Brief report

Bombay phenotype is associated with reduced plasma-VWF levels and an increased susceptibility to ADAMTS13 proteolysis

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ABO blood group is an important determinant of plasma von Willebrand factor antigen (VWF:Ag) levels, with lower levels in group O. Previous reports have suggested that ABO(H) sugars affect the susceptibility of VWF to ADAMTS13 (a disintegrin and metalloproteinase with thrombospondin type-1 repeats-13) cleavage. To further test this hypothesis, we collected plasma from individuals with the rare Bombay blood group. VWF:Ag levels were significantly lower in Bombay patients (median, 0.69 IU/mL) than in groups AB, A, or B ($P < .05$) and lower than in group O individuals (median, 0.82 IU/mL). Susceptibility of purified VWF fractions to recombinant ADAMTS13 cleavage, assessed using VWF collagen-binding assay (VWF:CB), was increased in Bombays compared with either group O or AB. Increasing urea concentration (0.5 to 2 M) increased the cleavage rate for each blood group but eliminated the differences between groups. We conclude that reduction in the number of terminal sugars on *N*-linked glycan increases susceptibility of globular VWF to ADAMTS13 proteolysis and is associated with reduced plasma VWF:Ag and VWF:CB levels. (Blood. 2005;106:1988-1991)

Introduction

The antigens of the ABO system (A, B, and H) consist of complex carbohydrate molecules. H (Fuc α1→2 Gal β1→4 GlcNAc β1→) antigen is an essential carbohydrate acceptor for either α-1,3-N-acetylgalactosaminyltransferase (A transferase) or α-1,3-galactosyltransferase (B transferase), which are both encoded by the ABO locus (9q34).[1] In group A, B, or AB individuals, the A and B transferases convert precursor H antigen into either A (GalNAc α1→3 [Fuc α1→2] Galβ 1→4 GlcNAc β1→) or B (Gal α1→3 [Fuc α1→2] Galβ 1→4 GlcNAc β1→) determinants, respectively. In group O individuals, the *O* allele does not encode any functional transferase enzyme so that they continue to express terminal H structures only.[2] In human tissues, H antigen can be synthesized by 2 distinct α-1,2-fucosyltransferases. One is the *H* gene *(FUT1)*–encoded H enzyme that regulates expression of ABH antigens in red blood cells.[3] The other is the *Secretor* gene *(FUT2)*–encoded Se enzyme that regulates expression of ABH antigens in the gastrointestinal tract and secretions.[4] Individuals with the very rare Bombay phenotype are non-Secretors and also fail to express H transferase *(FUT 1)*.[5] Such people cannot synthesize A or B antigenic structures regardless of their ABO blood group genotype, and ABH antigens are absent from both their erythrocytes and secretions.[6] Para-Bombay individuals also fail to express H transferase, but do express the *FUT2* (Secretor)–encoded α-1,2-fucosyltransferase, so that ABH antigens are present in their secretions but not on erythrocytes.[6,7]

It is well established that ABO blood group exerts a major quantitative effect on plasma von Willebrand factor (VWF) levels, with significantly lower levels in group O individuals.[8,9] Moreover, ABH antigenic determinants have been identified on the *N*-linked glycans of circulating VWF according to the blood group of the individual.[10] However, the mechanism through which these glycans influence plasma-VWF antigen (VWF:Ag) levels remains unclear. Animal studies have shown that VWF glycans may influence rate of hepatic clearance,[11] and previous data suggested it may be mediated by the H antigen.[12] On the other hand, Bowen recently reported that VWF of different ABO blood groups exhibited different susceptibility to specific cleavage by ADAMTS13 (a disintegrin and metalloproteinase with thrombospondin type-1 repeats-13) ($O \geq B > A \geq AB$).[13] To further investigate how glycan expression on VWF influences plasma VWF:Ag levels, we have collected plasma samples from a series of Bombay and para-Bombay individuals. As these individuals lack the H antigen, they provide a critical test of current hypotheses. We report the novel observation that Bombay phenotype is associated with plasma-VWF levels similar or lower than group O. In addition, we demonstrate that Bombay VWF demonstrates significant increased susceptibility to cleavage by ADAMTS13, via a conformation-dependent mechanism.

