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Clearance of von Willebrand Factor

Cécile V. Denis ¹, Olivier D. Christophe¹, Beatrijs D. Oortwijn², Peter J. Lenting^{2,3}

¹INSERM U770, Le Kremlin-Bicêtre, F-94276, France; Univ Paris-Sud, Le Kremlin-Bicêtre, F-94276, France

²Laboratory for Thrombosis and Haemostasis, Department of Clinical Chemistry & Haematology, University Medical Center Utrecht, Heidelberglaan 100, 3584 CX Utrecht, the Netherlands

³Crucell Holland B.V., Department of Protein Discovery, Archimedesweg 4-6, 2333 CN Leiden, the Netherlands

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Address correspondence to:

Dr. C.V. Denis

INSERM U770

80 rue du Général Leclerc

94276 Le Kremlin-Bicetre Cedex

Tel: 33-(0)1-49-59-56-05

Fax: 33-(0)1-46-71-94-72

Email: denis@kb.inserm.fr

Abstract

The life cycle of von Willebrand factor (*VWF*) comprises a number of distinct steps, ranging from the controlled expression of the *VWF* gene in endothelial cells and megakaryocytes to the removal of *VWF* from the circulation. The various aspects of *VWF* clearance have been the objects of intense research in the last few years, stimulated by observations that *VWF* clearance is a relatively common component of the pathogenesis of type 1 von Willebrand disease (*VWD*). Moreover, improving the survival of *VWF* is now considered as a viable therapeutic strategy to prolong the half-life of factor VIII in order to optimise treatment of haemophilia A.

The present review aims to provide an overview of recent findings with regard to the molecular basis of *VWF* clearance. A number of parameters have been identified that influence *VWF* clearance, including its glycosylation profile and a number of *VWF* missense mutations. In addition, *in vivo* studies have been used to identify cells that contribute to the catabolism of *VWF*, providing a starting point for the identification of receptors that mediate the cellular uptake of *VWF*. Finally, we discuss recent data describing chemical modification of *VWF* as an approach to prolong the half-life of the *VWF*/FVIII complex.

Introduction

For each organism, the regulation of protein levels is a key process that requires a delicate balance between biosynthesis and elimination. Defects in clearance mechanisms may result in deficiency or an accumulation of certain components, which may eventually provoke pathological manifestations. For instance, the accumulation of amyloid β peptide due to reduced LRP-mediated clearance contributes to the pathogenesis of Alzheimer disease^{1,2}, whereas defects in the clearance of lipoprotein particles are associated with familial hypercholesterolemia.³ These two examples illustrate the universal requirement for mechanisms that control the clearance of proteins, and the haemostatic system is not an exception in this regard.

One important player of the haemostatic system is von Willebrand factor (VWF). VWF is a multimeric protein which contributes to the initial recruitment of platelets to injured vessel by acting as a molecular bridge between the exposed subendothelial matrix and the platelet receptors GpIb/IX/V and α IIb β 3.⁴ Apart from its role in primary haemostasis, a number of other functions have been recognized as well. First, VWF and its propeptide are indispensable for the intracellular formation of endothelial-specific storage organelles, the Weibel-Palade bodies.^{5,6} These organelles are not only a storage-compartment for ultra-large VWF multimers, but also for a variety of other proteins, such as P-selectin, interleukin-8 and angiotensin-2 (for reviews see Michaux *et al.* and Rondaij *et al.*).^{7,8} Second, the presence of VWF has a suppressing effect on the metastatic potential of tumour cells.⁹ Third, VWF comprises binding sites for *Staphylococcal aureus*-surface proteins (Protein A), which may facilitate intravascular colonisation by these pathogens.¹⁰⁻¹² Further, VWF may promote the proliferation of smooth muscle cells.¹³ Finally, we have recently reported on the ability of VWF to act as an adhesive surface for leukocytes.¹⁴ VWF-leukocyte interactions involve P-selectin glycoprotein ligand 1 (PSGL-1) as well as the family of β 2-integrins.

