Clearance of Normal and Type 2A von Willebrand Factor in the Rat

By John H. Stoddart, Jr, Janet Andersen, and Dennis C. Lynch

A model for the in vivo clearance of normal and mutant forms of human von Willebrand factor (vWF) has been established using catheterized rats. vWF clearance rates in rat plasma were determined by quantitation of reduced vWF subunits on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and multimeric vWF was analyzed using nondenaturing SDS-agarose gels. Normal vWF derived from human umbilical vein endothelial cells displayed a biphasic pattern of clearance, with half times of 35 minutes (T1/2 a; SD 15. min) and 245 minutes (T1/2 b; SD 76. min.); metabolic clearance rate = 0.65%/minute. High molecular weight multimers of vWF were cleared more rapidly than dimeric vWF. vWF containing the S1613P mutation found in some type 2A von Willebrand disease (vWD) patients was observed to undergo proteolysis in vivo resulting in a reduction of high molecular weight vWF and concomitant appearance of rapidly-migrating satellite species, although the overall clearance rate of vWF antigen was similar to wild type vWF. These results provide direct in vivo evidence that the S1613P mutation causes the characteristic type 2A vWD phenotype. Full-length recombinant vWF produced from transfected Chinese hamster ovary cells was cleared at a similar rate to endothelial cell-derived vWF, and recombinant vWF devoid of O-linked carbohydrates was cleared significantly faster. vWF devoid of sulfate was cleared at a similar rate as wild type vWF, indicating the sulfate moiety of vWF does not regulate in vivo clearance. This animal model should prove useful in subsequent in vivo analysis of additional forms of vWD and in the development of protease inhibitor therapy for 2A vWD.

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(T5087C), resulting in the conversion of S1613P.13 The expression is under the control of the SV40 enhancer, immediate-early promoter, and includes T antigen splice and termination signals. The pHSVNEO plasmid, containing the bacterial neomycin-resistance gene, has been described.12

Cell culture, transfection, and cell line selection. HUVECs from normal individuals were provided by M. Gimbrone (Brighton and Women’s Hospital, Boston, MA). Endothelial cells from a type 2A VWD patient expressing the vWF mutation S1613P, to be referred to as 2A EC, have been described.13,14 A strain bearing the same mutation was derived from a sibling of the first child. EC were cultured as previously described.14 To generate vWF devoid of sulfate, HUVECs were cultured in sulfate-free minimal essential media in the presence of freshly-diluted 20 mmol/L sodium carbonate as described.15 LDL-D cells, provided by Dr Monty Krieger (Massachusetts Institute of Technology, Cambridge), were cultured as described.16 Production of vWF devoid of O-linked carbohydrates was performed as described.12 Transfections of LDL-D cells with vWF expression vectors and pHSVNEO were performed by lipofection16 using 25 μg of vWF plasmid and 5.6 μg NEO plasmid, as recommended by the manufacturer (Bethesda Research Laboratories, Gaithersburg, MD). Single-cell–derived colonies resistant to genetin were expanded and assayed for secretion of vWF by enzyme-linked immunosorbent assay (ELISA).12

Metabolic labeling. Radiolabeling of vWF–producing cells with 35S CYS was performed as follows. HUVECs or LDL-D cells expressing vWF were cultured to near confluence in normal media in 100-mm dishes, washed twice in serum-free minimal essential medium (MEM), incubated for 60 to 90 minutes in cysteine-, methionine-free MEM, then incubated for 24 to 36 hours in 3.5 ml labeling media. The labeling formulation consisted of 35S CYS (100 Ci/mmol), 0.29 μg/mL unlabeled CYS, and 7.5 μg/mL unlabeled MET (1.25% and 50% of normal concentration of cysteine and methionine in MEM, respectively). Labeled conditioned media from cells were collected, placed on ice, centrifuged at 2,000 g for 10 minutes to remove cellular debris, then concentrated 10- to 20-fold using microconcentration material. No significant differences were observed in the yields between the EC-derived or recombinant material.