Study design

VWF glycans, VWF antigen, and VWF multimer distribution

Plasma samples from 47 anonymized individuals with Bombay blood groups were collected from blood transfusion centers. No clinical details on these individuals were available. The Bombay (n = 30) and para-Bombay (n = 17) phenotype of each case was established by serologic testing. Plasma samples previously collected from a series of healthy volunteer

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Submitted February 25, 2005; accepted May 4, 2005. Prepublished online as *Blood* First Edition Paper, May 10, 2005; DOI 10.1182/blood-2005-02-0792.

Supported by grants from the British Heart Foundation (FS/03/001, FS/03/074, and RG/02/008).

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donors (n = 169; 64 group A; 18 group B; 15 group AB; and 72 group O) were used as controls. Bombay and para-Bombay plasma samples were tested for evidence of H on VWF expression by Western blotting, and using a modified sandwich enzyme-linked immunosorbent assay (ELISA) technique as previously described.[12] In preliminary experiments, we established that the polyclonal rabbit anti–human VWF antibody used in this ELISA was not influenced by VWF glycan (data not shown). Plasma VWF:Ag levels and multimer analyses were performed as previously described.[12] VWF collagen-binding assay (VWF:CB) was performed using a commercial ELISA method (Technoclone, Surrey, United Kingdom) in accordance with the manufacturer's recommendations.

Purification of VWF and ADAMTS13 expression

VWF was purified from human group AB, group O, and Bombay plasmas, as previously described.[13] In brief, group-specific plasma was cryoprecipitated. The pellet was then resuspended in TC buffer (20 mM Tris [tris(hydroxymethyl)aminomethane]–HCl, 10 mM sodium citrate, pH 7.4) and passed through a Sepharose CL-2B HiPrep 26/60 gel filtration column (AmershamPharmacia, Buckinghamshire, United Kingdom). Eluate fractions were assessed for VWF content, multimer distribution, and purity as previously described.

Recombinant human ADAMTS13 was purified and quantified following stable transfection of HEK293 cells. This method has recently been described in full.[14] In a series of parallel experiments, cryodepleted pooled (groups O, A, B, and AB) human plasma was used as the source of ADAMTS13.[13]

Proteolysis of purified VWF by ADAMTS13

ADAMTS13-VWF cleavage assays were performed using either recombinant human ADAMTS13 or plasma-derived ADAMTS13.[14] In brief, 5 to 20 nM ADAMTS13 was preincubated with 10 mM $BaCl_2$ for 10 minutes at 37°C. The activated ADAMTS13 was then incubated at 37°C with 8 nM of comparable blood group–specific (O or AB or Bombay) high-molecular-weight (HMW)–VWF in reaction mix containing urea (0.5-4 M), 10 mM $BaCl_2$, 5 mM NaCl, 0.5 mM $CaCl_2$, and 15 mM Tris-HCl (pH 7.8). At specific time points, subsamples were removed and VWF proteolysis analyzed using VWF:CB and VWF multimer pattern. For plasma-derived ADAMTS13, proteolysis of blood group–specific (O versus AB versus Bombay) HMW-VWF was carried out essentially as previously described.[13]

