von Willebrand factor (vWF) is synthesized exclusively by endothelial cells and megakaryocytes, and stored in the intracellular granules or constitutively secreted into plasma. ABO blood group antigens are covalently associated with asparagine-linked sugar chains of plasma vWF. The effect of ABO-mismatched bone marrow transplantation (BMT) or blood stem cell transplantation (BSCT) on the expression of ABO blood group antigens on the vWF was examined to obtain information on the origin of these antigens. In ABO-mismatched (HLA-matched) groups, 8 cases of BMT and 4 cases of BSCT were examined. In all cases, the ABO blood groups on red blood cells were gradually converted to the donor’s type within 80 to 90 days after the transplantation. The blood group antigens on the vWF were consistent with the recipient’s blood group for the period monitored by enzyme-linked immunosorbent assay (ELISA). When vWF was isolated from normal platelets and examined for the blood group antigens using ELISA or immunoblotting, it showed few antigens. However, vWF extracted from veins expressed blood group antigens. These findings indicate that platelet (megakaryocyte)-derived vWF does not contain blood group antigens and that these antigens may be specifically associated with vWF synthesized in endothelial cells and secreted into plasma. Furthermore, it is possible that the persistence of the recipient’s blood group antigens on plasma glycoproteins such as vWF, independent of the donor-derived erythrocytes, after ABO-mismatched stem cell transplantation, may influence the immunological system in the production of anti-blood group antibodies resulting in the establishment of immunological tolerance in the recipient plasma.

ABO Blood Group Antigens on Human Plasma von Willebrand Factor After ABO-Mismatched Bone Marrow Transplantation

By Taei Matsui, Taketo Shimoyama, Masanori Matsumoto, Yoshihiro Fujimura, Yoshinobu Takemoto, Masahiro Sako, Jiharu Hamako, and Koiti Titani
Enzyme-linked immunosorbent assay (ELISA). ELISA plate (Immu- no module; Nunc Intermed, Kamstrup, Denmark) was coated with 50 or 100 µL (in each well) of anti-vWF goat immunoglobulin G (IgG) (20 µg/mL; Medical and Biological Laboratory (MBL), Nagoya, Japan) in 100 mmol/L bicarbonate buffer, pH 9.6 overnight at 4°C, and blocked with 200 µL of Tris-buffered saline (TBS; 10 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl) containing 1% bovine serum albumin (BSA) overnight at 4°C. The coated plate was used within 3 weeks after preparation and washed twice with 200 µL of TBS containing 0.05% tween 20 (TwTBS) before use. Plasma samples were appropriately diluted (10 to 100-fold with TwTBS) and 50 or 100 µL of each was applied to the anti-vWF coated plate and incubated for 90 minutes at room temperature. The plate was washed five times with 200 µL of TwTBS and incubated with either 50 or 100 µL of anti-A, anti-B monoclonal antibody (MoAb) (Ortho Diagnostics Systems, Raritan, NJ) diluted with TwTBS (10-fold for anti-A, fivefold for anti-B), biotin-conjugated UEA-I lectin (5 µg/mL, EY Laboratories, San Mateo, CA) that recognizes the type-2 H structure rich in blood group O, or horseradish peroxidase (HRP)-conjugated anti-vWF rabbit IgG (1/1,000 diluted with TwTBS; Dakopatts, Glostrup, Denmark) for 60 minutes. After washing with TwTBS, the plate treated with anti-A, anti-B, and UEA-I was incubated with HRP-conjugated anti mouse IgM (1/1,000 diluted with TwTBS, Zymed Laboratories, San Francisco, CA), anti mouse IgG (1/1,000) plus anti mouse IgG (1/1,000; TAGO Immunologicals, Camarillo, CA) and streptavidin (1/1,000, Vector Laboratories, Burlingame, CA) for 45 minutes, respectively. Peroxidase reaction was performed with 100 µL of solution containing 100 mmol/L NaCl, 0.5 mg/mL ω-phenylene-diamine, 0.02% H₂O₂, and 50 mmol/L Tris-HCl, pH 7.5, for 30 to 60 minutes at room temperature in the dark. The absorbance at 490 nm was measured with a plate reader after the addition of 100 µL of 8 mol/L H₂SO₄. vWF concentration was measured by ELISA using the control plasma N (Dade Behring, Marburg, Germany) and expressed as U/dL. Protein concentration was determined by bichinonic acid protein assay kit (Pierce, Rockford, IL) using BSA as a standard.