Immunoprecipitation and gel electrophoresis of vWF. Rat plasma (350 μL) was precleared in 2.0 ml RIPA (20 mmol/L Tris pH 8.0, 150 mmol/L NaCl, 1% Triton-X 100, 0.10% SDS) with 10 μL of Sepharose C4B: gelatin-agarose (1:1 in RIPA) for 60 minutes at 4°C. Precleared samples were incubated with 0.14 μL of 2.29 anti-vWF monoclonal antibody (MoAb) PAS beads (50 μg 2.29 MoAb/PAS; 1:1 in RIPA) with gentle rocking for 18 to 24 hours at 4°C. Beads were washed three times with RIPA and samples were analyzed on 6.0% SDS polyacrylamide gels under reducing conditions as previously described.14 The dried gels were quantitatively analyzed using a Betascope (Betagen, Waltham, MA). Analysis of multimeric vWF was performed on a 1.8% discontinuous nonreducing SDS agarose gels as described.14

Data analysis. The clearance rate data were analyzed on a SPARC 10 (SUN Systems) with PROC NLIN in SAS (SAS Institute, Inc, Cary, NC) using Marquardt and a Moore-Penrose inverse for the estimation steps. The equation fit was:

\[ C = \frac{A}{t + B} \]

where C is the blood concentration, t is time, A and a are the fast component parameters, and B and b are the slow component parameters.19 The pharmacokinetic parameters were obtained from the slopes and intercepts of this function. The early phase and late phase half times were determined by:

\[ T1/2a = 0.693/a \]

\[ T1/2b = 0.693/b \]

The metabolic clearance rate (MCR) was determined from the formula:

\[ \text{MCR} = \frac{\text{dose}(A/a + B/b)}{P} \]

and is expressed as % per minute. All clearance experiments were performed at least in triplicate. The MCR values were compared using a Wilcoxon rank sum test with two-sided P values.

RESULTS

An animal model was developed to analyze the in vivo clearance of human vWF from the circulation. Rats were catheterized through the jugular vein, fitted with a swivel and tether harness to allow freedom of movement, and 18 to 24 hours later, used for clearance studies. Concentrated
conditioned media from $^{35}$S CYS labeled cells were infused into catheterized rats and blood samples were withdrawn at selected times from 10 minutes to 10 hours into a blood collection cocktail containing anticoagulant and protease inhibitors. Plasmas derived from blood samples were immunoprecipitated using an antihuman vWF-specific MoAb, run on polyacrylamide gels under reducing conditions and quantitatively analyzed using a Betascope (Betagen, Waltham, MA). Results of a representative clearance experiment of normal EC-derived vWF are shown in Fig 1. Polyacrylamide gels show the predominant vWF mature subunit persisting for greater than 10 hours in the plasma, with minor amounts of the slower-migrating provWF subunit detected at very early times (Fig 1A). Rapidly-migrating vWF-specific fragments of normal EC-derived vWF were not observed in polyacrylamide gels. The relative recovery of infused vWF (using the 10 minute point as 100% Activity Remaining) is shown in Fig 1B. The clearance rate of EC-derived wild type vWF best fit a two-compartment model, with a rapid initial clearance ($T_{1/2}^a$) of 35. minutes followed by a slower rate of clearance ($T_{1/2}^b$) of 245. minutes (Table 1). The metabolic clearance rate (MCR), a parameter which considers both early and late phase clearance rates, was determined to be 0.65%/minute (Table 1).

vWF-injected animals were also evaluated for vWF multimeric content by electrophoresis using 1.8% agarose gels under nonreducing conditions. After infusion of wild type EC-derived vWF, the vWF dimer species, tetramers, and higher molecular weight species were clearly evident (Fig 2). The relative amount high molecular weight vWF (above tetramers) as compared with vWF dimers was quantitated by Betascope. The ratio of HMW vWF to dimeric vWF for control (noninjected) samples was approximately 0.6. At early times in vivo (up to 1 hour), this ratio was maintained, but by 4 hours, decreased to approximately 0.05. Despite the reduction of high molecular weight species, the presence of rapidly-migrating

**Table 1. Metabolic Clearance Rates of Endothelial Cell Derived and Recombinant vWF in the Rat**

<table>
<thead>
<tr>
<th>vWF Source</th>
<th>n*</th>
<th>$T_{1/2}^a$ min (SD)</th>
<th>$T_{1/2}^b$ min (SD)</th>
<th>Metabolic Clearance Rate, %/min</th>
<th>$P$ Value†</th>
</tr>
</thead>
<tbody>
<tr>
<td>HUVEC: wild type</td>
<td>8</td>
<td>35. (15.)</td>
<td>245. (76.)</td>
<td>0.65</td>
<td></td>
</tr>
<tr>
<td>HUVEC: sulfate-free</td>
<td>6</td>
<td>19. (18.)</td>
<td>218. (47.)</td>
<td>0.64</td>
<td>(P = .949)</td>
</tr>
<tr>
<td>Recombinant: wild type</td>
<td>4</td>
<td>17. (6.9)</td>
<td>295. (25.)</td>
<td>0.66</td>
<td>(P = .932)</td>
</tr>
<tr>
<td>Recombinant: O-Minus</td>
<td>3</td>
<td>13. (7.8)</td>
<td>129. (60.)</td>
<td>1.60</td>
<td>(P = .018)</td>
</tr>
<tr>
<td>Type 2A vWF‡</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HUVEC</td>
<td>5</td>
<td>29. (13.)</td>
<td>221. (80.)</td>
<td>0.74</td>
<td>(P = .421)</td>
</tr>
<tr>
<td>Recombinant</td>
<td>5</td>
<td>47. (13.)</td>
<td>206. (41.)</td>
<td>0.60</td>
<td>(P = .826)</td>
</tr>
</tbody>
</table>

