Characterization of Leu777Pro and Ile865Thr Type IIA von Willebrand Disease Mutations

By Susan E. Lyons, Kathleen A. Cooney, Paula Bockenstedt, and David Ginsburg

Type IIA von Willebrand disease (vWD) is an autosomal dominant bleeding disorder characterized by a qualitative defect in von Willebrand factor (vWF). A number of missense mutations responsible for type IIA vWD have recently been identified. This report examines the type IIA vWD mutations Leu777→Pro and Ile865→Thr by expression of recombinant vWF containing mutant and wild-type (WT) sequences. Recombinant vWF containing the L777P mutation (vWF(L777P)) showed markedly impaired secretion compared with that for wild-type vWF (vWFWT) after DNA transfection into mammalian cells. Multimer analysis of secreted vWF(L777P) showed predominantly low molecular weight forms. In contrast, recombinant vWF containing the I865T mutation (vWF(I865T)) was processed in a pattern similar to vWFWT, with secretion of the full spectrum of vWF multimers. Thus, L777P and I865T are subclassified as type IIA group I and group II mutations, respectively. Analysis of platelet vWF from a patient heterozygous for the L777P mutation shows reduced large vWF multimers in a pattern similar to plasma, consistent with the intracellular transport defect predicted for a group I mutation. An increase in the proportion of high molecular weight multimers observed in type IIA vWD patient plasma, after renal transplantation from a normal donor, suggests that the kidney endothelium may be a major source of plasma vWF.

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degradation to lower molecular weight vWF forms in plasma (group II mutations). In this report, we characterize the biosynthesis of vWF containing each of 2 previously reported type IIA vWD mutations, Leu777 → Pro (L777P) and Ile865 → Thr (I865T), leading to their subclassification as group I and group II, respectively. In addition, analysis of plasma vWF from a Type IIA vWD patient with the L777P mutation after kidney transplantation suggests that renal endothelial cells are a major site of plasma vWF synthesis.

### MATERIALS AND METHODS

**Patient material**  
Patients A7 and A12 were diagnosed with type IIA vWD based on decreased plasma vWF antigen, decreased ristocetin cofactor activity (disproportionately decreased compared with vWF antigen level), and an abnormal vWF multimer pattern. Patient A7 was originally reported as part of a large pedigree with "variant von Willebrand disease" by Green and Philip (corresponding to individual IV-46 in the original pedigree). Later, clinical analysis of plasma vWF from a Type IIA vWD patient with the L777P mutation after kidney transplantation suggests that renal endothelial cells are a major site of plasma vWF synthesis.

### Table 1. Clinical Evaluation of Patient A7 Before and After Renal Transplantation

<table>
<thead>
<tr>
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<th>Before Transplant</th>
<th>After Transplant</th>
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</thead>
<tbody>
<tr>
<td>Factor VIII-C (normal, 50% to 150%)</td>
<td>129 (10/88)</td>
<td>178 (12/92)</td>
</tr>
<tr>
<td>vWF antigen (normal, 50% to 150%)</td>
<td>47</td>
<td>60</td>
</tr>
<tr>
<td>Ristocetin cofactor activity (normal, 50% to 150%)</td>
<td>28</td>
<td>41</td>
</tr>
<tr>
<td>Serum creatinine (normal, 0.6 to 1.0 mg/dL)</td>
<td>2.2</td>
<td>1.4</td>
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Expression vector construction. The type IIA vWD mutations identified in patients A7 (L777P) and A12 (I865T) were inserted into full-length vWF cDNA in the expression vector pMT2, under the control of the adenovirus major late promoter. Briefly, genomic DNA from patient A7 was amplified by PCR as previously described. An Nco I-Kpn I fragment (nts 4482-4752) containing the mutation was inserted into full-length vWF cDNA in pMT2 using standard methods. The mutation identified in patient A12 (I865T) was prepared by site-directed mutagenesis of a vWF Sac I fragment ([i]30866-([i]6676) subcloned into the phagemid pSELECT using a commercial kit (Promega Corp, Madison, WI). The 19-bp mutagenesis oligonucleotide, TGGTGGCCACCTGGAG-TGGG (mutation is underlined), corresponds to the vWF cDNA sequence starting at nt 4874. A Kpn I-Kpn I fragment (nts 4752-5134) containing the mutation was subcloned into full-length vWF cDNA as above. In both constructions, all segments derived by PCR or mutagenesis were sequenced to verify the presence of the corresponding type IIA mutations and to exclude polymerase errors. pMT2-derived plasmids containing the full-length vWF cDNA with wild-type sequence, L777P, or I865T mutations are designated pWT-vWF, pL777P, and pI865T, respectively. The β-galactosidase (β-gal)-expression plasmid (pPl3gal-Gal), which contains the SV40 origin and LacZ cDNA under the direction of an SV40 enhancer/promoter, was kindly provided by L. Spain (Whitehead Institute, Cambridge, MA).

