Pharmaceutical Research, Vol. 23, No. 8, August 2006 (© 2006)
DOI: 10.1007/s11095-006-9018-1

Research Paper

Effect of von Willebrand Factor on the Pharmacokinetics of Recombinant Human Platelet Glycoprotein Ibα-Immunoglobulin G1 Chimeric Proteins

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Received October 18, 2005; accepted March 23, 2006

Purpose. Recombinant human platelet glycoprotein Ibα-immunoglobulin G1 chimeric proteins (GPIbα-Ig) have varying levels of anti-thrombotic activities based on their ability to compete for platelet mediated adhesion to von Willebrand Factor (vWF). Valine substituted GPIbα-Ig chimeras, at certain position, increase the binding affinity to vWF over its "wild-type" GPIbα-Ig analog. The purpose of this study was to determine the pharmacokinetics of two valine substituted GPIbα-Ig chimeras, GPIbα-Ig/1V (valine substitution at 239 position) and GPIbα-Ig/2V (double valine substitution at 233 and 239 position), in mice, rats and dogs.

Methods. Head-to-head comparisons of pharmacokinetics of GPIbα-Ig/1V and GPIbα-Ig/2V were investigated in rats and dogs after intravenous administration. Since vWF precipitates in the serum but not in plasma preparation, the concentration-time profiles of GPIbα-Ig/2V in rats were examined from the same blood samples for determination of matrix effect. The disposition of GPIbα-Ig/2V was also compared in vWF-deficient *versus* wild-type mice.

Results. For GPIbα-Ig/2V, the serum clearances were 2.62 ± 0.27 ml/hr/kg in rats and 1.97 ± 0.24 ml/hr/kg in dogs. The serum clearances of less potent GPIbα-Ig/1V were 1.08 ± 0.08 and 0.97 ± 0.19 ml/hr/kg in rats and dogs, respectively. In addition, the serum clearance of GPIbα-Ig/2V of 1.53 ml/hr/kg in vWF-deficient mice was lower than that in wild-type mice of 2.79 ml/hr/kg.

Conclusion. The difference in disposition for valine substituted forms of GPIbα-Ig in laboratory animals are likely affected by their enhanced binding affinity for circulating vWF.

KEY WORDS: factor VIII; GPIbα_Ig; pharmacokinetics of recombinant protein; vWF; vWF antibody.

INTRODUCTION

Von Willebrand factor (vWF) plays a pivotal role in hemostasis and thrombosis. *In vivo*, vWF promotes platelet adhesion to exposed subendothelial matrix components after vessel wall injury, mediates platelet–platelet aggregation, and stabilizes coagulation Factor VIII (FVIII) by acting as a protein carrier through noncovalent association (1). VWF circulates as dimers (~500 kDa) and multimers (over 2,000 kDa) as well as being immobilized on the surface of endothelial cells and subendothelial basement membrane. Glycoprotein Ibα (GPIbα) is a component of the platelet membrane glycoprotein (GP) Ib-IX-V complex. This receptor is involved in tethering platelets to the damaged blood vessels in conditions of high shear blood flow via interaction with immobilized vWF. This is the first step in the control of bleeding after tissue trauma. VWF-GPIbα interactions occurring in partially occluded arteries also lead to a pathologic consequence such as development of acute thrombotic occlusions (2,3).

The first 290 amino acids of the N-terminal of human GPIbα, containing either wild type, or specific substituted amino acids, were linked via single proline residue to the Fc region of human immunoglobulin G1 (IgG1) to create GPIbα-Ig chimeric proteins (Sako *et al.*, manuscript in preparation). This strategy has been used for several recombinant receptor-Fc chimeric proteins with improved pharmacokinetic profiles as therapeutic agents (4–6). The GPIbα-Ig chimeras were designed to act as soluble competitive inhibitors for the platelet associated GPIbα and thus disable the capacity of immobilized vWF to capture platelets at the site of plaque rupture under high shear stress. Valine substitution at 239 (GPIbα/1V) or 233 and 239 positions (GPIbα/2V) increase binding affinity to vWF (7,8). *In vitro* binding assay demonstrated increased binding affinity to vWF for GPIbα-Ig/1V and GPIbα-Ig/2V compared to that of "wild-type" GPIbα-Ig. Thus, these protein constructs, GPIbα-Ig, GPIbα-Ig/1V and GPIbα-Ig/2V have varying levels of anti-thrombotic activity based on their ability to compete for platelet mediated adhesion to vWF following arterial injury in areas of high shear stress.