vWF from platelets. Platelets (three samples each for A and B) were prepared from platelet-rich plasma by centrifugation (2,800 rpm, 10 minutes at 25°C), washed with phosphate-buffered saline (PBS; 150 mmol/L NaCl, 10 mmol/L Na-phosphate, pH 7.2) containing 9 mmol/L EDTA and 15% acid-citrate-dextrose (ACD) and twice with 150 mmol/L NaCl containing 10 mmol/L HEPES buffer, pH 7.5, and stored at −80°C until use. Platelets were suspended in five volumes of TwTBS containing protease inhibitors as described above and disrupted by sonication at 0°C with a Branson Sonifier 250 (Danbury, CT). After centrifugation at 15,000 rpm at 4°C for 45 minutes, the supernatant was used for ELISA as described above.

In a separate experiment, the soluble fraction of platelets and plasma from normal adults with blood group A or B (200 µL each) were mixed with 20 µL of anti-vWF goat IgG (15 mg/mL, MBL), respectively, for 3 days at 4°C. The immunoprecipitates were collected by centrifugation (15,000 rpm, 100 minutes at 4°C) and washed with PBS.

Aliquots of the washed immunoprecipitates were resolved with 25 µL of sodium dodecyl sulfate (SDS) buffer (2% SDS, 25% glycerol, 62.5 mmol/L Tris-HCl, pH 6.5) containing 5% 2-mercaptoethanol at 95°C, subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane as described. The membrane was incubated with TwTBS containing anti-A (1/10 diluted), anti-B (1/5 diluted) MoAb, or HRP-conjugated anti-vWF antibody (1/1,000 diluted) for 90 minutes at room temperature. After washing with TwTBS, HRP-conjugated secondary antibodies were used for detection of anti-A and anti-B binding, as described above. HRP reaction was performed using 0.2 mg/mL of diaminobenzidine and 0.05% H₂O₂ as a substrate.

vWF from vein. Small pieces of renal vein from postmortem individuals with type A (two samples) and O (one sample), obtained with informed consent, were carefully cleaned and canurated with PBS before freezing. The frozen veins (0.2 g, wet weight) were cut into small pieces on ice, suspended in 1 mL of TwTBS containing 10 mmol/L EDTA and protease inhibitors, and disrupted by sonication at 0°C. After centrifugation (15,000 rpm, 2 hours at 4°C), the supernatant was used for ELISA. The concentration of fibrinogen, transferrin, α₂-macroglobulin and vWF in the vein extracts and normal plasma were measured by sandwich ELISA using the corresponding HRP-conjugated antibodies (MBL, Dakopatts).

Other methods. The blood group of the red blood cells and the anti-blood group antibodies were determined by conventional hemagglutination assay using anti-A and -B MoAbs and standard human type A1 and B red blood cells (Ortho Diagnostics) according to the manufacturer’s instructions.

RESULTS

ABO blood group antigens on red blood cells. ABO blood group antigens on the surface of red blood cells and the anti-blood group antibodies in the plasma of the recipient with acute lymphocytic leukemia in first relapse (blood group AB, male, 36 years old), who was transplanted from a sibling donor (blood group O, female, 35 years old), were monitored for 161 days at intervals before and after BMT (Fig 1A) (the recipient experienced complete remission, but relapsed 7 months after transplantation and died of fungal infection even though a second BMT was performed). The recipient’s red blood cells with type AB showed a mixed field with anti-A and -B antibodies 1 week after transplantation, but had completely lost their reactivity to the antibodies after 90 days indicating that the blood group of the recipient was gradually converted to the donor’s type by ABO-mismatched BMT. The anti-blood group antibodies assayed by standard red blood cells (type A1 and B) showed that there was no production of either of these antibodies for the duration of the monitoring period (130 days after transplantation) (Fig 1A). In all cases of ABO-mismatched BMT examined, the antibodies against the recipient’s original blood group were not found in the plasma (Table 1).

ABO blood group antigens on plasma vWF. vWF in plasma was monitored using ELISA. Concentration of the vWF in the patients receiving a BMT was significantly higher (204 ± 42 U/dL, n = 6, P < .001) when compared with the average concentration in normal adults (91 ± 22 U/dL, n = 9) regardless of ABO-matched or mismatched BMT (Table 1). Plasma vWF, especially, was transiently increased after transplantation and gradually decreased as reported25 (Fig 1A and 1B). The level of ABO blood group antigens on the vWF also varied with the concentration of vWF, but it never converted to the donor’s type after ABO-mismatched BMT (Fig 1A and 1B). UEA-I lectin reacts with vWF from blood group O because it has more H-substance than the other groups. Reactivity of the recipient’s plasma vWF against UEA-I was less than that of the donor’s vWF except for a short period after BMT (Fig 1A).