**Cell culture and transfection.** COS-7 cells were maintained in Dulbecco's modified Eagle's medium (JRH Biosciences, Lenexa, KS) supplemented with 7.5% fetal bovine serum ( Gibco-BRL Life Technologies, Inc, Gaithersburg, MD). On the day of transfection, cells were split 1:2 from a confluent plate and transfected within 1 to 4 hours by a modified standard calcium phosphate-mediated transfection method. For each transfection, 10 μg of vWF and 2 μg of β-gal expression plasmid were used. Approximately 36 hours after transfection, cells were rinsed twice with phosphate-buffered saline and transferred into 3-mL serum-free AIM-V medium (Gibco-BRL). After 24 hours in serum-free medium, cells lysates and cell media were harvested from transfections, essentially as previously described. A mixture of protease inhibitors was added to final concentrations of 0.5 mg/mL leupeptin, 0.2 mg/mL aprotinin, 10 mg/mL bovine trypsin inhibitor, 0.7 mg/mL pepstatin, and 5 mmol/L iodoacetic acid (all purchased from Sigma Chemical Co, St Louis, MO) for both lysates and medium. Phenylmethylsulfonyl fluoride and EDTA were also added to final concentrations of 2 mmol/L and 5 mmol/L, respectively. vWF in cell lysates and cell medium was quantitated by enzyme-linked immunosorbent assay (ELISA), as previously described, using a commercial reference plasma (Bio/Data Corp, Horsham, PA) as a standard (assuming 10 μg/mL vWF concentration in normal plasma). β-gal ELISA was also performed as described using a commercial β-gal standard (Sigma). Wild-type vWF and vWF containing the mutations L777P or I865T will be referred to as vWF WT, vWF L777P, and vWF I865T, respectively.

**Agarose gel electrophoresis.** Preparation of samples from transfections and electrophoresis in vertical 1.5% sodium dodecyl sulfate (SDS)-agarose multimer gels were performed, as previously described, using chemiluminescence (Amersham Corp, Arlington Heights, IL) for detection of vWF multimers. After obtaining informed consent, PB was collected from patients into EDTA. For plasma and platelet lysates, PB was centrifuged at 100g for 10 minutes to obtain platelet-rich plasma, followed by centrifugation at
RESULTS

Steady-state analysis of recombinant vWF containing type IIA vWD mutations L777P or I865T. The previously identified type IIA vWD mutations, T4619 → C (L777P) and T4883 → C (I865T), were inserted into full-length vWF cDNA in the expression vector pMT2. To examine the synthesis of vWF containing these mutations, COS-7 cells were transfected with the vWF expression constructs pWTvWF, pL777P, or pI865T, and vWF in cell media and in cell lysates from transfected cells was quantitated by ELISA. Cells were cotransfected with a β-gal expression vector (pβ3P-Gal) as an internal control for transfection efficiency. vWF-ELISA values in lysates and media were normalized for transfection efficiency using intracellular β-gal levels from each transfection. Comparisons of the vWF level in conditioned media between WT and each mutant showed that vWF865T secretion was similar to vWFWT, whereas the level of vWF777P was markedly reduced (Fig 1). Intracellular levels of each mutant were greater than that of vWFWT. The defective secretion of vWF777P is similar to that previously observed with the group I IIA mutations V844D, S743L, and G742R. Intracellular L777P was entirely sensitive to digestion with endoglycosidase H (data not shown), indicating that mutant vWF was retained in the ER, which is similar to results with the other group I mutations. In contrast, the relative quantity of extracellular vWF865T in steady-state analysis was indistinguishable from that of vWFWT, which is consistent with classification of this mutation as a group II defect. However, the apparent intracellular accumulation of vWF865T may suggest a subtle defect in transport from the ER.