Another intriguing aspect of VWF is that it functions as a carrier to transport other proteins in the circulation. A recent example of a molecule that uses VWF as a molecular vehicle is osteoprotegerin. This protein is co-expressed with VWF in endothelial cells, and upon secretion both proteins remain in complex.^{15,16} The physiological relevance of complex formation between VWF and osteoprotegerin is yet unknown and remains to be resolved. The necessity for complex formation with VWF is much clearer for coagulation factor VIII (FVIII). FVIII is a plasma protein that functions as a cofactor for activated factor IX, and functional deficiency of FVIII leads to impaired generation of activated factor X.¹⁷ This deficiency is associated with a severe bleeding tendency, known as haemophilia A.¹⁸ Patients lacking VWF are characterized by a secondary deficiency of FVIII¹⁹, demonstrating that VWF is essential for appropriate survival of FVIII.

The present review aims to summarize recent developments regarding clearance of VWF. Knowledge of the mechanisms that are responsible for the clearance of VWF complex is important, because modulated clearance of VWF has been found to contribute to the pathogenesis of certain forms of von Willebrand disease (VWD). Moreover, since VWF is critical for the survival of FVIII, manipulating VWF clearance may be an approach to improve survival of FVIII in order to reduce the treatment-frequency of haemophilia A patients.

Biosynthesis of VWF

VWF is produced in endothelial cells and megakaryocytes.^{20,21} Megakaryocytic production is responsible for the presence of VWF in the α -granules of platelets, whereas endothelial cells are the primary source of VWF that is found in the subendothelial matrix and in plasma. Biosynthesis generates a single-chain pre-pro-polypeptide of 2813 amino acids, in which a number of domainal structures can be distinguished: D1-D2-D'-D3-A1-A2-A3-D4-B1-B2-B3-C1-C2-CK.¹⁹ After removal of the signal peptide, pro-VWF subunits undergo tail-to-tail linkage via disulphide bonding within the cysteine-rich CK-domains, resulting in pro-VWF dimers. Further processing proceeds within the Golgi-apparatus and involves multimerization via intramolecular cysteine-bonding within the D'-D3 domains. This process produces a heterogeneous pool of differentially sized multimers, with molecular masses ranging from 0.5×10^6 to over 10×10^6 Da. Imperative to this multimerization is binding of propeptide to the D'-D3 region, an interaction which is optimal under slightly acidic conditions such as those present in the Golgi.^{20,22} Indeed, defective interactions between propeptide and D'-D3 domains lead to impaired multimerization.²³ Of interest, the slightly acidic conditions needed for optimal propeptide binding also apply to the binding of proteins that co-localize with VWF in the endothelial storage organelles, such as osteoprotegerin and IL-8.^{16,24}

Finally, limited proteolysis in the trans-Golgi network separates the propeptide from the mature VWF multimer. Proteolysis is believed to be mediated by furin, which recognizes a positively-charged sequence downstream the Arg763-Ser764 cleavage site.²⁵ Mutations in this area lead to incomplete removal of the propeptide, thereby generating pro-VWF multimers displaying suboptimal FVIII binding.^{26,27} Following proteolysis, VWF is targeted to the storage organelles. Noteworthy, both VWF and propeptide are absolutely necessary for Weibel-Palade body formation, whereas α -granule formation occurs in their absence. Endothelial cells obtained from animals with a complete VWF-deficiency indeed lack Weibel-Palade bodies^{5,28}, and the formation of these storage-organelles can be restored upon transfection with VWF.⁵ The importance of VWF for Weibel-Palade body formation is further underscored by the fact that mutations associated with impaired multimerization also affect size and number of Weibel-Palade bodies.⁷

Glycosylation of VWF

Apart from multimerization and proteolytic processing, VWF is also subject to extensive glycosylation. The amino acid sequence of VWF predicts the site of 12 *N*-linked and 10 *O*-linked oligosaccharide side-chains, and this extensive number of glycans contributes to almost 20 % of the molecular mass of the VWF molecule.²⁹ *N*-linked glycosylation is initiated within the endoplasmatic reticulum, and this process continues within the Golgi apparatus. *N*-linked glycans on VWF display a wide spectrum of complexity, and include bi- tri- and tetra-antennary structures.³⁰ Interestingly, VWF is one of the few plasma proteins that have blood group A, B and H antigenic structures incorporated in their oligosaccharide side-chains.³¹ It is important to stress that the blood group determinants are attached to the *N*-linked but not the *O*-linked structures.³² As for the *O*-linked glycans, the majority consists of sialylated T-antigen, a mucin-type core 1 structure.³² The contribution of glycosylation to VWF biology is still a poorly understood area, despite many years of research. It seems conceivable to assume that

optimal glycosylation is required for proper synthesis and secretion. Furthermore, the presence of the glycan structures also seems to be involved in regulating VWF function, an issue that is discussed in more detail elsewhere.^{33,34} Finally, the glycosylation profile of VWF has proven to be a prominent parameter that influences VWF plasma levels.