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In this report, we describe the dispositions of these two valine substituted human GPIbα Fc chimeric proteins, GPIbα-Ig/1V and GPIbα-Ig/2V, in mice, rats and dogs, and in particular, the effect of circulating vWF on the disposition of GPIbα-Ig.

MATERIALS AND METHODS

Materials and Animal Information

Test articles, GPIbα-Ig, GPIbα-Ig/1V and GPIbα-Ig/2V, were generated by the Biopharma division of Wyeth Pharmaceuticals (Andover, MA). The test articles were supplied as sterile solutions in phosphate buffered saline (PBS, pH 7.2). The purity was no less than 97%. The stock solutions were stored at −80°C. Unless specified, the chemicals and reagents were purchased from Sigma (St. Louis, MO) and used as received. The studies were performed at Wyeth Research (Andover, MA) or at a contract institution (DaVinci Biomedical Research, South Lancaster, MA) under the supervision of the Institutional Animal Care and Use Committee. All doses were freshly formulated in PBS on the day of study. No animal had received any prior treatment of any protein products, and therefore, no pre-existing neutralizing antibodies were expected.

In Vitro Binding Activity to vWF

A direct binding enzyme-linked immunosorbent assay (ELISA) was used to assess the vWF binding activity of GPIbα-Ig, GPIbα-Ig/1V and GPIbα-Ig/2V. Briefly, an ELISA plate (Immulon HB2, MTX Lab System, Vienna, VA) was coated with 5 μg/ml recombinant human vWF (Wyeth Biopharma, Andover, MA). After coating, 4% bovine serum albumin was added to minimize non-specific binding. Standard curves of GPIbα-Ig, native as well as valine substituted, were prepared. After incubation for 30 min, bound GPIbα-Ig was detected using murine anti-human IgG Fc horseradish peroxidase (Southern Biotech Associates, Birmingham, AL). A colorimetric read-out was provided by the addition of 3,3′,5,5′-tetramethylbenzidine (TMB, BioFX Laboratories, Owings Mills, MD). The optical densities were read at 405 nm. The *in vitro* vWF binding activities of GPIbα-Ig, GPIbα-Ig/1V and GPIbα-Ig/2V were assessed through the comparison of the binding standard curves.

Iodination of GPIbα-Ig/2V and Preparation of ^{125}I Traced GPIbα-Ig/2V Dose Solution

GPIbα-Ig/2V was labeled with ^{125}I using the Iodo-gen method (Pierce Biotechnology, Rockford, IL). The ^{125}I-labeled GPIbα-Ig/2V was then separated from the reaction mixture using a short size exclusion column (NAP™ 5 columns, Amersham Biosciences, Piscataway, NJ). The purity of the labeled material was confirmed by HPLC where a single peak with the same retention time as unlabeled GPIbα-Ig/2V was observed. The dose solution was prepared by mixing unlabeled GPIbα-Ig/2V with a trace amount of ^{125}I labeled GPIbα-Ig/2V (2%, v/v) in PBS. The final drug concentration was 62.5 μg/ml.