* n = number of determinations.
† $P$ value comparison with the MCR of normal EC vWF.
‡ S1613P 2A vWF.
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Fig 2. Multimeric structure of wild type EC-derived vWF after circulation in vivo. Blood samples from a rat injected with 35S CYS-labelled wild type vWF from HUVEC were removed at (1) 10 minutes, (2) 60 minutes, (3) 120 minutes, (4) 240 minutes, and (5) 300 minutes, immunoprecipitated, and electrophoresed on an SDS agarose gel under nonreducing conditions as described. Control lanes (c) represent noninjected vWF. The relative positions of the mature vWF dimer (d) and tetramer (T) are shown. The regions of high molecular weight vWF (HMW) and dimeric vWF (d) screened by Betascope for quantitation are indicated.

satellite species from the in vivo samples was not observed. This suggests that the clearance of EC-derived vWF in vivo was not mediated by a proteolytic mechanism.

We sought to determine if sulfation of vWF affects the in vivo clearance, since vWF is known to be modified post-translationally by sulfation of asparagine-linked carbohydrates, and sulfation is known to affect in vivo clearance of certain glycoprotein hormones such as lutropin. We have previously shown that vWF devoid of sulfate assembles into multimers and localizes into Weibel Palade bodies of endothelial cells correctly. Of further note, sulfation of Factor VIII is essential for the interaction of Factor VIII with vWF. As shown in Table I, the clearance rate of sulfate-free vWF (MCR = 0.64%/minute) was similar to wild type vWF, suggesting sulfation of vWF does not influence the metabolic clearance. Further, the multimeric pattern of sulfate free vWF as seen on nondenaturing agarose gels was similar to wild type vWF (data not shown).

We also investigated the in vivo clearance of normal and mutant recombinant full-length vWF produced by stably-transfected CHO cells. We have previously shown that this wild type recombinant vWF forms normal subunits and higher order multimers. The clearance rate of wild type recombinant vWF was similar to wild type EC vWF (MCR = 0.66%/minute; Table 1). The multimeric pattern of recombinant vWF exposed in vivo demonstrates that higher order multimers persist in the circulation, similar to EC-derived vWF.

Fig 3. In vivo clearance of normal and O-linked carbohydrate defective recombinant vWF. (A) Analysis of in vivo clearance of wild type recombinant vWF by agarose gel electrophoresis. Blood samples were removed at (1) 10 minutes (2) 60 minutes, (3) 120 minutes, (4) 240 minutes, and (5) 300 minutes. Control samples (c) indicate noninjected wild type recombinant vWF. The positions of the major dimeric (d) and tetrameric (T) vWF species are indicated. (B) Represents in vivo clearance of O-minus recombinant vWF. The times of blood withdrawal are as in (A). Controls (c) represent noninjected O-minus vWF.
vWF (Fig 3A). The absence of rapidly-migrating satellite species in agarose gels (Fig 3A) suggests the recombinant wild type vWF was not proteolyzed. Furthermore, proteolytic fragments of recombinant normal vWF exposed in vivo were not observed on polyacrylamide gels (data not shown).

The clearance of recombinant vWF defective in O-linked carbohydrates was tested, as the presence or absence of certain carbohydrates can greatly affect clearance of plasma proteins. vWF deficient in O-linked carbohydrates was produced by the same CHO LDL-D cells used for the production of wild type vWF described above. These cells lack the enzyme UDP-Gal and UDP-GalNAc epimerase, which renders them incapable of producing proteins with O-linked carbohydrates when the appropriate growth medium is given. We have previously shown that O-linked defective ("O-minus") vWF binding to heparin and collagen is normal, and binding to platelets in the presence of ristocetin is reduced. In addition, O-minus vWF contains higher order multimers, which migrate more rapidly than wild type through agarose gels. The clearance rate of O-minus vWF was significantly faster than the wild type EC or recombinant vWF (MCR = 1.60%/minute; P = 0.018; Table I). The rapid clearance of O-minus vWF was also observed in agarose gels (Fig 3B). Rapidly migrating satellite species of in vivo samples could not be detected in agarose gels (Fig 3B), or polyacrylamide gel electrophoresis (PAGE) (not shown), suggesting that the O-minus vWF was not rapidly proteolized.

