Human Plasma α2-Macroglobulin and von Willebrand Factor Possess Covalently Linked ABO(H) Blood Group Antigens in Subjects With Corresponding ABO Phenotype

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We recently identified ABO(H) blood group structures in Asn-linked sugar chains of human von Willebrand factor (vWF) purified from factor VIII concentrates (J Biol Chem 267:8723, 1992). We surveyed plasma glycoproteins carrying ABO(H) blood group antigens by Western blotting analysis and sandwich enzyme-linked immunosorbent assay using blood group-specific monoclonal antibodies (MoAbs) and a lectin. Two major plasma proteins showing apparent molecular weight of about 180 Kd and 270 Kd by sodium dodecyl sulfate polyacrylamide gel electrophoresis reacted with blood group-specific MoAbs and Ulex europeus lectin I in accordance with donor blood group. Direct sequence analysis of the protein bands showed their identity with the N-terminal sequences of α2-macroglobulin (α2M) and vWF, respectively. The two bands also reacted with anti-α2M and anti-vWF antibodies. The α2M and vWF prepared from plasma by immunoprecipitation showed the appropriate blood group antigenicity. After incubation with endoglycosidase F, both α2M and vWF lost almost all reactivity with anti-blood group reagents. About 90% of plasma vWF, but only ~10% of α2M, was immunoprecipitated with anti-blood group antibody. These results indicate that at least two plasma glycoproteins, vWF and α2M, possess Asn-linked ABO(H) blood group antigens in normal individuals with corresponding ABO phenotype. Therefore, ABO(H) blood group antigens in plasma glycoproteins should be considered during preparation of plasma materials for therapeutic use.

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Materials and methods

Materials and reagents. Plasma from healthy donors of known blood group was prepared from blood containing 0.38% sodium citrate immediately after collection, and stored at −80°C after addition of protease inhibitors (4 mmol/L EDTA, 4 mmol/L N-ethylmaleimide, 100 kallikrein inhibitor units/mL of aprotinin) until use. Anti-A and -B mouse MoAbs were obtained from Ortho Diagnostic Systems (Raritan, NJ) or Kokusai-shiyaku (Kobe, Japan). Anti-A mouse MoAb (A005) specific for type 2 chain [GalNacα1→3Galα1→4GlcNAc-R] was from BioCarb Chemicals (Lund, Sweden), and Ulex europeus lectin I (UEA-I) conjugated with biotin was from Honen Corporation (Tokyo, Japan). Anti-human vWF mouse MoAb (RGR) specific for reduced vWF and anti-vWF rabbit IgG were donated by Dr Z.M. Ruggeri (Scirpps Research Institute, La Jolla, CA) and Dr S. Miyata (Nara Medical College, Kashihara, Nara, Japan), respectively. Anti-vWF goat IgG and anti-α2M rabbit IgG were from Medical and Biological Laboratories (MBL) Company (Nagoya, Japan) and Dakopatts (Glostrup, Denmark), respectively. Other polyclonal antibodies (PoAbs) against human plasma glycoproteins, and second antibodies and avidin conjugated with horseradish peroxidase (HRP) were from MBL, Dakopatts, or Zymed Laboratories Incorporated (San Francisco, CA) unless otherwise indicated. Authentic human α2M was from Sigma Chemical Company (St Louis, MO), and vWF was purified from FVIII concentrates as previously described.

Western blotting. Plasma was mixed with an equal volume of sodium dodecyl sulfate (SDS) buffer (2% SDS, 3% 2-mercaptoethanol, 0.1% SDS, 5% 2-mercaptoethanol, 0.1% SDS, and 25 mmol/L Tris-HCl, pH 6.8) containing 0.1% SDS and 25 mmol/L Tris-HCl, pH 6.8) containing 0.1% SDS and 25 mmol/L Tris-HCl, pH 6.8). Plasma was added to the samples, and the mixture was heated to 100°C for 10 min. The samples were then subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using a Mini Protean III electrophoresis system (Bio-Rad Laboratories, Hercules, CA) with a running gel of 12.5% acrylamide and 0.1% SDS, and a stacking gel of 4% acrylamide and 0.1% SDS. The gels were stained with Coomassie Brilliant Blue R-250 or silver stain. The blots were washed with 0.1% SDS and 0.1% Triton X-100, then blocked with 5% nonfat dry milk and 0.1% Tween 20 in PBS. The blots were incubated with 1/500 diluted anti-α2M or anti-vWF antibodies, or 1/500 diluted anti-human vWF mouse MoAb (RGR) for 1 h at room temperature, followed by 1/2000 diluted rabbit anti-goat IgG or 1/1000 diluted goat anti-mouse IgG for 1 h at room temperature. The blots were then incubated with 1/500 diluted avidin conjugated with horseradish peroxidase (HRP) for 1 h at room temperature, followed by 1/1000 diluted 3,3′-diaminobenzidine (DAB) for 5 min at room temperature. The bands were visualized by enhanced chemiluminescence (ECL) reagents (Amersham, Arlington Heights, IL) or 3,3′-diaminobenzidine (DAB) for 5 min at room temperature. The bands were visualized by enhanced chemiluminescence (ECL) reagents (Amersham, Arlington Heights, IL) or 3,3′-diaminobenzidine (DAB) for 5 min at room temperature.
detected with HRP-conjugated second antibody or avidin (diluted blots were washed four times with TW/TBS, specific binding was buffered saline, pH 7.4 (TBS), containing 0.05% Tween 20 (TW/Ortho) anti-a,M (100 μL/TBS) and incubated with anti-blood group MoAbs (diluted 1:10 except for A005 at 1:100), biotin—UEA-I (5 μg/mL), or other antibodies diluted 1:500 with TW/TBS for 90 minutes at 20°C. After blots were washed four times with TW/TBS, specific binding was detected with HRP-conjugated secondary antibody or avidin (diluted 1:1,000), using 4-chloro-naphthol and H2O2 as substrates.