Disposition of ^{125}I-GPIbα-Ig/2V in vWF Deficient and Wild-Type Mice (9)

Male and female vWF-deficient mice of 4 to 8 weeks old were matched with similar age and sex of wild-type mice (n = 21 per group). Each mouse received a dose of 0.25 mg/kg of GPIbα-Ig/2V containing a trace amount of ^{125}I labeled material (5.6 to 7.3 million cpm per mouse) via tail vein injection. After dose administration, three mice per time point from each group were sacrificed for blood samples. The time points were 0.25, 3, 7, 24, 48, 72 and 168 h post dose administration. Serum GPIbα-Ig/2V concentrations, measured as nanogram equivalent per milliliter, were assayed for total and trichloroacetic acid (TCA)-precipitable counts with a gamma counter (1480 WIZARD™, WIZARD Inc., Gaithersburg, MD). The counts were corrected with the half-life of ^{125}I radioactivity decay, assuming only precipitable counts are associated with intact protein.

Comparison of Serum *vs* Plasma Pharmacokinetic Profiles of GPIbα-Ig/2V in Rats

Male adult Sprague–Dawley rats (Charles River, Wilmington, MA) had a jugular vein catheter surgically implanted prior to arrival at the laboratory. Rats were divided into high dose (5 mg/kg, n = 4) and low dose (0.1 mg/kg, n = 4) treatment groups. After tail vein injection, blood samples of approximately 0.3 ml were taken at 0.25, 6, 24, 72, 144 and 336 h through the catheter. The loss of blood was replaced with an equal volume of saline. Each blood sample was collected into two vials, one with and one without EDTA. Therefore, serum and plasma samples were generated from the same blood sample at each sampling time. Serum and plasma samples were stored at −80°C until assayed for GPIbα-Ig/2V concentration by ELISA.

Comparison of Pharmacokinetics of GPIbα-Ig/2V and GPIbα-Ig/1V in Rats and Dogs

Male adult Sprague-Dawley rats were divided into GPIbα-Ig/2V (0.25 mg/kg, n = 5) or GPIbα-Ig/1V (0.25 mg/kg, n = 5) treatment groups. After tail vein dose administration, blood samples of 0.15 ml were taken at 0.25, 1, 2, 4, 7, 24, 32, 48, 56, 72, 96, 168 and 240 h through the catheter. Similarly, six male Beagle dogs (n = 3 per group), weighing 8 to 10 kg, were treated with a similar dose of either GPIbα-Ig/2V (0.25 mg/kg) or GPIbα-Ig/1V (0.175 mg/kg). Blood samples of approximately 0.2 ml were collected at pre- and 0.25, 1, 3, 6, 12, 24, 48, 72, 96, 120, 144, 168, 192, 240, 336, 432 and 504 h post dose administration. Serum samples were frozen at −80°C until assayed for GPIbα-Ig concentration by ELISA.

Determination of GPIbα-Ig Levels in Serum or Plasma Samples

The GPIbα-Ig ELISAs were quantitative sandwich assays designed to measure GPIbα-Ig, as well as its valine substituted analogs, levels in serum or plasma of multiple species. In general, the test article, either GPIbα-Ig/1V or GPIbα-Ig/2V, was captured on ELISA plates by a commer-

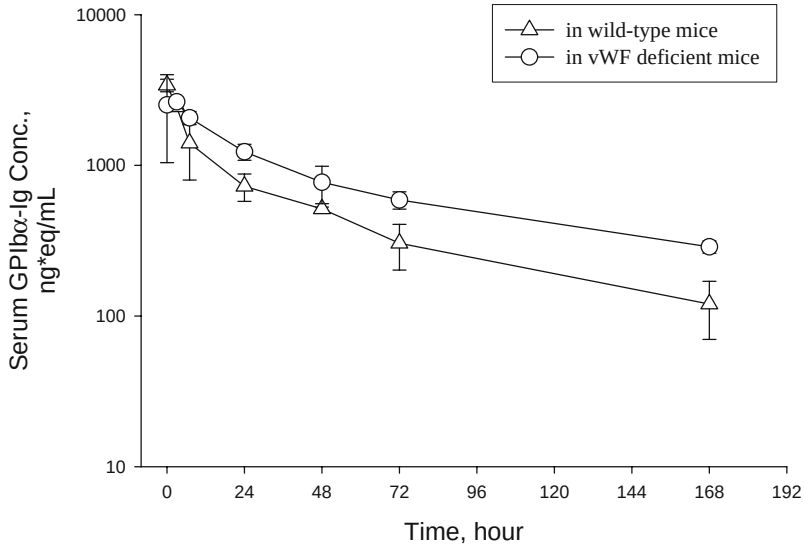

Fig. 1. Serum concentration *versus* time profiles of GPIbα-Ig/2V in wild type and vWF deficient mice after IV administration of 0.25 mg/kg.