In the case of major mismatched BMT from a type A donor to a type O recipient (Fig 2), plasma vWF of the recipient at 7 months after transplantation still showed UEA-I binding activity similar to the level before transplantation. No reactivity was shown to anti-A antibody, indicating neither a significant reduction of the vWF with blood group O, nor the production of vWF with blood group A antigen.

In the eight cases of ABO-mismatched BMT examined, there
plasma vWF was no change in the blood group of plasma vWF (Table 1). Examination 3 years, 9 months after transplantation showed that the vWF still continued to express the recipient’s blood group antigens but not the donor’s type.

In addition to bone marrow, BSCT was also examined. Three cases of allogeneic peripheral and one case of cord ABO-mismatched BSCT showed the same results as BMT (Table 2). Plasma vWF expressed the original blood groups in contrast to red blood cells, which were converted to express the donor’s type.

**Blood group antigens on vWF from platelets and vein.** To elucidate the origin of blood group antigens on plasma vWF, we examined vWF extracted from both platelets and the renal vein. vWF immunoprecipitated from platelets and plasma both showed the 270 kD subunit band with some minor degraded bands by immunoblotting with anti-vWF antibody (Fig 3). Plasma vWF showed the corresponding blood group, whereas platelet vWF had no or only a faint blood group antigenicity. The latter showed two bands at about 110 and 130 kD that were reactive to the corresponding anti-blood group antibody, but did not react with anti-vWF antibody. Also using ELISA, platelet vWF showed no significant binding to the anti-blood group antibody (Fig 4).

vWF in the renal vein extracts was examined using ELISA. Among the samples from two subjects with type A and one subject with type O, vWF from the type A subjects clearly showed blood group A antigenicity, although the reactivity was about half that of plasma vWF from normal type A subjects (Fig 4). Vein vWF from the type O subject showed only UEA-I reactivity (data not shown). To address the question of whether vWF in the vein extracts used was mostly derived from contaminated plasma, we measured the contents of several plasma proteins in the extracts and compared them with those in normal plasma. Fibrinogen, transferrin, and α₂-macroglobulin in the vein extracts used were estimated to be 0.4 ± 0.1, 0.4 ± 0.1, and 0.6 ± 0.3 U/dL, respectively (n = 3), whereas the vWF in the extracts was 12.2 ± 2.2 U/dL. These findings suggest that the vein extracts contained a greater amount of vWF compared with other plasma proteins and that vWF derived from plasma appeared to be less than 1%.

**DISCUSSION**

HLA-matched but ABO-mismatched BMT has no influence on marrow engraftment, graft rejection, and graft-versus-host disease if appropriate care, such as plasma exchange and antibody absorption, is performed to avoid acute hemolysis. However, delayed hemolysis, retarded growth of erythroblast, or undergrown erythrocytes have often been observed as complications. Production of anti-blood group antibody must be controlled by the remaining host antigens and the donor-derived lymphocytes. It has been reported that blood group antigens are covalently linked to vWF, FVIII, and α₂-macroglobulin in plasma. In the present study, the blood group antigens on erythrocytes gradually converted to the donor’s type, but the blood group on plasma vWF did not change after ABO-incompatible BMT (and BSCT). No antiblood group antibody against the recipient’s original blood group was detected after the transplantation. Wernet and Mayer reported that isoagglutinins against the recipient’s original red blood cell type are produced only during the early days after transplantation even though the patient has converted to the donor’s red blood cell type after ABO-mismatched BMT. Although it is probable that the immunosuppressing treatment might interfere with the production of antibodies in the recipient, the observed pattern of antibody production (Table 1) suggests that the remaining blood group antigens on plasma glycoproteins such as vWF might contribute to the establishment of immunological tolerance.