Immunoprecipitation. Plasma (1 mL) from each blood group donor was mixed with either blood group-specific MoAb (500 μL; Ortho) anti-a,M (100 μL; Dakopatts), or anti-vWF (rabbit IgG, 100 μL) PoAbs in microcentrifuge tubes and incubated for 24 hours at 4°C. Immunoprecipitates were collected by centrifugation at 15,000 rpm for 20 minutes, washed several times with TBS, and dissolved in 100 μL of SDS buffer containing 2 mol/L urea. Aliquots (2 to 5 μL) were subjected to Western blotting analysis. Plasma was mixed with 10 volumes of either anti-A or anti-B MoAb (Ortho) for 48 hours, followed by addition of one-tenth volume of anti-mouse IgM (MBL). After incubation for another 48 hours, each mixture was centrifuged, and amounts of vWF and α2M remaining in the supernatant were estimated by enzyme-linked immunosorbent assay (ELISA).

Endoglycosidase F treatment. Aliquots (5 μL) of α2M immunoprecipitated from group A plasma with anti-α2M, as described above, and purified vWF (10 μg) were incubated with or without 100 μU of endoglycosidase F (Boehringer Mannheim, Mannheim, Germany) in 50 μL of solution (50 mmol/L sodium-acetate buffer, pH 6.0, 40 mmol/L EDTA, 2% n-octylglucoside, 2% 2-mercaptoethanol, 0.1% SDS, 0.25% sodium azide) for 10 hours at 37°C. Reaction was stopped by heating at 100°C for 5 minutes after addition of an equal amount of SDS buffer, and proteins were analyzed as described above.

Organic solvent treatment. Immunoprecipitates of α2M and vWF were washed three times with chloroform:methanol:water (1:2:0.8 by volume) for 15 minutes and then subjected to Western blotting. Proteins on PVDF membrane were treated similarly before incubation with antibodies. ELISA plates (Becton Dickinson Co, San Jose, CA) were precoated with 20 μg/mL of PoAb against human vWF (MBL), α2M (Dakopatts), IgG, fibrinogen, fibronecetin, or α1-acid glycoprotein (MBL) in 50 mmol/L sodium bicarbonate buffer, pH 9.5, overnight at 4°C, followed by blocking with TBS containing 1% bovine serum albumin. Serially diluted plasma from healthy subjects (10 from each blood group, including 1 to 3 nonsecretors) was added to the plate and left for 90 minutes at room temperature. The plate was washed with TW/TBS, then incubated with anti-A or anti-B MoAb (Ortho; diluted 1:10 with TW/TBS) or biotin—UEA-I lectin (5 μg/mL) for 45 minutes. Binding of MoAb or lectin was detected by secondary antibody or avidin conjugated with HRP (diluted 1:1,000) using o-phenylenediamine-HCl and H2O2 as substrates. HRP reaction was continued in the dark for 30 minutes and terminated with sulfuric acid. Absorbance of reaction products at 490 nm was measured using buffer solution as a blank. To remove FVIII from FVIII/vWF complex, plates coated with anti-vWF were washed with 0.4 mol/L CaCl2 after incubation with plasma, as described.16

RESULTS

Plasma glycoproteins reactive with anti-blood group reagents. Western blotting analysis showed that two plasma components reacted with MoAbs specific for blood groups A, B, and AB (Fig 1). The major and minor reactive bands had apparent molecular weight values of 180 Kd and 270 Kd. Group O plasma components showed reactivity with UEA-I but not with anti-A or anti-B. UEA-I, which shows affinity for α-fucose residues (especially for H substance,20 which is usually expressed in all blood groups), reacted with the two components mentioned above and some other proteins, regardless of blood group (Fig 1, lanes 10 to 12).