cially available monoclonal antibody to GPIbα (GTI-N5P, GTI, Brookfield, WI). The captured GPIbα-Ig was then detected with a murine anti-human IgG horseradish peroxidase labeled secondary antibody which produces a colored end product with the addition of 3,3′5′5-tetramethylbenzidine (TMB) as substrate. The optical density (OD) was recorded spectrophotometrically at 450 nm. Serum or plasma samples were diluted to the linear range of the standard curve to allow quantitation. The lowest quantifiable sample concentration was approximately 1.5 ng/ml.

Data Analysis

The pharmacokinetic parameters were determined using WinNonlin (version 4.1, Pharsight, Mountain View, CA). Calculations were performed using non-compartmental analysis approach. The estimation of area under the concentration *versus* time curve (AUC) was based upon log trapezoidal rule. The terminal rate constant (λ) was derived from the slope of the terminal log-linear phase of serum concentrations-time curves. The apparent terminal half-life ($t_{1/2}$) was calculated as $0.693/\lambda$. No statistical analysis other than descriptive statistics was conducted.

RESULTS

Effect of Valine Substitution on the Binding Affinity of GPIbα-Ig to vWF

Valine substitution at the 233 and 239 positions within the GPIbα Cys^{209}–Cys^{248} disulfide loop increases affinity of platelet GPIbα for vWF (7,8). Stronger binding to vWF of these gain-of-function of valine substituted GPIbα has also

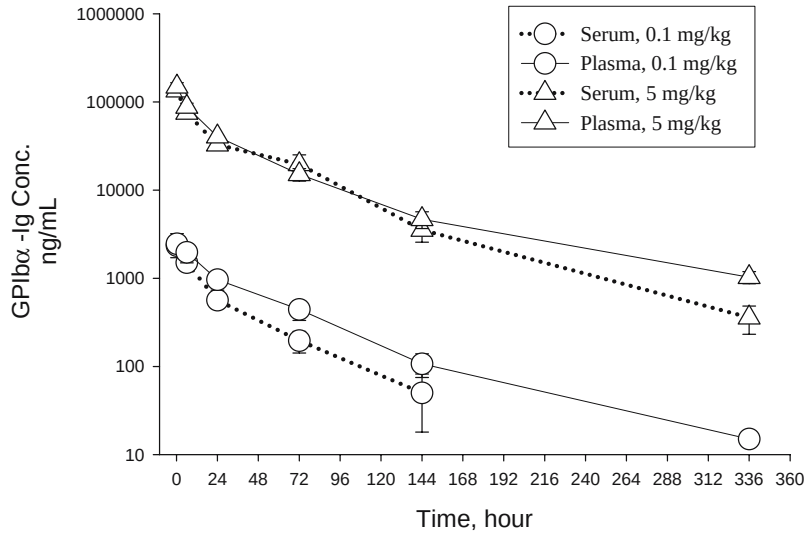

Fig. 2. Plasma and serum concentration *versus* time profiles of GPIbα-Ig/2V from the same blood samples after IV administration of 0.1 and 5 mg/kg to rats.

been observed in the presence of modulators, such as ristocetin (7). In order to confirm the function of valine substituted GPIbα Fc chimeric proteins, a direct binding ELISA was performed by passively coating vWF on the plate without using nonphysiological modulators. In the absence of shear force under this experimental format, GPIbα-Ig, GPIbα-Ig/1V and GPIbα-Ig/2V displayed increased vWF binding affinity at IC_{50} of 17.7, 1.8 and 0.5 μg/ml, respectively.