Glycosylation of VWF: effect of blood group determinants on VWF levels

More than two decades ago, it was already recognized that VWF levels are lower in individuals having blood group O compared to those having non-O³⁵, and this observation has been confirmed in many other studies (reviewed in³⁴). On average, VWF concentrations are approximately 25% lower in persons with blood group O than those in non-O individuals. Levels are even more reduced in persons with the Bombay-phenotype, who lack expression of ABO-antigens.³⁶

How to explain the lower levels of VWF in those who have blood group O? Studies by O'Donnell and colleagues have provided evidence that the effect is mediated by the ABH antigenic structures present on the VWF molecule self rather than via indirect mechanisms.³⁷ They found a correlation between expression levels of A-transferase, the amount of A antigenic determinants present on the VWF molecule and corresponding VWF levels. A similar relationship between the loading of VWF with A or B determinants and VWF levels has more recently been reported by Morelli and colleagues.³⁸ Thus, in some way the blood group determinants present on the VWF molecule affect biosynthesis, secretion, clearance or a combination thereof.

Attempts to address the effect of blood group determinants on biosynthesis and secretion *in vitro* have been proven to be difficult, because of the absence of A- and B-transferase activity in isolated human umbilical vein endothelial cells, the main primary cell type that is used to study VWF biology.^{39,40} However, a number of arguments are available that point against a major effect of the blood group determinants on biosynthesis and/or secretion. First, VWF concentrations in platelets are affected to a minor extent by blood group type⁴¹, indicating that the blood group determinants do not affect synthesis and storage in megakaryocytes. Second, the rise in VWF levels upon desmopressin treatment appears to be similar for patients with different blood groups⁴², suggesting similar efficiency of VWF secretion from endothelial storage pools.

Based on these observations, it seems conceivable that the effect of blood group determinants is most likely affecting clearance of VWF. Although not formally proven, there are a number of observations that are in support of this possibility. First, preliminary data were recently presented concerning the half-life of VWF following desmopressin treatment in healthy individuals.⁴³ It was found that that the half-lives were significantly shorter in O-subjects than in non-O-subjects. Another observation relates to the ratio between VWF propeptide and mature VWF. VWF propeptide and mature VWF are released simultaneously in a 1:1 molar ratio. However, propeptide is cleared 3- to 4-fold more rapidly than VWF, resulting in distinct propeptide/VWF ratio under steady-state conditions.⁴⁴ Interestingly, this ratio is dissimilar between individuals with different blood groups, in that blood group O individuals have an increased ratio compared to non-O persons.^{45,46} Given the notions that (1) VWF and propeptide are released in a 1:1 ratio and (2) propeptide levels are unaffected by blood group, it should follow that VWF has a shorter half-life in blood group O persons. This possibility has been

addressed by Nossent *et al.* using a model assuming that the propeptide half-life is more or less constant amongst individuals. This model predicts the half-life of endogenous VWF to be 2 h longer in non-O persons compared to blood group O persons.⁴⁶ Finally, several studies have reported that the half-life of infused FVIII correlates with steady-state VWF levels, its carrier protein.^{47,48} When we analysed this correlation in a cohort of 38 haemophilia A patients, we found that this correlation is strongly influenced by blood group (Fig. 1). This may explain that why the half-life of FVIII (irrespective whether it is recombinant or plasma-derived), is considerably shorter in haemophilia A patients with blood group O with FVIII half-lives being 11.5 ± 2.6 h and 14.3 ± 3.0 h ($p = 0.0044$) in O- and non-O patients, respectively. A similar effect of blood group on FVIII survival has previously been reported.⁴⁸ Since no blood group determinants are present on the FVIII molecule, the most logical explanation for the blood-group dependent half-life of FVIII is that the half-life of its carrier protein VWF is determined by the blood group determinants.