Results and discussion

In both Bombay and para-Bombay individuals, we found no evidence of H antigen on VWF, confirming that both phenotypes are associated with an alteration in the glycan structure of circulating VWF distinct to that observed in normal plasma VWF (Figure 1A). In keeping with previous reports,[8,9] we observed a significant effect of ABO blood group on plasma VWF:Ag levels, with significantly lower levels in group O individuals (Figure 1B) compared with non-O. However, we also demonstrated that VWF:Ag levels in Bombay patients (median VWF:Ag = 0.69 IU/dL) were significantly lower than in groups AB, A, or B ($P < .05$). Moreover, Bombay VWF:Ag levels were also lower than in group O individuals (median VWF:Ag = 0.82 IU/dL), although this difference failed to achieve statistical significance ($P = .133$; Mann-Whitney analysis) (Figure 1B). Bombay and para-Bombay phenotypes result from null mutations at the *FUT1* and *FUT2* loci, which are both located on chromosome 19,[3,4] remote from the *ABO* locus on chromosome 9. Consequently, this effect of Bombay phenotype on plasma-VWF levels is conclusive evidence that the effect of ABO group on plasma VWF:Ag levels is due to a direct functional effect of the ABH determinants on VWF, rather than linkage disequilibrium between the *ABO* locus and another unidentified VWF regulatory locus.

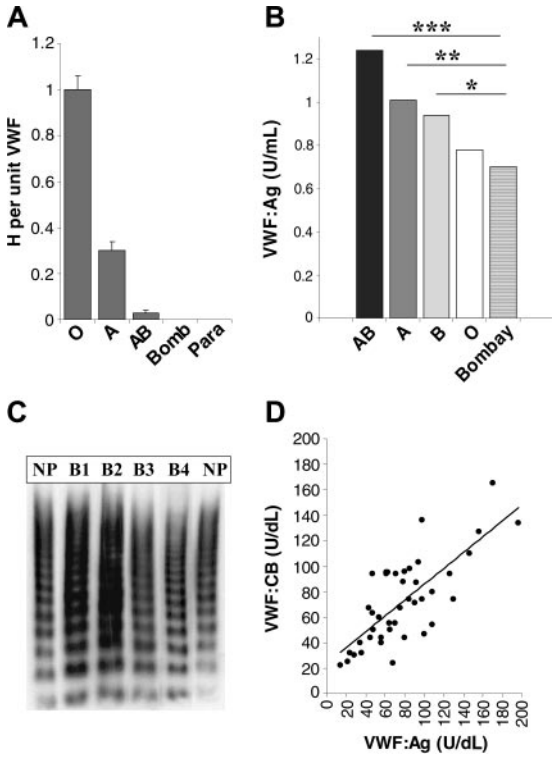

Figure 1. Effect of Bombay phenotype on plasma-VWF glycan expression, plasma VWF:Ag level, and plasma-VWF multimer composition. (A) The amount of *H antigen* expressed per unit VWF was measured in a series of healthy individuals (group O, n = 72; group A, n = 64; group AB, n = 15) and in Bombay (n = 30) or para-Bombay (n = 17) individuals using a modified sandwich ELISA. Each plasma sample was tested in duplicate at 3 dilutions, and results represent means ± SEM. Using similar methodologies, no *A or B antigen* expression was detected on Bombay or para-Bombay VWF (data not shown). In some cases the SEM cannot be seen due to its small size. (B) Plasma VWF:Ag levels were measured by ELISA. Median values for each group are shown. VWF:Ag levels were significantly lower in Bombay compared with groups AB, A, and B (***$P < .001$, **$P < .01$, and *$P < .05$, respectively). Among the blood group A individuals, genotype (A_1A_1, A_1O_1, or A_2O_1) at the ABO locus was determined by polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) analysis as previously described.[12] Bombay VWF:Ag levels were significantly lower than those in A_1A_1 homozygotes or A_1O_1 heterozygotes ($P < .01$). Although previous studies have demonstrated an effect of Secretor blood group on plasma-VWF levels, we found no difference in plasma VWF:Ag levels between para-Bombay (Secretor) and Bombay (non-Secretor) individuals (data not shown). (C) Plasma multimer analysis of 4 Bombay individuals (B1 to B4) compared with 2 healthy controls. No loss of HMW-VWF multimers was apparent in the Bombay individuals. (D) Plasma VWF:CB levels were also significantly reduced in Bombay plasmas (median VWF:CB = 71 IU/dL), compared with group O (median VWF:CB = 88 IU/dL; $P = .04$, Mann-Whitney). However, as shown for the 47 Bombay individuals, there remained a good correlation between VWF:Ag and VWF:CB. NP indicates normal plasma.