Effect of Circulating vWF on the Disposition of GPIbα-Ig/2V

Although the initial concentration was very similar, the overall serum exposure of GPIbα-Ig/2V was notably higher in vWF deficient than that in wild type mice (Fig. 1). There were two possible causes for such differences. One was that GPIbα-Ig/2V was simply cleared slower in vWF deficient mice. The other, only for wild type mice, was the formation of vWF×GPIbα-Ig/2V complex (or complexes), and that complex was precipitated in serum preparations and/or cleared faster than unbound GPIbα-Ig/2V.

There is ample evidence that vWF is involved in the blood clotting process. Apart from binding and stabilizing blood coagulation Factor VIII (FVIII), lack of vWF in von Willebrand disease patients exhibits significant bleeding (1,12). A higher affinity for vWF without the need for vWF binding and shear force effects would provide a potential greater neutralization of the vWF platelet GPIb interaction and thus a potentially more effective anti-thrombotic effect. This might be most easily assessed as the difference between plasma and serum recovery of the various forms of GPIbα-Ig.

From the same blood samples, showed in Fig. 2, the serum and plasma GPIbα-Ig/2V levels remained essentially the same at earlier time points after 5 mg/kg or 0.1 mg/kg

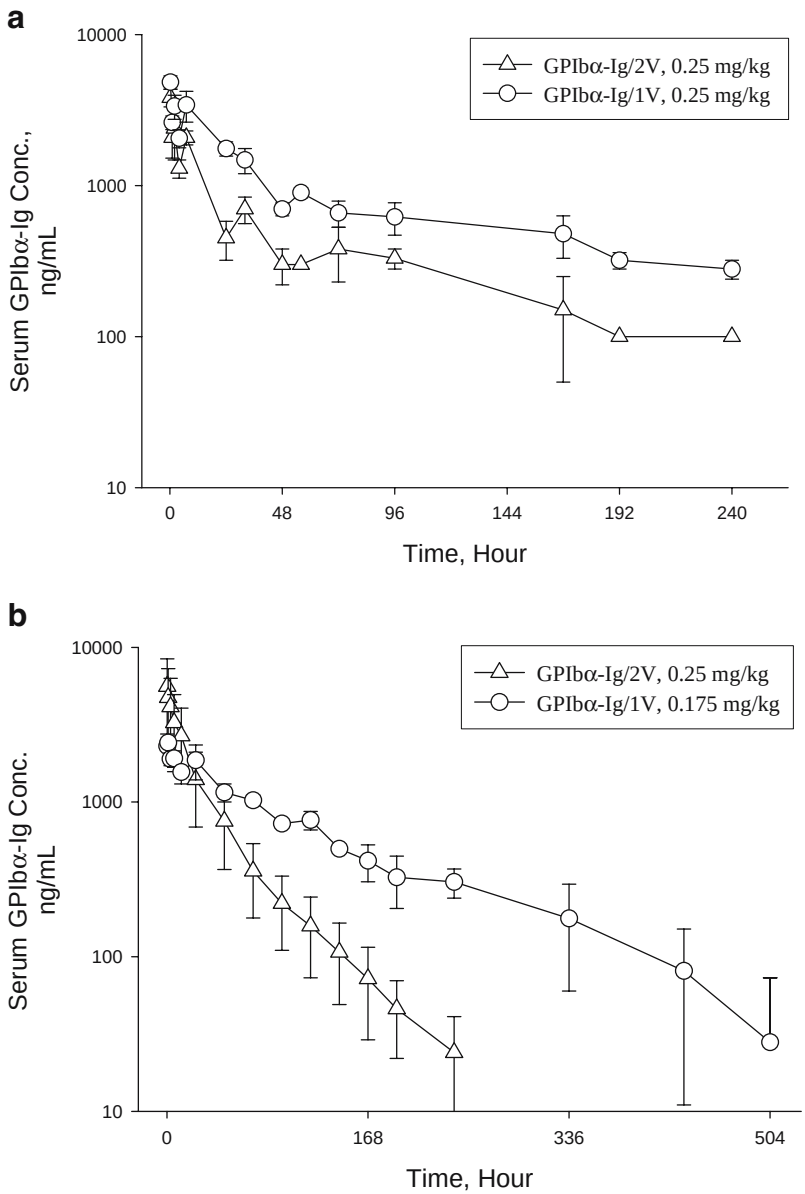

Fig. 3. Serum concentration *versus* time profiles of GPIbα-Ig/1V and GPIbα-Ig/2V in rats (a) and dogs (b) after IV administration.

injection to rats. However, once the concentration went below 1 to 3 μg/ml, serum GPIbα-Ig/2V levels were consistently lower than that in plasma. This indicated that part of GPIbα-Ig/2V was precipitated in the serum preparations, likely in the form of vWF×GPIbα-Ig/2V complex (or complexes).

Effect of Binding Affinity to vWF on the Pharmacokinetics of GPIbα-Ig

The serum exposures of GPIbα-Ig/2V and GPIbα-Ig/1V were directly compared in rats and dogs (Fig. 3). In both species, the serum exposures of more potent GPIbα-Ig/2V were consistently lower than that for lesser potent GPIbα-Ig/1V. Differing only by a single amino acid residue of these two structurally almost identical macromolecules, the difference in serum exposures indicated that binding affinity between GPIbα-Ig and vWF played a role in the disposition of GPIbα-Ig.