The clearance of a naturally occurring type 2A vWD mutant (S1613P) was also examined. We have previously shown that this mutant vWF derived from cultured endothelial cells has the ability to form a full spectrum of multimers and is susceptible to proteolysis. The clearance rate of 2A EC vWF-reduced subunits was similar to wild type EC vWF (Table I). The multimeric pattern of the type 2A mutant vWF after in vivo exposure is shown in Fig 4. The presence of rapidly migrating satellite species below the major tetramer and dimer bands demonstrates the generation of proteolytic fragment(s). As observed in polyacrylamide gels under reducing conditions, the major proteolytic fragment of 2A produced in vivo migrates at 176 kD (data not shown), in agreement with our previous observations. Of interest was the fact that mixing of 2A vWF with rat plasma ex vivo allowed for proteolysis to generate the apparently identical 176 kD fragment, unless the protease(s) was inhibited by high EDTA concentrations (data not shown). The relative ratio of HMW vWF: dimeric vWF for control noninjected 2A vWF (from gel shown in Fig 4) was approximately 0.15, significantly lower than wild type vWF. The ratio declined dramatically after in vivo exposure.

The 2A mutant, which is heterozygous in EC-derived cultures, and the recombinant 2A, homozygous for the mutation, behaved similarly in the rat model. Recombinant type 2A vWF derived from CHO cells stably transfected with the full-length vWF cDNA containing the identical mutation (S1613P) described above was also tested for clearance. The recombinant 2A vWF subunits were cleared from the rat plasma at a similar rate to wild type and 2A EC vWF, as shown in Table 1. Although the recombinant 2A vWF produced multimers, the extent of multimerization was not as great as for EC-derived material (Fig 5A). After in vivo exposure of recombinant 2A vWF, a reduction of high molecular weight material and concomitant appearance of rapidly-migrating satellite species were observed (Fig 5A). The migration of the major proteolytic fragment (≈176 kD) was similar to the EC-derived 2A vWF (Fig 5B and our previous observations), suggesting that the site of proteolysis of the recombinant material was similar or identical to that in EC-derived 2A vWF.

DISCUSSION

In the present study, we describe the plasma elimination kinetics of normal and mutant forms of human von Willebrand factor in the rat. Multimeric vWF, with molecular weight range estimated upwards of 10,000 kD, is perhaps the largest protein in plasma. Despite the progress made on the structure, biosynthesis, and function of vWF, relatively little is known about its clearance from the circulation. In the rat model described here, we used vWF derived from human endothelial cells or transfected hamster (CHO) cells. The clearance of vWF derived from normal EC followed biphasic kinetics, with a rapid initial distribution phase (T 1/2 a ≈30 minutes), and the slower elimination phase (T 1/2 b ≈40 minutes). The elimination phase may be more representative of the natural clearance state, because...
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A

T-.
d-.
S1
S2

provWF 180 Kd
mature vWF

Fig 5. Clearance of recombinant type 2A vWF in vivo. (A) vWF derived from type 2A recombinant (S1613P mutation) was injected into a rat, and blood samples taken at (1) 10 minutes, (2) 30 minutes, (3) 60 minutes, (4) 180 minutes, and (5) 240 minutes, then analyzed by agarose gel electrophoresis. The control (c) represents 2A vWF immunoprecipitated in the presence of rat blood under identical conditions as the samples. (B) SDS-PAGE under reducing conditions of recombinant 2A vWF either untreated (lane 1) or exposed in vivo for 10 minutes (lane 2). The provWF polypeptide, mature vWF subunit, and the position of the 180 kD marker are shown.

total amount of vWF antigen. Using nonreducing agarose gels, it was observed that the high molecular weight vWF is eliminated from the circulation faster than the corresponding dimeric vWF. It is reasonable to assume that the multiplicity of binding structures on vWF would make a large multimer a better target than a dimer for removal from the circulation. There were not significant differences in the clearance of wild type vWF derived from cultured human or hamster cells in this rat model. There is precedence for using the rat as a model of clearance for human proteins. For instance, human erythropoietin and homologous rat erythropoietin were found to be cleared at similar rates in the rat. Furthermore, the clearance rates of human vWF in the rat are very similar to the rates reported for the clearance of human vWF in a rabbit model.