Through cleavage at the Tyr1605-Met1606 bond within the VWF A2 domain, ADAMTS13 regulates plasma-VWF multimer composition.[15] In keeping with a previous report,[13] we found group O VWF was cleaved significantly more quickly than group AB. If the ABO effect on plasma-VWF level is mediated by susceptibility to ADAMTS13 cleavage, then cleavage of Bombay VWF should be at least as rapid as that of group O. In fact, we demonstrated that Bombay HMW-VWF is cleaved significantly faster than either group O or group AB ($P < .001$) (Figure 2A-C). This marked difference was apparent over the full range of ADAMTS13 concentrations studied (5-20 nM) (data not shown). In a parallel set of experiments, cryodepleted plasma was used as the source of

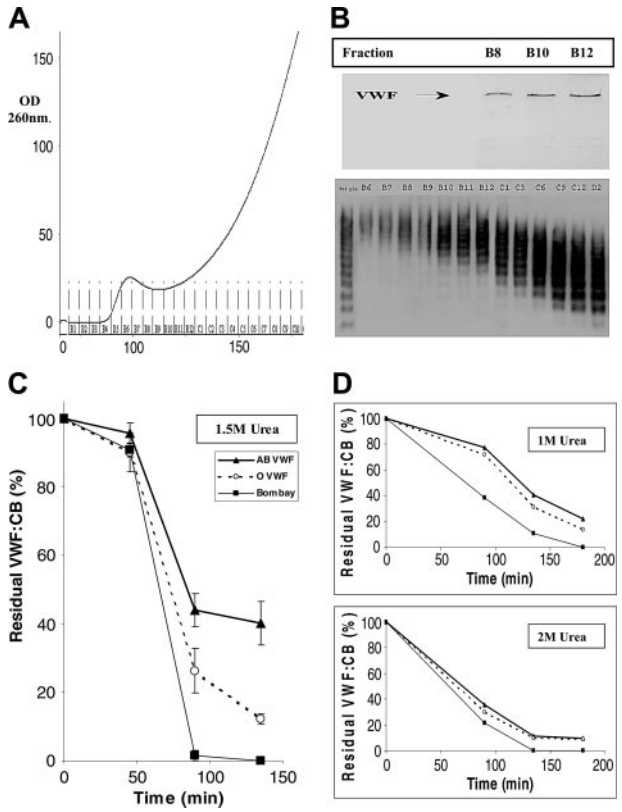

Figure 2. Purification of Bombay VWF and susceptibility to ADAMTS13 proteolysis. To investigate further the mechanism underlying the low plasma VWF:Ag levels associated with the Bombay phenotype, we purified HMW-VWF from 2 individual Bombay subjects and from normal pooled group AB and group O plasmas. (A) Serial analysis of eluate fractions from the Sepharose CL-2B gel filtration column demonstrated that VWF began to elute in fraction B6. OD indicates optic density. (B) Analysis of the early fractions (B6-B9) by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions followed by silver staining demonstrated a single major protein band at 250 kDa consistent with monomeric VWF. Multimer analysis showed that fractions also contained predominantly HMW-VWF. (C) In order to investigate susceptibility of HMW-VWF to ADAMTS13 proteolysis, comparable eluate fractions (range, B7-B9) were chosen for group AB, group O, and Bombay VWF purifications, containing comparable HMW-VWF. These were incubated with recombinant human ADAMTS13 (5-20 nM) at 1.5-M urea concentration, and rate of VWF cleavage was assessed by determining the rate of fall in VWF:CB. Results (mean of 6 experiments ± SEM) are expressed as a percentage residual collagen-binding activity. In some cases, the SEM cannot be seen due to its small size. After 90 minutes of incubation, the rates of proteolysis between groups AB, O, and Bombay were significantly different (O versus AB, $P < .01$; Bombay versus O, $P < .01$; and Bombay versus AB, $P < .001$). (D) The effect of ABO on susceptibility of VWF to ADAMTS13 proteolysis was investigated at different concentrations of urea (0.5 to 4 M). As urea concentration was raised from 0.5 M to 2 M, the rate of VWF proteolysis increased for each blood group. However, the difference between AB, O, and Bombay HMW-VWF, which was most marked at 1.5 M urea, became progressively less apparent as urea concentration was increased. In the presence of 4 M urea, proteolysis of HMW-VWF by ADAMTS13 was inhibited (data not shown).