DISCUSSION

Serum clearance and $t_{1/2}$ of two recombinant human GPIbα-Fc chimeric proteins, GPIbα-Ig/1V and GPIbα-Ig/2V, are listed in Table I. In summary, the serum clearance of GPIbα-Ig/2V was lower in vWF deficient than that in wild type mice. In rats and dogs, under the same or comparable dose levels, more potent GPIbα-Ig/2V exhibited higher serum clearance than its lesser potent analog, GPIbα-Ig/1V. The $t_{1/2}$ of GPIbα-Ig/2V was also shorter than the $t_{1/2}$ of lesser potent GPIbα-Ig/1V. In addition, the $t_{1/2}$ of GPIbα-Ig/2V of 57 h in mice, of 84 h in rats and of 48 h in dogs were comparatively shorter to the $t_{1/2}$ of human IgG or other recombinant Fc chimeric proteins in these lab animals (4–6,13,14).

In vivo, GPIbα-Ig/2V likely co-exists as unbound form and as vWF × GPIbα-Ig/2V complex (or complexes). The binding affinity ($k_{binding}$) and circulating vWF levels determine the plasma concentration ratio of vWF × GPIbα-Ig complex and unbound GPIbα-Ig. It has the following relationship:

$$k_{binding} \times [\nu WF] = \frac{[\nu WF \times GPIb\alpha - Ig]}{[GPIb\alpha - Ig]}$$

The plasma and serum concentration *versus* time profiles of GPIbα-Ig/2V from the same bloods samples (Fig. 2) indicated the existence of vWF × GPIbα-Ig complex (or complexes). This complex was precipitated in serum but not in plasma preparations. When the unbound GPIbα-Ig/2V was in great excess compared to the vWF × GPIbα-Ig/2V complex, the precipitation of vWF × GPIbα-Ig/2V complex in the serum preparation was negligible. Therefore, there was little difference between measured serum and plasma concentration of GPIbα-Ig/2V. As the ratio of GPIbα-Ig/2V to vWF × GPIbα-Ig/2V complex went down, the drop in serum GPIbα-Ig concentration became more significant than that in plasma. Given the circulating vWF levels of approximately 10 μg/ml (15,16) and average molecule weight of 500 kDa (as dimer) or higher (over 2,000 kDa as aggregates) for circulating vWF, the plasma concentration of approximately 2 μg/ml for GPIbα-Ig/2V appeared to be the threshold of "stoichiometric" binding between circulating vWF and GPIbα-Ig/2V.