The notion that ABO-determinants influence VWF clearance may be of relevance for the development of VWF preparations for therapeutic use, for instance by increasing the amounts of non-O VWF molecules in plasma-derived VWF concentrates. Moreover, in view of the development of a recombinant-derived VWF therapeutic preparation⁴⁹, it is of importance to investigate how the non-human glycosylation profile affects the survival of VWF in humans. In particular, it is of relevance to exclude that the glycan profile of recombinant VWF mimics that of the Bombay-phenotype, since this phenotype is associated with low VWF levels.³⁶

Apart from the ABO-blood group effect, other blood group-dependent modifications of the glycosylation profile may also affect VWF plasma levels. An example hereof is the *Secretor* system, which is involved in the ability to secrete blood type antigens in body fluids. Persons with blood group O and the SeSe-genotype have significantly higher VWF levels (approximately 20%) compared to those with the O-blood group combined with the Sese- or sese-genotype.⁵⁰ It is not known whether Secretor-dependent modifications affect VWF clearance, and propeptide-analysis would provide insight in this regard.

The effect of glycosylation on VWF clearance: Sialylation

As for most human glycoproteins, also oligosaccharides side-chains on VWF contain terminal sialyl-residues. For many proteins it has been demonstrated that sialyl-groups covering galactose-residues are needed to prevent premature clearance of these proteins by carbohydrate-recognizing receptors, such as asialoglycoprotein-receptor. Enzymatic removal of sialyl-residues from VWF results in molecules that have a markedly short half-life when injected in rabbits (240 min and 5 min for normal and de-sialylated VWF, respectively)⁵¹, suggesting that sialyl-groups are also needed to protect VWF from premature clearance. The physiological relevance of VWF sialylation has elegantly been demonstrated by Ellies *et al.*, who reported that in a patient group referred to the hospital for real or suspected bleeding disorder, reduced sialylation by sialyl-transferase ST3Gal-IV was associated with reduced VWF plasma levels.⁵² Also in a more recent study by Millar *et al.*, the half-life of VWF released upon desmopressin was found to correlate with the extent of sialylation.⁵³ Furthermore, the

half-life of endogenous VWF was reduced by 2-fold in mice lacking sialyl-transferase ST3Gal-IV, which display impaired sialylation.⁵²

The value of animal models to investigate the relationship between glycosylation and regulation of VWF levels, is most vividly illustrated by the work of Ginsburg and coworkers using the RIIS/J mouse strain. This mouse strain is characterized by VWF levels that are several-fold lower compared to other mouse strains.⁵⁴ Via a crossbreeding approach, it was demonstrated that the gene responsible for these low VWF levels was distinct from the VWF gene⁵⁵ More detailed studies identified this VWF-modifier gene as being *Galgt2* (currently renamed as *B4galnt2*), which encodes a *N*-acetylgalactosaminyltransferase.⁵⁶ The activity of this enzyme determines the expression of an oligosaccharide specific for a murine T cell subpopulation.⁵⁷ The human equivalent of this enzyme is responsible for expression of the blood-group antigen Sd^a. Murine expression of this glycosyltransferase is normally limited to intestine and kidney. In RIIS/J mice, a genetic defect relieves this restriction and endows endothelial expression, allowing it to enrich VWF with a surplus of terminal *N*-acetylgalactosamine residues.⁵⁸ Upon secretion into plasma, this form of VWF is readily recognized by the asialoglycoprotein-receptor and subject to rapid clearance.

Both the ST3Gal-IV-deficient and RIIS/J mouse models have provided important insight into the interplay between VWF glycosylation and survival, and it leaves no doubt that in the future more relevant information will be revealed using similar approaches.

The effect of glycosylation on VWF clearance: O-linked glycosylation

The complexity of VWF glycosylation is underscored by the presence of both *N*- and *O*-linked glycans. Whereas the *N*-linked side-chains are more or less evenly dispersed over the molecule, the *O*-linked glycans are mainly clustered around the platelet-binding A1 domain. It is not surprising therefore that several studies have demonstrated the regulatory role of *O*-linked glycans in VWF-platelet interactions.^{59,60} As for their role in regulating VWF clearance, initial studies revealed that suppression of *O*-linked glycosylation during synthesis gave rise to recombinant VWF preparations with a strongly reduced half-life in rats.⁶¹ This may indicate that the presence of *O*-linked glycans protects VWF from rapid clearance, either via prevention of VWF-binding to clearance receptors or by avoiding improper folding of VWF

