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

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| ABSTRACT |

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. Multimer length is regulated, in part, by the metalloprotease ADAMTS13 (a disintegrin and metalloprotease with thrombospondin motifs). Increased VWF proteolysis may predispose to bleeding, whereas insufficient proteolysis may predispose to thrombotic thrombocytopenic purpura. 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 and for heterozygous Y/C1584 VWF compared with homozygous Y/Y1584 VWF. Blood group O and C1584 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.

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), 4801G/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 Sau3AI (GATC) and HhaI (GCGG) restriction site. The primers used for the PCR were K2A and K1B.

**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. Details of the HG are available on request.

**Phenotype analysis**

VWF antigen (VWF:Ag) and VWF collagen binding activity (VWF:CB) 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.

**Proteolysis studies**

Cryoprecipitate (the source of plasma VWF) was prepared from 500 µL of plasma 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. 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=5h or 6h) of incubations. Proteolysis was expressed as the percent loss of VWF:CB (decrease in VWF:CB between T=0h and T=5h 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 homozygous 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).

<table>
<thead>
<tr>
<th>Single nucleotide polymorphism</th>
<th>Amino acid polymorphism</th>
<th>Frequency</th>
<th>95% CI</th>
<th>Genotype</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>4414G/C</td>
<td>G/H1472</td>
<td>0.86/0.14</td>
<td>±0.030</td>
<td>GG</td>
<td>0.74</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>GC</td>
<td>0.24</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>CC</td>
<td>0.02</td>
</tr>
<tr>
<td>4413T/C</td>
<td>T/H1477</td>
<td>0.998/0.002</td>
<td>±0.004</td>
<td>TT</td>
<td>0.996</td>
</tr>
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<td></td>
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<td>0.004</td>
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<td></td>
<td></td>
<td></td>
<td>CC</td>
<td>0</td>
</tr>
<tr>
<td>4435G/A</td>
<td>G/S1479</td>
<td>0.996/0.004</td>
<td>±0.006</td>
<td>GG</td>
<td>0.992</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>GA</td>
<td>0.008</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>AA</td>
<td>0</td>
</tr>
<tr>
<td>4693G/T</td>
<td>V/L1565</td>
<td>0.92/0.08</td>
<td>±0.024</td>
<td>GG</td>
<td>0.844</td>
</tr>
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<td></td>
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<td>GT</td>
<td>0.152</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>TT</td>
<td>0.00</td>
</tr>
</tbody>
</table>

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, therefore the representation of ABO blood groups in the V/V1565 and V/L1565 VWF 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 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, 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 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. The present data indicate that the VVF 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, 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.
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 proteolysis may be inversely related, 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
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