Although the changes in serum exposure may not necessarily reflect the clearance *in vivo* in these animal species, we believe vWF did alter the clearances of GPIbα-Ig. A proposed scheme for the catabolism/elimination of GPIbα-Ig *in vivo* is illustrated in following schematic:

$$\begin{array}{ccc} [GPIb\alpha - Ig] + [\nu WF] & \rightleftharpoons & [\nu WF \times GPIb\alpha - Ig] \\ \downarrow k_{GPIb\alpha - Ig} & \downarrow k_{\nu WF} & \downarrow k_{complex} \end{array}$$

In the absence of vWF, the elimination of GPIbα-Ig is, assuming first order, determined by unbound concentration and elimination rate constant of GPIbα-Ig ($k_{GPIb\alpha\text{-}Ig}$). However, in the presence of circulating vWF, the elimination of GPIbα-Ig *in vivo* in any given specie may depend upon combinatory factors such as (A) plasma concentration and the rate of elimination of unbound GPIbα-Ig ($k_{GPIb\alpha\text{-}Ig}$), (B) the plasma concentration of circulating vWF, (C) the binding affinities ($k_{binding}$) between GPIbα-Ig and circulating vWF in the chosen specie, and (D) the plasma concentration and elimination rate of the vWF × GPIbα-Ig complex ($k_{complex}$). Therefore, the apparent clearance of GPIbα-Ig should be the combination of the clearances of unbound GPIbα-Ig and vWF × GPIbα-Ig complex.

Since GPIbα-Ig/2V was cleared more slowly in vWF deficient mice, of which the formation of vWF × GPIbα-Ig/2V complex was not anticipated, higher clearance in wild-type mice indicated that the vWF × GPIbα-Ig complex cleared faster than the unbound form (i.e., $k_{complex} > k_{GPIb\alpha\text{-}Ig}$). For GPIbα-Ig in any given specie, higher circulating vWF level and/or higher binding affinity produce higher levels of vWF × GPIbα-Ig complex. Consequently, higher levels of vWF × GPIbα-Ig complex result faster apparent clearance of GPIbα-Ig. This may be the reason why the more potent GPIbα-Ig/2V exhibited faster clearance in both rats and dogs than less potent GPIbα-Ig/1V. This may also explain why the $t_{1/2}$ of these two GPIbα-Ig chimeras were shorter comparing to some other Fc chimeric proteins (4–6).

Unfortunately, the vWF levels are varied among animal species (15,16) and, the binding affinity between human GPIbα-Ig and vWF of various origins are likely not the same. Therefore, the *in vivo* concentration and clearance of vWF × GPIbα-Ig complex (or complexes) in various species are difficult to determine and/or predict. For example, GPIbα-Ig/2V had shorter $t_{1/2}$ in dogs (48 h) than that in mice (57 h) or in rats (84 h). One possibility is that dog has 3 to 4 times higher circulating vWF than that found in rats and mice

Table I. Pharmacokinetic Parameters of GPIbα-Ig/1V and GPIbα-Ig/2V in Mice, Rats and Dogs

Animals	Test articles	CL_{serum}±SD (ml/hr/kg)	$t_{1/2}$±SD (hour)
vWF-deficient mice	GPIbα-Ig/2V	1.53	87
wild type mice	GPIbα-Ig/2V	2.79	57
Rats	GPIbα-Ig/2V	2.62 ± 0.27	84 ± 22
Rats	GPIbα-Ig/1V	1.08 ± 0.08	111 ± 45
Dogs	GPIbα-Ig/2V	1.97 ± 0.24	48 ± 23
Dogs	GPIbα-Ig/1V	0.97 ± 0.19	90 ± 31

(15,16). Another possibility is that human GPIbα-Ig/2V may have higher binding affinity to dog vWF than that to rodents'. In either situation, the vWF × GPIbα-Ig/2V complex levels would be higher and, higher level of circulatory vWF × GPIbα-Ig/2V complex is likely resulted faster clearance of GPIbα-Ig.