Immunoprecipitation was performed to identify these two plasma components, and the immunoprecipitate from group A plasma with anti-A MoAb was analyzed by Western blotting. Two bands of 180 Kd and 270 Kd were again obtained (Fig 2, lanes 1 and 2). Bands indicated by arrows are those of the anti-A MoAb (mouse IgM) used as reagent. The 180-Kd and 270-Kd plasma proteins were identified as α2M and vWF, respectively, based on three lines of evidence. (1) Each protein band on the PVDF membrane was excised after staining with Coomassie blue and subjected to amino acid sequence analysis.17 N-terminal sequences of SVSGKXXXYMV and SLSXRPPPMVK were obtained for the 180-Kd and 270-Kd bands (X indicates no or unclear identification), which is consistent with N-terminal sequences reported for human α2M (SVSGKPOYTMV)21 and vWF (SLSRPPMKV).16 respectively. (2) The two bands reacted with anti-α2M PoAb and anti-vWF MoAb (RG8), respectively (Fig 2, lanes 4 and 5). (3) The α2M and vWF prepared from group A plasma by immunoprecipitation using anti-α2M and anti-vWF PoAbs clearly reacted with anti-A MoAb (Fig 2, lanes 6 and 8). The α2M and vWF prepared from donors of other blood groups also showed the appropriate blood group antigenicity in addition to UEA-I reactivity (data not shown). When commercially obtained authentic human α2M was examined for blood group specificity, it reacted with anti-A MoAb (Fig 2, lane 7) and UEA-I but only slightly with anti-B MoAb (data not shown). The vWF purified from FVIII concentrates also reacted with anti-A (Fig 2, lane 9), whereas reactivity with anti-B was slightly weaker. This weak reactivity may have been because of the small amount of B substance in the purified glycoprotein, or the problems of effective MoAb titer; however, different clonal MoAbs showed similar results (data not shown).

The two plasma proteins remaining in the supernatant after immunoprecipitation with anti-blood group specific MoAbs were roughly estimated by ELISA. About 90% of plasma vWF was precipitated with the antibody, but only about 10% of α2M was precipitable even with excess amounts of MoAb. These results suggest that almost all vWF molecules possess blood group antigens, whereas only a subset of α2M molecules possesses these antigens or reacts with corresponding antibodies, despite the fact that α2M is the major plasma glycoprotein carrying ABO(H) blood group determinants.

Effects of endoglycosidase F treatment. The possibility of contamination by noncovalently linked blood group-active glycolipids is minimal, because pretreatment of the protein before SDS-PAGE or the membrane after Western blotting with chloroform:methanol:water (1:2:0.8 by vol-
ABO BLOOD GROUP ANTIGENS IN α₂M AND VWF

Fig 1. Western blotting analysis of plasma glycoproteins with anti-blood group MoAbs and UEA-I lectin. Plasma proteins from blood group A (lanes 1, 2, 6, and 10), B (lanes 3, 7, and 11), O (lanes 4, 8, and 12), and AB (lanes 5 and 9) were incubated with anti-A MoAb (lanes 2 through 5), anti-B MoAb (lanes 6 through 9), and UEA-I lectin (lanes 10 through 12) on PVDF membrane after SDS-PAGE. Lane 1, protein stain by fast green (FG). Molecular weight values (Kd) of standard markers are shown at left. Two plasma components migrating at about 180 Kd and 270 Kd showed blood group antigenicity.

Anti-A MoAb A005, which is specific for type 2 chain, also reacted with α₂M and vWF, indicating the presence of blood group A determinant with type 2 chain in these plasma glycoproteins (data not shown).

ELISA for ABO(II) blood group antigens in plasma vWF. To confirm the occurrence of specific blood group antigens in plasma vWF, plasma samples from healthy Japanese subjects were examined using the ELISA system. The vWF from individual plasma samples was immobilized on
ELISA plates using anti-vWF PoAb, and tested for reactivity with anti-A, anti-B, and UEA-I. Results (Fig 4) were similar to those obtained by Western blotting. The vWF in plasma expressed the donor’s blood group antigen as well as H-antigen. Reactivity of UEA-I with group O plasma was stronger than that with other groups. Secretor status seemed to have no significant effect on reactivity. Although FVIII is noncovalently associated with vWF in plasma, reactivity with anti-blood group reagents was not reduced when plasma-incubated plates were washed with a high ionic strength buffer containing 0.4 mol/L CaCl₂ to remove FVIII from the complex. 19

Similar reactivity with anti-blood group reagents was observed when plates were coated with anti-α₂M PoAb, but no reactivity was observed when plates were coated with PoAbs against human IgG, fibrinogen, fibronectin, or α₁-acid glycoprotein (data not shown).