Multimer analysis of secreted recombinant vWF. Recombinant vWF secreted from cells transfected with pWTvWF, pL777P, and pI865T was analyzed by SDS-agarose gel electrophoresis to assess the effect of these mutations on multimer structure. The multimer pattern of extracellular vWF865T was indistinguishable from that of WT (Fig 2). However, only trace amounts of vWF777P were seen in the conditioned medium (Fig 2B), and these were predominantly low molecular weight multimer species. When equal amounts (as quantitated by ELISA) of vWF777P as compared with vWFWT and vWF865T were analyzed, larger multimers were observed, although reduced in quantity (Fig 2A).

Multimer analysis of plasma vWF from a type IIA vWD patient after renal transplantation. To evaluate the potential contribution of endothelial cells in the renal vasculature to circulating vWF, we analyzed plasma from patient A7 after kidney transplant. Because pretransplant plasma was unavailable for direct comparison in our laboratory, blood was obtained from patient A7’s mother (corresponding to individual III-25 in the original pedigree) who is also affected with a moderately severe bleeding diathesis. The T4619C mutation reported in this family results in a loss of a Pst I restriction site. DNA restriction analysis with Pst I, shown in Fig 3, shows that both patient A7 and her mother are heterozygous for the T4619C (L777P) mutation. Clinical analysis (Blood Center of Southeastern Wisconsin) of plasma vWF from patient A7 before renal transplant is shown in Fig 4A. Although analyzed in different gel systems, plasma from the mother of patient A7 shows a type II vWD multimer pattern similar to that of patient A7 before renal transplant, with decreased intermediate and HMW multimers when compared with that for normal controls (Figs 4B and C). Even on long exposure of the lane containing the mother’s plasma, intermediate molecular weight multimers are greatly decreased, and HMW multimers are absent (Fig 4C). By comparison, the vWF multimer pattern seen in patient A7 plasma after renal transplantation has a greater relative proportion of intermediate and HMW multimers (Figs 4B and C) compared with
plantation shows an increase in circulating HMW vWF multimers in the presence of the normal renal allograft.

**DISCUSSION**

In this report, two type IIA vWD mutations, L777P and I865T, are subclassified into groups I and II, respectively, based on steady-state analysis of recombinant mutant vWF expressed in heterologous cells. The group I type IIA mutations cause selective retention of HMW vWF in the ER. Expression of vWF L777P in COS-7 cells showed impaired transport/secretion, with more pronounced effects on the HMW vWF forms, which is consistent with the group I classification. This is similar to our previous observations for the mutations V844D, S743L, and G742R. The decrease in large vWF multimers observed in patient A7's platelet lysate is consistent with a transport defect in vivo that also affects the storage of HMW vWF multimers in the α-granule.

In contrast, I865T can be categorized as a group II mutation, similar to R834W and G742E, based on its normal synthesis and secretion in COS-7 cells. The location of the I865T mutation within the region of clustered type IIA mutations, the coinheritance with type IIA vWD in a large pedigree, and its absence from a panel of 134 normal alleles all support the characterization of this amino acid substitution as an authentic type IIA vWD mutation. However, the occurrence of a rare, private DNA sequence polymorphism cannot be completely excluded. To date, a total of 15 type
Fig 4. Multimer analysis of vWF from patient plasma and platelets is shown. Clinical multimer analysis (Blood Center of Southeastern Wisconsin) of patient A7 plasma [PtA7] before renal transplant as compared with normal plasma [NI] is displayed in panel (A). Panels (B) through (D) were all run on the same 1.5% nonreducing SDS-agarose gel and developed by chemiluminescence. In panel (B), 3 µL of each plasma sample are run in adjacent lanes. In panel (C), 10 ng of plasma vWF for each sample (quantitated by ELISA) are analyzed in adjacent lanes. Patient A7 plasma after renal transplant [PtA7] is compared with normal plasma [NI], plasma from a patient with type IIB vWD (Arg545Cys) [IIB], and plasma from patient A7’s mother [mother]. A longer exposure (t exp) of the lane containing plasma from patient A7’s mother’s sample is also included (30 sec; others, 5 sec). A total of 10 ng of vWF, quantitated by ELISA, from platelet lysates from patient A7 [PtA7] and a normal individual [NI] are analyzed in panel (D).

IIA vWD mutations have been reported.14,25 Of these mutations, 9 (including those in the current report) have been characterized by transfection analysis,17,26,27 with 5 mutations resulting in a normal group II pattern of secretion.