The majority of the *O*-linked glycans consist of the sialylated tumor-associated T-antigen.³² This structure consists of the disaccharide galactose-(β 1-3)-*N*-acetylgalactosamine and is sialylated through capping with two *N*-acetylneuramic acid residues (*i.e.* NeuAc(α 2-3)Gal-(β 1-3)-[NeuAc(α 2-6)]GalNAc). Previously, it has been shown that this glycan structure on VWF can be detected using peanut agglutinin-lectin, but only after removal of sialyl-residues.⁶² We used this lectin to quantify loading of VWF with *O*-linked glycans. Unexpectedly, there appeared to be an inverted relationship between loading with *O*-glycans and VWF levels in a cohort of over 100 healthy subjects; thus the more glycosylation, the lower the levels of VWF.⁶³ Also in cohorts of patients having either unusually high or unusually low levels of VWF, this correlation was observed (Fig 2). For instance, liver cirrhosis patients have levels of VWF that may be increased up to 20-fold of normal. In these patients, the extent of *O*-linked sialylated T-antigen was significantly reduced by more than 2-fold on average, and

near undetectable in some of them. In contrast, increased loading with O-linked glycans (up to 4-fold) was observed in patients with reduced levels of VWF and classified as VWD-type 1.

It remains to be investigated whether or not O-linked glycans affect VWF levels by modulating clearance of the protein. It should be noted that we found a linear correlation between loading with O-linked glycans and propeptide/VWF ratios in VWD-type 1 patients. Since this ratio may be considered a surrogate marker for clearance, it seems that O-linked glycans may indeed contribute to the *in vivo* survival of VWF. Further studies are needed to investigate whether manipulation of O-linked glycosylation may be used to alter the half-life of recombinant VWF molecules.

The effect of proteolysis on VWF clearance

VWF circulates as a heterologous set of differently sized molecules. Apart from the intact multimeric proteins, it has been recognized that also proteolytic fragments of VWF are present in plasma.⁶⁴ Proteolysis occurs within the A2-domain between residues Tyr1605 and Met1606, and is mediated by a VWF-specific protease from the ADAMTS-family (*A Disintegrin and Metalloprotease with ThromboSpondin motif*), *i.e.* ADAMTS13.^{65,66} It is not uncommon that proteolysis of proteins is taken by the body as a sign for removal, and it has been suggested that this may apply to VWF as well.⁶⁷ However, the available data are not in support of this hypothesis. First, no difference in survival was observed for intact VWF and proteolysed fragments that were present in plasma-derived VWF preparations, which we infused in VWF-deficient mice (Lenting & Denis, unpublished observations). Second, Stoddart and colleagues compared the survival of wild-type VWF and a proteolysis-sensitive VWF mutant in rats, and could not detect differences in survival of both proteins.⁶¹ In view of the low susceptibility of human VWF for proteolysis by murine ADAMTS13, it cannot be excluded that the observed similarity in clearance in the study by Stoddart originates from a low proteolytic activity of rat ADAMTS13 towards human VWF. To avoid potential complications caused by species-differences, we analysed steady-state VWF levels in ADAMTS13-deficient mice and their wild-type littermates, assuming that levels should be higher in ADAMTS13-deficient mice if proteolysis enhances clearance. However, no differences in VWF levels could be detected in this regard (Lenting & Denis, unpublished observations). The relationship between ADAMTS13 levels and VWF half-life has also been examined using patients with VWD-type 1 or haemophilia A.⁶⁸ This study could not establish a correlation between ADAMTS13 levels and post-desmopressin survival of VWF, thereby providing further evidence against ADAMTS13 contributing to clearance of VWF. Whether other proteases exist which are able to modify VWF survival is yet unknown, but deserves further studies.

Modified clearance in the pathogenesis of VWD

Inspection of the VWD database reveals a large number of missense mutations associated with qualitative (VWD-type 2 mutations) or quantitative (VWD-type 1 and type 3 mutations) consequences. In the previous decades, the effect of many mutations on synthesis and secretion of VWF has been studied, providing insight on why these mutations are associated with reduced VWF levels.¹⁹ It is only recent that it has been recognized that low levels of VWF in VWD patients may also originate from modified clearance.⁶⁹ The first report on the association between increased clearance and VWD