The finding that plasma vWF continued to express the recipient’s blood group after ABO-mismatched BMT suggested two possibilities for the origin of these antigens on the vWF. One was that vWF produced in megakaryocytes, differentiated from bone marrow stem cells, would not be secreted but stored in platelets. The other was that vWF in megakaryocytes and erythroblasts was not secreted but stored in megakaryocytes, and the latter was that vWF was secreted from megakaryocytes and erythroblasts. The former was that vWF was secreted from megakaryocytes and erythroblasts.
by crossed BMT of pigs, suggesting that the secretion of vWF from platelets is limited to the local area at thrombosis. Platelets have been known to have both covalently and noncovalently bound blood group antigens. Recently, the covalently bound antigens have been found in platelet membrane glycoproteins (GP) such as GPIa, Ib, IIa, IIb, IIIa, IV, and V, suggesting that platelets (megakaryocytes) have a machinery to assemble the blood group antigens. We have prepared platelet vWF, but it has no or only a faint blood group antigenicity. The 110 and 130 kD proteins observed in the immunoprecipitated platelet vWF (Fig 3) seem to be GPs coprecipitated with vWF. The absence of blood group antigens in platelet vWF has also been recently reported by two groups. The very faint blood group reactivity observed in the platelet vWF (Fig 3 and 4) might be a contamination from the plasma vWF adsorbed onto the platelets.

Another vWF producing site is endothelial cells. We found that the renal vein extracts contained vWF with blood group antigens. Expression of the blood group antigens by the vWF molecule was about half that of the plasma vWF, suggesting that vWF molecules with no or a small amount of blood group antigens also exist. It is possible that these vWFs are incompletely glycosylated. Alternatively, the glycosylation may be different between vWF that is constitutively secreted and that stored in the regulated Weibel-Palade body pathway. Recently, Yamamoto et al reported that the synthesis of vWF in endothelial cells varied among the organs in mice. Glycosylation is regulated by glycosyltransferases and trimming glycosidases in cells, suggesting that the blood group antigen production might also be altered by each organ. When we analyzed vWF extracted from cultured human umbilical vein endothelial cells, no significant blood group antigens were observed and neonatal plasma vWF showed a lower expression of these antigens (Matsui, unpublished observations), suggesting that the blood group antigens on plasma glycoproteins may also be developmentally regulated like embryonic antigens.

Our present findings, together with the recent findings of Brown et al showing that plasma vWF with blood group antigens was rapidly increased after administration of DDAVP to a type I von Willebrand disease patient, strongly suggest that vWF with blood group antigens is specifically glycosylated in endothelial cells but not in megakaryocytes. It is also possible that the blood group antigens are attached to vWF extracellularly by plasma glycosyltransferases after secretion. A or B
Table 2. Blood Group Antigens on Plasma vWF and RBC of the Recipients after ABO-Mismatched Allogeneic-Peripheral Blood Stem Cell (PBSCT) and Cord Blood Stem Cell Transplantation (CBT)

<table>
<thead>
<tr>
<th>Case</th>
<th>Recipient</th>
<th>Diagnosis</th>
<th>Blood Group</th>
<th>Time after Transplantation (months)</th>
<th>vWF Conc. (U/dL)</th>
<th>Blood Group after Transplantation</th>
<th>Anti-blood Group Antibody Production</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBSCT</td>
<td>N.T.</td>
<td>M (16)</td>
<td>CML</td>
<td>A O</td>
<td>7</td>
<td>247</td>
<td>A O</td>
</tr>
<tr>
<td></td>
<td>A.T.</td>
<td>M (3)</td>
<td>JMML</td>
<td>A O</td>
<td>21</td>
<td>169</td>
<td>A O</td>
</tr>
<tr>
<td></td>
<td>M.I.</td>
<td>F (8)</td>
<td>ALL</td>
<td>O A</td>
<td>15</td>
<td>117</td>
<td>O A</td>
</tr>
<tr>
<td>CBT</td>
<td>S.Y.</td>
<td>F (3)</td>
<td>FHL</td>
<td>B O</td>
<td>6</td>
<td>202</td>
<td>B O</td>
</tr>
</tbody>
</table>

JMML: juvenile myelomonocytic leukemia; FHL: familial hemophagocytic lymphohistiocytosis.

Although the biological function of the blood group antigens on vWF is still not clear, the different glycosylation between platelet and endothelial vWF might influence the function of each pool of vWF in hemostasis or in its association with FVIII. Recently, Sarode et al reported that the blood group sugar chains on vWF influenced the ristocetin-induced platelet agglutinating activity. Further studies on the relationships between thrombotic complications followed by ABO-mismatched BMT or BSCT and the presence of blood group antigens on vWF may contribute to more successful transplantation.
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