ADAMTS13. Once again, Bombay HMW-VWF was significantly more susceptible to proteolysis (data not shown). The mechanism through which ABO blood group influences susceptibility to cleavage by ADAMTS13 remains unknown, but Tyr1605-Met1606 bond is flanked by 2 *N*-linked (asparagine 1515 and 1574) and 5 *O*-linked (threonine 1468, 1477, 1487, and 1679, and serine 1486) potential glycosylation sites.[16]

Previous studies have demonstrated that *N*-linked glycan structures directly influence the folding of glycoproteins by reducing conformational freedom of the local peptide backbone.[17,18] We hypothesized that glycan changes may alter the conformation of VWF and thus alter accessibility to the ADAMTS13 cleavage site. To investigate this hypothesis, we repeated ADAMTS13 cleavage assays over a range of urea concentrations (0.5-4 M) to mimic changes in shear forces responsible for unraveling VWF multimer. As urea concentration increased, we found that the rate of VWF proteolysis increased for each of the different blood groups studied, but Bombay VWF continued to be cleaved most quickly (Figure 2D). However, as the concentration of urea progressively increased, the differential effects observed between AB, O, and Bombay became less apparent. This observation suggests that oligosaccharide chain composition may influence the conformation of VWF, such that removal of terminal sugars allows the A2 domain to adopt a conformation more permissive for cleavage by ADAMTS13. Alternatively, the glycan structure of VWF may influence the ability of HMW-VWF multimers to unwind in vivo in response to shear stress.

Reduction in the number of sugars on the oligosaccharide chains of VWF is clearly associated with an increased susceptibility to cleavage by ADAMTS13. Whether this susceptibility to ADAMTS13 proteolysis is responsible for a quantitative effect on plasma VWF:Ag levels remains to be determined, as the magnitude of the Bombay effect on cleavage is not clearly matched by a comparable effect on plasma-VWF level. Furthermore, it is interesting that despite the increased rate of cleavage, plasma multimer analysis in Bombay and para-Bombay individuals appeared normal. In particular, we observed no loss of HMW multimers as seen in typical patients with type 2A von Willebrand disease (VWD),[19] and the ratio of VWF:CB to VWF:Ag (CBA/Ag) was similar to that observed in other blood groups (Figure 1C-D).[9] This apparent paradox of increased susceptibility to ADAMTS13, yet normal plasma multimer distribution, has been previously described in association with the Tyr1584Cys polymorphism of VWF.[20,21] The paradox may reflect the fact that the increased ADAMTS13 susceptibility associated with both Tyr1584Cys and the Bombay phenotype is markedly less than that arising from classic type 2A mutations.[21] Further studies are required to clarify how VWF proteolysis by ADAMTS13, plasma multimer distribution, and VWF clearance are integrated in vivo.

Acknowledgments

The authors wish to acknowledge the following individuals who supplied Bombay and para-Bombay plasma samples: Dr J. Clarke and Professor W. Watkins, Imperial College London, United Kingdom; Ms J. Poole, International Blood Group Reference Laboratory, United Kingdom; Dr J. Harrison National Blood Service, United Kingdom; Professor M. Lin, Mackay Memorial Hospital, Taipei, Taiwan; Dr B. Fischer Australian Red Cross; and Professor George Garratty, American Red Cross Blood Services.

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