**DISCUSSION**

It is unclear which plasma protein(s) carry ABO(H) blood group antigens, or whether plasma glycoproteins have intrinsic blood group antigens, or whether blood group-specific glycolipids are adsorbed to certain plasma proteins. 1 The present results clearly indicate that at least two plasma glycoproteins, vWF and a part of α₂M, carry covalently associated ABO(H) blood group antigens in normal individuals with corresponding ABO phenotype. There is a possibility that FVIII might also react with anti-blood group reagents, because group A and H structures were reported in FVIII prepared from a group A donor; 23 however, we were unable to identify FVIII protein because of limited amounts available for Western blotting analysis.

In cases of ovarian cysts or stomach cancer, concentrations of blood group substances have been known to increase in plasma, 15,24 but this phenomenon has been ascribed to release of mucin-type glycoproteins from cystic or gastric mucus. 1 Such soluble mucin-type glycoproteins found in body fluids contain mostly Ser/Thr-linked ABO blood group antigens. 1,23 Their reactivity with type 2 chain (Galβ1 → 4GlcNAc-R)-specific anti-A MoAb and susceptibility to endoglycosidase F indicate that vWF and α₂M contain blood group determinants with type 2 chain among Asn-linked sugar chains. 14 Structural analysis of Asn-linked sugar chains of human vWF purified from FVIII concentrates has also demonstrated the presence of ABO(H) blood group structures based on type 2 chain. 14 Sugar compositional analysis of human α₂M prepared from pooled plasma did not show the presence of N-acetylgalactosamine residue, 26 which is a prerequisite for group A determinant. Because the proportion of α₂M carrying blood group antigens was rather small, it is possible that α₂M in pooled plasma containing group A or B antigens is precipitated by naturally occurring isoagglutinins in plasma, with the result that soluble α₂M has a lower content of blood group A and B antigens. Our results suggest the need to consider blood group status when preparing plasma glycoproteins from pooled sources for analysis. However, detailed quantitative structural analysis of α₂M sugar chains is obviously necessary.

Known as an endogenous protease inhibitor, 27,28 α₂M has been recently shown to bind cytokines, growth factors, and low-density lipoprotein receptors, 29,30 indicating that it is a multifunctional protein like vWF. Like many other plasma glycoproteins, α₂M seems to be synthesized mainly in the liver. 29,31 It is unclear how α₂M acquires blood group determinants during biosynthesis; α₂M carrying ABO(H) antigens may be synthesized by cells other than hepatocytes, eg, macrophages and fibroblasts. 30,31 Because vWF is synthesized in megakaryocytes and endothelial cells 32 that express blood group substances, 3,33,34 translated vWF may be glycosylated via the same mechanism.
Fig 4. Detection of blood group antigens in plasma vWF by ELISA. vWF in plasma samples from healthy Japanese subjects with blood groups A, B, O, and AB (10 of each) was immobilized on plates coated with anti-vWF, and reactivity with anti-A (●), anti-B (○), and UEA-I (▲) was examined. Ordinate indicates plasma concentration, where the undiluted is defined as 1.0. Data represent means ± SD of 10 samples.

The biologic significance of blood group antigens occurring on α2M and vWF is unclear, although a significant correlation between vWF concentration and blood group O phenotype has been reported. Blood group determinants may affect the stability or secretion of vWF. Consideration of ABO(H) blood group is crucial in blood transfusion and other clinical applications. FVIII/vWF concentrates of intermediate purity prepared from pooled plasma have been used for replacement therapy in hemophilia A and von Willebrand disease patients, because FVIII is essential for blood coagulation and vWF for hemostatic plaque formation as well as stabilization of FVIII. vWF in several commercial FVIII/vWF concentrates showed blood group antigenicity (unpublished results). Therefore, repeated use of such concentrates may result in pathogenic immunologic stimulation. Life-threatening adverse reactions such as hemolysis or hemagglutination have been observed after infusion of large amounts of these concentrates in some hemophiliacs with anti-FVIII antibody (inhibitor). These side effects have been assumed to be mostly caused by contamination by isoagglutinins. Blood group antigens present in the concentrates may potentiate these adverse reactions. Further study is needed on half-life of biologic activities after infusion of ABO-matched vWF (or FVIII/vWF) concentrates. Preparation of highly purified virus-free plasma materials is important for therapeutic use, but also the presence of ABO(H) blood group antigens in plasma glycoproteins must be considered during the preparation. Also, for recombinant proteins, the use of blood group A/B-deficient host cells may be beneficial in certain cases.

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