appeared in 2002, and described the rapid disappearance of VWF upon treatment with desmopressin in 7 VWD patients carrying the combined M749I/R1205H mutations.²⁶ Additional studies revealed that these patients were not unique with regard to decreased survival of VWF. Brown and coworkers demonstrated that the average survival of VWF was shorter in a large group of VWD-type 1 patients than in haemophilia patients.⁴² It should be noted that a detailed evaluation of the individual patients was not provided in this report. Nevertheless, these data provide an important indication of the common occurrence of increased clearance in the pathogenesis of VWD type 1. More detailed analysis of the correlation between certain mutations in VWF and their effect on VWF clearance has been provided in a number of other studies. Van Schooten *et al.* described the increased clearance of VWF in patients harbouring selective cysteine-mutations (Cys1130Phe, Cys1149Arg, Cys2671Tyr), whereas Haberichter *et al.* reported decreased survival in families having either a Tyr1144Gly or a Ser2179Phe mutation.^{23,45} Thus, 6 different mutations have currently been linked to abnormal VWF clearance, and it leaves no doubt that this spectrum of mutations will expand in the future.

It is important to realise that the abovementioned studies analyzed the survival of endogenous VWF following desmopressin treatment, making it difficult to distinguish between increased clearance being caused by the amino acid replacement, additional gene mutations or secondary defects in clearance receptors for VWF. In addition, the determination of the half-life is often based on a few time-points, which prevents accurate calculation of these half-lives. It is obligatory therefore, that additional studies are being performed in a more controlled environment. We have chosen to do so by addressing the survival of recombinant VWF mutants in VWF deficient mice.^{23,70} This approach not only provides information regarding pharmacokinetic parameters of wild-type and mutant molecules, but also allows us to study their cellular targeting.

As for the translation to the clinical environment, insight into the survival aspects of VWF in VWD patients is of relevance concerning the way by which these patients are treated. If the half-life of endogenous VWF is too short, it might be preferential to apply replacement therapy rather than desmopressin treatment in these patients. The question is then: how to identify patients that have reduced VWF levels because of a reduced survival of their VWF? To perform extensive half-life studies might be a major burden for patients. We feel that relevant information can be derived from the propeptide/VWF ratios. Increased propeptide/VWF ratios have been found in patients with increased clearance^{23,45} and this ratio could therefore serve as a surrogate marker for increased clearance.

Clearance receptors for VWF: an unexplored aspect of VWF biology

At present, no information is available about which cells and which receptors contribute to the removal of VWF from the circulation. In order to get such information, we used our VWF-deficient mouse model to identify tissues that are of importance for VWF clearance. Intravenous injection of VWF resulted in the accumulation of the bulk of VWF protein in the liver.⁷⁰ However, VWF was also found in a number of other organs, like spleen and kidneys. Taking the size of each of these organs into account, VWF was taken up relatively efficient by both liver and spleen in particular.⁷¹ We are currently trying to establish which cells within these tissues are responsible for the cellular uptake for VWF. Preliminary immuno-histochemical analysis revealed considerable co-localisation between VWF and specific

markers for macrophage cells, pointing to a role for macrophages in both liver and spleen as dominant cells that endocytose VWF.⁷¹ This observation is in line with an observation made by Sodetz *et al.*, who reported that blockade of the reticuloendothelial system (which consists of phagocytotic cells, such as Kupffer cells) resulted in a delayed clearance of the VWF/FVIII complex.⁵¹

The mechanism by which these cells take up VWF requires further investigation. The molecular size of multimeric VWF may span several hundreds nanometres when in a coiled conformation.⁷² It is unlikely therefore that VWF is able to enter cells via the classical pathways involving clathrin-coated pits or caveolae, simply because of its physical dimensions. In this respect, our finding that macrophages are able to take up this relatively large molecule is in line with the capacity of these cells to rapidly endocytose large particles such as microbes. Whether or not this involves a receptor-mediated or receptor-independent process remains to be established. However, we have recently found that VWF may act as an adhesive surface for leukocytes by interacting with the leukocyte-specific $\beta 2$ -integrin family. This family has been recognized as an important mediator involved in the uptake of bacteria and other pathogens by macrophages, and could therefore also serve as an endocytic receptor for VWF.

