber E, Cameron C, Notley CR, et al. Founder von Willebrand factor haplotype associated with type 1 von Willebrand disease. Blood 2003; 102:549-57.
13. Bowen DJ, Collins PW. Insights into von Willebrand factor proteolysis: clinical implications. Br J Haematol 2006; 133:457-67.
14. Savage DA, Wood NA, Bidwell JL, Fitches A, Old JM, Hui KM. Detection of b-thalassaemia mutations using DNA heteroduplex generator molecules. Br J Haematol 1995;90:564-71.
15. Short PE, Williams CE, Picken AM, Hill FG. Factor VIII related antigen: an improved enzyme immunoassay. Med Lab Sci 1982;39:351-5.
16. Gerritsen HE, Turecek PL, Schwarz HP, Lammle B, Furlan M. Assay of von Willebrand factor (vWF)-cleaving protease based on decreased collagen binding affinity of degraded vWF: a tool for the diagnosis of thrombotic thrombocytopenic purpura (TTP). Thromb Haemost 1999; 82:1386-9.
17. Raines G, Aumann H, Sykes S, Street A. Multimeric analysis of von Willebrand factor by molecular sieving electrophoresis in sodium dodecyl sulphate agarose gels. Thromb Res 1990;60:201-12.