The rat model described herein has been used to analyze the clearance of mutant derived from a patient with type 2A vWD. The results presented here show that the 2A (S1613P) vWF mutant behaved in the rat model with the characteristic type 2A phenotype in humans, ie, a reduced amount of high molecular weight multimeric species and an increased amount of rapidly-migrating satellite species. The metabolic clearance rate of this type 2A mutant is not significantly different from the wild type vWF. This correlates with the fact that, despite abnormal multimeric content, there is often a near normal amount of total vWF antigen in many type 2A vWD patients. The carboxy-terminal fragment of vWD15 is known to be elevated in the plasma of patients with 2A vWD11,25 is thought to arise from the proteolytic cleavage between residues TYR(1605) and MET(1606) of mature vWF.26 A calcium-dependent neutral protease (calpain) has been implicated in the cleavage process.26,27 Consistent with these observations, our results demonstrate a proteolytic fragment of ~176 kD from type 2A vWF in the rat generated by an EDTA-inhibited protease. This is the first direct evidence that a single point mutation of this type can confer increased proteolytic sensitivity and suggests the proteolytic mechanism might be conserved between species. The fact that the characteristic 2A fragment could be generated by mixing 2A vWF with rat plasma ex vivo suggests the protease is plasma-based. Finally, these results offer a formal "proof" that the S1613P mutation is responsible for the type 2A von Willebrand disease in these patients.

It is not known whether the clearance of vWF in vivo is mediated by a specific receptor, multiple receptors, proteolysis, or alternatively, extravasation into the tissue. It is possible that vWF found naturally in the extracellular matrix may, in part, be derived from vWF, which was extravasated from the plasma during tissue injury and/or inflammation. Given the known binding affinity of vWF for collagen and heparin, components of the extracellular matrix, this is an attractive possibility. The asialoglycoprotein receptor mediates the cellular uptake of a number of desialated proteins in vitro, including chorionic gonadotropin, alpha-2 macroglobulin, and erythropoietin, although its role in vivo remains to be determined. The asialoglycoprotein receptor recognizes galactose, N-acetyl galactosamine, and related galactosides that are exposed after the removal of terminal sialic acid of glycoproteins. It has been demonstrated that vWF with
terminal sialic acid removed by neuraminidase was rapidly cleared from the circulation of rabbits. Our results demonstrate that the O-linked carbohydrate moieties play a role in vWF clearance, because O-minus vWF was eliminated significantly faster than normal vWF. This suggests that a defect in carbohydrate metabolism and/or incorrect post-translational modification could lead to certain forms of von Willebrand disease. The absence of proteolytic fragments suggests the rapid clearance of O-minus vWF involves a receptor-based, rather than protease-based clearance mechanism. It is possible that the production of O-minus vWF by elimination of GaINAc from the medium also causes the production of certain N-linked carbohydrates truncated with galactose, serving as recognition structures for the asialoglycoprotein receptor. Receptor blocking studies could be performed to determine whether other carbohydrate-specific receptors play a role in clearance of vWF, such as the mannos/RNAc receptor, the fucose receptor, or the phosphomannosyl receptor (for review, see Ashwell and Harford). There is also precedence for sulfation moieties of glycoproteins playing a role in protein clearance. For instance, a receptor that recognizes a specific sulfated structure, SO4-4GalNAcB1,4GlcNAcB1,2-Man (S4GGnM) present on sulfated lutropin, has been characterized from liver cells. Removal of sulfate from lutropin results in rapid clearance in vivo, apparently through the asialoglycoprotein receptor. However, in this study, we demonstrated that the model may mimic clearance in humans, it is possible, between human and rat vWF, although expected to be high, is not known. There could exist differences in posttranslational modification that play a role in the clearance. We determined that the clearance rate of recombinant wild type vWF produced in a stably-transfected CHO cell line is cleared at a similar rate as wild type EC-derived vWF. The ability of recombinant vWF to persist in the circulation suggests that recombinant vWF might ultimately have therapeutic value. The current model may prove advantageous for evaluating therapeutic regimens aimed at the treatment of 2A vWD by means of proteolytic inhibition.

ACKNOWLEDGMENT

The authors wish to thank Dr. Z. Rügzer for the monoclonal antibody 2.2.9, Dr. M. Krüger for the LDL-D cells, Suzanne Quinn for help with the vWF ELISA, and Robert Hopkins for helpful discussions.

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