Kageyama *et al.* had studied the pharmacokinetics of a fully humanized monoclonal antibody to vWF, vWJ200, in cynomolgus monkeys using citrated plasma as the sample matrices (17). In their report, the elimination $t_{1/2}$ of vWJ200 ($t_{1/2,\text{antibody}}$) was approximately 20 to 40 h in monkeys and was dose dependent. The $t_{1/2,\text{antibody}}$ was substantially shorter than the average $t_{1/2}$ of 3 to 14 days reported for some other humanized monoclonal antibodies in cynomolgus monkeys (18,19). The clearance of the antibody was linear at lower doses, but decreased as the IV dose exceeded 1 mg/kg and the plasma levels of the antibody were above approximately 5 μg/ml. Associating with the finding in this report, we believe vWF played a role in the clearance of this antibody under the same mechanism proposed for GPIbα-Ig chimeric proteins. In circulation, like GPIbα-Ig, vWJ200 co-exists as unbound form and as vWF × antibody complex. The vWF × antibody complex is likely cleared faster than the unbound antibody (i.e., $k_{complex} > k_{antibody}$). At low dose, the vWF × antibody complex is the primary form of existence in the circulation. The clearance of the antibody is thus essentially determined by the clearance of vWF × antibody complex and therefore, it was linear. This also explains why the $t_{1/2,\text{antibody}}$ was shorter than some other humanized monoclonal antibodies in cynomolgus monkeys. As the dose increases, the availability of vWF limits to formation of the vWF × antibody complex. Hence, the plasma clearance of the antibody decreases as the fraction of unbound form increase.

It has been showed that circulating vWF improves the *in vivo* stability of blood clotting Factor VIII (FVIII). VWF protects FVIII from low-density lipoprotein receptor-related protein (LRP) mediated catabolism by binding to the A2 domain of FVIII. The exposure of FVIII to catabolism is then dependent upon the dissociation of FVIII from circulating vWF. Therefore, the clearance of FVIII *in vivo* is reduced as long as it binds to vWF (20,21). In fact, the half-lives of various FVIII products ($t_{1/2,\text{FVIII}}$) were either shorter or approximately equal to the 3–4 h half-life of vWF ($t_{1/2,\text{vWF}}$) in mice (22), the 4 to 5 h of $t_{1/2,\text{vWF}}$ in rats (23,24), the 22 h of $t_{1/2,\text{vWF}}$ in dogs (25–28) and the approximately 24 h of $t_{1/2,\text{vWF}}$ in humans (29). There has been no report of FVIII products exhibiting $t_{1/2,\text{FVIII}}$ significantly longer than the $t_{1/2,\text{vWF}}$ in animals or in humans. In addition, the $t_{1/2,\text{FVIII}}$ of infused human FVIII is approximately 15.3 h in blood type-O patients and 19.7 h in type-A patients (30). Coincidently, the circulating vWF levels are 25% lower for type-O over type-A in general population (31,32). One possibility is that lower circulating vWF reduces the level of vWF × FVIII complex and that, in turn increases the vulnerability of FVIII to catabolism. Thus, the *in vivo* fate of FVIII products may follow a similar scheme as we proposed for GPIBα-Ig. For example, the $t_{1/2,\text{FVIII}}$ of various FVIII products may relate to the plasma concentration and turnover rate of circulating vWF in human. The approximately one day turnover rate for vWF poses one of the challenges for development of long lasting FVIII products (20).

In conclusion, the disposition of GPIbα-Ig chimeras, perhaps as well as various FVIII products and vWF antibodies, are likely to be affected by their *in vivo* binding kinetics with, availability of, and turnover rate of vWF in different species.

ACKNOWLEDGMENTS

The authors thank Ms. Jessica Doherty, Ms. Cindy Clark, Ms. Amy Ignatowicz and Dr. Terrie Cunliffe-Beamer for their excellent support for the animal studies.

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