VWF as a tool to prolong the half-life of FVIII

Current treatment of haemophilia A mainly involves replacement therapy using purified plasma-derived or recombinant FVIII preparations. Although part of the patients is still treated on an “on-demand”-basis, prophylactic treatment has become more common during the previous decade and often initiated after the occurrence of 1 or 2 joint bleeds. Although prophylactic treatment has resulted in a marked improvement of quality-of-life for haemophilia A patients, its application is still hampered by the requirement of frequent infusion (up to 3 times per week) because of the relatively short half-life of FVIII. In order to improve half-life of FVIII a number of initiatives have been taken. Most of the approaches are directly targeting the FVIII molecule, for instance via chemical conjugation of FVIII using polyethylene glycol polymers (*ie.* PEGylation). For a number of other proteins, this approach has been shown to encompass a powerful tool to increase half-life. Preliminary data describing the analysis of PEGylated FVIII in mice show that direct PEGylation of FVIII indeed is associated with an increased half-life.⁷³ However, it also appeared that PEGylation of FVIII may result in loss of at least part of its activity, thereby requiring higher doses for infusion.⁷⁴

Given the observation that half-life of FVIII is strongly influenced by VWF, it is of interest to consider VWF as a target to indirectly modulate FVIII half-life. In the circulation, the majority of the FVIII molecules (95-98 %) are in complex with VWF.^{75,76} In view of the high affinity of the complex (the affinity constant is less than 1 nM^{77,78}), it seems reasonable to assume that VWF-bound FVIII follows VWF in its clearance pathway. This possibility has been explored by Turecek and coworkers, who reported that modification of VWF via polysialylation or PEGylation gave rise to VWF molecules that displayed increased half-life when tested in VWF-deficient mice.^{79,80} Moreover, the prolonged half-life of PEGylated VWF was associated with an increased survival of FVIII which was co-injected with the conjugated VWF molecules. It should be noted that the increase in FVIII half-life was less pronounced than the prolongation for VWF, probably because free FVIII that dissociates from the complex is

susceptible to rapid clearance via for instance LDL-receptor related protein-1.⁶⁹ Furthermore, the binding capacity of PEGylated VWF to bind FVIII appeared to be reduced, although the affinity of the complex remained unchanged.

One final consideration in this regard relates to the potential side-effects of chemically modifying either FVIII or VWF. Several studies have shown that PEGylated proteins often exhibit reduced immunogenicity *in vivo*.^{81,82} It has been suggested that PEGylation-dependent reduction of immunogenicity may also apply to FVIII.⁸³ However, it is also of importance to mention that PEGylation of protein may increase immunogenicity, as became apparent during clinical trials using a PEGylated recombinant fragment of human thrombopoietin (*ie.* PEG-rHuMGDF). The development of inhibitory antibodies against this PEGylated molecule forced the termination of these clinical trials.⁸⁴

Concluding remarks

Taken together, we feel that exciting new data have been generated in the last few years with regard to the clearance of VWF. These data constitute the prelude for a better understanding of the apparently complex catabolic pathway of this intriguing molecule. Elucidating the molecular basis of VWF clearance is of importance to explore new avenues to improve treatment of VWD and haemophilia A.

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Disclosure

P.J. Lenting is employee of Crucell Holland B.V.

Legends

Figure 1: Association between pre-infusion VWF levels and FVIII half-life depends on blood group. FVIII half-life was determined in a cohort of 38 severe haemophilia A patients⁸⁵ and observed half-lives are plotted *versus* pre-infusion VWF levels. In agreement with previous studies (see refs^{47,48}), a statistically significant correlation between FVIII half-life and VWF antigen levels is observed ($p = 0.001$). Surprisingly, this correlation appeared to be different for patients having blood group O (open circles; $n=19$) or blood group non-O (closed circles; $n=19$). These data further show that average half-life of FVIII is shorter in haemophilia A patients having blood group O (11.5 ± 2.6 h) compared to patients having blood group non-O (14.3 ± 3.0 h).

Figure 2: Correlation between extent of glycosylation with sialylated T-antigen (represented by PNA-binding) versus VWF antigen levels. From different cohorts plasma samples were analysed for the presence of sialylated T-antigen using the biotinylated Peanut agglutinin-lectin (for details on assay see reference⁶³). Cohorts included von Willebrand disease-type 1 patients (VWD patients) and their unaffected family members (unaffected FM), patients with liver cirrhosis (Cirrhosis patients) and healthy controls. It appears that the extent of sialylated T-antigen loading inversely correlates with VWF levels in a non-linear manner (please note the log-scale for VWF levels).

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