Several lines of evidence suggest that extracellular proteolysis of vWF multimers may result in the type IIA vWD phenotype in group II patients.12,13,28 The groups I and II mutations are clustered near the proteolytic cleavage site between Tyr842 and Met843.15 Cleavage at this site generates a 176-carboxyl-terminal fragment that is markedly increased in type IIA vWD plasma compared with that in normal plasma.8,10,28 In addition, the full spectrum of vWF multimers has been previously observed in platelet lysates from two individuals with group II mutations, supporting the hypothesis that a postsecretory defect accounts for the absence of plasma HMW multimeric species in these patients.17 Two additional type IIA vWD mutations (E875K and S850P), characterized via analysis of recombinant vWF, can also be classified as group II mutations.26,27 However, correlation with platelet vWF multimers from patients with these mutations is not available.

Group I or group II type IIA vWD mutations cannot be distinguished on the basis of the location or type of amino acid substitution.17 Both severe group I mutations, L777P and V844D, result from nonconservative amino acid substitutions. Other group I and II mutations include both nonconservative substitutions, such as G742E (group II) and G742R (group I), and conservative ones, such as I865T (group II) and S743L (group I). We hypothesize that all of these mutations perturb the same sensitive structure exposed on the surface of the vWF molecule.17 The altered structure that results from group I mutations interferes with intracellular transport, possibly through increased affinity for a resident ER protein(s). In contrast, the altered conformation produced by group II mutations escapes recognition by the ER machinery but results in increased susceptibility of vWF to a plasma protease(s) after secretion into plasma.

Most subtypes of type II vWD are currently classified based on differences in plasma vWF multimer patterns. The large number of multimer variants observed with both low- and high-resolution gel analysis and the occurrence of inconsistencies between clinical laboratory assays and multimer analyses have made subtype assignment difficult. Patient A7 shows the potential problem of vWD classification based largely on use of vWF multimer pattern. Although the multimers were initially interpreted as most consistent with type IIB or acquired vWD, the other clinical features, the location of the identified mutation (L777P)17 and the results of transfection analysis, taken together, are most consistent with the diagnosis of group I type IIA vWD.

The distinction between group I and group II may have important implications for the treatment of type IIA vWD. Type IIA vWD patients have a variable response to 1-deamino 8-D-arginine vasopressin (DDAVP), an analogue of
vasopressin used to treat various forms of vWD, via release of stored vWF. Some investigators have reported little effect of this drug in type IIA vWD patients, whereas others document good clinical responses. Intracellular storage pools in group I patients would not be predicted to contain HMW vWF multimers, and, thus, DDAVP would not be expected to increase the amount of circulating HMW vWF multimers. However, because storage granules from group II patients should contain the full range of vWF multimers, DDAVP would be expected to result in a transient increase in plasma HMW vWF multimers and improved hemostasis. Thus, the division of type IIA vWD into group I and group II may have significant implications for therapy; however, this hypothesis remains to be tested.

Of note, patient A7 had an improvement in her vWF antigen level and bleeding symptoms (menorrhagia and bruising) after transplantation of a normal kidney from her unaffected sister. In patients without vWD, previous investigators have reported that vWF antigen levels increase after placement of a renal allograft, but levels appear to return to baseline approximately 4 to 6 months after transplant. Others report that this increase in vWF may be partially caused by cyclosporine nephrotoxicity, although this is controversial. In addition to an increase in vWF antigen (persisting for >16 months after transplant), patient A7 also appears to have a greater percentage of circulating HMW vWF multimers (Fig 4). We hypothesize that the quantitative and qualitative changes in plasma vWF observed in this patient may be caused by the contribution of the transplanted kidney to circulating vWF. Webster et al previously reported no improvement in factor VIII levels or bleeding times in two pigs with severe vWD, who received normal porcine kidney allografts; however, these two animals lived for 6 and 7 days. During this time, the animals apparently suffered from continual hemorrhage that eventually led to their deaths. Similarly, Mannucci et al described a type III vWD patient who underwent a liver transplant for management of chronic hepatitis and hepatocellular carcinoma. Three months after transplantation, the patient's vWF antigen was approximately twenty times higher than before transplantation, although still remarkably low (1.02%; with normal range, 50% to 150%). However, no clinical information was provided to document the status of the transplanted organ or the drug regimen used for immunosuppression. It has been previously shown that endothelial cells remain of donor origin in transplanted organs, except in the setting of organ rejection and cyclosporine toxicity. In patient A7, we have documented both a quantitative as well as a qualitative improvement in circulating vWF after successful placement of a normal renal allograft, which was also associated with a subjective improvement in bleeding symptomatology. These observations suggest that, with continued improvement in organ transplantation and endothelial cell graft procedures, such approaches may eventually have a role in the management of severe forms of vWD.

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